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Department of Biosciences

**Development of Novel 3D Co-Culture Models of
the Human Intestinal Mucosa and their
Applications to Study Epithelial Transport and
Inflammatory Bowel Diseases**

Nicole Janay Darling

2021

A thesis submitted for the degree of Doctor of Philosophy

Abstract

Investigations into intestinal epithelial function frequently require the use of animal models or *ex vivo* human tissue samples. However, human tissue has limited availability while animal models exhibit species differences to the human intestinal structure and function. Additionally, in accordance with the principles established by the National Centre for the 3Rs (NC3Rs), there is a drive to move away from animal-based models. As such, several *in vitro* cell culture-based models have been developed to investigate epithelial function, the gold standard of these models is the Caco-2 monolayer, frequently employed throughout industry for the purposes of assessing absorption in the development of new chemical entities. Numerous limitations exist regarding this gold standard culture system: epithelial monolayers have differences in protein expression and activity, there is a lack communication between other intestinal cell lineages, and the *in vivo* microenvironment which provides structural support and signalling molecules to the epithelium is not considered using this reductionist approach to modelling the intestine structure and function.

This project aimed to address two key issues surrounding the Caco-2 epithelium: the lack of cell-cell communication, and absence of cell-extracellular matrix (ECM) communication. It was hypothesised that the culture of Caco-2 epithelial cells in combination with other cell lineages including, epithelial, stromal, and immune cells would enhance the structure and function of the model system to recreate the characteristics of the intestinal mucosa more accurately.

Through the use of 3D tissue culture technologies, it was demonstrated that fibroblast lineages produced *de novo* ECM representative of the intestinal lamina propria. Furthermore, differentiation of a Caco-2 monolayer on the surface of the lamina propria highlighted an altered epithelial polarisation, with greater apical-basolateral polarity as evidenced by the highly elongated, columnar morphology of the cells when compared to the gold-standard culture system. Moreover, this co-culture was shown to have functional properties of the epithelial barrier more like that of *in vivo* tissue. The complexity of this 3D co-culture system could be further enhanced by the addition of a mucus-producing cell lineage to represent the goblet cell component of the intestinal epithelium. Additionally, a constituent of the intestinal immune system was successfully incorporated into the lamina propria compartment of the model – specifically, macrophages differentiated from a monocytic cell line. Macrophages were selected for this purpose as they have principal roles in maintaining a state of anergy within the intestine, however perturbations of macrophage function can result in chronic inflammation and a subset of intestinal diseases collectively known as inflammatory bowel disease (IBD). The final immune-competent co-culture was demonstrated to instigate a proinflammatory response that altered the epithelial barrier integrity of the model when exposed to stimulatory effector molecules, recapitulating aspects of IBD.

Overall, this model has the potential to be utilised as a platform for further investigations regarding normal and inflammatory transport processes within the intestinal mucosa and a proof-of-concept study has demonstrated that the model has further applications in understanding the mechanisms underpinning IBD.

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This thesis is dedicated to the memory of Joe and Alex Liddle.

Declaration

The work described herein was carried out in the Department of Biosciences, Durham University between October 2016 and January 2021. All of the work is my own, except where specifically stated otherwise. No part has previously been submitted for a degree at this or any other university.

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Publications Arising from this Work

Original Research

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Abbreviations

2D	Two-Dimensional	CRP	C-Reactive Protein
3D	Three-Dimensional	CSF	Colony Stimulating Factor
5'AMP	5' Adenosine Monophosphate	c-SRC	Cellular Proto-Oncogene Tyrosine-Protein Kinase
ADME	Absorption, Distribution, Metabolism and Excretion	CYP	Cytochrome P450
AIEC	Adherent-Invasive <i>E. coli</i>	DC	Dendritic Cell
Aip-1	Actin-Interacting Protein 1	dH ₂ O	Deionised Water
AJ	Adherens Junction	DMEM	Dulbecco's Modified Eagle Medium
AJC	Apical Junctional Complex	DMSO	Dimethyl Sulfoxide
AMP	Antimicrobial Peptides	DNA	Deoxyribonucleic acid
APC	Antigen Presenting Cell	DSS	Dextran Sulphate Sodium
ATG16L1	Autophagy-Related Protein 16-1	<i>E. coli</i>	<i>Escherichia coli</i>
BCRP	Breast Cancer Resistance Protein	E-Cadherin	Epithelial-Cadherin
BCS	Biopharmaceutical Classification System	ECL	Enhanced Chemiluminescence
BiP	Immunoglobulin-Binding Protein	ECM	Extracellular Matrix
BMP	Bone Morphogenic Protein	EDTA	Ethylenediaminetetraacetic Acid
BSA	Bovine Serum Albumin	EGF	Epidermal Growth Factor
<i>C. jejuni</i>	<i>Campylobacter jejuni</i>	ENS	Enteric Nervous System
CD Molecule	Cluster of Differentiation	ER	Endoplasmic Reticulum
CD	Crohn's Disease	ERK	Extracellular Signal Related Kinase
cDNA	Complementary Deoxyribonucleic Acid	F-Actin	Filamentous Actin
CE	Carboxylesterase	FAE	Follicle-Associated Epithelium
CFTR	Cystic Fibrosis Transmembrane Regulator	FBS	Foetal Bovine Serum
CMV	Cytomegalovirus	FCγR1	High Affinity Immunoglobulin Gamma Fc Receptor
CO ₂	Carbon Dioxide	FD	Fluorescein Isothiocyanate Dextran
COX	Cyclooxygenase	FGF	Fibroblast Growth Factor
		Flt3-L	Fms-Related Tyrosine Kinase 3 Ligand

FoxP3	Forkhead Box P3	LPS	Lipopolysaccharide
GALT	Gut Associated Lymphoid Tissue	LSGS	Low Serum Growth Supplement
G-CSF	Granulocyte Colony Stimulating Factor	M Cell	Microfold Cell
GI	Gastrointestinal	M0	Unpolarised Macrophage
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor	M1	Classically Activated Macrophage
GRO α	Growth-Regulated Oncogene Alpha	M2	Alternatively Activated Macrophage
GWAS	Genome Wide Association Studies	MAP	Mitogen-Activated Protein
H&E	Haematoxylin & Eosin	MAPK	Mitogen-Activated Protein Kinase
HBSS	Hanks Balanced Salt Solution	MCP	Monocyte Chemotactic Protein
HDFn	Neonatal Human Dermal Fibroblasts	M-CSF	Macrophage Colony Stimulating Factor
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid	MDC	Macrophage-Derived Chemokine
Hh	Hedgehog	MDR	Multidrug Resistance
HIPE	High Internal Phase Emulsion	MHC	Major Histocompatibility Complex
IBD	Inflammatory Bowel Disease	MIG	Monokine Induced by Interferon-Gamma
ICAM	Intercellular Adhesion Molecule	MIP	Macrophage Inflammatory Protein
ICC	Interstitial Cells of Cajal	MLCK	Myosin Light Chain Kinase
IEC	Intestinal Epithelial Cell	MMP	Matrix Metalloproteinase
IFN	Interferon	mRNA	Messenger Ribonucleic Acid
Ig	Immunoglobulin	MRP	Multidrug Resistance Associated Protein
IL	Interleukin	MTT	Thiazolyl Blue Tetrazolium Bromide
IP-10	Interferon-Gamma Induced Protein 10	MTX	Methotrexate
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-Lined	MyD88	Myeloid Differentiation Primary Response 88
IRE1	Inositol-Requiring Enzyme	NBCS	New-Born Calf Serum
JAM	Junctional Adhesion Molecule	NCE	New Chemical Entities
LDH	Lactate Dehydrogenase	NEAA	Non-Essential Amino Acids
LPMC	Lamina Propria Mononuclear Cells	NF- κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NOD	Nucleotide-Binding, Oligomerisation Domain	STAT	Signal Transducer and Activator of Transcription
O.D.	Optical Density	TA	Transit Amplifying
OATP	Organic Anion Transporting Polypeptide	TBS-T/BRB-T	Blot Rinse Buffer
OCT	Organic Cation Transporter	TEER	Transepithelial Electrical Resistance
PAGE	Polyacrylamide Gel Electrophoresis	TEM	Transmission Electron Microscopy
Papp	Apparent Permeability Coefficient	TEMED	Tetraacetythylenediamine
PAS	Periodic Acid Schiff	TGF	Transforming Growth Factor
PBMC	Primary Blood Mononuclear Cells	Th	T Helper
PBS	Phosphate Buffered Saline	Tim-3/Gal-9	T-Cell Immunoglobulin Mucin 3/ Galectin-9
PEG	Prostaglandin		
PFA	Paraformaldehyde	TIMP	Tissue Inhibitor of Metalloproteinases
PGLA	Polylactic-glycolic acid	TJ	Tight Junction
P-gp	P-glycoprotein	TLR	Toll-Like Receptor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-Kinase	TNBS	Trinitrobenzene Sulfonic Acid Colitis
PMA	Phorbol-12-Myristate-13-Acetate	TNF	Tumour Necrosis Factor
qPCR	Quantitative PCR	Treg	Regulatory T Cell
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted	TREM	Triggering Receptor Expressed on Myeloid Cells
RNA	Ribonucleic Acid	TSLP	Thymic Stromal Lymphoprotein
RPMI	Roswell Park Memorial Institute Medium	UC	Ulcerative Colitis
		UPR	Unfolded Protein Response
<i>S. marcescens</i>	<i>Serratia marcescens</i>	VEGF	Vascular Endothelial Growth Factor
SC	Stem Cell	Wnt	Wingless-related integration site
SCFA	Short Chain Fatty Acid	XBP	X-Box Binding Protein
SCID	Severe Combined Immunodeficiency	YAP	Yes-Associated Protein
SDS	Sodium Dodecyl Sulphate	ZO-1	Zonula Occludens 1
SIS	Small Intestinal Submucosa	α -SMA	Alpha Smooth Muscle Actin
SNP	Single Nucleotide Polymorphism		

Chapter 1: Introduction

1.1. The Gastrointestinal Tract

The gastrointestinal (GI) tract is comprised of a series of interconnected organs extending from the mouth to the anus and encompasses the oesophagus, stomach, and the small and large intestines (Figure 1.1). It functions to facilitate the digestive process by catalysing the breakdown of ingested food and orally administered drugs; absorbing nutrients; and removing waste products through muscle contraction and the release of enzymes and hormones.

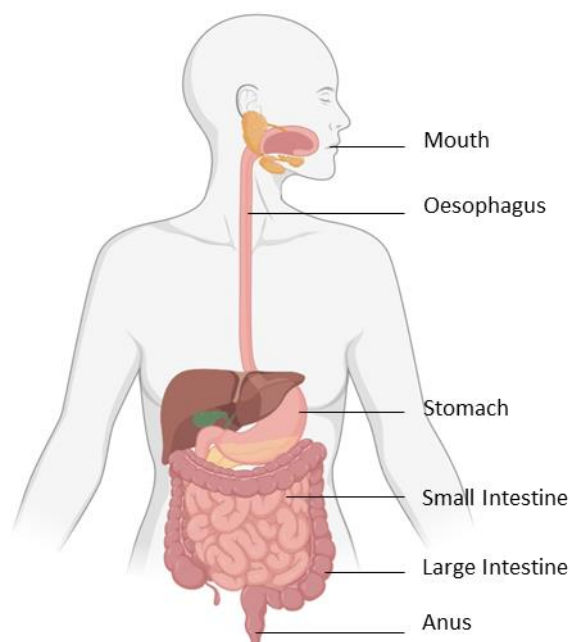


Figure Error! No text of specified style in document.1.1. The anatomy of the gastrointestinal tract. The GI tract is a series of organs encompassing the mouth, oesophagus, stomach, small and large intestines, and the anus. They form a continuous hollow tube structure throughout the body that facilitates digestion. Figure created with Biorender.com.

To enable the GI tract to function efficiently, there is a consistent quadruple layered structure with slight regional variations to the precise cellular composition depending on the specific function of the organ in the GI tract. The four conserved layers consist of the serosa, muscularis propria, submucosa and mucosa, that encircle a hollow lumen throughout the tract (Figure 1.2). Surrounding the entire digestive tract is the serosa, a visceral membrane containing blood and lymphatic vessels as well as nervous and adipose tissue; that functions to protect the organs of the GI tract. Adjacent to the serosa is the muscularis propria; a smooth

muscle wall arranged into two layers of an outer longitudinal and an inner circular smooth muscle. Within the loose extracellular matrix (ECM) between these muscular layers lies the myenteric plexus; a network of nervous tissue that is part of the enteric nervous system (ENS). The myenteric plexus regulates the intrinsic rhythmic contractions of the adjacent musculature through signals from the interstitial cells of Cajal (ICCs). Propagation of electrical waves from the ICCs allows the longitudinal muscle to contract and shortens the GI wall, resulting in increased mixing of the intestinal contents, whereas contraction of the circular muscle layer induces peristaltic transit of the contents across the epithelial surface (1,2).

The submucosa is a layer of fibroblasts and collagen-rich connective tissue that contains the blood and lymphatic vessels and functions to support the overlying mucosa layer. Within the submucosa lies the submucosal plexus, also known as Meissner's plexus: a highly ganglionated extension of the myenteric plexus that controls local blood flow, GI secretions and epithelial electrolyte and ion transport (3). The submucosal plexus is also responsible for co-ordinating intestinal motility by controlling the contractile activity of the muscularis mucosae; a thin layer of cylindrical muscle fibres that is part of the innermost layer of the GI wall - the mucosa. The function of the muscularis mucosae is poorly characterised but it is known to separate the mucosa from the deeper layers of the intestinal wall and provides local contraction. The mucosa also contains a supportive collagen-rich lamina propria, while the overlying absorptive and secretive epithelium separates the external environment of the lumen from the internal layers by acting as a protective barrier to bacteria and other foreign bodies and is also the principle site of absorption (4).

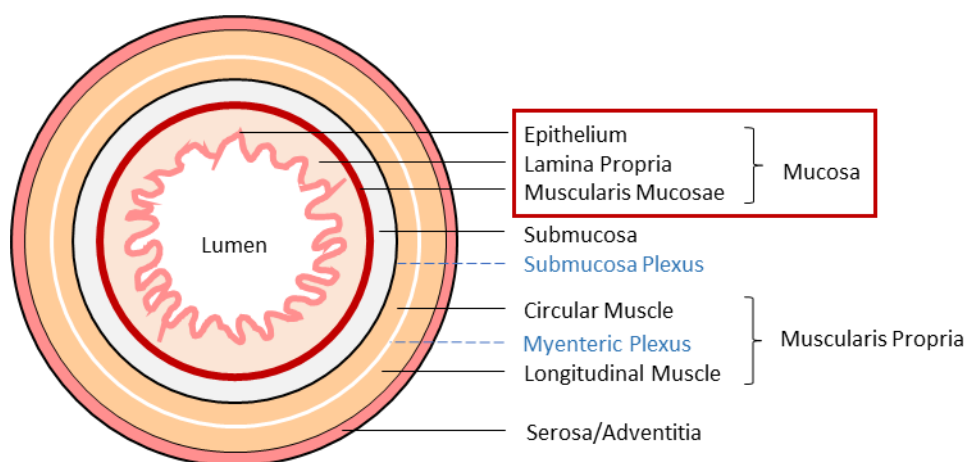


Figure 1.2. Structure of the gastrointestinal tract. A cross sectional view of the gastrointestinal tract indicating four distinct layers: the serosa, muscularis propria, submucosa and mucosa. Plicae folds are depicted within the walls of the mucosa surrounding the intestinal lumen. The neurones of the enteric nervous system are labelled in blue: the submucosal plexus and myenteric plexus, located within the submucosa and between the two layers of the muscularis externa respectively.

1.2. Intestine Anatomy and Physiology

The primary function of the GI tract is to facilitate the absorption and transport of nutrients, yet the majority of this occurs in the lower GI tract: the small and large intestines which function to absorb both nutrients and orally administered drugs into the body (Figure 1.3).

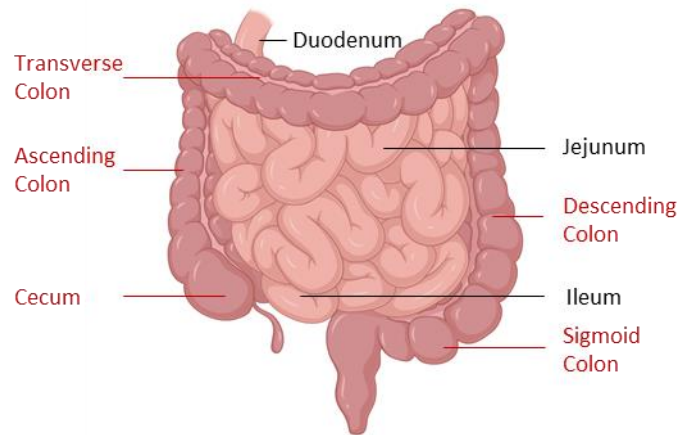


Figure 1.3. The gross anatomical structure of the small and large intestines. The small intestine consists of the duodenum, jejunum, and ileum; 5 %, 50 % and 45 % of the total small intestinal length respectively (black text). The large intestine is comprised of the cecum, ascending colon, transverse colon, descending and sigmoidal colons (red text). Figure created with BioRender.com.

The small intestine is 6 m long and has a diameter of 2.5-3 cm; it can be functionally separated into the duodenum, jejunum, and the ileum, although there are no anatomical differences between the two distal segments (5,6). The most proximal part of the small intestine is the duodenum, the shortest part of the small intestine. Partially digested food, known as chyme, enters the duodenum through the pyloric sphincter of the stomach. Secretions from the pancreas and liver are also received by the duodenum to assist in the degradation of chyme. The primary function of the duodenum is to neutralise stomach acid and to assist with this, Brunner's glands lie in the submucosa of the duodenum to produce a neutralising alkaline bicarbonate solution that also contributes to a layer of lubricating mucus lining the epithelial surface (7). The jejunum is the longest part of the small intestine and is where the majority of absorption occurs, while the most distal part of the small intestine, the ileum, contains the ileocecal valve that controls the flow into the cecum of the large intestine.

The large intestine is 1.5 m long and has a diameter of 6-7.5 cm. It can also be anatomically divided into different segments (5): the cecum, ascending colon, transverse colon, descending colon and sigmoid colon. As most nutrient and drug absorption has already taken place before reaching the large intestine, the large intestine does not require such a large surface area and therefore does not contain villi, resulting in a smoother surface than the small intestine. The major functions of the large intestine are water, vitamin and electrolyte absorption and removal of waste from the body by concentrating it into faeces.

1.2.1. Structural Adaptations to Increase the Surface Area for Absorption

Over 90 % of all absorption that takes place in the GI tract is carried out by the small intestine. To exponentially increase the surface area available for absorption, the small intestine has several adaptations, namely: villi, microvilli, and plicae circulares. Villi are finger-like projections that extend into the lumen and increase the surface area available for absorption 30-fold. Epithelial cells lining the villi have microvilli at their apical surface, which amplify the surface area a further 600-fold and promote efficient nutrient absorption (5). Plicae circulares or Kerckring's valves (depicted in Figure 1.2) are deep circular folds within the intestine walls that also increase the surface area available for absorption; they are a fixed structure that proximally have a thicker structure, while more distally in the intestine, they are less numerous and thinner (8). Within the mucosa lies an extensive network of capillaries to carry absorbed nutrients to the hepatic portal circulation and numerous lymphatic capillaries that assist in the transport of chylomicrons or ultra-low-density lipoproteins to venous circulation (Figure 1.4).

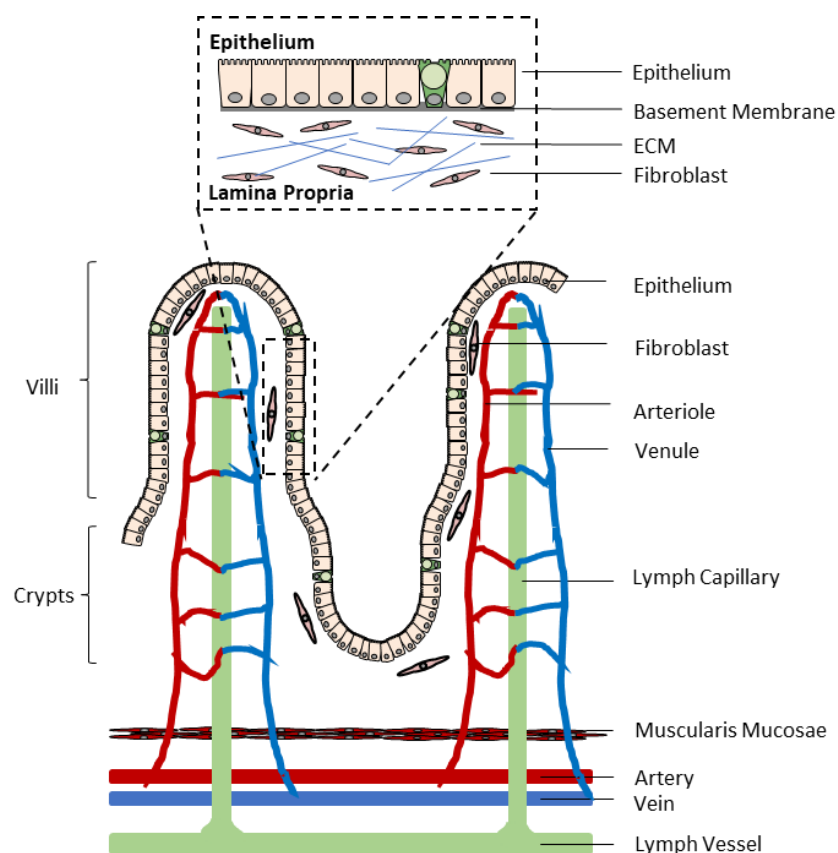


Figure 1.4. Cross section of the small intestine mucosa showing the crypt and villus structures and circulatory systems. Villi structures exist in the intestine to increase the surface area of epithelial cells available for absorption, and the blood and lymphatic circulatory systems extend through to the villi tip. The intestine is one of the most highly vascularised organs in the body to efficiently transport nutrients absorbed by the epithelial cells.

1.2.2. Stem Cells and the Crypt-Villus Axis

Located within the plicae circulares of the intestine, and lying between villi within the small intestine, are the crypts of Lieberkühn; tubular glands that descend into the connective tissue and home of the intestinal stem cells. Stem cells undergo a continuous process of self-renewal and are responsible for maintaining the

epithelial lining that is renewed every 3-5 days, due to the shedding of aged, apoptotic epithelial cells into the inner lumen from either the villi tip in the small intestine or at the surface epithelial cuff in the large intestine (9–12). The crypts of Lieberkühn provide a stem cell niche in which the cells reside, and where bone morphogenic protein (BMP) antagonists are produced by the subepithelial myofibroblasts and smooth muscle cells. As cells migrate upwards, they are exposed to active Wnt (wingless-related integration site)/ β -catenin signalling molecules resulting in the establishment of a proliferative compartment, and further migration up towards the villus tip exposes the cells to active BMP signalling and Hedgehog (Hh) signalling, inducing their differentiation (13,14). These signalling gradients across the length of the crypt and villi, result in crypt-villus axis where, generally, cells at the base of the crypt remain in an undifferentiated state and increased differentiation occurs as the cells migrate to the villus tip (Figure 1.5).

For many years intestinal stem cells had proved to be elusive. Two different models of intestinal stem cells were proposed in the 1970s (Figure 1.5) (13,15); the “+4 position” model and the “stem cell zone” model. In the “+4 stem cell model”, 4-6 stem cells reside in the +4 position above a cluster of Paneth cells and give rise to the rapidly dividing transit amplifying (TA) cells that beget the differentiated cells of the epithelium (16). The later model, however, postulates that the stem cells are interspersed within the Paneth cells. These stem cells are known as Crypt Base Columnar Cells and reside within a stem cell niche that is created by the Paneth cells (17,18). Recent evidence from label retention and lineage tracing experiments has supported the idea that there are two distinct populations of stem cells in the intestine that conform to the two proposed models. One population, the +4 stem cells, are extremely radiation sensitive and display a quiescent phenotype, while the crypt base columnar cells are Lgr5⁺ (leucine-rich repeat-containing G-protein coupled receptor 5) and are actively cycling; these Lgr5⁺ cells are considered to be responsible for the day-to-day turnover of the intestinal epithelium (15,19).

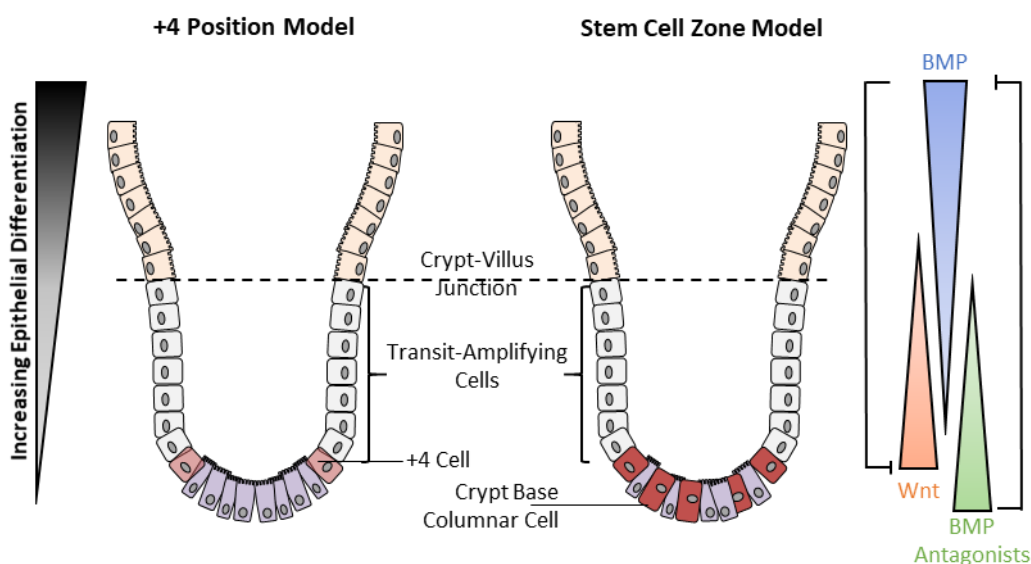


Figure 1.5. Proposed intestinal epithelial stem cell models. The +4 Position Model proposed that the base of the crypts is populated exclusively by Paneth cells that have terminally differentiated, while the stem cells lie above the Paneth cells in the +4 position in the crypt. In the Stem Cell Zone Model, there are undifferentiated cycling cells, known as crypt base columnar cells, that lie amongst the Paneth cells and these cells are the proposed stem cells. Adapted from Kosinski et al., 2007 and Barker et al., 2008.

1.2.3 Cells of the Intestinal Epithelium

The intestinal mucosa has a highly diverse population of cell types ranging from immune and bacterial cells to stromal and epithelial cells (Figure 1.6) (20), each highly specialised to carry out their specific function and maintain intestinal homeostasis.

1.2.3.1. Enterocytes

The most prevalent cell type in the small intestinal epithelium are enterocytes, which constitute > 80 % of the intestinal epithelial cells (IEC). Enterocytes are absorptive cells that are highly polarised columnar cells with rigid microvilli projections at the apical surface that form a brush border. Microvilli contain large, negatively charged integral membrane mucin-like glycoproteins that form a carbohydrate-rich glycocalyx. This brush border associated with glycocalyx creates a digestion-promoting microenvironment and contains absorbed pancreatic and glycoprotein enzymes that assist in terminal digestion (21,22). The brush border also contains different metabolic enzymes such as alkaline phosphatase, aminopeptidase and sucrase isomaltase, that can be exocytosed from the microvilli tip to the periapical space above enterocytes allowing the final stages of nutrient hydrolysis to take place immediately before absorption (23).

Along with the digestive and absorptive properties of enterocytes, these cells are also responsible for forming a barrier against the diffusion of foreign antigens and pathogens into host tissues (22,24). Enterocytes also have important secretory roles and are responsible for transporting immunoglobulin A (IgA); a molecule with essential roles in maintaining homeostasis between the host and intestinal microbiota, into the intestinal lumen (25,26). In the large intestine, the absorptive cells are known as colonocytes; these cells are very similar to enterocyte cells but are involved in absorption of lipid soluble molecules that are transported via passive diffusion and have fewer enzymes involved in terminal differentiation (20).

1.2.3.2. Goblet cells

The second most abundant cell type within the intestinal epithelium are goblet cells; 4-10 % of cells in the duodenum are goblet cells and their number increases distally through the intestines to approximately 16-24 % in the colon (10,27,28). Goblet cells produce trefoil peptides that are necessary for epithelial maintenance and mucins (29); highly glycosylated glycoproteins that hydrate and gel to form part of the glycocalyx discussed above in Section 1.2.3.1, upon secretion from goblet cells. Goblet cells also produce two types of mucins: membrane-bound mucins such as *MUC1* and *MUC3*, and secretory mucins such as *MUC2* and *MUC5AC*. *MUC3* is the most abundant membrane-bound mucin in the small intestine, while *MUC2* is the most prominent secreted mucin that forms part of the front-line defence against foreign antigens while still allowing for the transport and absorption of nutrients (28). Mucus acts as a lubricant for flow through the intestines and protects the epithelial lining from digestive enzymes. A single layer of mucus is present in the small intestine while a double layer is present in the large intestine; the inner layer is strongly adhered to the

epithelial cells and contains no microorganisms under normal, healthy conditions, while the outer layer is loosely attached and provides a microenvironment for microbial flora to live in (12,28,30).

1.2.3.3. Paneth cells

Paneth cells are only present in the small intestine and do not exist in the large intestine. They are unique in that upon differentiation, the cells migrate down to the base of the crypts of Lieberkühn, rather than towards the villus tip, where they contribute to the stem cell niche by producing Wnt and Notch signalling factors that assist in the regulation of intestinal epithelial cell self-renewal (31,32). Removal of Paneth cells also differs from other epithelial cell types; rather than being removed by exfoliation, Paneth cells are removed by phagocytosis via macrophages present in the lamina propria after 21-30 days (22). The principal function of Paneth cells is to maintain immune homeostasis through secretion of antimicrobial peptides such as defensins, lysozyme and lectins into the mucus layer, once signals from microbial antigens are detected (32–34).

1.2.3.4. Enteroendocrine cells

Enteroendocrine cells are isolated, hormone-secreting cells that make up < 1 % of the epithelial cell population (35). Enteroendocrine cells act as chemoreceptors by sensing luminal contents and secreting regulatory hormones such as glucagon-like peptide and gastric-inhibitory peptide to control digestion and intestinal motility (25,36). Hormones secreted by enteroendocrine cells can act either locally in the surrounding cells or more systemically by releasing hormones and peptides into the bloodstream to activate nervous responses. Enteroendocrine cells are also able to detect harmful antigens and can initiate host defence responses (10,37).

1.2.3.5. Microfold cells

Microfold cells (M cells) are specialised antigen sampling cells that are predominantly located at inductive sites in gut-associated lymphoid tissues (GALT) such as Peyer's Patches, Colonic Patches and Isolated Lymphoid Follicles, where approximately 10 % of the cells present in these lymphoid tissues are M cells (38,39). Phenotypically, M cells have fewer microvilli than other intestinal epithelial cells and display irregular brush border at their apical surface (40,41). M cells function to transport luminal antigens and microorganisms across the epithelium, via transcytosis, to more specialised antigen presenting cells (APCs), namely dendritic cells, that are then able to induce immune cell tolerance or initiate an immune response (38,42). Although M cells form part of the intestinal defence system, they lack an overlying mucus layer and are thus a weak point in the epithelial barrier that can be exploited by microorganisms to gain entry into the host (39).

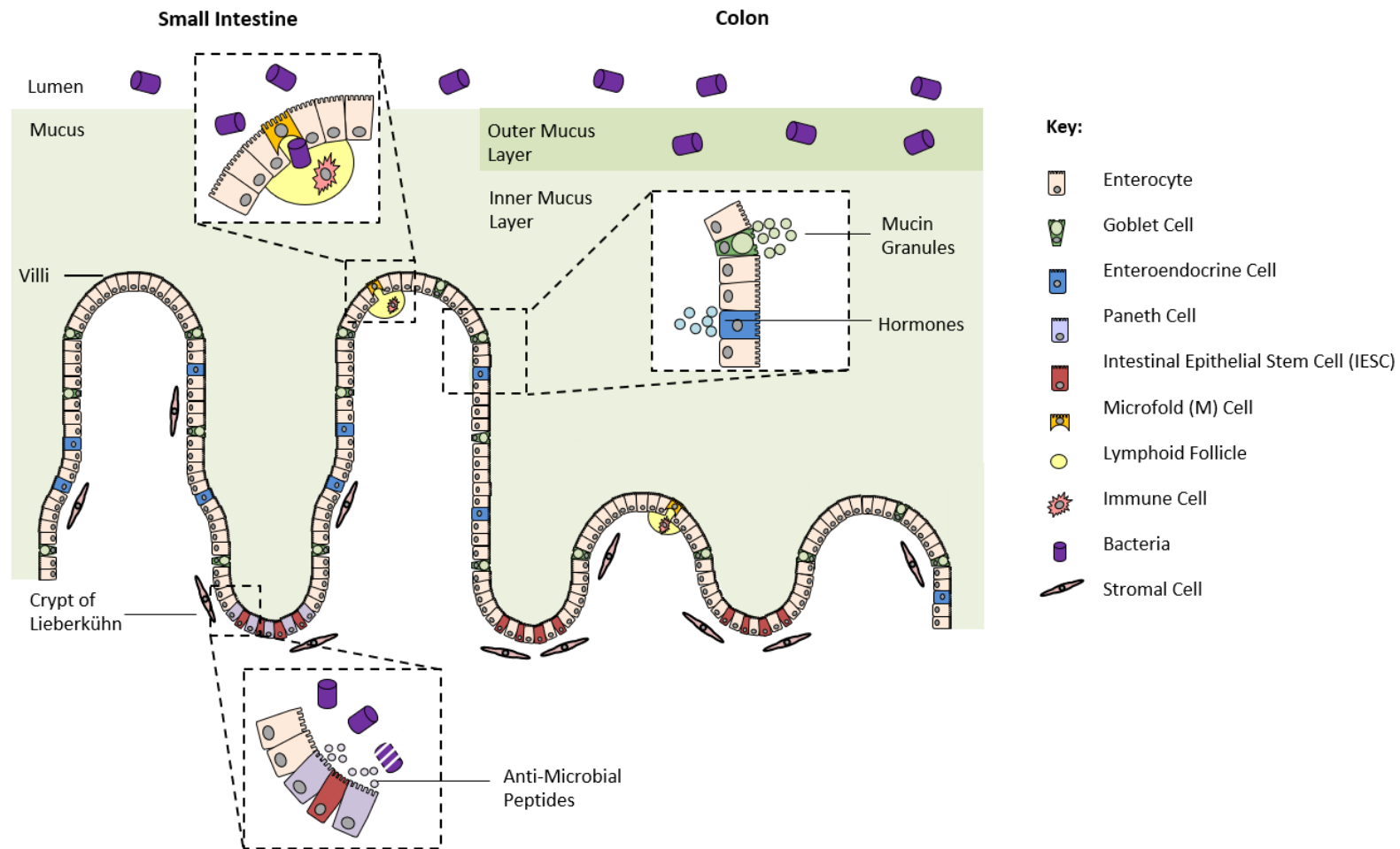


Figure 1.6. Cells of the intestine mucosa. The intestines have numerous cell types including stem cells, epithelial cells, and cells of the lamina propria. In addition, there are numerous immune cells located within the lymphoid follicles of the intestine. Adapted from Peterson and Artis 2014.

1.2.4. Structural Organisation and Function of the Lamina Propria

The lamina propria lies below the intestinal epithelial layer and constitutes the second major part of the intestinal mucosa. It primarily functions to support the epithelium and assist in the generation a mucosal immune response (discussed in further detail in Section 1.4) and has a specialised structure and cellular composition to complete its role.

1.2.4.1. Mesenchymal architecture

The lamina propria of the intestine displays a layered architecture along the crypt-villus axis (43). Epithelial cells rest on a basement membrane; a layered structure comprised of a basal lamina and an underlying reticular sheet known as the fibroreticular lamina with five main extracellular components: collagen IV, laminin, fibronectin, heparin sulphate proteoglycan and entactin (44,45). The basal lamina can be further divided into two layers; the lamina lucida which lies adjacent to the epithelial cells and the lamina densa which juxtaposes the fibroreticular lamina.

Within the intestine, the basal lamina is continuous throughout the crypts but has pores between 0.5-5 μm in diameter, known as fenestrations, in the villi (46). The fibroreticular lamina consists mainly of collagen and sub-epithelial fibroblasts and also contains pores with a diameter of 3-7 μm that are known as foramina (47). A dense network of fibroblast-like cells exists at the base of the crypts, while fibroblast-like cells of the upper villi display a stellate shape that are connected to the fenestrations and foramina in the basement membrane to form a 0.3-5 μm cellular sieve, that together, allow passage of absorbed nutrients (48,49). It is also thought that these pores allow for immune cell trafficking; this is evidenced by the presence of large foramina in the fibroreticular lamina below the intestinal lymphoid follicles (47,49).

1.2.4.2. Mesenchymal cells of the lamina propria

Many mesenchymal cells exist in the intestinal lamina propria including fibroblasts and myofibroblasts, vascular mural cells known as pericytes, bone marrow stem cells, smooth muscle cells of the muscularis mucosae, and lymphatic lacteals. The following section will focus on the ECM-producing fibroblast and myofibroblast component of the lamina propria, due to their importance in supporting the epithelium; for an in depth review of the other components, see Powell et al., (50).

Within the intestinal lamina propria, two types of fibroblast cells exist: fibroblasts and myofibroblasts that exhibit some properties of smooth muscle cells such as gap junctions and myofilament bundles. Fibroblasts are a source of myofibroblasts in the intestine. Transforming growth factor- β (TGF- β) is thought to be a key inducer of myofibroblast differentiation, particularly during wound healing (51,52). Myofibroblasts have typically been characterised by the presence of α -smooth muscle actin and this has led to controversy over whether myofibroblasts are actually present at all in the intestine, as more in-depth immunostaining and electron microscopy characterisation revealed that the perceived myofibroblasts are more akin to fibroblasts or immature smooth muscle cells. This has led to stricter characterisation of myofibroblasts that are defined

as being vimentin⁺, desmin⁻, α -smooth muscle actin⁺, CD45⁻, CD31⁻, and CD90⁺ (Thy-1⁺), with an abundant rough endoplasmic reticulum (ER) and Golgi, as well as myofilament bundles being connected with fibronectin through a fibronexus junction, leading to the hypothesis that there are no myofibroblasts in the healthy intestine (49,50,53–55).

However, others have postulated that myofibroblasts are present in the intestine and are present in subepithelial locations within the villus tip making them more like the smooth-muscle pericytes of the vasculature, while fibroblasts cluster below the epithelium at the villus tip (50). Myofibroblasts have also been shown to constitute an essential part of the stem cell niche by secretion of BMP antagonists; for example, myofibroblasts support the growth of isolated crypt organoids and were able to maintain their *in vitro* culture for over 50 days. Without myofibroblasts, the organoids began to degrade after 3 days in culture (56).

1.3. Transport Across the Intestinal Epithelium

Nutrients and drug compounds that are absorbed by the intestinal epithelium encounter a series of barriers: the mucus layer or aqueous boundary; the epithelial cell layer; and the lamina propria - though the effects of this layer are minimal as it is relatively permeable to absorbed compounds (57). The mucus layer is an unstirred layer in contact with the apical surface of the epithelium. The importance of this unstirred layer as a barrier to absorption has been demonstrated in *in vitro* models of the intestinal epithelium. Compounds that are rapidly absorbed by epithelial cells had altered apparent permeability coefficients upon reduction of the thickness of the unstirred layer through agitation of the models, while more slowly absorbed compounds remained unaffected. *In vivo* the effect of the aqueous boundary is reduced by the continuous mixing of the luminal contents (58).

Although the mucus layer acts as a barrier to the passage of molecules, it is not completely impenetrable; large molecules are able to move slowly through the mucus layer while small hydrophilic molecules can rapidly diffuse through this layer and encounter the next barrier to transport: the epithelium. Transport across the intestinal epithelium occurs via four main routes: passive transcellular, passive paracellular, carrier-mediated transport and endo-/exocytosis vesicle-mediated transport (Figure 1.7) (57). Lipophilic molecules migrate through the epithelial cells via the passive transcellular route; they are distributed into the cell membrane of the epithelial cells and are rapidly absorbed. Many orally administered drugs are lipophilic to encourage their fast absorption and effect (57), yet the main transport routes of drug compounds are the passive paracellular and carrier-mediated transport routes which will be discussed in greater detail.

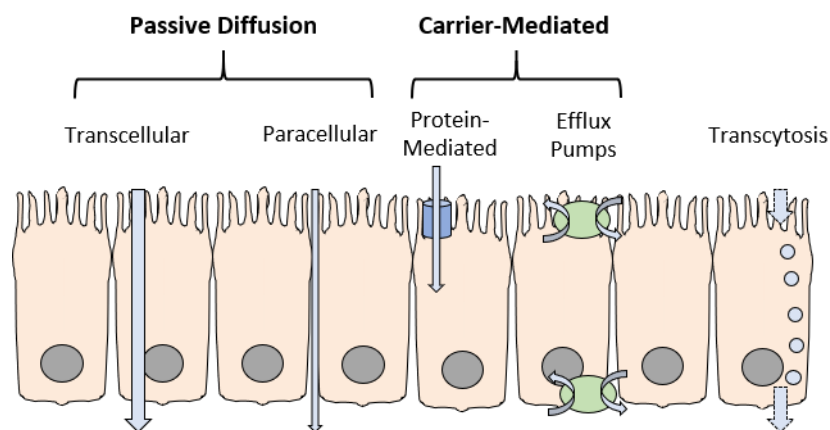


Figure 1.7. Absorption routes across the intestinal epithelium. The majority of drugs are passively absorbed through the epithelium by the trans- and paracellular transport routes. Other molecules are transported via protein mediated systems which can be either active or passive. Macromolecules undergo a transcytosis method of transport wherein they are transported across membranes in endocytosed vesicles. Adapted from Artursson 1991.

1.3.1. Paracellular Transport and the Role of Epithelial Junctions

Unlike lipophilic molecules, molecules that are charged or hydrophilic cannot cross the lipid membrane and must be transported across the epithelium in another manner. The second method of passive, energy-independent transport is the paracellular route. This occurs through water-filled channels between the lateral membranes of the epithelial cells and can occur in one of two ways: passive diffusion between cells, or convective solvent drag whereby compounds are carried across the epithelium by the flux of water (58).

The paracellular route is controlled by the apical junctional complex (AJC) that comprises of tight junctions (TJ), directly below the apical membrane, and subjacent adherens junctions (AJ) that provide connections to the actin cytoskeleton (Figure 1.8). Disruption of the AJC is a contributing factor to the pathogenesis of inflammatory bowel diseases and will be discussed further in Section 1.4.

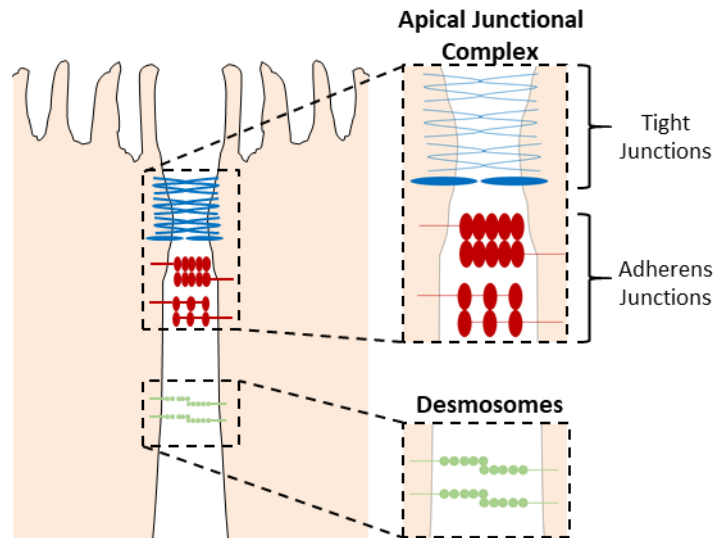


Figure 1.8. Schematic representation of the epithelial junctional complexes. Tight junctions and adherens junctions are located in the apical region of epithelial cells and together form the apical junctional complex responsible for regulating epithelial permeability and adhesion of adjacent cells. Desmosomes link to the intermediate filament cytoskeleton and are responsible for resisting mechanical stress.

1.3.1.1. Tight junctions

Tight junctions are a network of protein fibrils that appear as thin bands that form a continuous ring around the epithelial cells. They are localised to the apical most part of the cell, just below the plasma membrane and form a barrier to the passage of extracellular fluid and ions between adjacent epithelial cells. Not only do tight junctions form a barrier to transport, they are also crucial to the establishment and maintenance of cell polarity by blocking the movement of proteins and lipids between the apical and basolateral domains of the cell membrane (59). Tight junctions take up roughly 0.01 % of the surface area of the intestine, yet despite this constitute a major route for drug and nutrient absorption (57). The radii of the pores in humans is 0.8 and 0.3 nm in the duodenum and colon respectively, reflecting the higher permeability of the small intestine compared to the colon and potentially increasing the solvent drag (57,60).

Tight junctions are composed of a series of transmembrane proteins that seal adjacent cells and cytoplasmic plaque proteins that anchor the transmembrane proteins and form links to the cytoskeleton (Figure 1.9) (61). Plaque proteins are PDZ domain-containing proteins such as zonula occludens (ZO)-1, -2 and -3, that mediate interactions between other proteins with a PDZ motif (59). They function as scaffolds that bring together the cytoskeleton, signalling proteins, transcription factors and integral membrane proteins (62).

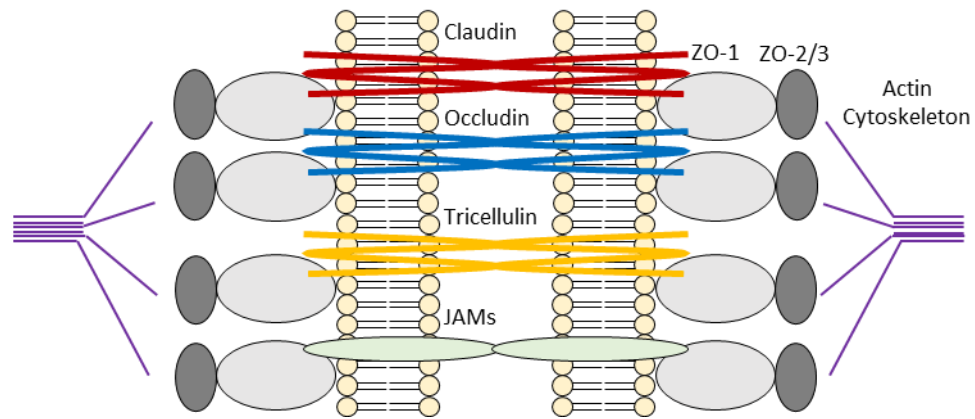


Figure 1.9. Simplified structure of the intestinal tight junctional complex. The two principle transmembrane proteins of tight junctions are claudins and occludins. Tricellulin and JAMs are also involved in bridging the gap between cells. Zonula occludin proteins, along with other adaptor proteins including cingulin and AF6, provide an intracellular scaffold to anchor the transmembrane proteins to the actin cytoskeleton. Adapted from Robinson et al., 2015.

One of the first integral membrane tight junction proteins to be identified was occludin; a tetraspan transmembrane protein that has roles in sealing the epithelial barrier, evidenced through studies where fibroblasts transfected with occludin were able to form tight junctions (63,64). However, the precise role of occludin is not fully understood as mouse knockout experiments demonstrated that mice deficient in occludin were still able to form structurally relevant tight junctions (65).

Claudin proteins were recognised around the time of occludin identification. Claudins comprise a family of at least 27 proteins that have a similar tetraspan structure to occludin. They are generally considered to be the main factors regulating barrier properties and have two major functions depending on their charge (62,66). The major type of claudins are the sealing proteins, such as claudin-1 and -3, while the main pore-forming proteins in the intestine are the selective cation-permeable claudin-2 and -15 (62). Current understanding of claudin structure is that the first, larger extracellular loop is critical for the “tightness” and selective ion permeability and the second, smaller extracellular loop is responsible for narrowing the cleft between adjacent epithelial cells (66).

Two further integral membrane proteins identified in tight junctions are tricellulin and junctional adhesion molecules (JAM). Tricellulin proteins are found where three neighbouring cells meet and are therefore form a tricellular tight junction structure (67). The structure of tricellulin, is again reminiscent of occludin, and has been shown to compensate for some occludin functions (67). Its importance in tight junctions has also been highlighted in *in vitro* intestinal models using Caco-2 cells where knockdown of tricellulin resulted in decreased TEER and delayed barrier development (68). JAM proteins are single transmembrane immunoglobulin proteins whose roles in tight junctions are not fully understood but it is thought that they are important for immune cell trafficking (69).

1.3.1.2. Adherens junctions

Adherens junctions lie directly subjacent to tight junctions within the apical junctional complex and their formation precedes the development of TJs as they are responsible for recruiting TJ proteins to the lateral

membrane (70). Along with initiating the development of cell-cell contacts, adherens junctions have major roles in maintaining tissue integrity as well as controlling cell proliferation and motility.

Structurally, adherens junctions are less complex than tight junctions. They consist of an adhesive core formed by homotypic interactions of two types of integral membrane proteins: cadherins and nectins (71). The major cadherin present in the intestine is the calcium-dependent classical type cadherin known as epithelial (E-) cadherin. Nectin associations are calcium-independent and the function and regulation of nectin intercellular adhesions within the intestine is poorly understood.

The assembly and stability of adherens junctions is regulated by intracellular catenin proteins including α -catenin, β -catenin and p120 catenin. Catenins are responsible for controlling the trafficking and retention of E-cadherin at the plasma membrane and link adherens junctions to the filamentous (F)-actin cytoskeleton to form a continuous circumferential belt along the epithelium (72,73). This occurs either directly or indirectly, thorough binding of adaptor proteins such as afadin (74).

1.3.1.3. Additional junctions in epithelial cells

Several other types of epithelial junctions exist within the mucosal barrier. These include gap junctions and desmosomes which are involved in the formation of cell-cell contacts, as well as hemidesmosomes that are responsible for epithelial cell attachment to the underlying ECM.

Desmosomes are responsible for forming mechanical cell-cell contacts through interactions of the integral membrane desmosomal cadherins: desmoglein and desmocollin (75). The only isoform identified in the intestine epithelium is desmoglein 2 and recent studies have postulated that desmoglein 2 has roles in controlling epithelial permeability through p38 mitogen-activated protein (MAP) kinase signalling (76). The intracellular domains of the desmosomal cadherins form links to the intermediate filament cytoskeleton through a series of plaque proteins such as plakoglobin and desmoplakin, allowing cells to resist mechanical stress. Loss of desmoplakin from the intestinal epithelial layer does not alter adhesion between adjacent cells and further highlights the importance of the AJC in barrier integrity (77). Desmoplakin, is however, critical to controlling microvilli length (77).

Gap junctions are the final type of cell-cell junction. They are highly dynamic protein complexes composed of hexameric associations between individual connexin integral membrane proteins (78). Gap junctions are responsible for mediating intercellular communication through direct transfer of ions along with other signalling molecules and thus have a role in electrical and metabolic communication between cells (79).

The intestinal epithelium overlies the ECM-rich lamina propria and so in addition to cell-cell adhesion complexes, there are also junctions between the cell-ECM known as hemidesmosomes. Hemidesmosomes link the intermediate filament cytoskeleton to the underlying ECM in a junctional complex primarily consisting of integrin proteins which firmly adhere the epithelium to the ECM (80).

1.3.2. Carrier-Mediated and Transcytosis Transport Across the Epithelium

Two final transport routes are used in the absorption of nutrients and chemical compounds in the intestine: vesicle transport and carrier-mediated transport. The least studied methods of transport across the intestinal epithelium are vesicle-mediated endo- and exocytosis. However, recent studies have highlighted the importance of this transport route in macromolecular transport. Once a high concentration of macromolecules is in contact with the cell membrane, energy-dependent invagination and vesicle formation occurs via a process known as endocytosis (81). Inhibition of glycolysis and oxidative phosphorylation prevents the membrane invagination mechanisms as cell membrane re-synthesis is a process that requires energy (82). Membrane invagination results in the macromolecules being encased in phagosomes that migrate to the supranuclear region of the cell whereupon lysosomes merge with the phagosome to form a phagolysosome where the macromolecules are digested. Any undigested molecules that escape digestion migrate to the basolateral membrane and are exocytosed into the lamina propria (81).

Carrier-mediated transport is the final transport route for nutrient and drug absorption in the intestine (Figure 1.10) (83). Uptake transporters present on the apical membrane of enterocytes and efflux transporters present on the basolateral membrane of enterocytes are responsible for the absorption of molecules into the blood stream such as organic anion transporting polypeptide (OATP) and multidrug resistance associated protein 1 (MRP-1) respectively. Other transporter proteins have roles in detoxification of the intestine and prevent absorbed molecules from entering the circulation. Efflux transporters located on the apical surface of the epithelium such as P-glycoprotein (P-gp) and uptake transporters on the basolateral membrane such as organic cation transporter (OCT) work together to perform detoxification processes. The different proteins involved in carrier mediated transport are highlighted in Table 1.1.

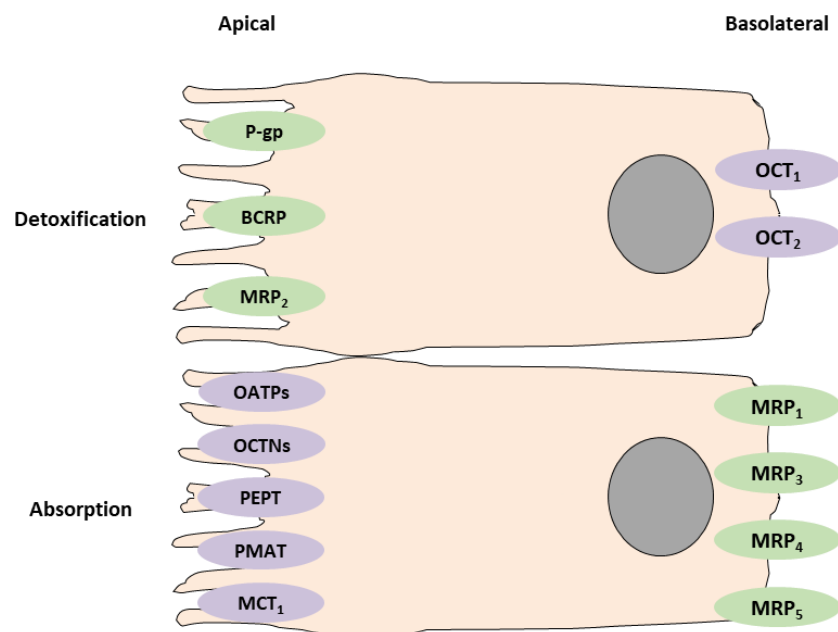


Figure 1.10. Transporter proteins in the intestinal epithelium. Uptake transporters are depicted in lilac, while efflux transporters are shown in green. Uptake transporters on the basolateral membrane in collaboration with efflux transporters on the apical membrane are responsible for detoxification. Uptake transporters on the apical surface and efflux transporters on the basolateral plasma membrane carry out the carrier-mediated absorption process. Adapted from Estudante et al., 2013.

Table 1.1. Transporter proteins involved in carrier-mediated transport within the intestine.

Transporter Protein	Membrane Localisation	Function	Reference
Breast Cancer Resistance Protein (BCRP)	Apical	Efflux Transporter – excretory transporter, limits oral bioavailability of drugs with diverse substrate range with overlap between MRP-2 and P-gp.	(83)
Monocarboxylate Transporter 1 (MCT1)	Apical	Uptake Transporter – absorptive transporter of short chain fatty acids (SCFA) that are produced from microbes in the intestinal lumen.	(84)
Multidrug Resistance-1 (MDR-1)/ P-glycoprotein (P-gp)	Apical	Efflux Transporter – excretory role with broad substrate specificity of chemical compounds, preferences for hydrophobic, amphipathic, or cationic molecules. Naturally occurring substrates include biologically active compounds within normal diet such as plant chemicals and endogenous compounds including bile salts and steroid hormones.	(83–85)
Multidrug Resistance-Associated Protein (MRP)-1, -3, -4, -5	Basolateral	Efflux Transporter – absorptive transporter of organic ions including glutathione conjugated drugs and endogenous compounds including prostaglandins.	(86)
Multidrug Resistance-Associated Protein-2 (MRP-2)	Apical	Efflux Transporter – excretory transporter protein of organic ions and conjugates of heavy metals including arsenic, along with naturally occurring bile salts.	(87)
Organic Anion Transporting Polypeptides (OATP) 1A2 and 2B1	Apical	Uptake Transporter – absorptive transporter protein. Transports solutes and organic compounds.	(83,84)
Organic Cation Transporters (OCT) N1 and N2	Basolateral	Uptake Transporter – Secretory transporter of small hydrophilic ions.	(83)
Plasma Membrane Monoamine Transporter (PMAT)	Apical	Uptake Transporter – absorptive transporter of monoamines.	(88)
Proton Dependent Oligopeptide Transporter (PEPT)	Apical	Uptake Transporter – absorptive solute transporter.	(83)

1.4. Maintenance of Intestinal Homeostasis and Inflammatory Bowel Diseases

The gut-lumen interface has the highest population of microorganisms in the body with over 10^{12} microorganisms/mL of luminal contents and as such requires a highly co-ordinated and regulated immune response (12). Disruptions to immune homeostasis in the intestines can lead to chronic inflammation and contribute, not only to inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC), but is also thought to play a role in the development of other autoimmune diseases at sites other than the intestines, such as asthma and type 1 diabetes (89–92).

1.4.1. Inflammatory Bowel Diseases

Inflammatory bowel diseases consist of two major phenotypes: Crohn's disease and ulcerative colitis. The prevalence of IBD exceeds 0.3 % in Europe and North America with an increase in incidence in newly industrialised countries throughout the rest of the world, highlighting the importance of research in understanding the mechanisms underlying IBD (93). IBDs are multifactorial diseases that occur as a result of genetic susceptibility; environmental factors such as the microbiota; barrier dysfunction with elevated epithelial permeability; and immune dysregulation, resulting in a chronic inflammatory response in the GI tract.

Crohn's disease can affect any part of the GI tract but is most prevalent in the ileum. As the small intestine can be affected in CD, malnutrition of CD patients can occur as the damaged organ can no longer effectively absorb nutrients. Inflamed areas of tissue appear as patches surrounded by areas of healthy tissue and inflammation can occur throughout the thickness of the GI tract wall. CD is associated with abdominal pain and rectal lesions (94). CD patients exhibit areas of both ECM deposition and degradation: excessive ECM deposition results in stricturing fibrosis of the wall of the GI tract while exacerbated ECM remodelling and depletion results in the formation of fistulae – penetrating holes that destroys the boundaries between tissues (95).

Unlike CD, ulcerative colitis is restricted to the large intestine; if a patient is in a state of active UC, the entire large intestine is affected and there are no areas of healthy tissue. A further distinguishing feature of UC is that inflammation only occurs in the innermost mucosal layer of the intestine. UC patients experience severe, intermittent pain, diarrhoea, and blood in stool. UC patients also have a higher propensity than CD patients to develop colorectal cancer (94,96).

Evidence for the role of different susceptibility genes involved in IBD comes from genome wide association studies (GWAS) and murine models of IBD. Five principal murine models of IBD exist (Table 1.2) and are used to address scientific questions, evaluate pharmacokinetics and examine the preclinical efficacy and safety testing of new chemical entities to treat IBD (97,98). The use of GWAS studies and animal models has helped identify the role of specific cell types involved in the pathogenesis of IBD.

Table 1.2. The five commonly used experimental mouse models of IBD. The nature of the insult to the model and its phenotypic response is described along with the major functions and therapies addressed by each individual model.

Mouse Model	Nature of Insult and Response	Model of:
Dextran Sulphate Sodium (DSS) Colitis	Chemical destruction of IEC layer results in invasion of commensal bacteria and the onset of acute colitis followed by restitution and repair of the epithelial barrier.	Epithelial barrier function
Trinitrobenzene Sulfonic Acid Colitis (TNBS)	Renders colonic proteins immunogenic to the host immune system and drives acute colitis.	Effect of anti-cytokine therapies
Interleukin (IL)-10 Knockout	Deletion of the <i>IL-10</i> gene locus in mice results in accumulation of adaptive immune cells and chronic inflammation.	Immunoregulation and macrophage function
Adoptive Cell transfer	Transfer of naïve CD4 ⁺ T cells from donor mice to immunodeficient mice resulting in chronic inflammation and wasting disease.	Immunoregulation and regulatory T cells
Oxazolone	Chemical agent that elicits a chronic inflammatory response resembling ulcerative colitis in the mouse colon.	Model of ulcerative colitis

1.4.2. The Role of Microorganisms in Homeostasis of the Intestine

Until birth, the lumen of the gastrointestinal tract is devoid of microbes. However, in the neonate, the GI lumen is quickly colonised by many microflorae and is dominated by two phyla of bacteria: the Firmicutes and the Bacteroidetes (99–101). This results in the intestinal immune system co-evolving with the microbiota in a mutually beneficial relationship. For example, bacteria residing in the intestinal lumen synthesise vitamins B and K which are utilised by epithelial cells for the metabolism of fat and sugar molecules. Bacteria also produce short chain fatty acids (SCFA) as metabolites, and these are used as an energy source by the host cells to enhance the production of mucus (102).

The microbial compartment is also critical in the establishment and function of GALT in the intestine mucosa. This is evidenced by studies using germ-free mice that lack commensal microorganisms; hypoplastic Peyer's patches are observed in these animals along with a reduced number of CD4⁺ T cells. However, introduction of commensal bacteria to these mice, leads to colonisation of the gut lumen and the structural and functional immune abnormalities previously observed in these mice is corrected (12,103).

Studies from germ-free mice also highlight the importance of microorganisms in determining the “inflammatory tone” of the intestine and maintenance of a continuous low-level physiological inflammatory response. In germ-free mice, there is almost a complete lack of inflammatory cells, whereas in the healthy

mice that have a gut lumen colonised by commensal bacteria, approximately 1/3 of cells in the lamina propria are T cells (104–106).

1.4.3. The Epithelium as a Physical Barrier

A major contributor to intestinal homeostasis is the epithelium itself which acts as a physical barrier to prevent the entry of microorganisms into the host tissues. Disruption and impairment of this barrier results in microbial entry and induction of an inflammatory response; a phenotype observed in patients with inflammatory bowel diseases. As such, regulation of epithelial junctional integrity and paracellular permeability is crucial for maintaining immune cell homeostasis and is an area which can be enhanced by the commensal microbiota or exploited by pathogens. For example, the expression of the tight junction proteins claudins and occludins can be enhanced by a bacterial metabolite known as indole (107). Toxins released by pathogenic bacteria often target the apical junctional complex, which can also be manipulated by viruses: rotaviruses can alter the levels of phosphorylation on occludin proteins thereby changing the localisation of occludin to tight junctions (108).

IECs are partially responsible for transducing signals from microorganisms to immune cells that then co-ordinate local immune responses at the mucosal barrier or more systemic immune responses. Both the innate and adaptive immune cell responses are directly influenced by signals derived from, not only the intestinal epithelium, but also mesenchymal cells of the lamina propria. Together IECs and stromal cells can locally regulate the immune response at the mucosal barrier or induce more systemic effects.

1.4.3.1. The mucus barrier

The first physical barrier blocking both commensal and pathogenic bacteria from entering the host is the mucus layer. As discussed previously, microorganisms in healthy individuals are only present in the loose, outer layer of mucus in the intestine, while the inner mucus layer remains devoid of bacteria, in part due to IgA. IgA is produced by mature B cells, also known as plasma cells, and is exocytosed into the intestinal lumen by enterocytes where IgA then acts as part of a defensive mechanism to prevent the residing of microorganisms in the inner mucus layer (109,110).

MUC2 is the most abundant mucin protein present within the mucus and is used as a source of nutrients for the commensal microbiota, which in-turn produce butyrate, a SCFA utilised by IECs, most notably by the goblet cells to increase MUC2 production (102,111). The importance of the mucus layer and of glycan proteins in particular, is highlighted by genetic studies of mice. Mice lacking O-glycan, the core structural protein of MUC2, display bacterial invasion and colonisation within the colonic mucosa reminiscent of ulcerative colitis, while mice completely deficient in MUC2 spontaneously develop spontaneous colitis as a result of bacterial invasion (112,113). Loss of mucus-producing goblet cells and a decrease in mucin production is a characteristic feature of ulcerative colitis (114).

1.4.3.2. Paneth cells and the role of autophagy and the unfolded protein response

Paneth cells are essential for maintaining homeostasis in the small intestine. They are responsible for the production and secretion of AMPs such as defensins and c-type lectins, into the inner mucus layer and, in combination with the activity of IgA, allow it to remain a microbial-free compartment. Both defensins and c-type lectins function by binding to the cell membrane of microbes and inducing the formation of pores, resulting in bacterial cell lysis and death (34). Deficiencies in the production of α -defensins by Paneth cells have been associated with mutations in the *NOD2* (nucleotide-binding oligomerisation domain) genes in both mice and humans and this, along with Paneth cell dysfunction, has been observed in patients with ileal Crohn's disease (115). *NOD2* mediates the sensing of peptidoglycan and its signalling is mediated via the myeloid differentiation primary response gene 88 (MyD88) to induce the production of AMPs by Paneth cells. Mice deficient in MyD88 display deficient production of AMPs and mucus, with an increased predisposition to colitis, while mice lacking proper *NOD2* function have increased susceptibility to bacterial infection (109,116,117).

Correct Paneth cell activity relies on the proper functioning of the unfolded protein response (UPR) and autophagy pathways. Autophagy controls the catabolism of intracellular pathogens through the formation of autophagosomes that target the cargo for lysosome-mediated degradation (Figure 1.11) (118). *NOD2* has a role in activating the autophagy pathway by interacting with ATG16L1 (autophagy-related protein 16-like-1) (119). ATG16L1 is an autophagy protein that has been linked to an increased predisposition for Crohn's disease; furthermore, mice lacking this protein have alterations in Paneth cell secretion, resulting in the accumulation of abnormal granules within the cells and reduced AMP production (120–122).

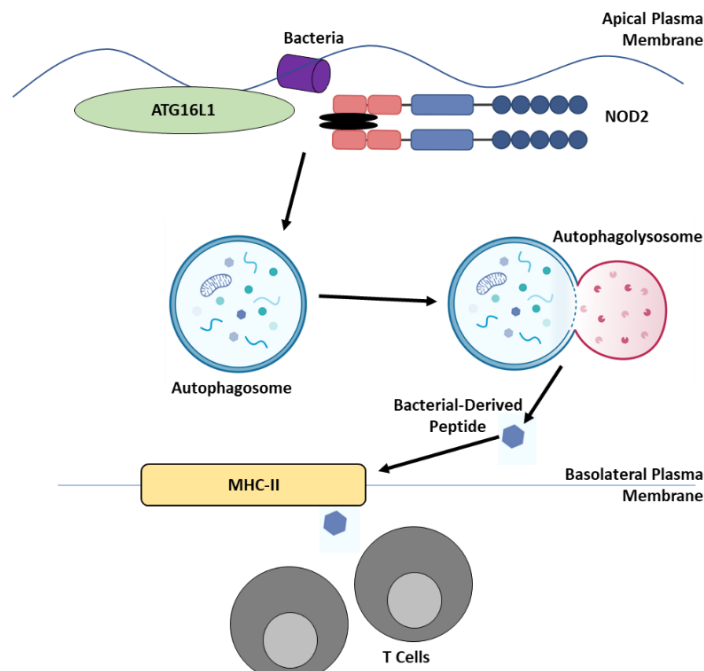


Figure 1.11. The role of ATG16L1 and NOD2 in the autophagy pathway. *NOD2* recruits ATG16L1 to the plasma membrane when it recognises the bacterial protein, muramyl dipeptide, resulting in the formation of an autophagosome followed by autophagolysosome formation and degradation of internalised bacteria. Bacterial peptides from the autophagolysosome are presented on MHC-II molecules to T cells to induce an appropriate immune response. Mutations in *NOD2* or ATG16L1, prevent recruitment of ATG16L1 to the plasma membrane and lysosomal degradation does not occur and an appropriate immune response is not generated. Adapted from Henderson and Stevens 2012. Figure created with BioRender.com.

A further characteristic of IBD is ER stress and upregulation of the UPR. A co-ordinated protein folding response and trafficking of secretory proteins by the ER and Golgi apparatus is required for maintaining the proper secretory functions of both Paneth cells and goblet cells; both of which are highly susceptible to ER stress. Accumulation of unfolded or misfolded proteins in the ER leads to the initiation of the UPR. Mice with mutations in the protein IRE1 (IEC-restricted isoform of the UPR effector enzyme-1) have increased vulnerability to DSS-induced colitis (96). IRE1 normally functions to activate X-box Binding Protein 1 (XBP1); a transcription factor involved in protein quality control (123). Loss of XBP1 resulted in decreased lysozyme production and secretion by Paneth cells and increased prevalence of *Salmonella typhimurium* infection (12,123). XBP1 loss also results in basal activation of the transcription factor Nuclear Factor κ -light-chain-enhancer of activated B cells (NF- κ B) which induces a proinflammatory response to various stimuli such as Tumour Necrosis Factor- α (TNF- α) (12,123).

1.4.3.3. Toll-like receptors

IECs express a range of innate immune receptors known as pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) and Nod-like receptors (NLR). TLRs are transmembrane receptors that recognise specific antigens; for example, TLR2 recognises lipoproteins and TLR4 recognises lipopolysaccharide (LPS) while NLRs such as NOD1 and NOD2 recognise peptidoglycans (124). TLRs are responsible for inducing proliferative and anti-apoptotic factors that regulate the intestinal epithelial barrier and limit microorganism entry into the lamina propria. Activation of TLR and NOD responses occurs via the MyD88-dependent signalling pathway. In the healthy intestine mucosa, low basal PRR activation is required to maintain proper barrier function. However, mice deficient in MyD88 or lacking TLRs have an increased susceptibility to DSS-induced colitis and exhibit defective tissue repair processes (109,116,117).

1.4.3.4. The apical junctional complex in inflammatory bowel diseases

Disruption of the apical junctional complex that forms the underlying basis of the epithelial barrier has been implicated in the pathogenesis of inflammatory bowel diseases. It is believed that altered epithelial permeability is an event secondary to proinflammatory signals in ulcerative colitis and is a primary factor in the initiation of Crohn's disease (125,126). This is highlighted through examination of first-degree relatives of CD patients, where 10-54 % of relatives displayed increased epithelial permeability in the absence of clinical CD symptoms and evidence from some animal models that exhibit increased permeability before inflammation (125,127–129). Dilation of the AJC and increases the distance between adjacent epithelial cells and has been observed in mucosal biopsies of both CD and UC patients resulting in a "leaky gut" with increased epithelial permeability and microbial infiltration, further exacerbating the release of proinflammatory cytokines from immune cells (71,130).

Three potential mechanisms for the disruption of the AJC structure and resulting increase in permeability have been proposed: decreased AJC junctional protein expression, altered vesicle trafficking of junctional components, and remodelling of the peri-junctional actin cytoskeleton (71). Decreased expression of both AJ

and TJ proteins has been observed in IBD patients. For example, decreased E-cadherin expression and expression of the AJ stabilisation protein, catenin has been identified in the inflamed mucosa of IBD patients along with the loss of occludin in the colon of inflamed tissue (130,131). Mouse models have also identified that reduction of E-cadherin expression results in the development of a spontaneous colitis that resembles CD in humans (129). Further loss of junctional proteins is demonstrated through cadherin and claudin switching in active IBD. Increased levels of placental (P-) cadherin are found in biopsies from IBD patients and increased levels of pore-forming claudin-2 with a concurrent decrease in the sealing claudins -3, -4, -5, -7 and -8 resulting in increased epithelial permeability (132,133).

Altered trafficking of junctional proteins has also been observed in patients with IBD. Vesicle trafficking is crucial for the continuous regulation of junctional turnover through exocytosis of newly synthesised proteins and endocytosis and removal of mature junctional components. These mechanisms normally exist in a state of equilibrium during normal intestinal homeostasis, however in IBD, increased endocytosis and decreased exocytosis has been detected. For example, the proinflammatory cytokine interferon- γ (IFN- γ) induces the endocytosis of both TJ and AJ transmembrane proteins through the Rho/Rock GTPase signalling pathway causing rapid disassembly of the AJC and increased epithelial permeability (134–137).

Cytoskeletal abnormalities have also been observed in *in vitro* models of IBD and patients themselves. Perturbations in actin turnover can produce alterations in permeability; reduction in the F-actin turnover protein, actin-interacting protein (Aip)-1, is correlated with an increase in permeability and mice with mutations in Aip-1 exhibit chronic inflammation (138,139). Increased actomyosin contractility and alterations in the function of the myosin II motor protein have also been associated with increased epithelial permeability and chronic inflammation (71).

1.4.4. The Role of Stromal Cells in Intestinal Homeostasis

Mesenchymal stromal cells, predominantly fibroblasts and myofibroblasts, have crucial roles in tissue regeneration and wound repair within the intestinal mucosa. In response to injury, cyclooxygenase (COX)-2 expressing stromal cells initiate proliferation of intestinal epithelial stem cells through MyD88 and prostaglandin E2 (PGE2) signalling pathways (140). Concurrent with stem cell activation, stromal cells are also responsible for ECM remodelling during wound healing. Further roles for stromal cells in maintaining homeostasis are via the induction of B cell differentiation. Fibroblasts and myofibroblasts bind to IgM expressing B cells and induce their differentiation into plasma cells that secrete IgA (141). IgA is then transcytosed to the inner mucus layer via enterocytes and acts to maintain a microenvironment free of microorganisms.

Stromal cells are also key regulators of the innate and adaptive immune responses. Recent studies have provided evidence that fibroblasts and myofibroblasts within the intestinal lamina propria can act as non-professional antigen presenting cells; TLR signalling in these cells induces the production of proinflammatory cytokines such as IL-6 and IL-8 along with adhesion molecules such as intercellular adhesion molecule-1

(ICAM-1). Expression of these proteins and through stromal cell expression of class II major histocompatibility complex (MHC) and the co-stimulatory molecules CD80 and CD86 which can induce the recruitment and activation of T cells (142–144).

Mesenchymal cells are also responsible for maintaining a microenvironment enriched in anti-inflammatory molecules and are a source of TGF- β , IL-10 and PGE2 (49,50). These are important for maintaining a tolerant environment to commensal bacteria and self-antigens by promoting the expansion of regulatory T cells (Tregs) that dampen down proinflammatory responses (145,146).

Stromal cells also play a role in the pathogenesis of IBD. Overexpression of the proinflammatory cytokine TNF- α is a hallmark of IBD. It induces the secretion of type I and IV collagens, matrix metalloproteins (MMPs) and tissue inhibitors of matrix metalloproteins (TIMPs) (147,148). Enhanced MMP-3 expression has also been demonstrated in stromal cells that have an increased oxidative stress, which is the case in CD (149,150). Increased levels of MMPs have been found in the inflamed mucosa of IBD patients while higher levels of TIMPs are found in areas of fibrosis (151).

1.4.5. Cells of the Innate and Adaptive Immune Responses

Many complex interactions occur between the innate and adaptive immune systems to generate an immune response within the intestinal mucosa. A brief overview of the function of various immune cells (dendritic cells, macrophages and T cells) and their potential contributions to IBD is described herein, however for more detailed reviews see: Garret et al., Macdonald et al., Okumura et al., Kmiec et al., and Imam et al., (12,152–155)

1.4.5.1. M cells and dendritic cells

Along the intestinal epithelium, there are inductive sites that are home to the M cells. Although no studies have postulated a role for M cells in IBD, they have a crucial role in the continuous immunosurveillance of the gut lumen contents where they translocate antigens across the mucosal barrier to the dome region of the follicle associated epithelium. Antigens are then processed by immature dendritic cells (DCs) that then differentiate into mature DCs that migrate to T cell zones at inductive sites or to the mesenteric lymph nodes where they function to activate T cells and induce the adaptive immune response (Figure 1.12) (156). At inductive sites, DCs are responsible for activating the differentiation of B cells into IgA secreting plasma cells (20). However, there are also DCs resident within the lamina propria of the mucosa that can directly sense luminal contents by extending processes known as dendrites between IECs and into the lumen and sampling the antigens present (157).

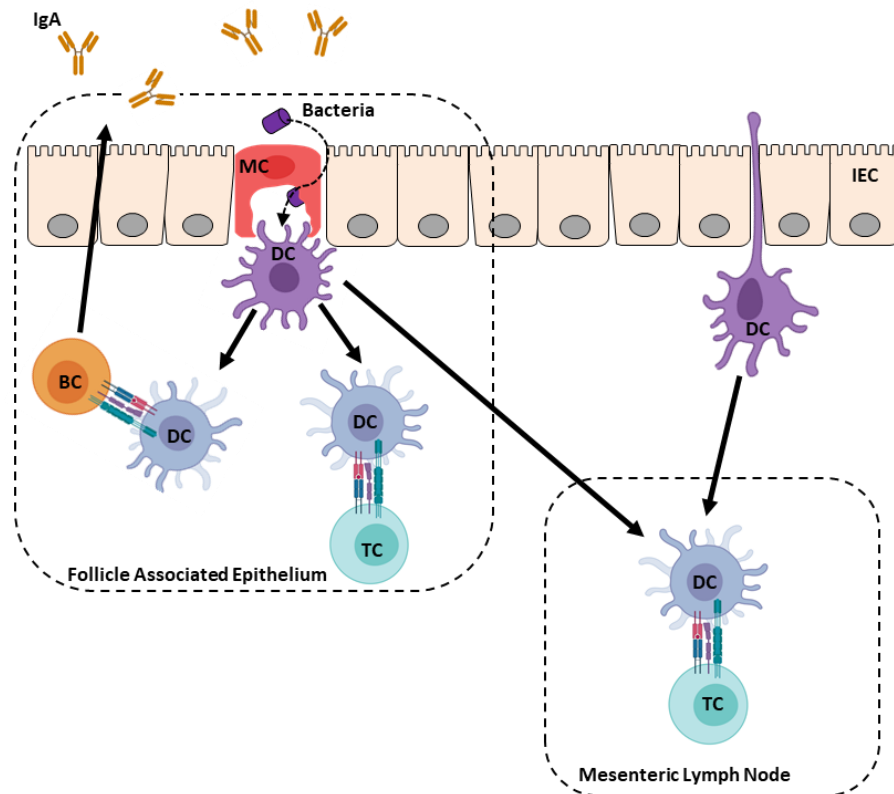


Figure 1.12. The role of dendritic cells in intestinal immune regulation. Dendritic cells (DC) within the follicle associated epithelium (FAE) receive luminal antigens from M cells (MC). DC cells process and present antigens to lymphocytes within the FAE: B cells (BC) are induced to differentiate into IgA secreting plasma cells and T cells (TC) are activated to generate an appropriate immune response. DCs in the FAE can also migrate to the mesenteric lymph nodes through the lymphatic system and activate T cells there. DCs also directly sample the luminal contents by extending processes between intestinal epithelial cells (IEC) and migrate to lymph nodes to present the antigens to T cells. Adapted from Coombes and Powrie 2008. Figure created with BioRender.com.

IECs and DCs work together to control immune cell tolerance to commensal bacteria. IECs secrete thymic stromal lymphopoietin (TSLP) which inhibits interleukin (IL)-12 production in response to bacteria and drives the development of T helper cell (Th) 2 polarising, CD103⁺ DCs that down-regulate the adaptive immune response (158). However, disruption of NF- κ B signalling in mice results in reduced TSLP production, increased IL-12, fewer Th2 cells and an inability to properly control infection (159). Crohn's disease patients have IECs that are unable to produce TSLP and are therefore unable to properly control the immune response generated by DCs (158,160).

Regarding UC, DC function and contribution is not well characterised. However, in patients with UC, CD80⁺ cells resembling activated DCs are present in aggregates, along with lymphocytes (161). These DC-like cells produce macrophage inhibitory factor resulting in the recruitment and activation of neutrophils, contributing to chronic inflammation (162).

1.4.5.2. Macrophages

Intestinal macrophages constitute the largest pool of tissue-resident macrophages in the entire body. They are found within inductive sites and in the lamina propria of the intestinal mucosa adjacent to the epithelium

and are continuously replenished by CD14⁺/Ly6C⁺ blood monocytes (163). They are responsible for the clearing of cellular debris and pathogens through phagocytosis. Macrophages also facilitate wound repair processes, induce the expansion of Tregs and are involved in the establishment and maintenance of immune tolerance to commensal microbiota (164).

Two types of macrophage subtypes exist: classically activated M1 proinflammatory macrophages and alternatively activated M2 anti-inflammatory macrophages. Under homeostatic conditions, tissue-resident intestinal macrophages exist in a state of anergy where they exhibit a non-inflammatory profile when exposed to microbial stimuli i.e., they do not produce proinflammatory cytokines or upregulate co-stimulatory molecule expression, such as CD40, in response to TLR signalling (165). This is associated with the reduced expression of TLR, CD14, FCγR1 (high affinity immunoglobulin γ FC receptor), and complement receptors 3 and 4 on the cell surface of macrophages and as such the cells exist in a hyporesponsive state conducive to immune tolerance (152,166).

In healthy individuals, intestinal macrophages also exhibit a lack of triggering receptor expressed on myeloid cells-1 (TREM-1), a cell surface molecule that normally functions to enhance proinflammatory responses through upregulation of proinflammatory cytokines such as TNF and IL-6 (167,168). However, in IBD patients, high levels of macrophages expressing TREM-1 and high TREM-1 mRNA expression levels have been observed and consequently, high TLR-induced inflammatory responses are detected (169).

Patients with active IBD also present with an increase in the total number of macrophages within the lamina propria. Both M1 and M2 macrophages have an amplified number, yet the balance of macrophages favours the M1 proinflammatory phenotype (170). Studies of mouse models with alterations in the balance of M1 and M2 macrophages indicated that M2 macrophages have a protective effect against colitis while M1 macrophages contribute to the disease pathogenesis (171–173). M1 macrophages mediate the chronic inflammation observed in IBD patients through secretion of proinflammatory cytokines and the release of ECM proteases and reactive oxygen and nitrogen species. For example, CD patients have increased levels of CD14⁺ macrophages which produce IL-23 and TNF in response to commensal bacteria, thus abolishing immune tolerance (174). TNF production also impairs the structure of tight junctions between epithelial cells, resulting in disruption of the epithelial barrier and exacerbating the inflammatory response due to microorganism infiltration (170,175).

Further evidence for a role of macrophages in chronic inflammation comes from mouse models. Macrophages produce large quantities of IL-10, an anti-inflammatory cytokine that normally functions to dampen down the immune response and to maintain Treg cells. In IL-10^{-/-} mice, there is an increase in production of the proinflammatory cytokine IL-12 and the mice spontaneously develop colitis (176). Severe combined immunodeficient (SCID) mice, lacking T and B cells and therefore an adaptive immune system, still develop colitis and chronic inflammation upon DSS treatment (177). This indicates that DCs and macrophages must both play a role in the pathogenesis of this disease.

1.4.5.3. T cells

CD4⁺ T cells are believed to play a key role in the initiation and pathogenesis of IBD though their response and initial activation is primarily dictated by the antigen presenting, innate immune cells. Evidence for this comes from the high numbers of CD4⁺ T cells present in the inflamed intestinal mucosa of IBD patients, the effectiveness of anti-CD4⁺ T cell antibodies in the treatment of IBD, and the high numbers of remission cases of IBD observed when patients have a concomitant human immunodeficiency virus (HIV) infection (178,179). Naïve CD4⁺ T cells can be differentiated into various subpopulations of T helper (Th) cells depending on the signals present in the microenvironment (Figure 1.13) (154,155,180). Traditionally CD was characterised by a Th1 response while UC was characterised as a Th2 response. However, this paradigm has now shifted with the greater understanding of further CD4⁺ subtypes including, regulatory T cells (Treg), Th17, Th22 and Th9 cells.

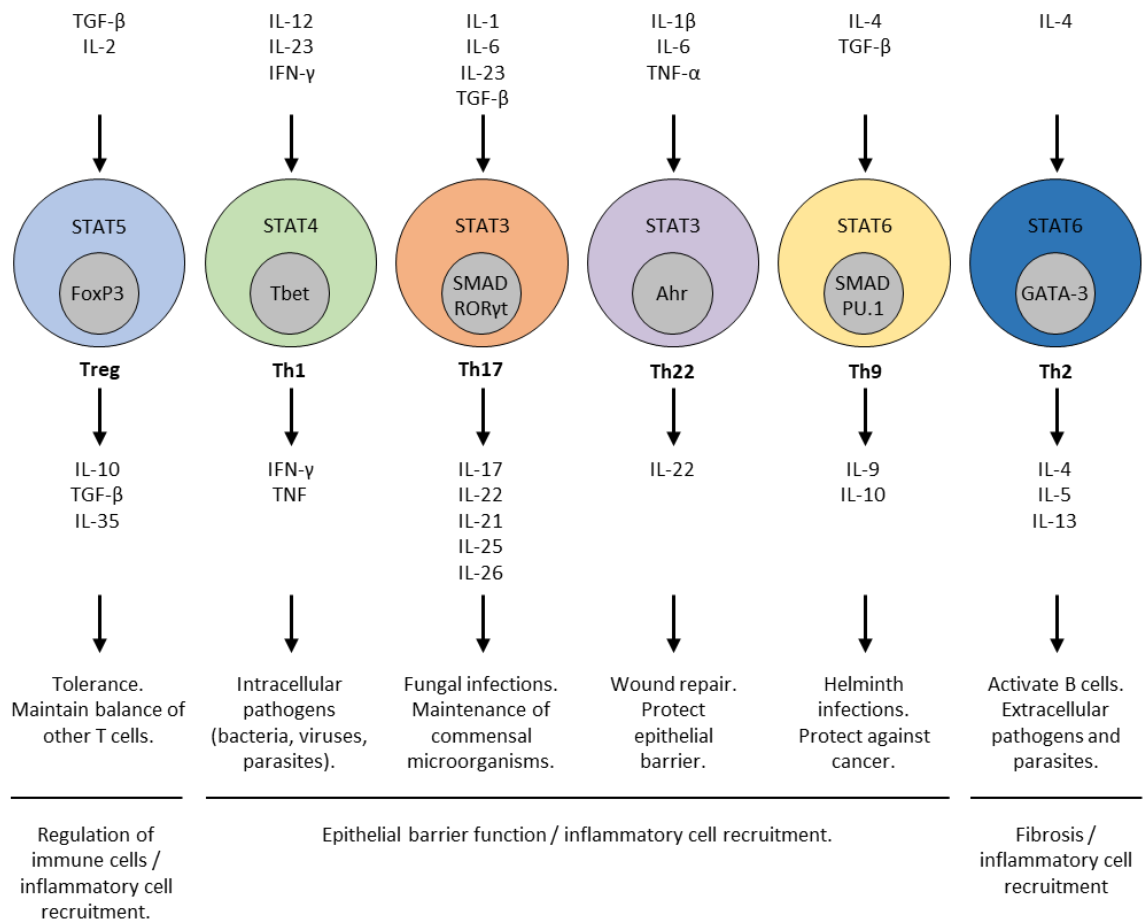


Figure 1.13. Signalling pathways involved in the differentiation of the different CD4⁺ T cells present in the intestine. Cytokines present in the microenvironment of naïve CD4⁺ T cells influence their differentiation program by initiating signal transducer and activator of transcription (STAT) signalling pathways. Activation of STAT signalling induces the expression of the appropriate nuclear transcription factor that drives the differentiation of the different T cell subsets that go on to produce pro- and anti-inflammatory cytokines specific to the T cell type. Adapted from Raphael et al., 2015, Kmiec et al., 2017 and Imam et al., 2018.

1.4.5.3.1. Th1 cells

Th1 cells are responsible for stimulating a proinflammatory immune response against intracellular pathogens. Th1 cell differentiation occurs in the presence of IL-12, IL-23 secreted by DCs, or IFN- γ produced by Th1 cells themselves, through the signal transducer and activator of transcription (STAT)4 signalling pathway to activate the transcription factor Tbet (154,181,182). GWAS studies have indicated the presence of single nucleotide polymorphisms (SNPs) associated with the *IL12B* gene that correlate with amplified disease pathogenesis, while SNPs on the *STAT4* gene have been linked to UC (183,184). Th1 cells secrete IFN- γ and TNF, that respectively, activate macrophages and stimulate the cytotoxic responses of CD8⁺ T cells. Contradictory evidence exists as to whether these two cytokines influence the inflammatory responses observed in IBD. For example, IFN- γ has been shown to be protective in some mouse models including IFN- γ -deficient mice where they displayed increased susceptibility to DSS-induced colitis; while other studies have demonstrated that IFN- γ is required for the development of colitis in an alternative DSS-induced model (185,186). In regard to the human situation, SNPs have been found around the *IFNG* gene in both CD and UC patients, linked to and enhanced expression of IFN- γ (187,188). Like IFN- γ , the contribution of TNF to inflammatory mouse models is contradictory, however evidence from IBD patients provides a much stronger indication that TNF is involved in chronic inflammation. For instance, *TNF* SNPs have been associated with instances of IBD and anti-TNF therapy is used as a successful treatment in some cases of IBD, which has the potential to result in complete remission of the disease (189,190).

1.4.5.3.2. Th2 cells

Th2 cells function to remove extracellular pathogens. Differentiation of Th2 cells is induced by the presence of IL-4, produced by DCs, which activates the STAT6 signalling pathways resulting in the activation of the transcription factor GATA binding protein 3 (GATA3) (191). Th2 cells have conventionally been associated with UC, partially due to the high phosphorylation and therefore activation levels of STAT6 and higher expression levels of GATA3 correlating with disease severity (192,193). GATA3 overexpression is also implicated in mouse models of UC, where its overexpression results in acute colitis (194,195). Th2 cells predominantly produce IL-4 but also produce IL-5 and IL-13 which help induce B cell differentiation into IgA secreting plasma cells. Possible loss of function mutations have been observed in the *IL4* gene in UC patients, along with an increase in the expression of IL-5 and IL-13 (155,196).

1.4.5.3.3. Th9 cells

Although not considered to be one of the main factors, Th9 cells have been implicated in the progression and pathogenesis of IBD. The presence of TGF- β and IL-4 induce the differentiation of naïve CD4⁺ T cells into Th9 cells that secrete IL-9 and IL-10 (197,198). An increase in IL-9 production has been observed in both UC and CD patients (198,199). Murine models of induced colitis have also displayed an increased expression of IL-9, conversely, mice with deficient IL-9 expression appeared to have resistance to the onset of colitis (198,200).

1.4.5.3.4. Th17 cells

Th17 cells are a subset of CD4⁺ T cells that are abundant in the healthy intestinal mucosa where they function to protect the epithelial surface from bacterial and fungal infections. The main driver of Th17 differentiation is the proinflammatory cytokine IL-6 which mediates the activation of the ROR γ t transcription factor through STAT3 activation (201,202). TGF- β and IL-23 are also required to initiate the differentiation of Th17 cells (203). IL-23, also involved in the differentiation of Th1 cells, is secreted by both DC and macrophages; it links the innate and adaptive immune responses in both homeostatic and diseased states. Levels of IL-23 are elevated in patients with IBD and in murine models of colitis: mice deficient for the IL-23 receptor did not exhibit symptoms of colitis, indicating that IL-23 signalling is critical to the onset of the disease (204–206). Th17 cells predominantly produce IL-17 and IL-22, both of which are elevated in the intestinal mucosa of UC and CD patients; additionally, Th17 cells themselves are amplified in CD (207–210). The pathogenicity of IL-17 occurs through its stimulation of myeloid cells, IECs and stromal cells and induces them to produce further proinflammatory cytokines leading to the recruitment of macrophages and neutrophils promoting more Th17/Th1 type immune responses (210). Th17 cells also exert their pathogenic effects by induction the induction of MMPs that degrade the lamina propria ECM (211).

1.4.5.3.5. Th22 cells

Differentiation of Th22 cells is initiated by IL-6, TNF- α and IL-1 β but is suppressed by the presence of TGF- β (212,213). Th22 cells are predominantly involved in wound healing and produce IL-22, a member of the IL-10 anti-inflammatory cytokine family. The IL-22 receptor only exists on non-haematopoietic cells in the intestine; thus, the effects of IL-22 are restricted to IEC and stromal cell populations (208). In the healthy intestine, IL-22 induces AMP, mucin, and IL-10 secretion by the epithelial cells. It also functions to alter epithelial barrier properties through upregulation of expression of the pore-forming protein, claudin-2 (214). Expression of IL-22 confers a protective effect in animal models of colitis, and this is supported by the reduced number of Th22 cells present in the intestinal mucosa of UC patients due to upregulated TGF- β expression (154,207).

1.4.5.3.6. Regulatory T cells

The final type of CD4⁺ T cell present in the intestinal mucosa are regulatory T cells (Tregs). These constitutively present T cells are unique in that they are responsible for controlling the balance of other T cell subtypes and mediating immune tolerance and recognition of cell; therefore, they are crucial for maintaining intestinal homeostasis (215). Under conditions where there are high levels of TGF- β present, naïve CD4⁺ T cells differentiate into Treg cells due to the induced expression of the forkhead box P3 (FoxP3) transcription factor (216). Loss of function mutations in FoxP3 results in a lack of Treg cells and consequent severe autoimmune diseases including immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, along with colitis and chronic inflammation of the small intestine (154,217). The importance of TGF- β for Treg differentiation is highlighted by murine models of T cell transfer-induced colitis, where administration of antibodies directed against TGF- β prevented the Treg-mediated suppression of colitis (218). Treg cells

normally produce high levels of the anti-inflammatory molecules IL-10 and TGF- β that are responsible for suppressing effector T cells and maintaining a tolerant environment towards commensal microorganisms and self. Effector T cells from IBD patients have resistance to Treg control, this is thought to be due to high levels of the TGF- β inhibitor: SMAD7. When SMAD7 is diminished, the cells regain susceptibility to Treg-mediated suppression (219).

Dendritic cells are important in controlling the balance between Tregs and Th17 cells (152). An imbalance of Th17 and Tregs is thought to be a contributing factor to the development and progression of Crohn's disease, resulting in an increase in Th17 cells (154). This, in turn, results in an inflamed microenvironment, and it is thought that under these conditions, Treg cells exhibit plasticity in their behaviour and could potentially become proinflammatory Th1 or Th17 cells (220,221). This would explain the enrichment of FoxP3⁺ cells and IL-17 producing cells detected in IBD patients.

1.5. Modelling Intestinal Tissue

A variety of methods exist to model the function of the intestine mucosa including *in silico* computational studies, physiochemical methods, *in vitro* cell cultures, *in vivo* animal experiments and *ex vivo* intestinal tissue of animal or human origin. *In silico* and physiochemical methods are used very early in the initial drug discovery phase to synthesise compounds and assess the physical and chemical properties of the newly developed compounds respectively. Cell and animal-based models are then used in the drug evaluation process before clinical trials and will be discussed in further detail.

1.5.1. *Ex Vivo* Intestinal Models

Ex vivo studies can be performed on either human or animal tissues, however the availability of human tissue is limited so many studies are performed using animal models. Different *ex vivo* models have varying disadvantages but use of animal tissue has limitations common to all models including interspecies differences in the anatomy, microbiota and functions to humans, resulting in difficulties upon data extrapolation to the human intestine (222).

Inverted intestinal rings and segments were some of the first *ex vivo* models used to examine differences in absorption mechanisms between different regions of the intestine. These high throughput techniques are relatively simple to perform and involve submerging an intestinal ring or segment in culture medium containing the chemical or molecule of interest. However, these models cannot measure transport through the epithelium and instead measure the accumulation of the compound within the absorptive enterocytes (223).

The use of everted sac models was also first reported around the same time as intestinal rings and segments and were originally used to measure the transport of glucose and amino acids (224). Everted sacs can be used to measure intestinal transport and to understand the metabolic and transporter protein functions. They are,

however, low throughput and do not accurately measure transport rates due to the presence of muscle layers and a thick unstirred water layer (222).

Ussing chamber systems are used to measure the transport of molecules across the intestinal epithelium and can also be used to study transport across other epithelial tissues (225). Different intestinal segments can be used in Ussing chamber's so absorption can be compared across all parts of the intestine (226). However, tissues isolated for Ussing chamber experiments do not survive long-term and they are a very low throughput system to use to study absorption.

1.5.2. *In Vitro* Models of the Intestine

Cell culture-based models of the intestinal epithelium are utilised in the pharmaceutical industry during the drug development process to characterise transport characteristics of new chemical entities before testing in animals and humans. *In vitro* models began as simple monolayer cultures of an individual cell type but have since advanced to incorporate many aspects of intestinal biology including the addition of multiple cell types, mimicking the architecture of the intestine, and introducing flow to models (Figure 1.14) (227,228).

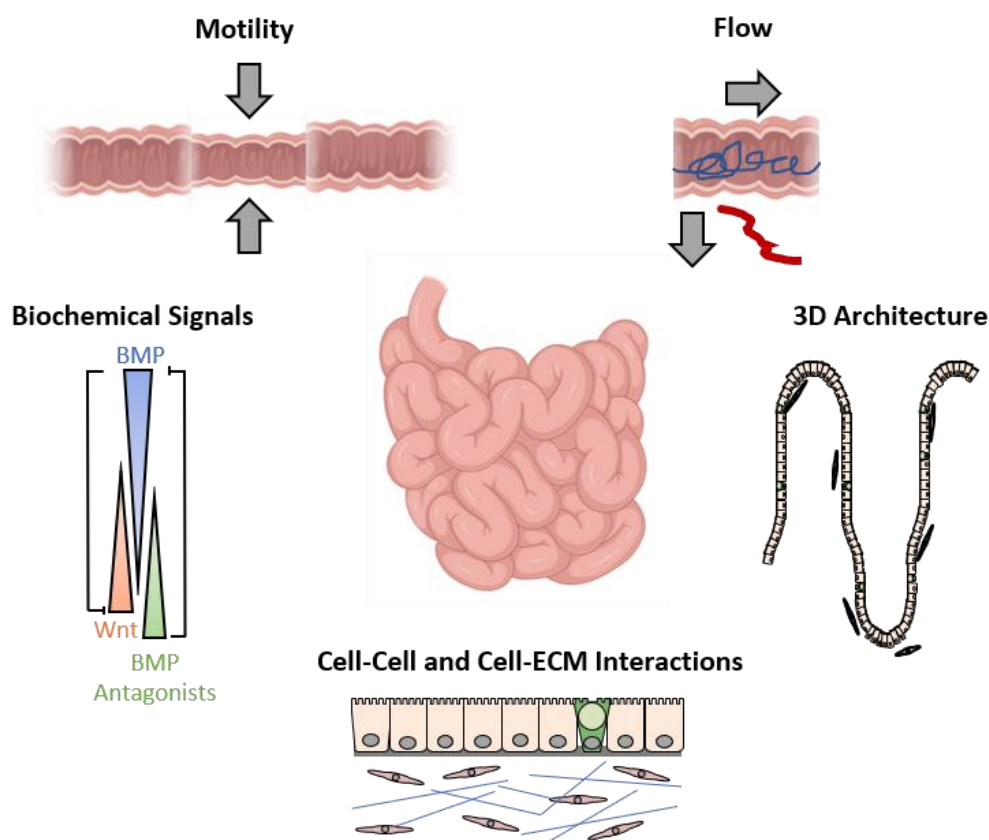


Figure 1.14 Intestine microenvironment factors that affect cell growth and behaviour. Microenvironment factors that need to be considered when designing novel *in vitro* intestinal models. Motility is the stretching and contracting of the GI muscles that leads to the flow of luminal contents through the GI tract. Flow of the bloody and lymphatic systems is an additional microenvironmental consideration. The intestine has a 3D architecture comprising of villi and crypts, within which cellular and ECM interactions occur. Signalling molecules, such as those maintaining the proliferation and differentiation balance within the intestinal crypt, can be incorporated into *in vitro* intestinal models. Adapted from Yamada and Cukierman 2007 and Costa and Ahluwalia 2019. Figure created, in part, with BioRender.com.

1.5.2.1. Monoculture *in vitro* models

Initial *in vitro* models of the intestine followed a reductionist approach, comprising of a single monolayer of cells typically cultured on 10 µm thick Transwell® polycarbonate or polyester membranes. Both animal (Table 1.3) and human cell lines have been isolated and utilised as models of intestinal transport. Due to difficulties in culturing primary intestinal epithelial cells, *in vitro* cultures using human cell lines have been established from cells isolated from adenocarcinomas.

Table 1.3: Animal-based *in vitro* models of the intestinal epithelium.

Cell Line	Animal Origin	Characteristics and Uses	Reference
MDCK	Dog kidney	Differentiate into columnar epithelial cells with tight junctions after 3/4 days in culture. Passive permeability correlated well with human absorption. Low transepithelial electrical resistance (TEER) compared to some human <i>in vitro</i> models, but possible differences in transporter protein expression to human intestine.	(229–231)
2/4/A1	Rat foetal intestinal cells	Differentiated monolayers that express brush border enzymes and form tight junctions that have a similar radius to human small intestine. Exhibit a TEER of 25-50 Ω.cm ² like the small intestine. Transport of passive paracellular compounds comparable to transport in the human jejunum. Absence of active drug transport processes.	(6,232–234)
IEC-6 & IEC-8	Rat small intestine	Small intestine origin. Expression of cytokines and TLRs. Useful for studying interactions with microbiota and bacterial adhesion.	(235–237)
IPEC-J2	Neonatal porcine small intestinal epithelial cells	Unlike many <i>in vitro</i> models, IPEC-J2 cells are non-transformed and are of a small intestinal, non-cancerous origin. Pig intestine closely resembles the structure and function of human intestine compared to murine models. Often used to study microbiota interactions and infection.	(237,238)

Four different types of human cell lines exist that are used to model the intestinal epithelium that are classified according to their manner and extent of differentiation (239). Type 1 cells spontaneously differentiate into enterocyte-like cells that express brush border enzymes, form domes and tight junctions between adjacent cells. The only cell line that belongs to the type 1 group are the Caco-2 cells. Type 2 cells are capable of differentiating into intestinal epithelial cells but require specific culture conditions; HT29 cells belong to this group as they differentiate into enterocyte-like cells upon addition of galactose to the culture medium. Type 3 cells, such as T84 cells, can also spontaneously differentiate and form domes but they lack the full complement of differentiation markers such as expression of brush border hydrolases. The final group of type 4 cells do not differentiate into enterocyte-like cells and have limited use as models of the intestine epithelium; SW480 cells fit into this category (239).

1.5.2.1.1. Caco-2 monolayers

Caco-2 monolayers are the most widely used *in vitro* model for drug permeability studies and are generally considered to be the “gold standard” model used by pharmaceutical companies and the relevant regulatory bodies. Caco-2 cells were first isolated from a human colorectal adenocarcinoma and are typically cultured on Transwell® membranes to generate epithelial models (240). Cells begin to spontaneously differentiate between 7-10 days after seeding, once they have formed a confluent layer of cells, thus they are the only cell type belonging to the type 1 category of human *in vitro* models of the intestine epithelium (239,240).

Despite their colonic origin, differentiated Caco-2 cells most closely resemble foetal ileal enterocytes based on their enzyme expression profile and are an invaluable tool in predicting the permeability of lipophilic molecules that are transported via the passive transcellular pathway. However, they are limited in their ability to predict the passive paracellular permeability of hydrophilic compounds due to the size of their tight junctions, 3.7-4.4 Å, reflecting their more impermeable, colonic nature rather than the small intestine which have tight junction sizes of 3 Å and 8 Å respectively (241,242). This is further highlighted by the transport characteristics of atenolol, a compound that displayed significantly lower transport across Caco-2 monolayers when compared to the human jejunum (243,244). Transepithelial electrical resistance (TEER) of Caco-2 monolayers, a measure of barrier integrity and a reflection upon the “tightness” of the junctions formed, is also significantly higher than the *in vivo* intestine which ranges from 250-2500 Ω.cm² in the monolayer and 40-100 Ω.cm² throughout the small and large intestines (6,240,242,245–247).

Further limitations of the Caco-2 monolayer include the lack of cytochrome P450 (CYP)3A4 enzyme, one of the most important intracellular metabolising enzymes present in intestine enterocytes and as such Caco-2 cells frequently overestimate the fraction absorbed of CYP3A4 substrates (248). However new Caco-2 subclones have successfully been transfected to express CYP3A4 enzymes and therefore more accurately estimate the fraction absorbed of CYP3A4 substrates (249,250).

In addition to the lack of CYP3A4, Caco-2 cells also greatly overexpress the efflux protein P-gp and have variable expression of pharmaceutically important transporter proteins such as OCT and OAT (232,251–255). This is highlighted by transport studies of β-lactam antibiotics which are a substrate of the underrepresented dipeptide transporters. β-lactam compounds exhibit poor permeability in the Caco-2 monolayer but are completely absorbed *in vivo*, thus it is possible that the Caco-2 monolayer could generate false-negative data of compounds transported via carrier-mediated transport pathways (256).

Caco-2 monolayers are time-consuming and labour intensive to culture, leading to a number of groups attempting to reduce the cell culture period. To accomplish this, experimental conditions including medium composition have been changed, such as reduced serum levels, addition of growth factors and hormones or the addition of an ECM component (257–259). Although the culture time of these models can be reduced from 3 weeks to 1 week, monolayers express fewer efflux pump proteins and it is unclear if appropriately sized tight junctions are formed (232,257).

Monolayers of Caco-2 cells exhibit wide inter- and intra-lab variability depending on the age of the cells (passage number and culture duration), culture conditions (seeding density, media change regimen and media composition) and experimental conditions (composition, pH and osmolarity of transport buffer) (6,247,260). For example, the permeability of the hydrophilic compound mannitol, which is transported via the paracellular pathway, can vary up to 100-fold depending on the source of Caco-2 cells (242). Despite the many limitations of this model, it remains the most widely used epithelial model of intestinal transport.

1.5.2.1.2. Caco-2 TC-7 subclone models

TC-7 cells are a Caco-2 subclone that differentiate into an epithelial monolayer with morphologically similar properties to the parental cell line including the presence of a microvilli brush border and associated enzymes in addition to the presence of tight junctions between adjacent cells (261). The functional characteristics of the TC-7 subclone are also comparable to the Caco-2 parent cells, making these models the second most commonly used cell type to predict intestinal permeability; for example, the permeability of mannitol and PEG-4000 were found to be identical in both cell lines (261,262). TC-7 monolayers express CYP3A4 mRNA, thus may more accurately predict first-pass metabolism (263). They also express CYP3A5, a key metabolic protein in the human colon and so may prove useful for studying colonic drug transport and metabolism (264). However, recent studies have demonstrated that the TC-7 subclone is unsuitable to predict the absorption of poorly absorbed drugs, highly lipophilic drugs, and compounds which require transporter-proteins for transport (232,265).

1.5.2.1.3. HT29 cells and subclones

HT29 cells belong to the type 2 group of *in vitro* models of the human intestinal epithelium. Under standard culture conditions with high glucose and 10 % serum, HT29 cells display an undifferentiated phenotype (222). However, upon the replacement of glucose to galactose, HT29 monolayers resembled enterocytes with a brush border and hydrolase expression (266). Further supplements to the culture medium of HT29 cells induced the differentiation of mucin-producing subclones such as HT29-H and HT29-MTX. HT29-H cells are a heterogenous cell population consisting primarily of enterocyte-like cells with small quantities of mucus producing cells (267). The better characterised mucus-producing HT29 subclone, are the HT29-MTX cells which were established by stepwise addition of methotrexate up to 10^{-6} M to the culture medium (268). HT29-MTX monolayers successfully produce mucus and exhibit an apical brush border with intestinal hydrolases however, they lack the expression of the P-gp efflux pump and so do not accurately predict the fraction absorbed of chemical compounds (268). The main advantage of the HT29-MTX monolayer is the ability to study molecule interactions within the mucus and they also have the potential to be used to examine epithelial-microbiota interactions (237,269).

1.5.2.1.4. T84 cell monolayers

T84 cells belong to the type 3 category of human *in vitro* models. They are a human colonic carcinoma cell line that spontaneously differentiate upon reaching confluence, approximately 1-2 weeks. Unlike Caco-2 cells, they retain their colonocyte phenotype and resemble cells of the colonic crypt (270). Phenotypically, T84 cells are shorter than Caco-2 cells, produce mucin proteins and have little to no brush border enzyme expression, however they do strongly express the monocarboxylate transporter that is responsible for the uptake of short chain fatty acids produced by the microbiota in the colon (271). Although T84 monolayers are more reproducible than Caco-2 cells, they are almost exclusively used to study epithelial chloride secretion, rather than transport, due to the limitations discussed herein (272).

1.5.2.2. Epithelial co-culture models

One of the primary limitations of the gold standard Caco-2 monolayer is the lack of other cell types. To overcome this, numerous co-culture techniques have been employed to represent the cellular architecture of the intestine more accurately. The simplest co-culture intestinal models consist of multiple types of epithelial cells. A co-culture model consisting of Caco-2 and mucus-producing HT29-MTX cells is widely utilised to mimic the interaction of enterocytes and goblet cells respectively *in vitro*, enabling the influence of mucus on the transport of compounds to be studied. Validation studies of this co-culture model revealed that the permeability coefficients of hydrophilic, passively absorbed compounds correlated well with the fraction absorbed *in vivo* (273,274). It is believed that this enhanced correlation is the result of weaker tight junction formation from the HT29-MTX cells (275). However, the absorption rates of actively transported drugs were underestimated in comparison to both the Caco-2 monolayer and the fraction absorbed in humans (273), possibly due to the varying levels of transporter proteins expressed in HT29-MTX cells.

Alternative epithelial co-culture models have been established to incorporate an M cell phenotype. The first study reporting the conversion of Caco-2 cells to cells displaying an M cell phenotype was carried out using lymphocytes isolated from Peyer's patches (276). These lymphocytes infiltrated into the Caco-2 monolayer and induced the reorganisation of the brush border to resemble the structural phenotype of M cells and an increase in transcytosis of latex beads was observed (276). Further M cell models have been established using the Caco-2 cell line in co-culture with Raji B cells, isolated from human Burkitt's lymphoma. Raji B cells also induce an M cell phenotype in the Caco-2 cells which exhibit a sparse and irregular microvilli brush border and have increased transcytosis of nanoparticles (277,278). Such M cell models have the potential to be used in transport studies of encapsulated compounds and assist in the development of novel drug delivery systems.

A triple co-culture model incorporating Caco-2, HT29-MTX and Raji B cells has also been developed. These models have characteristics of enterocytes, goblet cells and M cells, thus have a greater resemblance to the small intestinal cellular architecture (275,279,280). Functional characterisation of such triple co-cultures revealed that they have an increased ability to transport insulin encapsulated in nanoparticles compared to

“free” insulin and the models also exhibited strong permeability coefficients with *ex vivo* transport data from porcine intestinal mucosa (275,280).

1.5.2.3. Organoid cultures

Models discussed previously rely on the use of cancerous cell lines due to difficulties in culturing primary intestinal epithelial cells *in vitro*. However, isolation of Lrg5⁺ stem cells from intestinal crypts, lead to the development of new intestinal cultures known as organoids or “mini-guts” (31,281). Organoids are self-organised three-dimensional (3D) structures that contain all cell types of the intestinal epithelium and recapitulate the crypt-villi structure observed *in vivo*, with the outer folds, consisting of Lrg5⁺ stem cells and Paneth cells, representing the intestinal crypts; and the inner folds are comprised of enterocytes, goblet cells and enteroendocrine cells and resemble villi (Figure 1.15) (282).

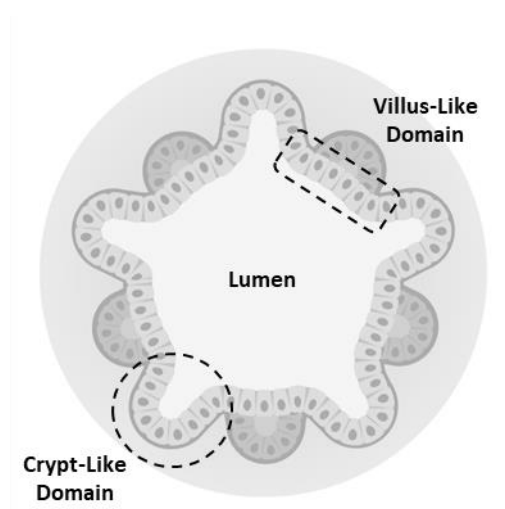


Figure 1.15. Intestinal organoid morphology. Lrg5⁺ stem cells cultured *in vitro* self-assemble into organoid structures with a hollow inner lumen. Lrg5⁺ stem cells differentiate into all intestinal epithelial cell types and locate to the outer folds of the organoids. Together with Paneth cells, they form the crypt-like domains. The other epithelial cell types: enterocytes, goblet cells and enteroendocrine cells are located in the inner folds and resemble villus-like domains. Figure created with BioRender.com.

Organoids have many different uses including the study of intestinal developmental process, disease modelling, host-microbe interactions and have the potential to be used in regenerative medicine as they have successfully been engrafted into mice (283–287). However, their use in studying the transport of different compounds and drug screens is limited due to their spatial arrangement wherein the apical surface of the cells is completely enclosed in a central hollow region (227,288,289). However, recent studies have altered the culture method of organoid to grow them in 2D manner as a monolayer, allowing for the introduction of drug compounds or other non-epithelial cell lineages, apically or basolaterally (290–292). Further limitations of organoid cultures include their heterogeneity in terms of size, shape and viability in addition to the lack of biophysical and biochemical cues experienced *in vivo* (227,288,289).

1.5.3. Modelling Diseased Intestinal Tissue

To study inflammatory bowel disease, animal models are primarily used, as discussed in Section 1.4.1. However, use of *in vitro* models has provided insights into the effects of specific inflammatory mediators or cytokines upon the intestine epithelium. A comprehensive, in-depth discussion of the varying IBD *in vitro* models will be provided in Chapter 6, a brief discussion will be provided here highlighting some of the key discoveries made using *in vitro* models.

1.5.3.1. The reductionist approach to modelling IBD *in vitro*

In vitro models of IBD have traditionally followed a reductionist approach and tended to look at the effects of inflammatory mediators on a single epithelial cell type (293). Models typically employ the use of T84, HT29 or Caco-2 cells cultured on a Transwell® insert and introduce an inflammatory effector molecule apically or basally. *In vitro* models have highlighted that intestinal epithelial cells can become activated by inflammatory signals and in response to their activation, produce their own cytokines and chemokines to recruit other immune cells and regulate an immune response at the epithelium and facilitate repair processes. Epithelial processing of antigens has been demonstrated to be critical to the outcome of the immune response, as they can induce proliferation of CD4⁺ T cells through expression of both MHC-I and MHC-II proteins (294–296).

Caco-2 monolayers specifically, have been employed to understand the mechanisms underpinning the role of TNF- α in damaging the epithelial barrier. TNF- α , a primary driver of IBD, was discovered to reduce the expression of both occludin and ZO-1 via activation of the myosin light chain kinase (MLCK) and NF- κ B signalling pathways; similar conclusions were found using murine models where TNF- α decreased the integrity of the epithelial barrier of the small intestine (297,298). Furthermore, alternative anti-TNF- α therapies have been tested using the Caco-2 monolayer; a novel compound named CT-P13 displayed similar biological activity to the widely used infliximab antibody (299). These studies have demonstrated that simplistic, reductionist approaches can be used to great effect *in vitro* and have the potential to be used to assess novel therapeutic compounds.

1.5.3.2. The use of co-cultures to model characteristics of IBD *in vitro*

The reductionist approach to studying IBD *in vitro* allows for the precise control of experimental conditions, yet it does not recapitulate the complex and dynamic interactions that are occurring *in vivo*. However, more complex model systems have been developed that have introduced immune cells into co-culture with an epithelial monolayer. For simplicity and ease of use, co-cultures frequently rely on the use of immunocyte cell lines often derived from cancers. Two key human cell lines include the THP-1 and U937 lineages which are representative of monocytes, however, they can be differentiated into dendritic cells or, more commonly, macrophages. Co-cultures using an epithelial cell lineage and macrophages derived from THP-1 or U937 cells are often used to investigate the effects of lipopolysaccharide (LPS) a bacterial endotoxin present in the cell wall of Gram-negative bacteria. LPS levels are elevated in IBD and are thought to be a contributing factor to

disease pathogenesis. Caco-2 epithelia alone are hyporesponsive to LPS, yet the addition of immune cells induces an increase in proinflammatory cytokines upon LPS stimulation (300,301). Furthermore, these proinflammatory effects have been shown to be augmented by IFN- γ indicating that in the IBD intestine *in vivo*, elevated IFN- γ levels may exacerbate the immune response to microbial peptides that would not normally drive inflammation (301). Moreover, these co-cultures have recently been utilised to assess potential therapeutic compounds; the pigment-protein, phycocyanin, was found to have protective effects against LPS stimulation, attenuating the levels of the proinflammatory cytokines IL-6 and IL-8 (302).

Alternative immune cell populations – peripheral blood mononuclear cells (PBMC) – isolated from the blood of healthy or diseased individuals have been employed in co-cultures of the intestinal mucosa. One key finding from an *in vitro* model incorporating neutrophils from a PBMC isolation was the postulation of a mechanism for diarrhoea that can be concurrent with IBD. Neutrophil introduction to a T84 epithelial monolayer resulted in an increase in chloride secretion due to the release of 5'-adenosine monophosphate (AMP) by the neutrophils. 5'-AMP binds to the receptor on the apical membrane of the epithelium and induces chloride secretion, driving the movement of cations and water (293,303).

Further studies have explored the effects of pro- or anti-inflammatory cytokines on an *in vitro* culture of epithelial cells and patient-isolated immune cells. IL-10, an anti-inflammatory cytokine, has been shown to down-regulate proinflammatory cytokine release from immune cells isolated from patients with Crohn's disease, leading to clinical studies of IL-10 and its related cytokines in the treatment of IBD (304–306). Conversely, TNF- α and IFN- γ were shown to disrupt the epithelial barrier *in vitro* and anti-TNF- α therapy is now used in treatment of IBD (307,308). More recent *in vitro* studies have emphasised a potential mechanism for anti-TNF- α treatments, through an induced increase in IL-10 production by macrophages (309).

Many investigations focus on the epithelium or immune cell contribution to IBD and do not always consider the effects of stromal cells in the lamina propria, which, as discussed previously, are known contributors to the pathogenesis of IBD. One model that has incorporated fibroblasts has been utilised to investigate the effects of dietary proteins on inflammation (310,311). This study demonstrated that wheat proteins can induce a proinflammatory response and have cytotoxic effects on the epithelium, which may aggravate inflammation in patients with active IBD (310). Although the role of fibroblasts during this inflammatory response was not fully investigated, the cells were demonstrated to have increased migratory potential following the induction of inflammation (310). To provide a more holistic insight into the proinflammatory response generated in IBD, fibroblasts are a crucial lineage that ought to be incorporated into *in vitro* models.

1.5.4. 3D Cell Culture Models of the Intestine

The most valuable models in used in the drug discovery process discussed so far have been cultured on Transwell® inserts that allow for the separation of the apical and basolateral surfaces of the epithelium and represent the intestinal lumen and blood vessels respectively (227). Although widely used, they are considered to be a reductionist approach as they do not incorporate the complex heterogeneous

microenvironment and the appropriate biomechanical and biochemical signals observed *in vivo* that function to regulate cell activity and behaviour. Therefore, the development of novel 3D models is considered to be an alternative culture technique that can be used to bridge the gap between the more two-dimensional (2D)-like Transwell® monolayers and animal models and have the potential to reduce animal use in research in accordance with the 3R principles: replacement, reduction, and refinement.

Numerous different techniques have been employed to recreate the 3D architecture of the intestine; from co-culture with stromal or immune cells, to using scaffolds to recreate more intestine-like structures and to bioreactors that can introduce flow or movement to the models.

1.5.4.1. Co-culture with non-epithelial cell lineages

In addition to co-culture of Caco-2 cells with other epithelial cell types, models have also been developed that include components from the lamina propria such as stromal cells or immune cells (discussed in further detail in Chapters 3 and 5 respectively). Many of these models employ the use of an ECM-based scaffold such as hydrogels to embed the stromal and/or immune cells into. Epithelial cells, predominantly Caco-2 or Caco-2 and HT29-MTX cells, are then seeded onto the surface of these cell-containing scaffolds to create multi-layered intestinal models. Such models can be used to assess stromal-epithelial interactions along with examination of drug permeability.

3D models comprising of Caco-2, HT29-MTX and stromal fibroblast-like cells recapitulate the structure of the epithelium and lamina propria components of the intestinal mucosa while functionally, the permeability of insulin was shown to be more representative of the human scenario than in the monolayers alone (312). A model similar to this triple co-culture also included an immune component of macrophage-like cells, in addition to the stromal cells and again resulted in a more physiologically relevant model both structurally and functionally (313). A reduction in TEER was observed, closer to *in vivo* values, as well as a high correlation of permeability and the fraction absorbed in humans (313). Though these co-culture models recreate a more physiological intestinal model, they lack the native villi-crypt and tubular architecture of the intestine (227,232).

1.5.4.2. Scaffold technologies for intestinal tissue engineering

A major shortcoming of many of the aforementioned cell culture-based models is the use of the Transwell® membrane. Transwell® inserts are frequently made out of polycarbonate or polyester and provide a flat surface for the establishment of epithelial monolayers however, the inserts are unable to support the 3D organisation of cells and instead are more representative of a 2D cell culture system. Investigations have therefore focused on the use of alternative scaffold technologies to recreate a 3D microenvironment *in vitro* to enhance cellular proliferation, differentiation, structure, and function.

1.5.4.2.1. Natural biomaterials in intestinal tissue engineering

Natural biomaterials are derived from plants and animals and can include proteins such as collagen, silk, or fibrin, or may also be polysaccharide based such as alginate or chitosan. Many hydrogels used in intestinal *in vitro* models are derived from type I collagen due to its ease of use and ability to support the growth and differentiation of numerous different cell types. Collagen gels have been used in intestinal co-culture models to embed specific cell lineages within the collagen layer. For example, human intestinal fibroblasts embedded within collagen gels have been demonstrated to support the growth and differentiation of a Caco-2 HT29-MTX 3D co-culture. These models exhibited evidence of ECM deposition and remodelling by the fibroblasts in addition to improving the paracellular permeability correlation to the human intestine (314). However, other studies have demonstrated that type I collagen gels can inhibit the differentiation and promote proliferation of Caco-2 cells, while phenotypically the cells displayed a flattened morphology highly unlike *in vivo* (315).

Commercially available hydrogels such as Matrigel have also been extensively used in intestinal. Matrigel is derived from the ECM of mouse sarcoma cells and as such contains many components of the native ECM and basement membrane. Matrigel is frequently utilised to culture of 3D intestinal organoids and has been demonstrated to provide the proper mechanical properties to induce the formation of 2D epithelial monolayers of such organoids enabling their use in studies of drug transport (316). However, high batch-to-batch variability and their animal origin severely limit the reproducibility and usefulness of natural hydrogels in tissue engineering. Furthermore, such gels can contract and degrade after polymerisation. Although this is a desirable property for transplantation purposes, long-term *in vitro* experiments are not always feasible due to altering structural and physical properties of the scaffold throughout the culture period.

1.5.4.2.2. Synthetic biomaterials in intestinal tissue engineering

Synthetic hydrogels have also been used for purposes of intestinal tissue engineering, as they have greater reproducibility than natural polymers due to their chemically defined structure. Biodegradable polymers such as PLGA (polylactic-glycolic acid) have been utilised in co-culture models using Caco-2 and HT29-MTX cells where investigators determined that the scaffold induced an increase in proliferation and differentiation of the two cell lineages (317,318). Culture of Caco-2, HT29-MTX and Lrg5 stem cells has also been performed on non-biodegradable synthetic hydrogels such as L-pNIPAM (a hydrogel composed of N-isopropylacrylamide, Laponite® and water) which has been demonstrated to support the long-term culture of these cells (319,320). Interestingly the location of the epithelial cells had a significant effect on their response to the hydrogel; culture of Caco-2 cells in layers on the surface of the polymer increased their viability, while their maintenance within the hydrogel reduced the viability (319). This was in contrast with HT29-MTX cells which remained viable under all culture conditions (319). Introduction of dynamic flow into such models induced the spontaneous formation of villus-like structures, more accurately re-creating the 3D intestinal architecture (319).

1.5.4.2.3. Decellularised scaffold technology

Alternative biological scaffolds that have been used in cell culture-based models of the intestine are small intestinal submucosa (SIS) scaffolds. These scaffolds are usually of animal origin; however, some tissue engineering models of other tissues and organs such as the skin and cornea have successfully utilised human decellularised scaffolds of the appropriate tissue origin (321,322). Interestingly, decellularised dermal scaffolds have been shown to support the growth of both cardiac progenitor cells and bronchial epithelial cells *in vitro*, though no such studies have as of yet been performed using human intestinal cells (323,324). For intestinal tissue engineering, the most frequently used decellularised scaffolds are porcine jejunal segments and are used in combination with hydrogels. Culture of Caco-2 cells on SIS scaffolds encourages the cells to grow in the native 3D crypt-villus intestinal architecture and enhanced growth and differentiation of the cells has been identified (325,326). An enhanced *in vivo* correlation regarding paracellular permeability was also identified on SIS scaffolds (325,326). Limiting the usefulness of such scaffolds is the harsh decellularisation processes that can disrupt the composition of the ECM which can degrade the scaffold and alter the cell-ECM signalling occurring in such models and also the high levels of heterogeneity between scaffolds (327,328).

1.5.4.2.4. The use of inert scaffolds in *in vitro* intestinal models

Inert scaffolds such as nanofibers have also been used in *in vitro* models of the intestine as they do not contain exogenous proteins of animal origin and provide mechanical support to the cells. Advantages of inert scaffolds include their high reproducibility and stability enabling consistency between experiments and long-term studies to be performed. Furthermore, their inert nature does not impact on the cellular growth or function and therefore reduces uncontrollable variables within cultures. Examples of such scaffolds include the extensively used Transwell® inserts and nanofiber scaffolds that are similar to the synthetic hydrogels. Culture of Caco-2 cells on 3D electrospun nanofibrous scaffolds reduced the abnormally high TEER of the cells cultured on Transwell® to levels similar to *ex vivo* porcine tissue segments and enhanced paracellular permeability of lucifer yellow and dextrans was observed (329).

1.5.4.2.4.1. Alvetex® Scaffold Technology

Alvetex® is an inert scaffold similar to the Transwell® cell culture platform; it is a porous polyHIPE (high internal phase emulsion) made from polystyrene. Inserts have over 90 % porosity and each membrane has a thickness of 200 µm ensuring that cells are never further than 100 µm away from nutrients allowing for nutrient and waste exchange via passive diffusion, thus mimicking *in vivo* conditions.

Although Alvetex® technology has not been used in cell culture-based intestinal models, it has been successfully employed in the development of several other mucosal models including the skin and respiratory system that exhibit similarities in their overall structure to the intestine (330,331). Within these models, fibroblasts are cultured within the Alvetex® Scaffold membrane and the overlying epithelial cells are

maintained on the surface, accurately recreating the 3D structure of their respective tissues. It is therefore hypothesised, that Alvetex® Scaffold could be utilised in *in vitro* intestinal models to recreate the structure and function of the lamina propria and epithelium.

1.5.4.3. Recreating intestinal architecture

Recent engineering techniques have attempted to recreate the structure of the villi or the hollow lumen *in vitro*. Recreation of villi structures is important as they are critical in the prediction of transport of slowly absorbed drugs which are thought to diffuse down the villi length to be absorbed by the crypts (332,333). The first studies to reconstruct this structure *in vitro* utilised a collagen hydrogel scaffold and laser ablation techniques with Caco-2 cells representing the enterocytes. These models exhibited varying degrees of cell differentiation down the villi axis and displayed a higher permeability of hydrophilic drugs, more like *in vivo* and the cellular morphology of the Caco-2 cells resembled enterocytes of the human jejunum (334). However, models formed multilayers due to Caco-2 cell invasion into the hydrogel as it degraded leading to adaptations of the model using a synthetic PGLA scaffold and incorporating in the epithelium HT29-MTX cells along with the Caco-2 cells (317). This recreated the mucus layer and allowed for co-cultures with bacterial species to be established (318). The most recent models recreating the villi structure incorporate a micropatterned chemical gradient to resemble the crypt-villus axis and promote the development of a stem cell-like zone in the crypts (335).

Other studies have focused on recreating the structure of the hollow lumen surrounded by the intestinal epithelial cell layer and have developed *in vitro* models in tube-like structures. Initial tube-like models were developed using a silk scaffold protein and could incorporate epithelial cells including Caco-2 and HT29-MTX co-cultures (336). The porous nature of the silk scaffold allowed researchers to incorporate stromal cells embedded in a collagen gel into the model. A low oxygen tension was established in the lumen of this tube-like model due to the accumulation of mucus secretions by HT29-MTX cells, accurately mimicking the more hypoxic microenvironment of the gut lumen and allowing studies with gut microbiota to occur (336).

1.5.4.4. Bioreactors in intestinal engineering

Further strategies to create more complex and dynamic *in vitro* intestinal models typically employ the use of bioreactors. The addition of flow to cell cultures is an important consideration as the intestine is one of the most highly vascularised organs in the body to transport absorbed nutrients. Flow can be added to cultures in numerous ways through controlled shear stress in perfusion systems to microfluidic chambers. The addition of perfusion to the a co-culture of Caco-2 cells and human microvascular endothelial cells on decellularised porcine intestinal segments in a bioreactor resulted in the Caco-2 cells morphologically appearing more like normal small intestinal enterocytes and displaying increased transport of low permeable compounds compared to static cultures, highlighting the importance of the addition of flow (325).

Other techniques to introduce flow into models use millifluidic chambers or microfluidic devices. Culture of Caco-2 cells in a millifluidic chamber correlated with an increase in barrier integrity and tight junction protein expression, yet also exhibited a higher permeability compared to static controls (337). Microfluidic devices or “organ-on-a-chip” models have enabled the development of co-cultures resembling different organ systems. For instance, intestine and liver organ-on-a-chip models using Caco-2 and HepG2 cells respectively allow for the study of absorption and first-pass metabolism to more accurately predict oral bioavailability of drugs and can be used in toxicity testing (338). One of the most complicated organ-on-a-chip models not only incorporates intestine and liver, but also has skin and kidney equivalents and has the potential to be used in more complete absorption, distribution, metabolism and excretion (ADME) studies as well as repeated dose systemic toxicity testing of new chemical entities (339).

While some studies have focused on the addition of flow and perfusion to mimic blood flow, other strategies have attempted to mimic peristalsis. Culture of Caco-2 cells on an elastic membrane allows for the repetitive deformation of the epithelium. Caco-2 cells had enhanced proliferation, altered differentiation and modulated expression of brush border enzymes (340). The apical junctional complex between the epithelial cells was disrupted and resulted in an increased paracellular permeability (341). However, as the increased permeability is likely due to the damage associated with tight junctions, models such as this have more use in differentiation studies to further elucidate signalling pathways involved in enterocyte differentiation (227).

1.6. Conclusions

In conclusion, the intestinal mucosa is a complex and dynamic structure that is comprised of an epithelium overlying a lamina propria and a muscularis mucosae. Many epithelial, stromal, and immune cells communicate with a commensal microbial compartment to maintain homeostasis and a healthy gut environment. Breakdown of proper communication can lead to the establishment of inflammatory bowel diseases that severely impact the intestinal biology.

Healthy and diseased intestinal modelling frequently relies on the use of animal models or their *ex vivo* tissues. With escalating demand to reduce and replace animals, the use of *in vitro* intestinal alternatives is becoming increasingly popular as a tool to study complex cellular processes such as drug permeability, toxicity, and inflammation in a controlled and simplified manner. Many factors must be taken into consideration when selecting the appropriate technologies to develop new intestinal models including, the addition and influence of multiple cell types, an appropriate scaffold to maintain the cells on and the addition of any dynamic factors, such as flow and peristalsis experienced *in vivo*. However, there remains a requirement for *in vitro* models that incorporate epithelial, stromal, and immune cells in a format that allows for simple permeability assays to be performed.

1.7. Project Aims and Objectives

This project aims to develop a novel *in vitro* model of the intestinal mucosa that incorporates an epithelial monolayer on the surface of a lamina propria component containing fibroblasts, an endogenous extracellular matrix, and the eventual addition of an immune cell component. The model will be created using a solid membrane of Alvetex® Scaffold to allow for simplistic transport experiments to be performed. It is hypothesised that the resulting model will have a more physiologically relevant structure to the human intestine mucosa and that the permeability of passively transported compounds will have increased predictive validity to the human *in vivo* scenario.

1.7.1. Objectives

- Develop and characterise a model of the healthy intestinal mucosa using the gold standard Caco-2 cell line in co-culture with fibroblasts to represent a lamina propria.
- Adapt the model to incorporate a mucus-producing cell line, HT29-MTX, and characterise the structure of the resulting model alongside human tissue and characterise the permeability of passive paracellular compounds and P-gp substrates.
- Optimise the development of a novel co-culture a novel co-culture model containing Caco-2 cells, fibroblasts and macrophage-like cells and characterise the model structurally and functionally.
- Stimulate the immune-cell co-culture model to mimic aspects of inflammatory bowel disease and characterise the resulting structural changes and alterations in permeability.

Chapter 2: General Materials and Methods

2.1. Routine Cell Culture of 2D Cells

2.1.1. Epithelial Cell Culture

2.1.1.1. Routine cell culture of Caco-2 cells

Caco-2 (86010202, ECACC, Salisbury, UK) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies, Invitrogen, Thermo Fisher Scientific, Cramlington, UK) with 10 % foetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM L-Glutamine (Thermo Fisher Scientific), 1% Non-Essential Amino Acids (NEAA, Thermo Fisher Scientific) and 100 Units/mL Penicillin, 100 µg/mL Streptomycin (Thermo Fisher Scientific) at 37 °C and 5 % CO₂ in a humidified environment. Cells were grown in 175 cm² culture flasks (Greiner Bio-One, Stonehouse, UK) and subcultured once the cells had reached 70-80 % confluency by washing with phosphate buffered saline (PBS, recipe in Appendix 1) and dissociation with 0.25 % Trypsin EDTA (Thermo Fisher Scientific) before neutralising with culture media. The cell suspension was centrifuged at 163 x g for 3 minutes and resuspended in growth medium. Cells were sub-cultured at a density of ~1 x 10⁵ cells/cm² and the medium was replaced every 2-3 days.

2.1.1.2. Routine cell culture of HT29-MTX cells

HT29-MTX-E12 (12040401, ECACC) were maintained in DMEM with 10 % FBS, 2 mM L-Glutamine, 1 % NEAA and 100 Units/mL Penicillin, 100 µg/mL Streptomycin at 37 °C and 5 % CO₂ in a humidified environment. Cryopreservation and revival were carried out according to the protocol outlined in Section 2.1.4. Cells were grown in 175 cm² culture flasks and subcultured once the cells had reached 70-90 % confluency by washing the PBS and dissociation with 0.25 % Trypsin EDTA before neutralisation with growth media. Cell suspensions were centrifuged at 163 x g for 3 minutes and resuspended in complete growth medium. Cells were sub-cultured at a density of ~ 1 x 10⁵ cells/cm² and the medium was replaced every 2-3 days.

2.1.2. Fibroblast Cell Culture

2.1.2.1. Routine cell culture of HDFn cells

Neonatal human dermal fibroblasts (HDFn, C-004-5C, cell line numbers #1366356 and #1366434, Thermo Fisher Scientific) cells were originally cultured in Medium 106 (M106, Thermo Fisher Scientific) supplemented with Low Serum Growth Supplement (LSGS, Thermo Fisher Scientific), 10 µg/mL Gentamicin and 0.25 µg/mL Amphotericin B (Thermo Fisher Scientific) at 37 °C and 5 % CO₂ in a humidified environment. Cells grown in M106 were sub-cultured by washing with PBS and dissociation with 0.025 % Trypsin EDTA (Thermo Fisher Scientific) which was then inhibited by Trypsin Neutraliser Solution (Thermo Fisher Scientific) containing calf serum and centrifuged at 163 x g for 3 minutes and resuspended in growth medium. Cells were sub-cultured at a density of $\sim 2.5 \times 10^3$ cells/cm² in 175 cm² cell culture flasks and the medium was replaced every 2-3 days.

HDFn cells were also maintained in DMEM with 10 % foetal bovine serum (FBS), 2 mM L-Glutamine, 1 % NEAA and 100 Units/mL Penicillin, 100 µg/mL Streptomycin. Cells cultured in DMEM were passaged between 70-80 % confluency by washing with PBS and dissociation with 0.25 % Trypsin EDTA before neutralising with culture media and were seeded in the same manner as described for M106 cells.

2.1.3. Immune Cell Culture

2.1.3.1. Routine suspension culture of U937 Cells

U937 cells (85011440, ECACC) were maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Sigma-Aldrich) with 10 % FBS, 2 mM L-glutamine and 100 Units/mL Penicillin, 100 µg/mL Streptomycin at 37 °C and 5 % CO₂ in a humidified environment. Cells were grown in Corning™ T-75 untreated suspension culture flasks (Thermo Fisher Scientific) and were maintained at densities between $2-9 \times 10^5$ viable cells/mL as determined by a trypan blue exclusion assay (Section 2.6.2) performed every 2-3 days of culture. Cultures were maintained by the addition of fresh RPMI media or by the replacement of media through centrifugation at 163 x g for 3 minutes and resuspension at an appropriate density.

2.1.3.2. Differentiation of U937 cells

For differentiation, a trypan blue exclusion assay was performed, and a single cell suspension was produced by centrifugation at 163 x g for 3 minutes and resuspension in complete RPMI to give a final density of 0.25×10^6 cells/mL. Cells were then seeded into either 75 cm² culture flasks (5×10^6 cells) or 6-well plates (1×10^6 cells) and were treated with either 10 or 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for a minimum of 48 hours. Cells were then rinsed two times with sterile PBS and fresh RPMI or DMEM was added. Cells were left for a further 24-hour rest and recovery period before use in further assays.

2.1.3.3. Subculture of differentiated U937 cells

Upon PMA treatment cells become adherent. To allow their use in downstream studies and models, U937 cells must be detached from the underlying tissue culture plastic through passaging techniques. Differentiated U937 cells were washed with sterile PBS followed by dissociation with 0.25 % trypsin EDTA before neutralising with culture media. The cell suspension was centrifuged at 163 x g for 3 minutes and resuspended in growth medium. Cells could then be seeded at the desired seeding density as specified in each experiment.

2.1.4. Cryopreservation and Revival of Cells

2.1.4.1. Cryopreservation and revival of adherent cells

For cryopreservation, a single cell suspension of adherent cells (Caco-2, HT29-MTX, HDFn) was produced as described above and cells were resuspended in either Synth-a-Freeze for M106 HDFn cells or 90 % complete DMEM containing 10 % Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, Dorset UK) for all other cells in cryovials (Thermo Fisher Scientific). Cells were frozen down at 2×10^6 cells/mL. Cryovials were gradually cooled using a Mr Frosty™ (Thermo Fisher Scientific) to -80 °C, before transferring to -150 °C for long-term storage.

To revive cells from cryopreservation, vials were warmed in a water bath until almost thawed. 9 mL of culture medium was then added to the cell suspension before transferring to a culture flask and the media volume was adjusted to 25 mL/175 cm² flask. Medium was replaced ~24 hours later and cells were maintained as described above.

2.1.4.2. Cryopreservation and revival of suspension cells

For cryopreservation of U937 suspension cells, a trypan blue exclusion assay was performed, and a single cell suspension was produced by centrifugation at 163 x g for 3 minutes and resuspension in 90 % complete RPMI with 10 % DMSO at a density of 5×10^6 cells/mL. Cells were then placed in cryovials and gradually cooled using a Mr Frosty™ to -80 °C, before transferring to -150 °C for long-term storage.

To revive cells from cryopreservation, vials were warmed in a water bath until almost thawed. 9 mL of culture medium was then added to the cell suspension. To remove DMSO from the culture, cells were centrifuged at 163 x g for 3 minutes and resuspended in an appropriate volume of media to give a final seeding density of $2-9 \times 10^5$ cells/mL. Cells were then maintained as described above.

2.1.5. Seeding Cells onto Glass Coverslips

To visualise markers expressed by 2D cells, samples were cultured as previously described on 16 mm coverslips (Thermo Fisher Scientific) in 12-well plates (Greiner). Coverslips were sterilised with 70 % ethanol (EtOH) and were either uncoated or coated with 10 µg/mL of Poly-D-Lysine (PDL, Sigma-Aldrich) solution (for

HDFn cells only) at 4 °C overnight before being washed 3 times in PBS. Cells were then seeded onto the coverslips and maintained as normal at 37 °C and 5 % CO₂ in a humidified environment.

2.2. Culture of Cells in 3D

2.2.1. Pre-Treatment of Alvetex® Substrates

Alvetex® Scaffold, Strata, and Polaris 12-well cell culture inserts (Reprocell Europe, Glasgow, UK) were rendered hydrophilic by submersion in 70 % EtOH for a minimum of 5 minutes before being washed in PBS. Scaffolds were then submerged in media in 6-well plates (Greiner), until ready for cell seeding.

2.2.2. Seeding of U937 Cells onto Alvetex® Substrates

Alvetex® Scaffold, Strata and Polaris 12-well cell culture inserts (Reprocell Europe) were rendered hydrophilic as described in Section 2.2.1. To seed differentiated U937 cells onto Alvetex® inserts, cells were passaged as described above and the cell suspension volume was adjusted to a density of 5×10^6 cells/mL, 100 µL was then seeded onto the surface of the membrane (0.5×10^6 cells/insert). Cells were left to adhere to the scaffold for a minimum of 2 hours before 10 mL of culture medium was added to the wells. Cells were maintained at 37 °C and 5 % CO₂ in a humidified environment in complete RPMI for a further 48 hours before fixation and wax embedding prior to histological analysis

2.3. Generation of Intestinal Models Utilising Transwell® Membranes

2.3.1. Formation of the Caco-2 Monolayer on Transwell®

A single cell suspension of Caco-2 cells was obtained as previously described. Cells were counted using a trypan blue (Sigma-Aldrich) exclusion assay and the volume was adjusted to a density of 0.5×10^6 cells/mL. 500 µL of the Caco-2 cell suspension was seeded onto each 12-well Transwell® insert (12 mm diameter, 0.4 µm pore size # 3401, Costar, Sigma-Aldrich) at a density of 2.2×10^5 cells/cm² (~ 250 000 cells/insert). 1.5 mL of medium was then added to the basolateral chamber. Models were cultured for a minimum of 21 days at 37 °C and 5 % CO₂ in a humidified environment and the medium (complete DMEM) was replaced every 2-3 days (Figure 2.1).

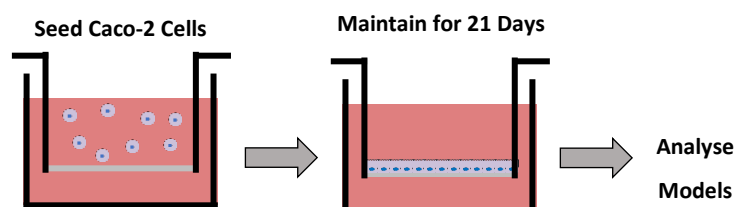


Figure 2.1: Schematic protocol of the Caco-2 Transwell® model culture system. Caco-2 cells were seeded onto Transwell® inserts and cultured for a minimum 21 days to allow epithelial differentiation, before harvesting and analysis of models.

2.3.2. Formation of the HT29-MTX monolayer on Transwell®

HT29-MTX-E12 cells were seeded onto Transwell® inserts in the same manner outlined for Caco-2 cells in Section 2.3.1. Briefly, a single cell suspension of HT29-MTX-E12 cells was obtained as previously described. Cells were counted using a trypan blue exclusion assay and the final media volume was adjusted to a density of 0.5×10^6 cells/mL. 500 μ L of the cell suspension was then seeded onto each 12-well Transwell® insert at a density of 2.2×10^5 cells/cm² (~250 000 cells/insert). 1.5 mL of complete DMEM was then added to the basolateral chamber and culture proceeded for a minimum of 21 days at 37 °C and 5 % CO₂ in a humidified environment and the medium was replaced every 2-3 days (Figure 2.2).

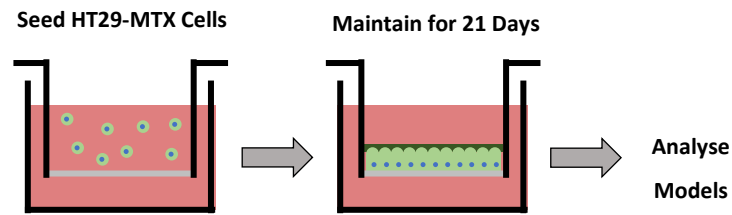


Figure 2.2: Schematic protocol of the HT29-MTX Transwell® model culture system. HT29-MTX cells were seeded onto Transwell® inserts and cultured for a minimum 21 days, before harvesting and analysis of models.

2.3.3. Formation of Caco-2 HT29-MTX Co-Cultures on Transwell®

Single cell suspensions of Caco-2 and HT29-MTX-E12 cells were obtained as previously described and cells were counted using a trypan blue exclusion assay. Cells were then mixed prior to seeding to yield cell ratios of 90:10 and 70:30 for Caco-2:HT29-MTX-E12 cells respectively. A total of 2.2×10^5 cells/cm² were seeded onto membranes in a final volume of 500 μ L per each 12-well Transwell® filter. 1.5 mL of complete DMEM was then added to the basolateral chamber and models were maintained for a minimum of 21 days at 37 °C and 5 % CO₂ in a humidified environment and the medium was replaced every 2-3 days (Figure 2.3).

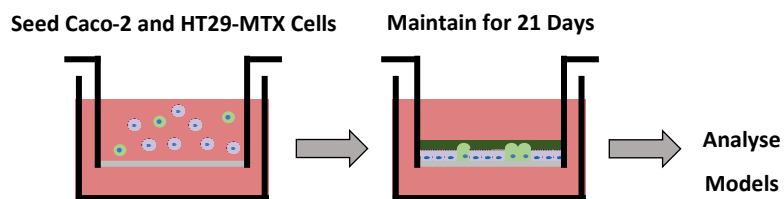


Figure 2.3. Schematic protocol of epithelial co-cultures of Caco-2 and HT29-MTX cells on Transwell® scaffolds. Caco-2 and HT29-MTX cells were seeded onto Transwell® inserts in ratios of 90:10 or 70:30 Caco-2 to HT29-MTX and cultured for a minimum 21 before harvesting and analysis of models.

2.3.4. Collection and Use of Fibroblast Conditioned Media

HDFn cells were grown to confluency in complete DMEM following standard culture procedures outlined in Section 2.1.2. Fibroblasts were then cultured in fresh DMEM for 24 hours before media was collected, centrifuged at $163 \times g$ for 10 minutes at 4°C before filtering with a $0.2 \mu\text{m}$ syringe filter (Thermo Fisher Scientific), media was then designated conditioned medium.

Before addition to the cells cultured under conventional 2D conditions or to Transwell® monolayers, conditioned media was diluted at a 1:1 ratio with fresh complete DMEM to ensure the media contained enough glucose and L-Glutamine to support cell growth. Transwell® models were set up as previously described and were treated with conditioned medium 2 days post-seeding and media was replaced every 2 days. Models were maintained at 37°C and 5 % CO_2 in a humidified environment for a minimum of 21 days.

2.3.5. Generation of a Caco-2 U937 Co-Culture Model using Transwell® Membranes

Caco-2 Transwell® monocultures were developed as outlined in Section 2.3.1. At day 11 of epithelial culture 0.5×10^6 U937 cells were differentiated in a fresh 12-well plate following the aforementioned protocol outlined in Section 2.1.3.2. Following the 24-hour rest period in complete DMEM, Transwell® inserts with Caco-2 cells at day 14 of the growth were added to the wells containing differentiated U937 cells. Co-cultures were maintained at 37°C and 5 % CO_2 in a humidified environment in complete DMEM until day 21 of epithelial cell culture. Models were then harvested as described in Section 2.7.1. for further analysis. A schematic protocol detailing the Transwell® U937 co-culture procedures is outlined in Figure 2.4.

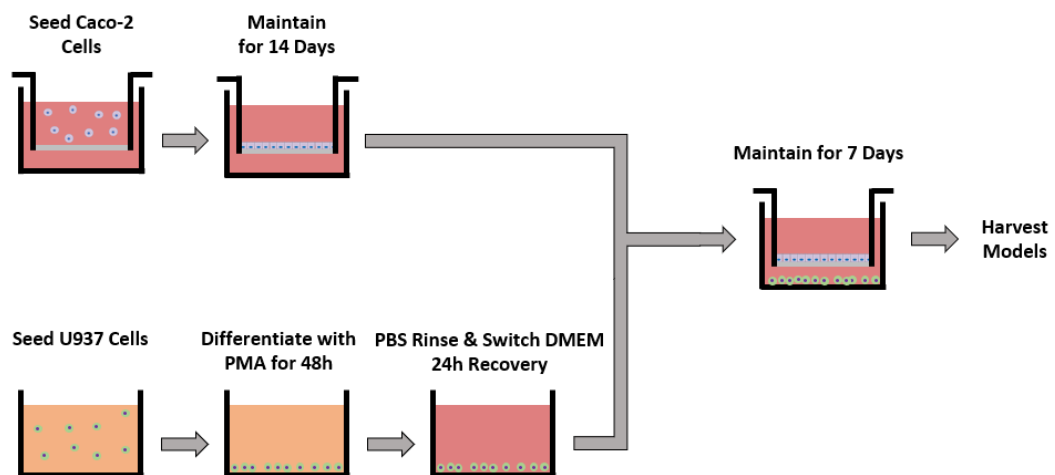


Figure 2.4. Schematic protocol detailing the co-culture of Caco-2 Transwell® monolayers and differentiated U937 cells. Caco-2 cells were seeded onto Transwell® inserts and cultured until day 14 as normal. U937 cells were differentiated for 48 hours with PMA treatment in RPMI culture media in 12-well plates. U937 cells were then rinsed twice in PBS and maintained in complete DMEM for a further 24 hours. 14-day-old Caco-2 epithelia were then transferred to the 12-well plate containing differentiated U937 cells at the base of the well. Culture then proceeded for a further 7 days.

2.4. Generation of Lamina Propria Equivalents Using Alvetex® Scaffold

2.4.1. Formation of a Lamina Propria Component using Dermal Fibroblasts

A single cell suspension of HDFn was obtained as previously described and cells were counted using a trypan blue exclusion assay. The cell suspension volume was adjusted to a density of 5.0×10^6 cell/mL and 100 μ L was seeded onto the surface of the scaffold (0.5×10^6 cells/insert). Cells were left to adhere to the scaffold for a minimum of 2 hours before 10 mL of medium was added to the wells. Models were maintained for 7-28 days, at 37 °C and 5 % CO₂ in a humidified environment in complete medium supplemented with 5 ng/mL Transforming Growth Factor- β 1 (TGF- β 1 PeproTech, London, UK) and 100 μ g/mL ascorbic acid (Sigma-Aldrich). Medium was replaced every 3-4 days and the culture plate was changed after the first media change to avoid the accumulation of cells on the base of the well that had fallen through the scaffold (Figure 2.5).

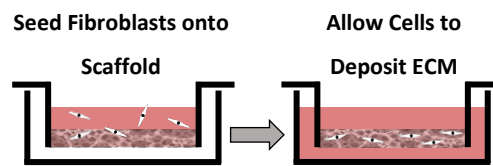


Figure 2.5. Schematic protocol detailing dermal fibroblast model development on Alvetex® Scaffold. Fibroblasts were seeded onto Alvetex® Scaffold and were maintained for 7-28 days in DMEM with TGF- β 1 and ascorbic acid supplemented to the media to allow cells to produce ECM. Models were then either harvested for downstream analysis or an epithelial monolayer was seeded onto the apical surface of the lamina propria equivalent.

2.4.2. Formation of a Lamina Propria Component using CCD-18co Fibroblasts

An intestinal model using CCD-18co (CRL-1459™, ATCC®, Guernsey, UK) fibroblasts was developed within the Przyborski lab by Dr Matthew Freer. 2D cultured CCD-18co fibroblasts were maintained in complete DMEM culture media and were cultured following the HDFn DMEM protocol discussed in Section 2.1.2. Cells were trypsinised as previously described and counted via a trypan blue exclusion assay; 220 000 CCD-18co cells were then seeded in 100 μ L into 24-well Alvetex® Scaffold (Reprocell Europe) in 12 well plates, allowed to adhere to the membrane for a minimum of 2 hours before adjusting the media volume (complete DMEM without any additional supplements) to 4.5 mL. Models were maintained for 7 days with a complete media change every 48 hours. An additional 220 000 cells were seeded onto the models at days 7, 9 and 11 of fibroblast culture (Figure 2.6). Models were maintained until day 14 of fibroblast culture, at 37 °C and 5 % CO₂ in a humidified environment. Culture of models performed by Dr Matthew Freer.

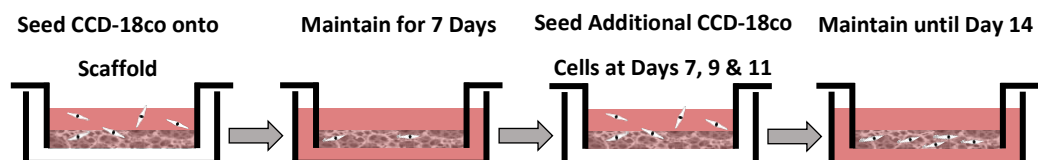


Figure 2.6. Schematic protocol detailing the culture of a co-culture model using CCD-18co fibroblasts. CCD-18co cells were seeded onto Alvetex® Scaffold and were maintained for 7 days before additional fibroblasts were seeded at days 7, 9 and 11 of culture. At day 14 of fibroblast culture models were then used as a support for the culture of Caco-2 cells on the apical surface. Models cultured by Dr Matthew Freer.

2.4.3. Seeding of Differentiated U937 cells onto Lamina Propria Equivalents

Lamina propria equivalents were generated as previously described with HDFn cells in Section 2.4.1. Differentiated U937 cells were passaged as described above and the cell suspension volume was adjusted to varying seeding densities ($1 \times 10^4 - 2 \times 10^7$ cells/mL) and 100 μ L ($1 \times 10^3 - 2 \times 10^6$) was seeded onto lamina propria equivalents that had been cultured for 7-10 days. Cells were left to adhere to the membrane for a minimum of 2 hours before 10 mL complete DMEM without additional supplements was added to the wells. Media was replaced every 2-3 days of culture. Models were maintained at 37 °C and 5 % CO₂ in a humidified environment in complete DMEM until day 14 of HDFn culture before harvesting for analysis or use in downstream applications (Figure 2.7).

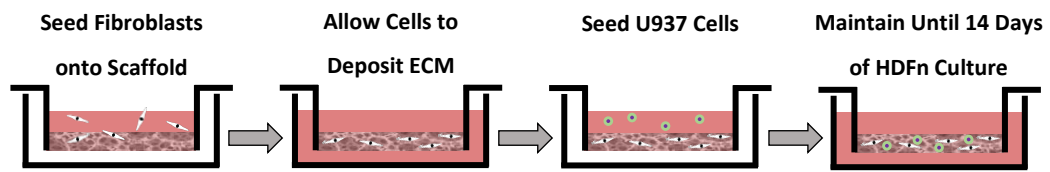


Figure 2.7. Schematic protocol detailing U937 co-culture with HDFn and Caco-2 cells. Lamina propria equivalents were generated as normal and U937 cells were seeded between days 7-10 of HDFn culture. Models were maintained until HDFn had been cultured for 14 days and then were either harvested for analysis or Caco-2 cells were seeded on the surface and maintained for a further 21 days.

2.5. Formation of an Epithelium on Alvetex® Membranes

2.5.1. Formation of a Caco-2 monolayer on Alvetex® Polaris

A single cell suspension of Caco-2 was obtained as previously described and cells were counted using a trypan blue exclusion assay. The cell suspension volume was adjusted to a density of 4.0×10^6 cells/mL and 100 μ L was seeded onto the apical surface of 12-well Alvetex® Polaris. Cells were left to adhere for a minimum of 2 hours before 10 mL of complete DMEM was added to the wells. Models were maintained for a further of 21 days, at 37 °C and 5 % CO₂ in a humidified environment and the medium was replaced every 3-4 days. The culture plate was changed once a week to avoid the accumulation of cells on the plastic that had fallen through the scaffold.

2.5.2. Seeding Caco-2 Cells onto the Surface of Lamina Propria Equivalents

2.5.2.1. Seeding Caco-2 cells onto dermal fibroblasts

Following HDFn pre-culture, a single cell suspension of Caco-2 cells was generated as previously described and cells were counted by a trypan blue exclusion assay. The cell suspension volume was adjusted to a density of 4.0×10^6 cells/mL and 100 μ L was seeded onto the apical surface of 12-well Alvetex® Scaffold containing dermal fibroblasts. Cells were left to adhere for a minimum of 2 hours before 10 mL of complete DMEM was added to the wells. Models were maintained for a further of 21 days, at 37 °C and 5 % CO₂ in a humidified environment and the medium was replaced every 3-4 days. The culture plate was changed once a week to avoid the accumulation of cells on the plastic that had fallen through the scaffold (Figure 2.8).

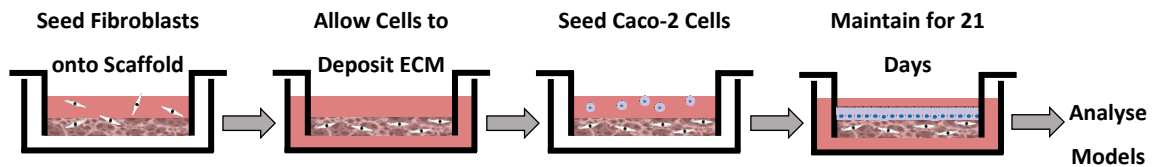


Figure 2.8. Schematic protocol Caco-2 cell culture on lamina propria equivalents. Fibroblasts were seeded onto Alvetex® Scaffold and were maintained for 7-28 days to allow cells to produce ECM. Caco-2 cells were seeded on the surface of the lamina propria-equivalent containing fibroblasts and maintained for a further 21 days allowing enterocyte differentiation to occur before harvesting and analysis of models.

2.5.2.2. Seeding Caco-2 cells onto CCD-18co fibroblasts

At day 14 of CCD-18co fibroblast culture, 220 000 Caco-2 cells were seeded onto the apical surface of the Alvetex® membrane following the protocol discussed in Section 2.5.2.1 and models were maintained following the same standardised protocols outlined in Section 2.5.2.1 and Figure 2.8. Culture of models performed by Dr Matthew Freer and images for analysis kindly provided by Dr Matthew Freer.

2.5.3. Seeding of HT29-MTX Cells onto the Surface of Lamina Propria Equivalents

A single cell suspension of HT29-MTX-E12 cells was obtained as previously described and cells were counted using a trypan blue exclusion assay. The cell suspension volume was adjusted to 4×10^6 cells/mL and 100 μ L was seeded onto the apical surface of 12-well Alvetex® Scaffold containing dermal fibroblasts (Section 2.4.1). Cells were allowed to adhere for a minimum of 2 hours before 10 mL of complete DMEM was added to the wells. Models were maintained at 37 °C and 5 % CO₂ in a humidified environment and the medium was replaced every 3-4 days. The culture plate was changed once a week to avoid the accumulation of cells on the plastic that had fallen through the scaffold (Figure 2.9).

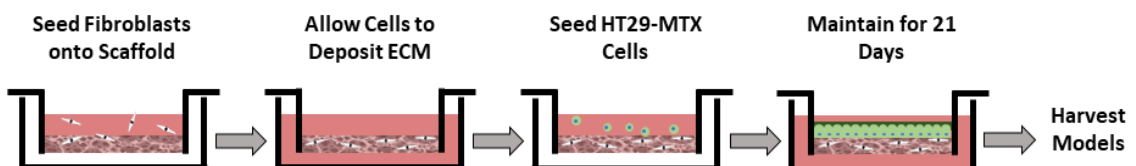


Figure 2.9. Schematic protocol detailing HT29-MTX model development on Alvetex® Scaffold. Dermal fibroblasts were seeded onto Alvetex® Scaffold and were maintained for 14 days to allow cells to produce ECM. HT29-MTX cells were seeded on the surface of the lamina propria-equivalent containing fibroblasts and maintained for a further 21 before harvesting and analysis of models.

2.5.4. Formation of an Enterocyte-Goblet Cell Co-Culture on the Surface of a Lamina Propria Equivalent

Single cell suspensions of Caco-2 and HT29-MTX-E12 cells were obtained as previously described and cells were counted using a trypan blue exclusion assay. Cells were then mixed prior to seeding to yield a cell ratio of 90:10 for Caco-2 to HT29-MTX-E12 cells respectively with a total of 0.4×10^6 cells seeded onto each Alvetex® Scaffold containing dermal fibroblasts (Section 2.4.1.) in a volume of 100 μ L. Cells were allowed to adhere for a minimum of 2 hours before 10 mL of complete DMEM was added to the wells. Models were maintained at 37 °C and 5 % CO₂ in a humidified environment and the medium was replaced every 3-4 days.

The culture plate was changed once a week to avoid the accumulation of cells on the plastic that had fallen through the scaffold (Figure 2.10).

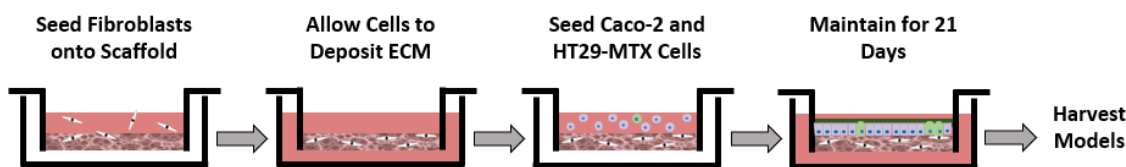


Figure 2.10. Schematic protocol of epithelial co-cultures of Caco-2 and HT29-MTX cells on lamina propria equivalents. Dermal Fibroblasts were seeded onto Alvetex® Scaffold and were maintained for 14 days to allow cells to produce ECM. Caco-2 and HT29-MTX cells were seeded on the surface of the lamina propria-equivalent containing fibroblasts in a ratio of 90:10 Caco-2 to HT29-MTX. Models were maintained for a further 21 before harvesting and analysis of models.

2.6. Characterisation of Cell Growth

2.6.1. Phase Contrast Imaging of 2D Cultured Cells

Cells maintained under traditional culture conditions on tissue culture plastic were routinely imaged by phase contrast microscopy using either a Nikon Eclipse TS100 inverted microscope (Nikon, Tokyo, Japan) with a Nikon DXM1200 digital camera attachment (Nikon) or an EVOS FL digital inverted brightfield and phase contrast microscope (Thermo Fisher Scientific).

2.6.2. Trypan Blue Exclusion Assay

To determine cell viability, cells were counted with a trypan blue exclusion assay. Trypan blue was added to cell suspensions at a ratio of 1:1 or 1:10 and applied to a haemocytometer (Agar Scientific, Essex, UK). Cells were then counted, and viability was expressed as a percentage of live cells.

2.6.3. Calculation of Population Doubling Time

Population doubling time was calculated by performing a growth assay of cells. Cells were cultured on tissue culture plastic and the number of cells retrieved after subculture was calculated by a trypan blue exclusion assay. When the cell growth was in the exponential phase, the cell numbers were applied to the following equation:

$$DT = Ln2 / Ln \left(\frac{Xe}{Xb} \right)$$

where DT = doubling time, T = incubation time, Xe = cell number at end of culture, Xb = cell number at the start of culture.

2.6.4. MTT Assay

Cells grown upon Alvetex® membranes or on 12-well traditional tissue culture plastic plates were washed in PBS and 0.5 mL 1 mg/mL Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich) solution in PBS was added to each sample including a control well without any cells. Plates were incubated at 37 °C, 5 % CO₂ for 1 hour

then 0.5 mL acidified isopropanol (1 μ L concentrated HCL/mL isopropanol, Thermo Fisher Scientific) was added for a 10-minute incubation period in the dark on an orbital shaker at 50 rpm at room temperature. Samples were placed into a flat-bottomed 96-well plate (Greiner) either diluted 1:1 in isopropanol (100 μ L:100 μ L) or neat (200 μ L). Absorbance was then measured at 570 nm using a BioTek ELx800 microplate reader (Thermo Fisher Scientific).

2.7. Assessment of Model Morphology and Structure

2.7.1. Fixation of Models for Paraffin Wax Embedding

To observe the structure of the models by histological staining, cells cultured on Transwell® and Alvetex® inserts were removed from their plastic support and washed in PBS before being placed in 4 % paraformaldehyde (PFA, Thermo Fisher Scientific) for either 2 hours at room temperature or overnight at 4 °C. Fixed samples were then washed 3 more times in PBS before being dehydrated through a series of ethanol solutions (30, 50, 70, 80, 90 and 95 % EtOH) for 15 minutes each. Samples were then immersed in 100 % EtOH 30 minutes before being transferred to embedding cassettes (VWR International, Lutterworth, UK) and submersion in Histo-Clear II (National Diagnostics, Lichfield, UK) for a further 30 minutes. Models were placed into a 1:1 mixture of Histo-Clear II and paraffin wax (Thermo Fisher Scientific) at 60 °C and then in wax only for a minimum of 1 hour each. Samples could then be embedded in wax using plastic moulds (Solmedia Ltd., Shrewsbury, UK) and embedding cassettes and were left to set at room temperature overnight. Transverse sections of the embedded samples were cut at 5 μ m using a Leica RM2125RT Microtome (Leica, Milton Keynes, UK) and then were mounted onto charged SuperFrost Plus (Thermo Fisher Scientific) microscope slides and left to dry overnight.

2.7.2. Histology

2.7.2.1. Haematoxylin and Eosin staining

Paraffin wax sections were deparaffinised in Histo-Clear I (National Diagnostics) for 15 minutes before being rehydrated through a series of ethanol solutions: 100 % EtOH for 2 minutes, 95 % EtOH and 70 % EtOH for 1 minute each. Samples were then washed with distilled water (dH₂O) for 1 minute followed by Mayer's Haematoxylin (Sigma-Aldrich) for 5 minutes before being washed in dH₂O for 30 seconds. The nuclei were then stained blue by immersion of slides in alkaline ethanol for 30 seconds. Samples were rehydrated through 70 % and 95 % EtOH for 30 seconds before staining in Eosin Y (2 % w/v in 95 % EtOH, Sigma-Aldrich) for a further 30 seconds. Slides were then dehydrated by 2 washes in 95 % EtOH for 10 seconds each followed by 100 % EtOH for 15 seconds then 30 seconds. Samples were then cleared by immersion in Histo-Clear I 2 times for 5 minutes each. Slides could then be mounted in Omnimount (Scientific Labs Ltd., Nottingham, UK) before imaging using a Leica ICC50 high-definition camera (Leica) mounted on a Leica DM500 microscope (Leica).

2.7.2.2. Periodic Acid Schiff / Alcian Blue staining

To visualise mucus production, samples were stained with Periodic Acid Schiff (PAS)/Alcian Blue staining kit (Atom Scientific, Cheshire, UK). Slides were deparaffinised and rehydrated as described for H&E staining and then stained in Alcian Blue for 10 minutes. Slides were then washed with dH₂O and incubated in 1 % Periodic Acid solution for 10 minutes and washed again in dH₂O. Slides were then transferred to Schiff reagent for 10 minutes and washed in hot water for 1 minute then in running water for 10 minutes. Nuclei were stained in Haemalum Mayer for 30 seconds before rinsing and differentiation in running water. Samples were then dehydrated in 70 %, 95 % and 100 % EtOH for 30 seconds each and then cleared 2 times for 5 minutes in Histo-Clear I and mounted in Omnimount. Samples were imaged on a Leica DM500 microscope.

2.8. Assessment of Cell Phenotype

2.8.1. Immunostaining

2.8.1.1. Immunostaining of 2D cells

Cells cultured on coverslips were fixed in either 4 % PFA for 10 minutes at room temperature or in ice cold 1:1 methanol-acetone (Thermo Fisher Scientific) for 10 minutes at -20 °C followed by 3 washes in PBS. Samples fixed in PFA had an additional permeabilisation step before continuing with the staining protocol; cells were permeabilised with 0.4 % Triton-X-100 (Thermo Fisher Scientific) in PBS for 15 minutes at room temperature. Samples were then blocked in 10 % new-born calf serum (NBCS, Thermo Fisher Scientific) in PBS for 1 hour at room temperature followed by an hour-long incubation in primary antibody diluted in blocking solution at room temperature. A negative control sample was included for each primary antibody whereby the sample would remain in blocking solution for the duration of the primary antibody incubation. Samples were washed 3 times in PBS for 10 minutes and all samples were incubated in secondary antibody and Hoechst 33342 (Thermo Fisher Scientific) diluted 1:1000 in blocking buffer for 1 hour at room temperature. Samples were then washed 3 more times in PBS for 10 minutes before mounting in Vectashield (Vector Labs, Peterborough, UK) and imaged using a Zeiss 880 confocal microscope (Zeiss, Cambridge, UK) or Zeiss Axioskop 40 (Zeiss). Laser settings were adjusted to the negative control samples to minimise background staining.

2.8.1.2. Immunostaining of paraffin sections

Paraffin wax sections were deparaffinised in Histo-Clear I for 15 minutes before being rehydrated through 100 % EtOH and 70 % EtOH for 5 minutes. This was then followed by immersing the slides in PBS for 5 minutes before antigen-retrieval in citrate buffer (recipe in Appendix 1) with 0.5 % Tween 20 (Sigma-Aldrich) at 95 °C

for a further 20 minutes. Samples were then blocked in 20 % NCBS in either PBS or 0.4 % Triton-X-100 PBS if permeabilisation was required for 1 hour before incubation in primary antibody at 4 °C overnight in a humidified chamber. To ensure antibody specificity, negative controls were included by omitting the primary antibody and keeping the sample immersed in blocking buffer. All samples were then washed 3 times in PBS for 10 minutes before being incubated with secondary antibody and Hoechst 33342 (1:1000) for 1 hour at room temperature. Slides were then washed 3 more times in PBS for 10 minutes before mounting in Vectashield. Images were acquired using a Zeiss 880 confocal microscope and laser settings were adjusted to minimise any background staining on the negative control sample.

2.8.1.3. Whollemount immunostaining of models

Whollemount immunostaining was carried out on Transwell® or Alvetex® samples that had been fixed in ice-cold 1:1 methanol-acetone for 20 minutes at -20 °C and washed 3 times in PBS. Samples were blocked for 1 hour at room temperature in 20 % NCBS in PBS before being incubated at room temperature for 2 hours in primary antibody diluted in blocking buffer. Samples were then washed three times in PBS for 10 minutes and incubated in secondary antibody and Hoechst 33342 (1:1000) in blocking buffer for 1 hour. This was followed by a further 3 washes in PBS and mounting in Vectashield. Negative control samples were included by omitting the primary antibody incubation step, samples remained in blocking buffer for the duration of the primary antibody incubation. Image acquisition was completed using a Zeiss 880 confocal microscope. Laser settings were adjusted to the negative control sample to minimise background staining.

2.8.1.4. Optimisation of antibody concentration

To determine optimal primary antibody concentrations (outlined in Table 2.1) titration experiments were conducted to determine the dilution that provided the best positive antibody staining with minimal background. Titration experiments were performed using Alvetex® full thickness intestinal models that had been paraffin fixed due to their higher levels of background staining. A series of antibody dilutions were assessed based on the product datasheet provided ranging from 1:50 to 1:500 and incubating samples in primary antibody overnight at 4 °C. Staining was conducted following the protocol outlined in Section 2.8.1.2.

Table 2.1. Primary antibodies used for immunofluorescent staining.

Antibody Target	Dilution	Species	Supplier	Product Code
CD14	1:100	Rabbit	Abcam	Ab183322
CD11b	1:100	Rabbit	Abcam	Ab52478
CD68	1:100	Mouse	Abcam	Ab955
Claudin-1	1:100	Rabbit	Abcam	Ab15098
Claudin-2	1:100	Rabbit	Abcam	Ab53032
Collagen I	1:100	Rabbit	Abcam	Ab34710
Collagen III	1:50	Rabbit	Abcam	Ab7778
Collagen IV	1:50	Rabbit	Abcam	Ab6586
E-Cadherin	1:100	Mouse	BD Biosciences	610181
Fibronectin	1:100	Rabbit	Abcam	Ab23750
Ki67	1:100	Rabbit	Abcam	Ab16667
Occludin	1:100	Mouse	Santa Cruz	Sc-133256
Villin	1:100	Rabbit	Abcam	Ab130751
Vimentin	1:100	Mouse	Santa Cruz	Sc-6260
ZO-1	1:100	Rabbit	Invitrogen	402200
α -Smooth Muscle Actin (α -SMA)	1:100	Rabbit	Abcam	Ab5694
β -Catenin	1:100	Mouse	Abcam	Ab22656

Table 2.2: Secondary antibodies used in immunofluorescent staining.

Antibody Target	Dilution	Supplier	Product Code
Donkey anti-Mouse Alexa Fluor 594	1:1000	Invitrogen	A21203
Donkey anti-Rabbit Alexa Fluor 488	1:1000	Invitrogen	A21206
Goat anti-Mouse Alexa Fluor 594	1:1000	Invitrogen	A11005
Goat anti-Rabbit Alexa Fluor 488	1:1000	Invitrogen	A11034

2.8.2. Electron Microscopy

2.8.2.1. Fixation of models and embedding in resin

Models were removed from their inserts and washed in PBS before fixation in for 1 hour at room temperature in Karnovsky's Fixative: 2 % PFA, 2.5 % glutaraldehyde and 0.1 M Cacodylate Buffer pH 7.4 in deionised water. Samples were then washed 3 times in 0.1 M Cacodylate Buffer, pH 7.6, before post-fixing in 1 % Osmium Tetroxide (Agar Scientific). Samples were dehydrated through a series of ethanol solutions as follows: 50 %, 70 %, 95 % and 100 % EtOH for 3 lots of 5-minute incubation periods for each ethanol or 10 minutes in 100 %. Samples were then infiltrated with a 1:1 mixture of 100 % Ethanol: Propylene Oxide (Agar Scientific), followed by 100 % Propylene Oxide and then a 1:1 mixture of Propylene Oxide: Epon Resin (Agar Scientific)

for 15 minutes each. Models could then be placed in Epon Resin solution on a rotator 3 times for 1 hour each to allow the resin to fully infiltrate into the sample. Samples were embedded into moulds filled with Epon Resin and left overnight in an oven set at 37 °C. Once hardened, samples could be sectioned on a Leica UC6 ultramicrotome (Leica).

Semi-thin sections (1 µm) were placed on SuperFrost Plus slides and stained with Toluidine Blue O for ~2 minutes (Koch-Light Research Laboratories Ltd., Gauteng, South Africa). Slides were mounted in DPX (Agar Scientific) and imaged using a Leica DM500 microscope. Ultra-thin sections of 60-70 nm were placed onto Copper 150 mesh grids (Agar Scientific) and were stained with 1 % alcoholic uranyl acetate dihydrate (Ted Pella Inc., California, US) for 10 minutes followed by washing in deionised water and post-staining in lead citrate trihydrate (Ted Pella Inc.) for a further 10 minutes and further washing in deionised water. Samples were left to dry at room temperature for a minimum of 20 minutes and were imaged using a Hitachi H7600 transmission electron microscope (Hitachi High-Tech, Oxford, UK). Human tissue samples processed and imaged by Ariana Gonz  les-Hau.

2.8.3. Western Blotting

2.8.3.1. Sample preparation

Models were washed in ice-cold PBS and lysed using Mammalian Protein Extraction Reagent (M-PER, Thermo Fisher Scientific) with 1 % HaltTM Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were homogenised by vortexing for 10 seconds every 5 minutes for a 30-minute period before sonication for 30 minutes and centrifugation at 13 200 x g for 20 minutes at 4 °C. Supernatants from 3 technical replicate models were pooled together and stored at -20 °C until use.

Protein concentration was determined using a Bradford Assay according to manufacturer protocols. Briefly, 5 µL of each sample were incubated with 250 µL Bradford Dye Reagent (Bio-Rad, Oxford, UK) for 5-60 minutes and then samples were read at 600 nm using a BioTek ELx800 plate reader and the concentration of protein present in each lysate was determined using a standard curve of known BSA protein standards (Bio-Rad).

2.8.3.2. Polyacrylamide gel electrophoresis and tank electro-transfer

Protein lysates were diluted 4:1 with 90 % Laemmli Buffer (Bio-Rad), 10 % 2-Mercaptoethanol (Sigma-Aldrich) and heated to 95 °C for 5 minutes. Gel electrophoresis was performed by loading 10-30 µg protein into polyacrylamide gels (recipe in Appendix 1), cast using the Mini Protean Gel Casting System (Bio-Rad); 8 % gel for proteins up to 200 kDa in size and 10 % for proteins up to 120 kDa in size. Samples were then electrophoresed at 100 V for approximately 2 hours, until the protein bands had run off the gel.

Following SDS-PAGE, wet electro-transfer of proteins onto a nitrocellulose membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) was performed. 8 % gels were transferred at 15 V for ~16-20 hours then increased to 45 V for 2 hours at 4 °C, while 10 % gels were transferred at 250 mA for 2 hours at room temperature.

2.8.3.3. Membrane blocking and immunoblotting

Membranes were stained with Ponceau S (Sigma-Aldrich) for 5 minutes then washed in deionised water before being blocked in 5 % milk/ TBS-T (recipe in Appendix 1) for 1 hour at room temperature. Primary antibody diluted in 5 % milk was applied overnight at 4 °C. Optimal antibody concentrations were determined through a series of titration experiments based on manufacturers data sheets provided for each antibody; concentrations ranged from 1:200 – 1:10 000. The optimal concentration was selected based on the signal to noise ratio of the antibody. Following primary antibody incubation, membranes were then washed 3 times for 10 minutes in TBS-T and incubated in secondary antibody diluted in 5 % milk for 1 hour before again being washed 3 times for 10 minutes in TBS-T. Clarity Enhanced Chemiluminescence (ECL, Bio-Rad) was applied to the membrane and incubated for 5 minutes followed by membrane exposure with CL-Xposure™ film (Thermo Fisher Scientific).

Table 2.3: Primary antibodies used in western blotting.

Antibody Target	Dilution	Species	Supplier	Product Code
Collagen I	1:1000	Goat	Southern Biotech	1310-01
Collagen III	1:500	Rabbit	Protein-Tech	22734-1-AP
E-Cadherin	1:1000	Mouse	BD Biosciences	610181
GAPDH	1:1000	Rabbit	Abcam	Ab9485
MMP-1	1:1000	Rabbit	Protein-Tech	10371-2-AP
TIMP-1	1:5000	Rabbit	Abcam	AB211926
α-SMA	1:500	Mouse	Abcam	AB7817
β-Actin	1:5000	Mouse	Abcam	Ab8224

Table 2.4: Secondary antibodies used in western blotting.

Antibody Target	Dilution	Supplier	Product Code
Donkey anti-Goat HRP	1:10 000	Merck	AP18P
Donkey anti-Rabbit HRP	1:10 000	Merck	AP182P
Goat anti-Mouse HRP	1:10 000	Merck	A4416

2.8.4. Quantitative PCR

2.8.4.1. RNA isolation

The ReliaPrep™ RNA Cell Miniprep System (2D cells) and ReliaPrep™ Tissue Miniprep System (*in vitro* models) (Promega, Southampton, UK) was used to isolate RNA following manufacturer's instructions. To isolate RNA from 2D cell cultures, cells were trypsinised to a single cell suspension and washed in ice-cold PBS and

resuspended in BL lysis buffer with 2 % 1-Thioglycerol. For model RNA isolation, 3 technical replicates were pooled together and washed in ice-cold sterile PBS and placed into LBA lysis buffer with 2 % 1-Thioglycerol. Lysates were then vortexed for 10 seconds every minute for 5 minutes before sonication for 15 minutes followed by centrifugation at 13 000 x g at 4 °C and transferred to a clean Eppendorf tube. Following these initial RNA extraction processes, both 2D and 3D samples were processed in the same manner.

Lysates were then passed through a 20-gauge needle (Sigma-Aldrich), 4-5 times to shear the genomic DNA. 100 % isopropanol was added to lysates and samples were mixed by vortexing. Samples were then transferred to Minicolumns in collection tubes and centrifuged at 13 000 x g for 30-60 seconds at room temperature; washed with RNA Wash solution and further centrifuged at 13 000 x g for 30 seconds. 30 µL DNase I incubation mix: 24 µL Yellow Core Buffer, 3 µL 0.09 M MnCl₂ and 3 µL DNase I enzyme was added to each sample and incubated for 15 minutes at room temperature. Lysates were then washed with Column Wash solution and centrifuged at 13 000 x g for 15 seconds followed by two further washes in RNA Wash solution. Collection tubes and eluate were discarded and the Minicolumns were transferred to Elution tubes. Nuclease Free Water was added to the columns and centrifuged at 13 000 x g for 1 minute for RNA collection. RNA yield and quality were assessed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific) and samples were stored at -80 °C until cDNA synthesis.

2.4.4.2. cDNA synthesis

cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems, Thermo Fisher Scientific). A 20 µL reaction was used to generate 1 µg of cDNA with 10 µL 2X RT Master Mix and 10 µL of RNA template.

Table 2.5: Components used in 2X RT master mix kit per 20 µL reaction.

Component	Volume/Reaction (µL)
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
Multiscribe™ Reverse Transcriptase	1.0
Nuclease-Free Water	4.2

Table 2.6: Thermocycler settings used to reverse transcribe the cDNA.

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 minutes	120 minutes	5 minutes	∞

2.4.4.3. Quantitative PCR

Pre-designed KiCqStart® SYBR® Green primers were obtained from Sigma-Aldrich (Table 2.7) and were reconstituted at 100 µM in Tris-EDTA buffer solution (Sigma-Aldrich). A stock solution of each primer was created by combining 10 µL of each forward and reverse primer with 80 µL nuclease-free water. Prior to use in the PCR reaction, primer stock solutions were further diluted 1:1 in nuclease-free water to give a working solution. Melt curves were generated for each primer utilised and are shown in Appendix 2.

Table 2.7. Primer sequences used for PCR analysis of gene expression.

Primer Pair ID	Gene / Protein	Forward Primer	Reverse Primer
H_ACTB_1	<i>ACTB</i> / Actin	GACGACATGGAGAAAATCTG	ATGATCTGGGTCATCTTCTC
H_GAPDH_1	<i>GAPDH</i> / GAPDH	TCGGAGTCAACGGATTTC	CAACAATATCCACTTTACCAGAG
H_ABCB1_1	<i>ABCB1</i> / P-gp	AGTGAAAAGGTTGTCCAAG	AGTCTGCATTCTGGATGG
H_ABCC2_1	<i>ABCC2</i> / MRP2	AAATGGCTGATCTCCTTTGC	GATAGCTGTCCGTACTTTTAC
H_ABCG2_1	<i>ABCG2</i> / BCRP	AAAGCCACAGAGATCATAGAG	GATCTTCTTCTTCTTCTCACC
H_CLDN1_1	<i>CLDN1</i> / Claudin-1	TTGGCATGAAGTGTATGAAG	ACCTGCAAGAAGAAATATCG
H_CLDN2_1	<i>CLDN2</i> / Claudin-2	CTGAGGAATGACTACTTCCC	ATCTAGAAGACCCTGAATGGC
H_CDH1_1	<i>CDH1</i> / E-Cadherin	CCGAGAGCTACACGTTC	TCTTCAAATCAACTCTGCC
H_OCLN_1	<i>OCLN</i> / Occludin	GGACTGGATCAGGGAATATC	ATTCTTTATCCAAACGGGAG
H_TJP_1	<i>TJP1</i> / ZO-1	TTGTCTTCAAAAACCTCCAC	GACTCACAGGAATAGCTTTAG
H_CD14_1	<i>CD14</i> / CD14	GATTACATAAACTGTCAGAGGC	TCCATGGTCGATAAGTCTTC
H_CD68_1	<i>CD68</i> / CD68	GTAAGTGAACCCCAACAAAAC	ATGTAGCTCAGGTAGACCAAC
H_IFNG_1	<i>IFNG</i> / IFN-γ	GGTAACTGACTTGAATGTCC	TTTTCGCTTCCTGTTTTCAG
H_TNF_1	<i>TNF</i> / TNF-α	AGGCAGTCAGATCATCTTC	TTATCTCTCAGCTCCACG
H_IL1B_1	<i>IL1B</i> / IL-1β	CTAAACAGATGAAGTGCTCC	GGTCATTCTCCTGGAAGG
H_IL6_1	<i>IL6</i> / IL-6	GCAGAAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
H_IL8_1	<i>CXCL8</i> / IL-8	GTTTTTGAAGAGGGCTGA	TTTGCTTGAAGTTTCACTGG
H_IL10_1	<i>IL10</i> / IL-10	CGGTTTAATAAGCTCCAAGAG	ATCTTCATTGTCATGTAGGC
H_TGFB1_1	<i>TGFB1</i> / TGF-β1	AACCCACAACGAAATCTATG	CTTTTAACCTGAGCCTCAGC

A 10 µL reaction using the SsoAdvanced™ Universal SYBR™ Green Supermix dye (Bio-Rad) was used to detect PCR products. 5 µL SYBR™ Green, 1 µL primer working solution, 20 ng cDNA were loaded into Hard-Shell PCR plates (Bio-Rad) and topped up to 10 µL with nuclease-free water. The following conditions were applied to the Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad) for 40 cycles:

Table 2.8. Bio-Rad CFX Connect qPCR Settings

	Initial Denaturation	Denaturation	Annealing and Extension
Temperature (°C)	95	95	55
Time (s)	30	15	15

Data was normalised to Actin or GAPDH and analysed using the $\Delta\Delta C_t$ method. Due to the low numbers of replicates (usually $n=6$ from 2 independent experimental occasions each with 3 technical replicates per PCR plate), no statistical analysis was performed on qPCR data.

2.8.5. Flow Cytometry

A single cell suspension was produced as previously described and cells were washed 3 times in PBS before being resuspended in 0.1 % bovine serum albumin (BSA, Sigma-Aldrich) in PBS blocking buffer. Cells were counted using a haemocytometer and 0.2×10^6 cells were added to wells in a round-bottomed 96-well plate (Sigma-Aldrich). Cells were then centrifuged at $163 \times g$ for 3 minutes at 4 °C and resuspended in 50 μ L of primary antibody diluted in blocking buffer. Cells were left for 1 hour on ice in primary antibody before washing 3 times in blocking buffer and resuspending in 50 μ L of secondary antibody. The plate was incubated for 1 hour on ice, in the dark before washing 2 more times. Cells were finally resuspended in 200 μ L blocking buffer and the plate was analysed using a Guava Flow Cytometer.

Table 2.9: Primary antibodies used in flow cytometry. Optimal primary antibody concentration determined using a series of titration experiments (1:50 – 1:1000) based on the individual product data sheet.

Antibody Target	Dilution	Species	Supplier	Product Code
CD 14	1:100	Rabbit	Abcam	Ab183322
CD11b	1:100	Rabbit	Abcam	Ab52478
CD68	1:100	Mouse	Abcam	Ab955
CD15	1:1000	Mouse	Abcam	Ab20137

Table 2.10: Secondary antibodies used in flow cytometry.

Antibody Target	Dilution	Supplier	Product Code
Donkey anti-Rabbit Alexa Fluor 488	1:100	Invitrogen	A21206
Donkey anti-Mouse Alexa Fluor 594	1:100	Invitrogen	A21203

2.4.5.1. Flow cytometry controls

A series of control wells was included in each flow cytometry experiment. A negative control well containing cells only with no primary or secondary antibody solution was included to visualise any autofluorescence produced by the cells themselves and to visualise the entire cell population. Additional negative controls included wells with secondary antibody only; during the primary antibody incubation period, cells within these wells remained in blocking buffer to again check for autofluorescence. Species specific IgG isotype controls were also utilised to identify non-specific binding interactions with cellular proteins and lipids. IgG was applied to samples during the primary antibody incubation, the secondary antibody step was carried out as normal. No difference was observed between the fluorescence levels of isotype and secondary antibody only controls.

2.4.5.2. Flow cytometry data analysis and gating

Raw data was first gated to exclude cellular fragments, clumps, and debris by utilising the forward and side scatter of the cells in an unstained well (Figure 2.11). Species-specific sera were then used to assess non-specific primary antibody binding and thereby gate for positive antibody binding. Data could then be expressed as % gated to quantify the proportion of positive cells.

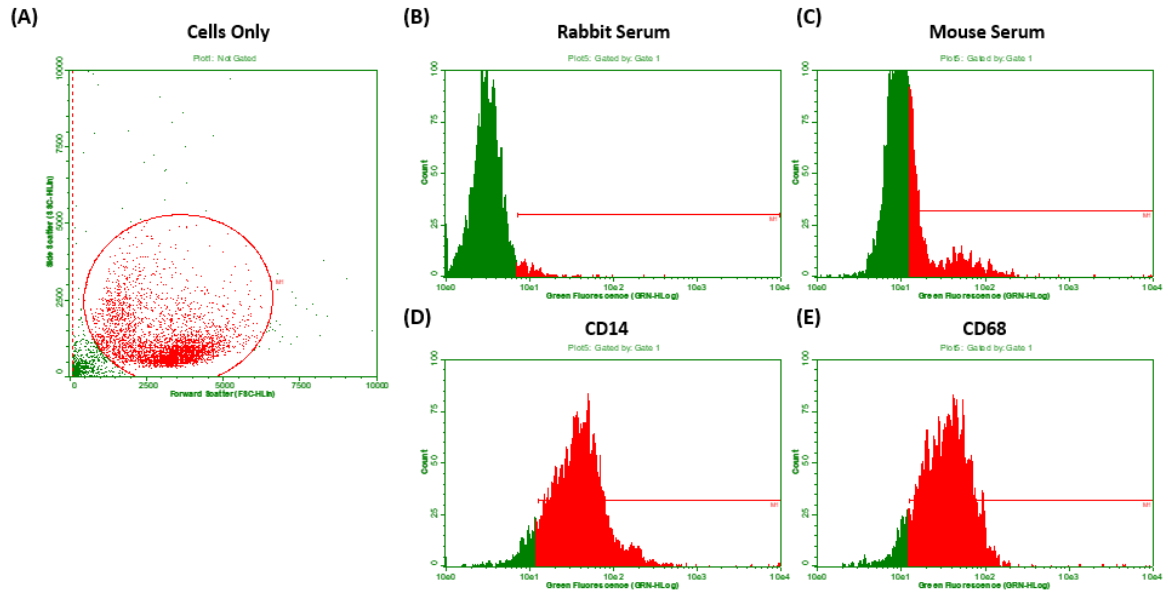


Figure 2.11. Representative control settings and antibody histograms from flow cytometry analysis. The living cell population, contained within the circle, was gated using the scatterplot (A). Representative rabbit (B) and mouse (C) serum control histograms were then gated to allow for the selection of positively stained cell populations. Example images of positively stained CD14 and CD68 rabbit (D) and mouse (E) antibodies respectively are shown for reference.

2.5. Biometric Analysis Using Fiji

2.5.1. Quantification of Ki67 Positive Cells

HDFn fibroblasts were seeded onto glass coverslips as described in Section 2.1.5 and were cultured for 4 days in either complete M106 or complete DMEM growth media with one media change at day 2 of culture. Cells were then fixed in 4 % PFA and immunofluorescent staining was performed using the protocol described in Section 2.8.1.1 for the proliferation marker Ki67 and counterstained with Hoechst to visualise nuclei. 10 images were taken using a Zeiss Apotome (Zeiss) microscope from random points on the coverslips per culture condition for a total of 3 independent test repeats; 30 cells were counted for each of the 3 test repeats, giving a total $n=90$. The total number of fibroblast cells were counted based on the expression of Hoechst and the number of Ki67-positive cells were counted in each image using the multi-point tool in the imaging processing software, Fiji (Figure 2.12). The percentage of Ki67-positive cells was determined.

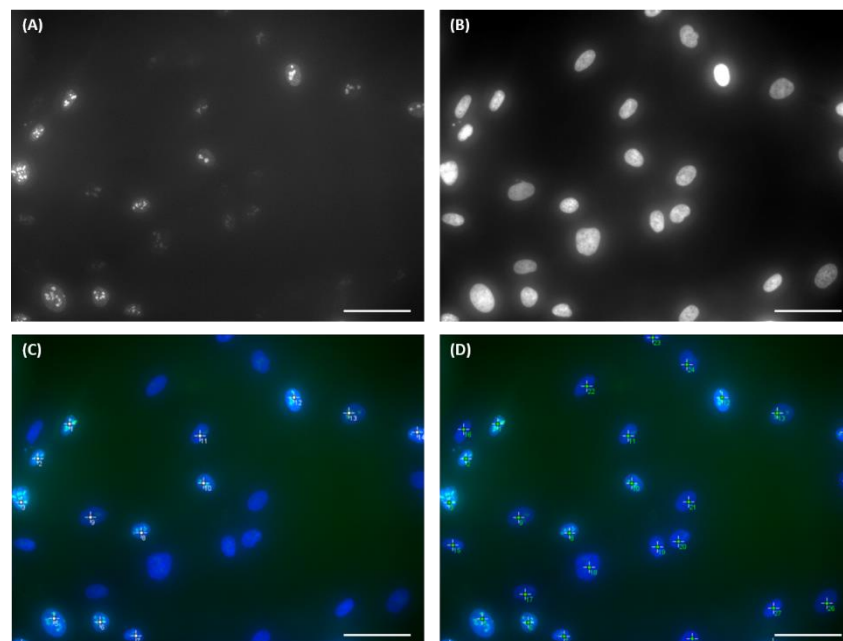


Figure 2.12. Quantification of Ki67-positive cells. HDFn cells were stained for the proliferation marker Ki67 (A) and nuclei were counterstained with Hoechst (B). The number of Ki67-positive cells was counted using the multipoint tool in Fiji (C) and the total number of cells in each image was also quantified (D) to determine the percentage of Ki67-positive cells. Split channel images are depicted in A and B while merged images with the associated multipoint tool used to count cells are depicted in C and D. Representative image of HDFn maintained in DMEM shown from 10 images of 3 different cultures. Scale bar: 100 μm .

2.5.2. Fibroblast Layering Measurements

H&E-stained images of HDFn cells cultured in Alvetex[®] Scaffold were used to measure the level of fibroblast layering on the base and surface of the Alvetex[®] membrane. Representative images of a total of 9 different models set up over 3 days were utilised in biometric analysis. Fiji software was used to set the scale and the straight-line tool was used to measure the height of cell layering from the edge of the scaffold (Figure 2.13). A total of 50 measurements were taken regularly along the entire length of the models from 3 random images along each of the 9 models.

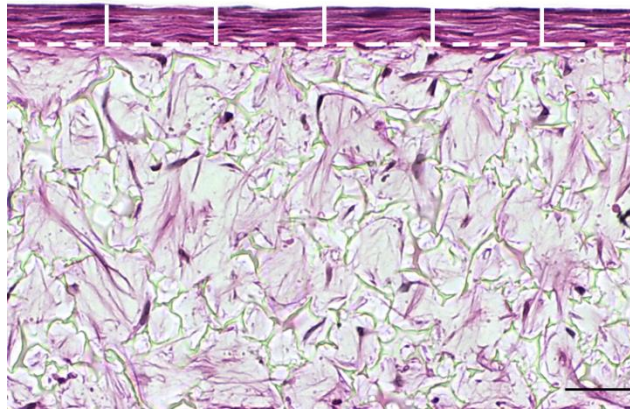


Figure 2.13. Biometric analysis of fibroblast layering. The edge of the Alvetex® Scaffold was traced with a dashed line and measurements of the fibroblast layering thickness were taken at regular intervals across each image as depicted by the white vertical lines. Representative image of a 28-day dermal fibroblast lamina propria equivalent, from a total of 9 different models. Scale bar: 50 μm .

2.5.3. Morphometric Analysis of Epithelial Cells

TEM images of Transwell® Caco-2 monolayers, Caco-2 HDFn co-cultures and human intestine tissue samples were used to perform biometric analysis of the epithelial cell shape (Figure 2.14). Images of human tissue used in analysis were kindly provided by Ariana Gonz  les-Hau. Fiji software was then used to set the scale before the straight-line tool was used to take measurements of the height and width of the samples from a minimum of 3 different areas on the same cell and an average value was determined per cell. Additional measurements were taken regarding the length of the epithelial lateral membrane which was carefully traced and measured using the Fiji freehand line tool. Ratios were determined for epithelial cell height: width and lateral membrane length: epithelial cell height for each individual cell measured. A total of 7-9 cells were measured for each sample.

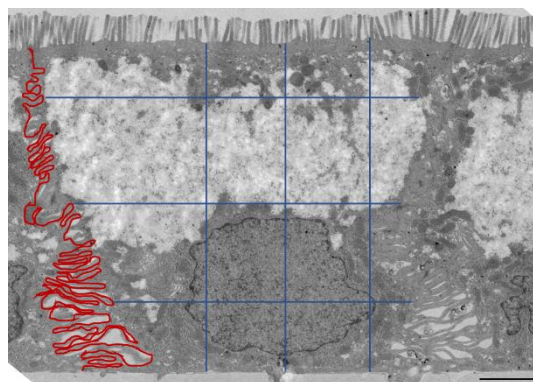


Figure 2.14. Biometric analysis of epithelial cell structure. Fiji software was used to measure the height and width of the epithelial cells from TEM images (blue lines) and to carefully trace and measure the length of the lateral membrane (red lines). Representative image of a Caco-2 cell in a Transwell® monolayer depicted. Scale bar: 2 μm .

2.6. Assessment of Epithelial Cell Function

2.6.1. Assessment of Epithelial Barrier Function

2.6.1.1. Transepithelial electrical resistance measurements

Barrier integrity was measured throughout epithelial cell culture by measuring the Transepithelial Electrical Resistance (TEER) across the cells (Figure 2.15). TEER measurements were taken using a Millicell ERS-2 Volt-Ohm Meter (World Precision Instruments, Hertfordshire, UK) using chopstick probes with silver/silver chloride pellets on the electrode tip. MERSSTX01 electrodes (World Precision Instruments) were used to measure TEER of cells cultured on Transwell® inserts while, a “bent-leg” probe created by the Department of Engineering at Durham University was used to measure the TEER of cells cultured on Alvetex® inserts. A total of 3 measurements were taken and averaged for each individual model and the total number of models measured outlined in individual figure legends.

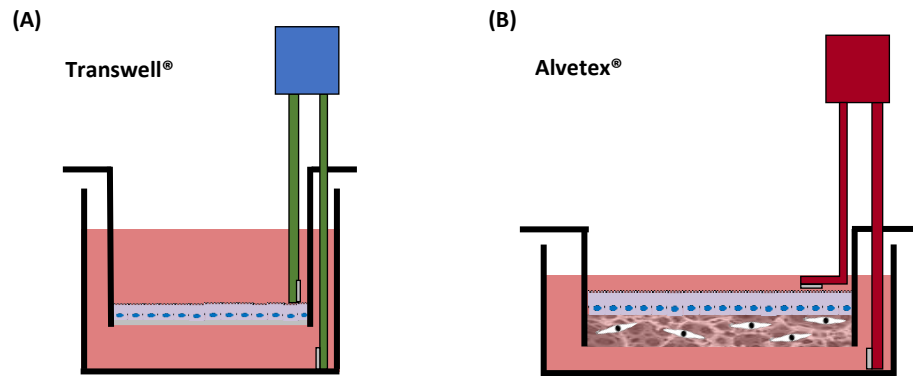


Figure 2.15: TEER probes used to measure epithelial electrical resistance across the Caco-2 monolayers. TEER measurements are taken by chopstick probes with silver/silver chloride pellets; the long leg, touches the cell culture plastic while the shorter leg rests above the surface of the epithelial cells. Transwell probes have two straight legs (A) and Alvetex® probes have a shorter “bent-leg”.

To calculate the resistance of the epithelial cells, the following equations were used:

Transwell®:

$$TEER (\Omega.cm^2) = (\Omega \text{ Cell Layer} - \Omega \text{ Blank Insert}) \times \text{Insert Surface Area } cm^2$$

Alvetex®:

$$TEER (\Omega.cm^2) = (\Omega \text{ Cell Layer} - \Omega \text{ Fibroblasts Only}) \times \text{Insert Surface Area } cm^2$$

The resistance of a blank Transwell® insert was subtracted from the Transwell® cultures while the resistance of fibroblasts cultured in Alvetex® was subtracted from the Alvetex® cultures. The resulting values were then multiplied by the surface area of the insert.

2.6.1.2. Lucifer yellow rejection assay

To measure barrier integrity a lucifer yellow rejection assay was carried out. Models were washed three times in PBS warmed to 37 °C then 100 µM of lucifer yellow dipotassium salt (Sigma-Aldrich) diluted in Hanks Balanced Saline Solution (HBSS, Sigma-Aldrich) was added to either the apical or basolateral compartment of an insert.

Table 2.11: Transport assay volumes. Volumes of HBSS added to the apical and basolateral sides of models during a typical transport assay.

	Apical (µL)	Basolateral (mL)
Transwell®	400	1.2
Alvetex® Scaffold	400	1.5

Models were incubated for an hour at 37 °C in a humidified chamber on an orbital shaker at 50 rpm for 1 hour before duplicate 100 µL samples were taken from both the apical and basolateral sides of the inserts and transferred to a 96-well plate. The plate was read at 485/535 nm excitation/emission using a BioTek Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Thermo Fisher Scientific). Lucifer yellow rejection was calculated using the following equation using average fluorescence readings from the duplicate samples:

$$Rejection (\%) = 100 \times \left(1 - \left(\frac{Receiver}{Donor} \right) \right)$$

Models with over 90 % rejection of lucifer yellow were considered to have an intact barrier.

2.6.2. Assessment of Drug Transport

Transport assays were performed between days 21 and 29 of epithelial cell culture in the same manner as described above for lucifer yellow for 1-2 hours. To inhibit P-glycoprotein activity when conducting rhodamine 123 transport assays, 100 µM Verapamil hydrochloride (Abcam) was added to HBSS.

Table 2.12: Test compounds used in transport assays.

Chemical Compound	Final Concentration	Excitation/Emission Wavelength	Supplier
Fluorescein isothiocyanate-dextran (FD)-20	1 mg/mL	480/530	Sigma-Aldrich
Fluorescein isothiocyanate-dextran (FD)-4	1 mg/mL	480/530	Sigma-Aldrich
Lucifer yellow	100 µM	485/535	Sigma-Aldrich
Rhodamine 123	5 µM	511/534	Sigma-Aldrich

Duplicate 50 µL samples were taken from the receiver compartments prior to starting and following the incubation. Duplicate sample readings were averaged, and the apparent permeability coefficient was determined by the following equation:

$$P_{app} (cm/s) = \left(\frac{dQ}{dT} \times V_r \right) / (C_0 \times A)$$

Where dQ/dT is the rate of transport of a compound, V_r is the volume of the receiver compartment, C_0 is the initial compound concentration at $T = 0$ and A is the surface area (cm^2).

2.7. Assessment of Immune Cell Function

2.7.1. Stimulation of 2D Cells and *In Vitro* Models

Lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) serotype O55:B5 was purchased from Sigma-Aldrich and used at 0.1, 1 and 10 µg/mL. Recombinant human interferon-gamma (IFN-γ) was purchased from PeproTech (London, UK) and was used at 10 and 100 IU/mL. Recombinant human tumour necrosis factor α (TNF-α) was purchased from R&D Systems (Minneapolis, USA) and used at 10, 50 and 100 ng/mL. All solutions were reconstituted in sterile PBS with the addition of 0.1 % bovine serum albumin (Sigma).

The cell culture media of all cells and models was replaced with fresh media 24 hours prior to stimulation. Unstimulated control cells and models received a vehicle control with sterile PBS. Effector molecules (LPS, IFN-γ and TNF-α) were added directly to the culture media of 2D cultured cells at the desired concentration. For Transwell®-based co-cultures, compounds were added to the basolateral media (1.5 mL) unless otherwise specified. Alvetex®-based models were normally cultured and maintained in 6-well plates, however, to stimulate the models, they were transferred to 12-well plates. This was achieved by snapping off the three arms of the apical insert holder and transferal with sterile forceps. 2 mL of complete DMEM was then added basolaterally and 0.4 mL was added apically. Unless otherwise stated all stimulants (LPS, IFN-γ and TNF-α) were added to the media of the basolateral compartment, while the apical media remained complete DMEM. Cells and models were incubated at 37 °C and 5 % CO₂ in a humidified environment; incubation periods were generally either 24, 48, or 72 hours unless otherwise stated.

2.7.2. Phagocytosis Assay

A Vybrant™ phagocytosis assay (Thermo Fischer Scientific) was performed according to manufacturer's instructions. Briefly, a fluorescent *E. coli* BioParticle suspension was prepared by thawing one vial of fluorescent particles and one vial of concentrated HBSS followed by brief sonication and resuspension in 4.5 mL dH₂O. In addition, a vial trypan blue solution was thawed and transferred to a 4 mL water with sonication to homogenise the solution.

Differentiated U937 cells were passaged, and cells were resuspended to a final concentration on 10×10^6 cells/mL and 100 µL was placed into individual wells of a 96-well plate and a further 50 µL medium was added

to each well. Negative control wells were prepared by the addition of 150 μ L culture medium to wells. Plates were then incubated for 1 hour at 37 °C and 5 % CO₂ in a humidified environment to allow cells to adhere to the microplate wells. After incubation, culture media was removed from the wells and 100 μ L of fluorescent BioParticle suspension was added to all wells, including the negative control and the plate was incubated for a further 2 hours. Following the second incubation, the BioParticle solution was removed from the wells and 100 μ L trypan blue solution was added for 1 minute at room temperature. Trypan blue was then removed, and the plate was read at 480/520 nm excitation emission using a BioTek Synergy™ H4 Hybrid Multi-Mode Microplate Reader.

2.7.3. Human Cytokine Array

To quantify the levels of proinflammatory cytokines produced following model stimulation, the cell culture media of models was collected and transferred to EveTechnologies (<https://www.evetechнологies.com/> Calgary, Canada) for analysis.

2.7.3.1. Collection of cell culture media

Following the appropriate incubation period of model stimulation, the cell culture media was collected in 1.5 mL Eppendorf tubes (Thermo Fisher Scientific). Media was then passed through a 0.2 μ m filter (Thermo Fisher Scientific) and centrifuged at 12 000 x g for 20 minutes at 4 °C. The supernatant was then collected in a fresh 1.5 mL Eppendorf tube and samples were stored at -80 °C.

2.7.3.2. Analysis of samples by EveTechnologies

Media samples were shipped to EveTechnologies in dry ice and the human cytokine array / chemokine array 48-plex (HD48) was conducted to quantify the concentration of proinflammatory cytokines and chemokines. Samples were ran in duplicate via bead-based multiplex technology also known as addressable laser bead immunoassay (ALBIA). A sample of fresh, complete DMEM was analysed as a control and no cytokines were detected within the media alone.

2.8. Statistical Analysis

GraphPad Prism 5 software was used to assess the statistical significance of results and the relevant statistical test was used depending on the dataset. Data was checked for normality by creating box and whisker plots and the Shapiro-Wilk test was used to confirm Gaussian distribution, a P value greater than 0.05 was considered to be normally distributed data. The type of statistical test performed was stated within each experiment, with statistical significance between groups highlighted on graphical figures.

2.8.1. Analysis of Normally Distributed Data

Normally distributed data (Figure 2.16) was analysed by either Student's unpaired t-test when two independent experimental groups were directly compared, and one-way and two-way analysis of variance (ANOVA) were used to compare multiple data sets. Following one-way ANOVA, Tukey's post-test was used to directly compare the mean of each sample to the mean of every other sample while Dunnett's post-test was used to compare the mean of each sample to the control sample only. Following two-way ANOVA, Bonferroni's post-test was used to compare the mean of each individual sample to all samples.

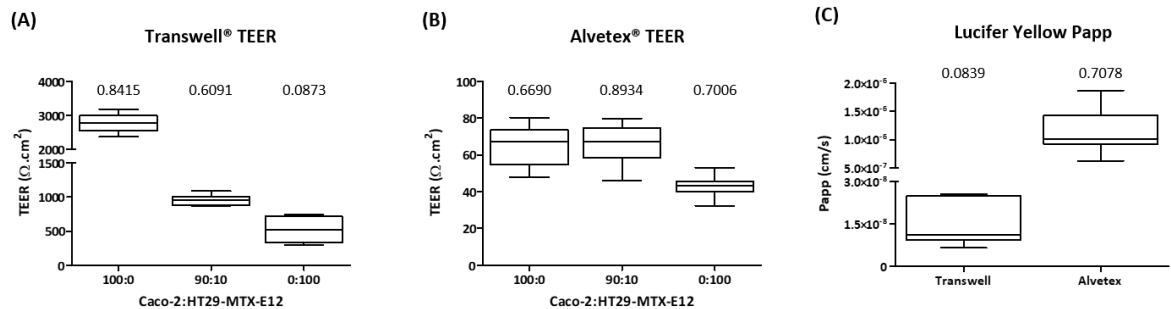


Figure 2.16. Box and whisker plots of normally distributed data. Examples of box and whisker plots are depicted of various parameters assessed within this thesis. The P value as determined by Shapiro-Wilk test is indicated above each column in every graph. TEER of Transwell® (A) and Alvetex® (B) co-cultures are shown. The apparent permeability coefficient of lucifer yellow in the apical-basolateral transport direction across Caco-2 monolayers maintained on the two different cell culture platforms is also depicted (C). Values are taken from a total of 9 models ($n=9$) that were established over 3 different experimental repeats, i.e., 3 models were established per experimental set up.

2.8.2. Analysis of Non-Normally Distributed Data

Data that was not confirmed to be normal through box and whisker plots (Figure 2.7) or where replicate numbers were too low to calculate Gaussian distribution by the Shapiro-Wilk test, alternative statistical analyses were performed. To directly compare differences in the median between two independent variables, the Mann-Whitney test was used.

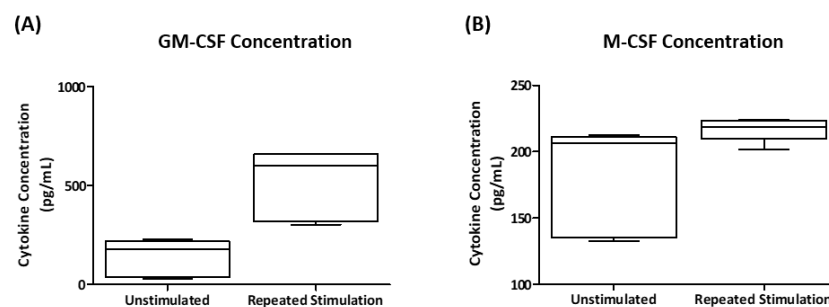


Figure 2.17. Box and whisker plots of non-normally distributed data. Examples of box and whisker plots are depicted of various parameters assessed within this thesis. The Shapiro-Wilks test could not be used to calculate normal distribution due to lower numbers of replicates assessed. Example data includes the concentration of GM-CSF (A) and M-CSF (B) as determined by the ALBIA assay conducted by EveTechnologies. A total of 6 media samples were analysed by the ALBIA assay from 3 separate experimental repeats of models conducted over 3 different days.

2.8.3. Replicate Numbers

Replicate numbers for each experiment are outlined in the experimental design section of each chapter. The total n number defines the total number of all repeats analysed per experiment. Two HDFn isolates from different individuals were utilised throughout the work as biological repeats, however all other cells are of cancerous origin, thus no true biological repeats were possible. Therefore, experiments were conducted 2-3 times independently utilising cells of different passage numbers (within the range specified for each cell line as outlined in Section 2.1.1). Within each independent experiment, a minimum of 3 models or cultures were established to provide intra-experimental technical repeats.

2.9. Human Tissue

Human intestinal tissue samples were collected by Reprocell Europe under appropriate ethical protocols in compliance with local laws and regulations that were reviewed and approved by the West of Scotland Research Ethics Committee (REC Ref:17/WS/0049). All tissue donors provided informed written consent. Tissues were received at Durham University under a formal material transfer agreement and were processed following relevant UK Human Tissue Act rules and guidelines and according to protocols outlined elsewhere within this chapter.

Chapter 3: Development of a 3D Intestinal Mucosa Co-Culture Model

3.1. Introduction

The development of *in vitro* models that mimic the structure and function of the intestine mucosa is of vital importance to the drug development process. Cell culture-based models are frequently used within the pharmaceutical industry to characterise the transport of new chemical entities (NCEs) before testing in animal models and human clinical trials. The most predominantly used *in vitro* model is the Caco-2 monolayer (discussed further in Sections 1.5.2. and 3.1.1.), however this system does not accurately recreate the structure and function of the intestine and can provide inaccurate results regarding the absorption properties of chemicals being tested. Therefore, new *in vitro* models are required that provide better predictive validity of NCEs to bridge the gap between *in vitro* models and animal testing and thus reduce the use of animals in this process.

3.1.1. The Gold Standard Caco-2 Monolayer

As discussed in Chapter 1, Caco-2 monolayers on Transwell® are considered to be the gold standard *in vitro* intestinal model for use in drug permeability studies. This is due to their simplistic nature and ease of use, while phenotypically monolayers express apical tight junctions, brush-border enzymes and transporter proteins that are characteristic of absorptive small intestinal enterocytes. However, Caco-2 monolayers have numerous drawbacks including differences in transporter protein expression and activity when compared to the *in vivo* intestine and a labour-intensive culture period. Caco-2 models have wide inter- and intra-lab variability due to several differences in culture protocols, from seeding density to alterations in media composition and feeding routines (Figure 3.1) (342). Cell age is known to have extensive consequences on the function of Caco-2 cells. For example, TEER and both passive transcellular and paracellular permeability have been demonstrated to widely vary with passage number (343,344). It is thought that long-term culturing of Caco-2 cells results in genetic drift and selection of specific Caco-2 subpopulations that have extensive variations from the parental cell line (344).

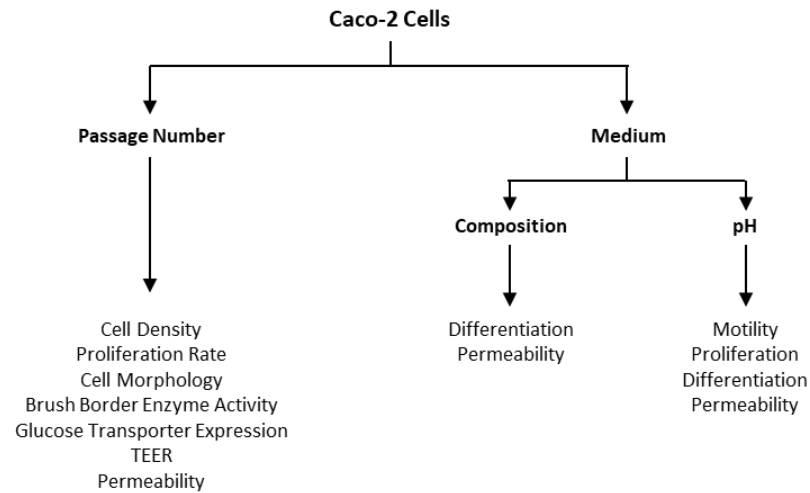


Figure 3.1. Factors affecting the performance of Caco-2 cells. Passage number and the culture media have far-reaching consequences on the function of Caco-2 monolayers. Adapted from Sambuy et al., 2005.

One of the major limitations of the Transwell® culture system stems from its reductionist approach, resulting in a 2D-like culture of the epithelial layer that does not represent the dynamic and complex microenvironment that cells reside in within the *in vivo* tissue (Figure 3.2). Recent advances in intestinal tissue engineering have sought to introduce additional cell types to the system or utilise more complex tissue culture substrates.

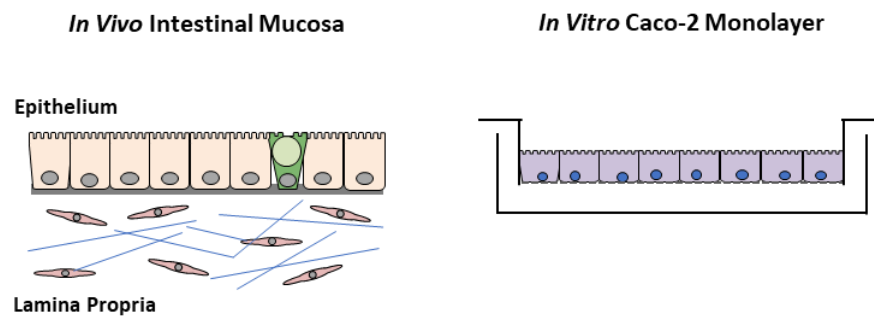


Figure 3.2. Caco-2 monolayers are a reductionist approach to intestinal tissue engineering. Cells *in vivo* exist in a complex 3D microenvironment with multiple different cell types and ECM. When Caco-2 cells are cultured *in vitro* they are grown in 2D-like conditions; a monolayer of cells that tend to flatten and only have contact with the same cell type.

3.1.2. Introduction of Cell-Matrix Interactions

One of the simpler methods of introducing complexity to an *in vitro* intestinal model is through the use of more complex biological scaffolds that facilitate cellular interactions with an ECM component. Biological scaffolds include hydrogels, silk protein scaffolds and decellularized small intestinal submucosa (SIS) scaffolds. Although widely used, one of the main disadvantages of biological scaffolds is that they are of animal origin due to lack of human tissue availability, which may introduce interspecies variation.

3.1.2.1. Hydrogels

Hydrogels are frequently used in intestinal tissue engineering. Hydrogels are hydrophilic polymer networks that are swollen with water and can be manipulated to introduce 3D intestinal geometry into the culture

system to more accurately represent the architecture of the *in vivo* small intestinal tissue (345). One of the most commonly used hydrogel constituents is type I collagen as it is abundant *in vivo* and is easy to extract and gel (346). Culture of Caco-2 cells on a collagen gel fabricated to accurately recreate the density and height of intestinal villi displayed a higher growth rate and a more *in vivo*-like barrier function (347,348). Absorption of the paracellular compounds fluorescein and dextran was higher in 3D models compared to Transwell® equivalents due to a decrease in tight junction protein expression. Also observed was a decrease in rhodamine 123 efflux, a substrate of P-gp that is typically overexpressed in monocultures (348). Together, these results highlight the importance of recreating a 3D architecture and the communication of cells with ECM proteins in tissue engineering to recapitulate the structure and function of *in vivo* tissues more accurately.

However, type IV collagen, not type I, is the predominant collagen in the intestine basement membrane and it is this that the epithelium is in direct contact with. Hydrogels rarely consist of type IV collagen as its properties are not conducive to gelling. Other constituents of note in the basement membrane are laminin and fibronectin. To study the effects of laminin and fibronectin on Caco-2 cell growth, Wang and colleagues developed a type I collagen scaffold pre-incubated with either laminin or fibronectin in a crypt-like topography. Although no permeability studies were performed, they demonstrated that gels containing fibronectin enhanced Caco-2 cell spreading, while laminin containing scaffolds induced differentiation (349). Other studies have shown that Caco-2 cells cultured on pure collagen are highly proliferative and motile compared to those maintained on fibronectin films and exhibited a more cancerous-like phenotype (350). Such studies emphasise the need for scaffolds containing the appropriate ECM constituents for *in vitro* cultures.

In addition to the limitations discussed above hydrogels have other drawbacks. Natural hydrogels such as Matrigel are poorly defined and have batch to batch variability, limiting the reproducibility of studies as they cannot be fully standardised. Furthermore, hydrogels may contract and degrade after polymerisation and alter the physical properties and structure of the scaffold

3.1.2.2. Decellularised scaffolds

Small intestinal submucosa (SIS) scaffolds are an additional biological scaffold that are typically of animal origin and the most frequently used are porcine jejunal segments in combination with a hydrogel coating. Culture of Caco-2 cells on porcine SIS permits cells to grow along crypt-villi structures and induces morphological changes in the cells. In various studies, Caco-2 cells exhibited an accelerated growth and differentiation on SIS scaffolds and a more polarised columnar phenotype reminiscent of enterocytes *in vivo* concurrent with an increase in the passive permeability of tested compounds (325,326). Culture of Caco-2 monolayers on SIS scaffolds has proved to be useful in the study of host-pathogen interactions, for instance, Caco-2 cells on Transwell® and SIS scaffolds displayed different phenotypes upon infection with *Campylobacter jejuni* (*C. jejuni*); SIS scaffolds highly resembled animal models indicating they could be an invaluable tool in studying further pathogenic bacterial interactions (351). Furthermore, SIS scaffolds have

been shown to support the growth and differentiation of small intestinal organoid cultures when used in co-culture with fibroblasts, and have the potential to be used in xenografts and regenerative medicine studies (352–354). Significant drawbacks to using SIS scaffolds remain, namely the harsh decellularisation processes of such scaffolds can disrupt the ECM composition which can result in rapid degradation and heterogeneity between scaffolds (327,328). In addition, such culture systems are not fully humanised, therefore have elements of interspecies variation within the models.

3.1.2.3. Inert scaffolds

Although they do not recreate cell-ECM interactions, the use of inert materials as scaffolds for intestinal tissue engineering is an attractive alternative to biological scaffolds as they contain no exogenous proteins of animal origin. Basement membrane-like structures have been successfully fabricated using electrospun nanofibers; culture of Caco-2 cells using such nanofibers reduced TEER values to levels similar to those in *ex vivo* porcine tissue. Additionally, absorption properties were generally improved over traditional Transwell® cultures and had a greater *in vivo* correlation for the chemical compounds lucifer yellow, dextran and rhodamine 123 (329). Evidence from models utilising inert scaffolds stresses the importance of recreating simplistic *in vivo*-like structures and not just biochemical interactions.

3.1.3. Epithelial-Stromal Cell Co-Cultures

In addition to cell-ECM interactions, another important consideration when developing novel *in vitro* intestinal models is the inclusion of other cell types to mimic cell-cell interactions experienced *in vivo*. This discussion will focus on *in vitro* co-cultures using epithelial cells and stromal cells. Co-cultures with different epithelial cell types will be discussed further in Chapter 4 and co-cultures with immune cells will be discussed in Chapters 5 and 6.

Numerous techniques have been used to investigate the effects of fibroblasts on intestinal epithelial cells. Indirect co-cultures have proved useful for studying the growth and differentiation of IECs while also identifying novel signalling compounds involved in the regulation of cell behaviour. Other studies utilise direct co-cultures of epithelial cells and fibroblasts. Direct co-culture models have a wide range of applications from studying the *in vivo* microenvironment, to wound healing experiments as well as permeability and toxicity studies. However, one of the most prevalent uses of direct co-cultures using cancerous epithelial cell lines such as Caco-2, is the study is migration of cells and ECM-breakdown to investigate cancer growth and metastasis, although this will not be covered herein.

3.1.3.1. Indirect co-culture techniques

The simplest of co-culture techniques typically employ paracrine methodologies: conditioned medium from fibroblasts or indirect co-culture of fibroblasts and epithelial cells. Such techniques have provided invaluable insights into growth factors involved in epithelial cell growth, differentiation, and maintenance. For example, hepatocyte growth factor from both CCD-18co intestinal fibroblasts and normal skin explant fibroblasts

stimulated proliferation of Caco-2, T84 and IEC-6 intestinal epithelial monolayers. This was demonstrated using conditioned medium from fibroblasts as well as indirect co-culture techniques where epithelial cells are cultured on Transwell® inserts and fibroblasts are seeded onto the base of the well that the insert rests in (355). Further studies using conditioned medium and indirect co-culture techniques of CCD-18co, neonatal dermal fibroblasts or lung fibroblasts with Caco-2 cells indicated that keratinocyte growth factor also stimulated proliferation and differentiation of the intestinal epithelial cells (356).

3.1.3.2. Direct co-culture models

One of the initial studies that involved direct co-culture of fibroblasts and intestinal epithelial cells was performed primarily using animal cell lines. Rat2 fibroblasts were embedded in a range of collagen gels and different epithelial cell lines (IEC-6 – rat, IPI-21 – boar, CRL2102 – human) were seeded on the surface (357). Epithelial cells grew in homogenous layers and exhibited morphological similarities to intestinal tissue. Based on this initial work, various other groups have successfully co-cultured human stromal and intestinal epithelial cells. One study involved the culture of Caco-2 cells on nanofilms with 1 or 8 layers of normal human dermal fibroblasts (358). The experiments demonstrated that Caco-2 cells had enhanced differentiation and tight junction protein expression when co-cultured with fibroblasts compared to monocultures. They also showed a switch in the predominantly expressed carboxylesterase (CE) from higher CE1 expression in Transwell® monolayers to higher CE2 expression in 3D co-cultures which more accurately reflects the expression patterns observed in the human small intestine (358).

Further studies have utilised stromal cells, Caco-2 cells, and the mucus-producing HT29-MTX cell lines. Using mouse embryonic fibroblasts embedded in a collagen gel on a Transwell® insert, Li and colleagues revealed that epithelial cells had a decreased TEER and P-gp expression and an improved correlation of compound permeability to the fraction absorbed in humans (313). A similar study using CCD-18co stromal cells in Matrigel more accurately predicted the apparent permeability coefficient of insulin than Transwell® monolayers (312). Other studies have taken a different approach and abandoned the use of Transwell® scaffolds completely. Through use of a silk scaffold protein, Chen et al. created a tube-like structure to recreate the hollow lumen of the intestine. They cultured human intestinal myofibroblasts embedded in a collagen gel on the silk scaffold and seeded Caco-2 and HT29-MTX cells on the surface. The epithelial lining accumulated mucus secreted by the HT29-MTX cells and induced a low oxygen tension that was shown to be more physiologically relevant and enabled additional co-culture with commensal gut bacteria (336).

3.1.3.2.1. Primary cell co-cultures

Not only have stromal cells been used in co-cultures with cancerous cell lines, but they have also enabled the culture of primary intestinal epithelial cells, a feat which is notoriously difficult to accomplish. Human organoids have been cultured on porcine SIS scaffolds in a perfused bioreactor; inclusion of subepithelial fibroblasts shortened the lengthy differentiation time of the organoids to 7 days and also stabilised the barrier integrity of the epithelium. Without the fibroblasts, the barrier was to be weakened (352). Further studies

have cultured human small intestine epithelial cells in the presence of growth factors and intestinal subepithelial myofibroblasts to recreate a crypt-like microenvironment for the epithelial cells. Absences of myofibroblasts resulted in diminished survival of the epithelial cells even in the presence of growth factors, emphasising the need for direct contact between different cell types to ensure efficient survival and function (56).

Bioprinting has been successfully employed to co-culture intestinal myofibroblasts and primary intestinal epithelial cell lines in a 3D architecture resembling the human intestine. Epithelial cells exhibited a polarised morphology with appropriate tight junction protein expression. Cytochrome P450 (CYP) enzyme expression and function was shown to be more *in vivo* like than Caco-2 monolayers which have wide variations in CYP450 expression compared to the human scenario. The bioprinting technique also demonstrated that co-culture of primary epithelial cells with stromal cells could replicate an injury response when exposed to chemicals that induced toxicity or inflammation (359). However, few co-cultures with primary cells have focused on the transport function of the epithelium.

3.1.4. TEER as a Measure of Barrier Integrity

Measurements of transepithelial electrical resistance (TEER) are used as a proxy to gather information on the uniformity of the monolayer and to quantify the dynamics of tight junction and epithelial barrier integrity. TEER reflects the conduct of ions through the paracellular pathway in epithelial monolayers and is generally considered to be a sensitive and reliable method to determine the epithelial integrity and confirm permeability of cultures (360).

Typically, in Caco-2 monolayers, TEER increases from the day of cell seeding and reaches a maximum and plateaus from approximately day 10-14 when a confluent monolayer of cells has formed. Caco-2 monolayers have exhibited TEER values that vary drastically from lab to lab depending on various factors in the culture set up, feeding regime and culture time. Generally, a leaky monolayer exhibits a TEER of 50-100 $\Omega \cdot \text{cm}^2$, an intermediate monolayer has a TEER of 300-400 $\Omega \cdot \text{cm}^2$, and a TEER of 2000 $\Omega \cdot \text{cm}^2$ and over is considered to be a tight barrier (360). Values typically reported are much higher than the *in vivo* intestine, which is stated to range from 40-100 $\Omega \cdot \text{cm}^2$ (6,245,246).

There are numerous other factors that affect TEER including medium composition, cell culture period, shear stress and temperature. To correct for temperature alterations, measurements are either performed on a hot plate set to 37 °C or are allowed to equilibrate to room temperature for a minimum of 20 minutes. A mathematical technique to correct for TEER related temperature changes has also been developed and shown to correlate directly with dextran flux (361). Cell passage number is another factor that greatly impacts the TEER of Caco-2 monolayers, though it is unclear whether TEER increases or decreases with age. For example, Yu and colleagues showed that as the passage number of Caco-2 cells increased, so too did the TEER (344,362). This was in agreement with a further study by Lu, yet Ranaldi demonstrated a decrease in TEER as

cells aged, while a further study indicated that TEER initially increased with passage number but after passage 60, started to decline (343,363,364).

TEER can be measured using Ussing chambers for tissue segments or for *in vitro* cultures, however their use for cell-based models are not widespread due to requirements for specialist equipment and complexities in the system set up (365). The preferred method for measuring TEER in *in vitro* models is through the use of chopstick probes. However, the chopstick technique is highly dependent on the position of the probes; they must be held at 90 ° and refrain from touching the cells as this may damage the monolayer (360). Probes also have limitations regarding scaffold size as they cannot accurately be used over large membranes (360); as such in this study, TEER measurements were taken from three different areas of the culture insert and an average value was calculated to ensure the full surface area of the membrane was covered.

Despite the many limitations, TEER continues to be used in GI models as it provides a non-invasive technique that can be continuously employed to monitor live cultures of cells during the growth and differentiation period and provides some insight into the tight junction dynamics and barrier integrity. TEER can also be used before and after drug permeability studies to ensure that the monolayer has not suffered any damage. However, in addition to the drawbacks discussed above, TEER cannot be used to quantify toxicity and cell viability. A study utilising chemical enhancers to drug absorption, indicated that enhancers induced toxic effects in Caco-2 cells, but this was not detected through TEER (366). Thus, caution must be used when stating that TEER is a measure of a healthy epithelial barrier.

3.1.5. Conclusions

While the Caco-2 monolayer provides an invaluable tool to examine the transport characteristics of novel drug compounds and for studying intestinal epithelial biology, it has numerous shortcomings that limit its ability to accurately recapitulate the *in vivo* small intestine. The colonic origin of the Caco-2 cell lineage predisposes the cells to form a more restrictive barrier than would naturally occur in the small intestine, ensuing an underestimation of the absorption of paracellular transported compounds.

Fundamental principles exist stating that structure is strongly related to function, consequently further improvements are required to improve the anatomical structure of the Caco-2 monolayer to generate models that better recreate the structure of the intestinal mucosa. Thus, combining the Caco-2 monolayer with stromal cell lineages that recreate the subepithelial compartment of the mucosal tissue has the potential to promote morphological changes in the Caco-2 epithelium and thereupon induce changes regarding their function.

3.2. Hypotheses and Aims

This chapter aims to optimise and characterise the development of a 3D intestinal co-culture model with a lamina propria compartment and an absorptive epithelial compartment to resemble the intestinal mucosa. It is hypothesised that Alvetex® will be a suitable platform for model development as it has been successfully used for the culture of several other *in vitro* mucosal models (330,331). It was further postulated that culture of fibroblasts within Alvetex® Scaffold will form a network of cells that secrete endogenous ECM proteins that recapitulate the lamina propria of the *in vivo* intestine. Finally, it was hypothesised that addition of absorptive epithelial cells, Caco-2, on the surface of the lamina propria equivalent would recreate the structure of the intestinal mucosa and with the potential to be used for permeability assays.

3.2.1. Objectives

- Characterise the structure of both the small and large intestine mucosal tissues.
- Recreate the gold standard Caco-2 monolayer and determine the suitability of Alvetex® Polaris as an alternative culture substrate for Caco-2 cells.
- Optimise the development of a lamina propria compartment through culturing HDFn cells in Alvetex® Scaffold and characterise the production of ECM.
- Optimise the co-culture of Caco-2 cells on the surface of the lamina propria equivalent.
- Characterise the structure and function of the 3D co-culture model in comparison to the Caco-2 monolayer, human tissue, and a population of intestinal fibroblasts; CCD-18co.

3.3. Experimental Design

The overarching aim of this chapter was to develop a 3D co-culture model of the human intestinal mucosa that recapitulated the enterocyte component of the epithelium and the lamina propria to enhance the cellular structure and therefore alter the function of the Caco-2 monolayer. Prior to the establishment of the co-culture system, the Caco-2 monolayer was initially re-created on Transwell® (Section 2.3.1) and Alvetex® Polaris (Section 2.5.1) membranes to provide control data for later comparison studies. Models were subject to structural analysis through histological staining and whole-mount immunostaining for components of the apical junctional complex. Additional functional assessment of the epithelial barrier properties was conducted via TEER, lucifer yellow rejection and assessment of the transport processes across the epithelium. A total of 9 models, that were set up over 3 different days, were analysed via each of these different methods.

To optimise the culture of a full-thickness intestinal mucosa model, Alvetex® Scaffold was employed as a platform to build the supporting lamina propria component. Fibroblasts of human dermal origin were initially established and maintained within Alvetex® Scaffold following previously established protocols (Section 2.4.1) (330). Media composition and maturation time of the lamina propria compartment was then further optimised to establish consistency in model feeding regimen throughout the full culture period and enhance model throughput respectively. A total of 9 lamina propria equivalents from 3 independent experiments set up over 3 days were analysed via H&E, immunostaining, and western blot. For western blot analysis, lysates of 3 technical replicates were pooled together, and 3 separate SDS-PAGE gels were carried out and analysed using lysates generated over 3 different days. Co-cultures of Caco-2 and HDFn cells (Section 2.5.1) were also assessed in the same manner as the lamina propria equivalents and were additionally evaluated functionally to characterise the properties of the epithelial barrier as described above. Like the previous models, 3 separate experiments were conducted with 3 models for each condition providing a total $n=9$.

To ensure that dermal fibroblasts were suitable for use within the model, Caco-2 Transwell® monolayers and Alvetex® co-cultures were compared structurally to both human tissue and an alternative Caco-2 fibroblast co-culture model via histological and immunofluorescent analysis. The alternative co-culture was established with intestinal-derived stromal cells known as CCD-18co, as such provided a useful comparison to demonstrate the similarity of the skin and intestinal fibroblasts with regards to ECM deposition *in vitro* and the ability to support an overlying epithelium. All co-culture models created with CCD-18co fibroblasts were established by Dr Matthew Freer, who kindly provided images for comparison (Section 2.4.2). Furthermore, Caco-2 monolayers and dermal fibroblast co-cultures were processed for TEM imaging and subject to in-depth biometric analysis ($n=7-9$, Section 2.5.3) of the morphology of the epithelial cells to highlight any structural alterations between the monoculture and co-culture epithelium. Samples were also compared to human compared to human tissue to determine if there was an improved structural correlation with co-culture epithelium and the *in vivo* intestine (human tissue samples kindly processed and imaged by Ariana Gonzáles-Hau).

3.4. Results

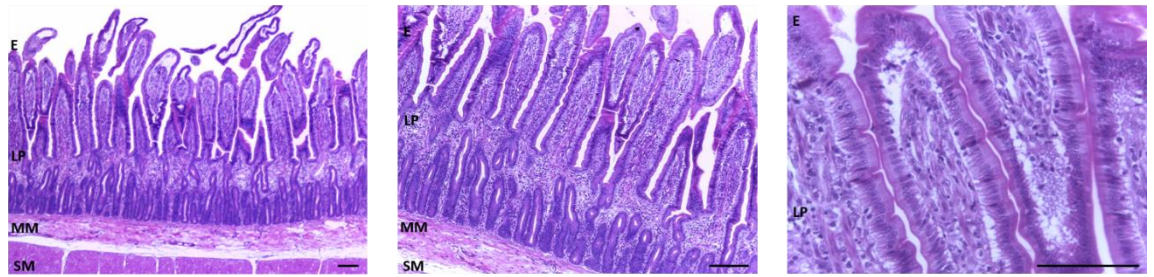
3.4.1. Characterisation of the Human Intestinal Mucosa

Structural analysis of healthy intestinal tissue samples was undertaken to characterise the gross morphology and ultrastructural anatomy of the small and large intestine mucosa. All small intestinal samples were kindly provided pre-stained by Professor Stefan Przyborski. Large intestine tissue samples were kindly provided as fresh tissues from Reprocell under a formal HTA agreement and were subsequently processed for wax embedding and TEM.

3.4.1.1. Histological characterisation of healthy intestine tissue

The overall morphology of the intestine mucosa is demonstrated through H&E staining and is similar between the small and large intestine (Figure 3.3). The mucosal layer consists of an epithelium and lamina propria that is separated from the underlying ECM-rich submucosa by the muscularis mucosa. Individually, epithelial cells in the small intestine have a densely stained apical membrane indicative of the presence of microvilli. Long villi processes can be observed in the small intestinal tissue, along with the narrow crypts of Lieberkühn that extend down towards the muscularis mucosa. This is in contrast with colonic tissue, where villi are lacking giving the appearance of a smoother luminal surface. Additionally, the crypts within colonic tissue appear longer than that of small intestinal tissue and the epithelial cells lining the crypts have a more bulbous and transparent apical cytoplasm suggestive of goblet cells that function to secrete mucus. Further H&E staining of the small intestine duodenum highlights the unique glands within the submucosa (Figure 3.4); these tubular structures are known as Brunner's glands and also have the primary function of producing alkaline mucins to neutralise the acid from the stomach.

(A)



(B)

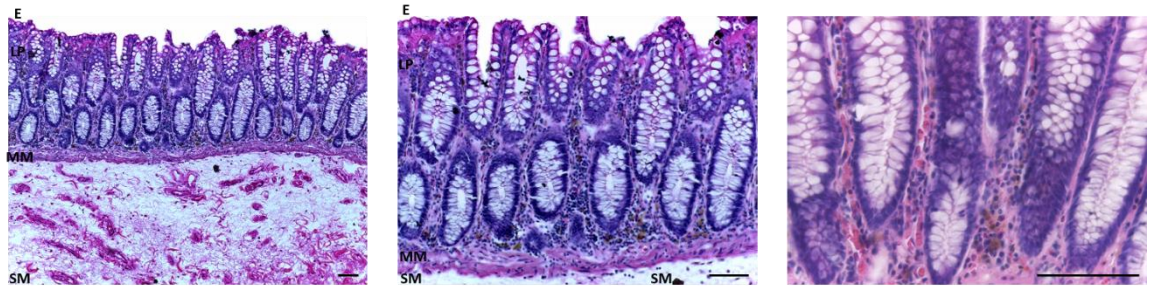


Figure 3.3. H&E staining of the human intestine mucosa. Small intestinal samples were provided pre-stained by Professor Stefan Przyborski (A). Villi structures can be observed extending apically toward the luminal surface. Human large intestine tissue samples were wax embedded and sectioned prior to histological staining (B). H&E staining shows the large intestine mucosa and submucosa. E=epithelium, LP=lamina propria, MM=muscularis mucosa, SM=submucosa. Scale bars: 100 μ m.

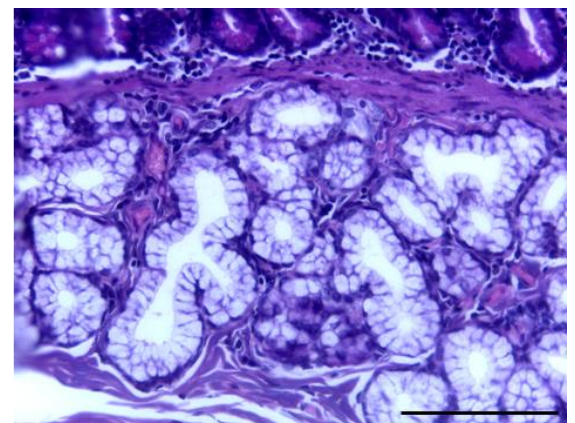
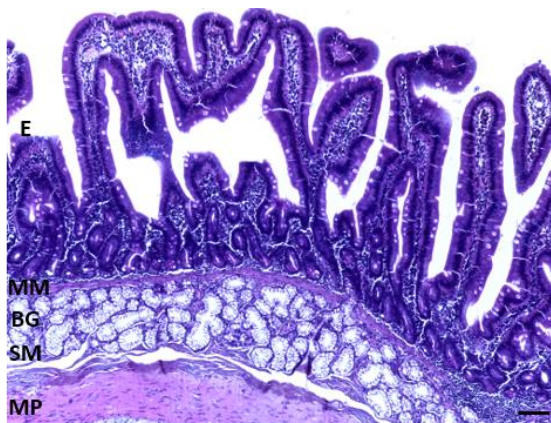


Figure 3.4. Histological analysis of the duodenum. Samples were provided pre-stained by H&E by Professor Stefan Przyborski. Tubular structures known as Brunner's glands are visible within the submucosa of the duodenum. E=epithelium, LP=lamina propria, MM=muscularis mucosa, SM=submucosa, MP=muscularis propria and BG=Brunner's glands. Scale bars: 100 μ m.

In addition to H&E staining, human small intestinal samples pre-stained with H&E and PAS were assessed (Figure 3.5) and large intestinal tissue samples were stained for PAS/Alcian Blue (Figure 3.6). Both PAS and Alcian blue staining were used to identify areas rich in glycoproteins and mucosubstances. Within the small intestine samples, there is evidence of dark blue staining within the apical cytoplasm of some epithelial cells, suggesting the presence of mucus producing goblet cells within the mucosa (Figure 3.5). Regarding the colonic tissue, PAS/Alcian blue staining highlighted neutral mucins a magenta colour due to being stained by the Schiff reagent while acidic mucins were identified by Alcian blue (pH 2.5) and were coloured blue. As seen in Figure 3.6, the crypts of the large intestine are elongated and extend to the bottom of the mucosal layer of tissue and are heavily stained by Alcian blue. Evidence of mucus producing cells can be observed lining the epithelial surface resulting in a secreted mucus gel layer that can be observed covering the apical surface of the epithelium.

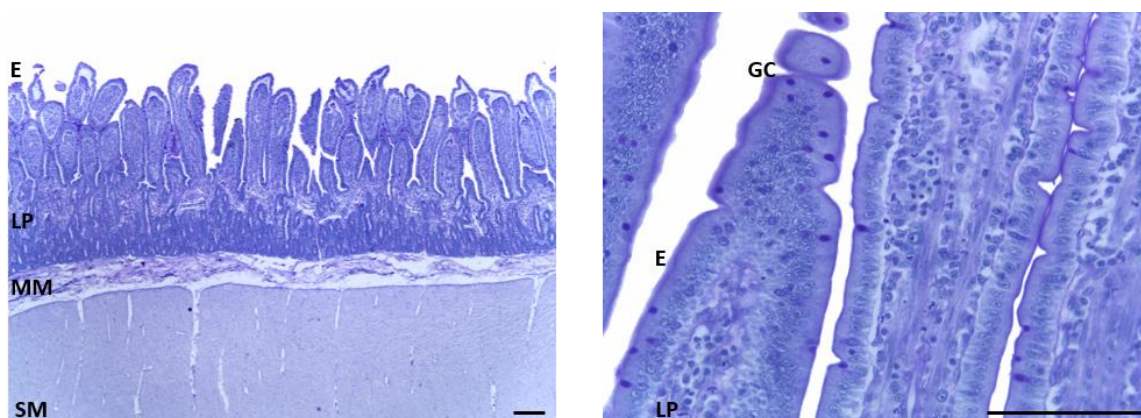


Figure 3.5. H&E and PAS staining of the human small intestine. Small intestinal samples were provided pre-stained by both H&E and PAS by Professor Stefan Przyborski. Dark blue intracellular staining indicates that the cells are mucus producing goblet cells. E=epithelium, LP=lamina propria, MM=muscularis mucosa, SM=submucosa and GC=goblet cell. Scale bars: 100 μ m.

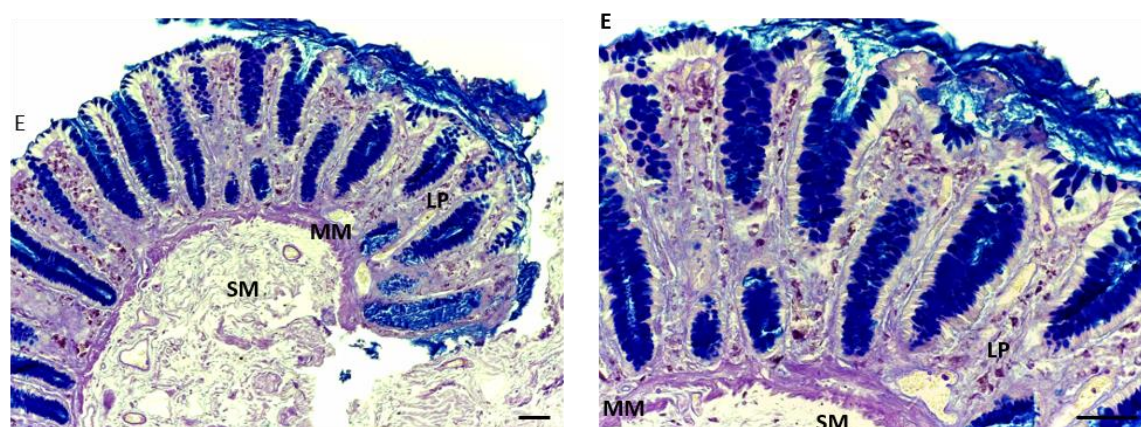


Figure 3.6. PAS/Alcian Blue staining of the human large intestine. Human large intestine tissue samples were wax embedded and sectioned prior to PAS/Alcian blue staining. Blue staining is indicative of the production of mucus. E=epithelium, LP=lamina propria, MM=muscularis mucosa, SM=submucosa. Scale bars: 100 μ m.

3.4.1.2. Ultrastructural characterisation of the large intestine epithelium

The presence of mucus-producing cells was evident in large intestinal tissue, as such ultrastructural TEM characterisation was undertaken to enable goblet cells within the epithelium to be individually recognised. Figure 3.7 depicts areas along the colonic epithelium wherein enterocytes were visible adjacent to goblet cells. The goblet cells can be identified due to their abundance of secretory granules located at the apical surface of the cells. Although they are more apparent and densely packed along the apical surface of enterocytes, goblet cells also express microvilli projections. Apical junctional complexes can be detected between adjacent enterocytes and between enterocytes and goblet cells demonstrating the presence of a barrier along the epithelium.

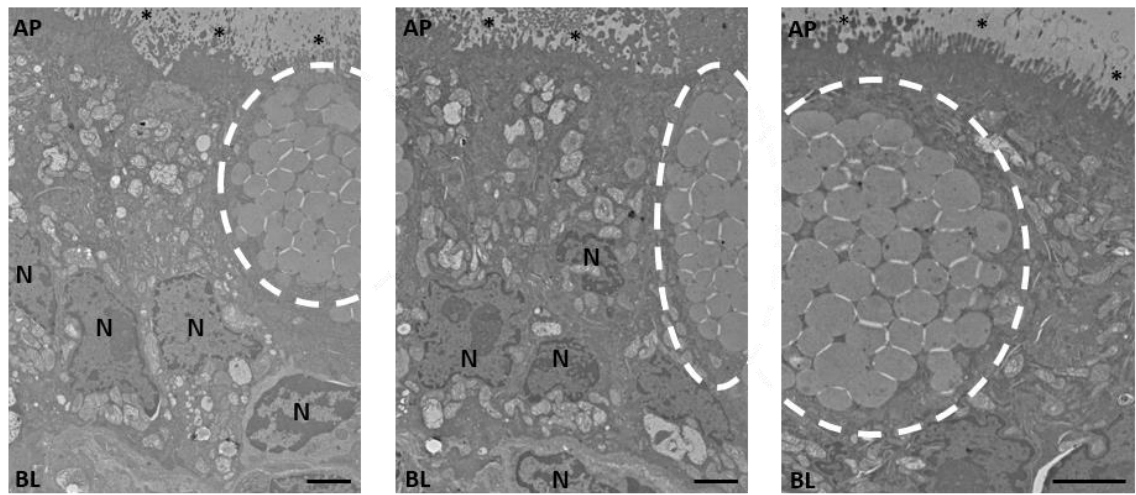


Figure 3.7. Ultrastructural analysis of human intestinal tissue. Human colonic tissue samples were processed and stained for TEM analysis. The intestinal epithelium comprises of both absorptive cells, that typically possess a dense layer of microvilli (*) at the apical surface, and goblet cells that produce mucus and store the mucus glycoproteins in vesicles (dashed circle) located apically in the cell cytoplasm before exocytosis into the apical lumen. Junctional complexes (#) can also be seen between adjacent enterocytes and adjacent goblet and enterocyte cells. AP=apical, BL=basolateral, and N=nucleus. Images kindly provided by Ariana González-Hau. Scale bars: 200 nm.

3.4.2. Characterisation of the Caco-2 Monolayer

3.4.2.1. Morphological characterisation of 2D cultured Caco-2 cells

The Caco-2 cell line was first isolated from a 72-year-old Caucasian male in the 1970s by Fogh. They are the most widely used intestinal cells for studying the absorption function of the intestine. Culture of Caco-2 cells on traditional 2D tissue culture plastic demonstrated that cells grow in colonies that exhibit a regular cobblestone morphology typical of epithelial cells (Figure 3.8A). Upon reaching high confluency, Caco-2 colonies express proteins present in the apical junctional complex when cultured in 2D on glass coverslips (Figure 3.8B). The tight junction proteins Claudin-1 and ZO-1 and the adherens junction protein E-cadherin clearly outline the apical surface of adjacent cells and highlight that cells grow as single monolayers in 2D culture and do not form multilayers.

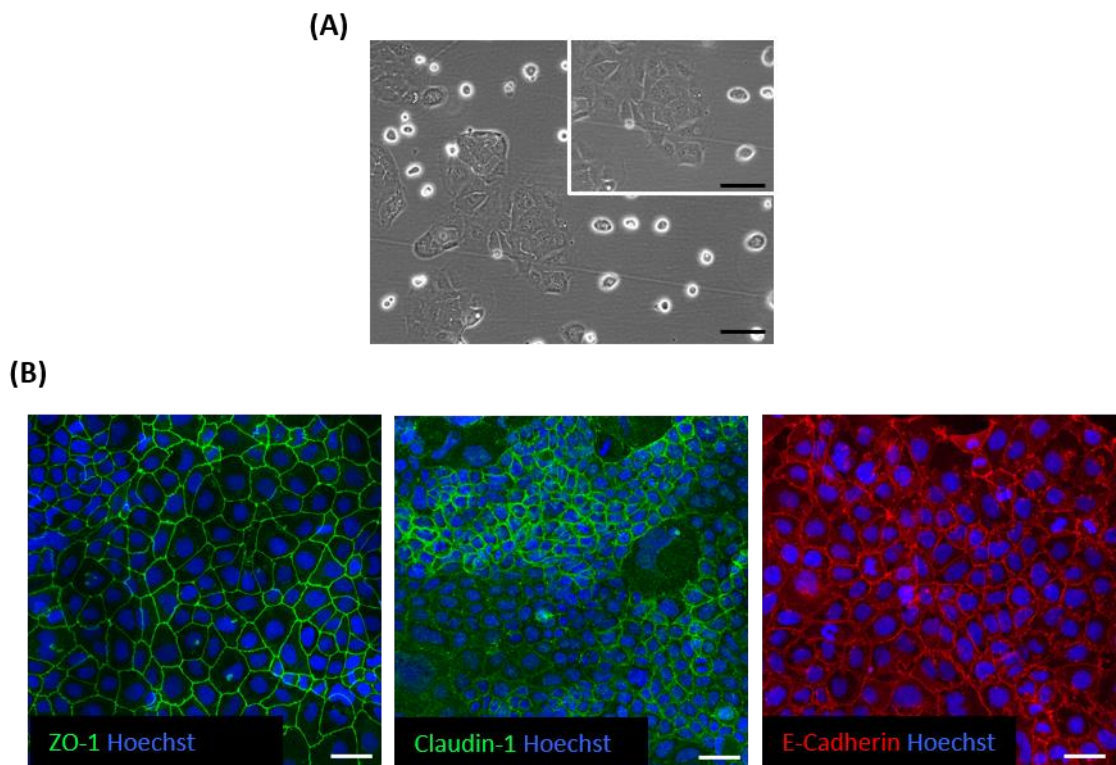


Figure 3.8. Caco-2 cells cultured in 2D display a cobblestone morphology. Phase contrast images of Caco-2 cells cultured on traditional 2D tissue culture plastics grow as colonies with a distinct cobblestone morphology (A, A-inset). Confocal images of Caco-2 cells cultured on glass coverslips for 4 days (B); tight junctions are depicted in green with immunolabelling for ZO-1 and Claudin-1, and adherens junctions are shown in red with immunolabelling for E-cadherin. Nuclei are counterstained by Hoechst (blue). Images shown representative of 3 cultures in total. Scale bars: 50 μ m.

3.4.2.2. Structural characterisation of the Caco-2 monolayer

To model the intestinal epithelium Caco-2 cells are cultured upon a Transwell® membrane for a minimum of 21 days to allow epithelial differentiation into absorptive enterocyte-like cells of the small intestine epithelium. Transwell® membranes used in this study are made of polycarbonate and are 10 µm thick with a pore size of 0.4 µm to prevent cells from falling through the membrane. Histological staining of Caco-2 Transwell® cultures reveals a monolayer of cells that have a cuboidal appearance (Figure 3.9A). This is unlike the *in vivo* small intestine where the epithelium rests on the surface of a lamina propria and is clearly polarised with a columnar morphology and a distinct brush border (Figure 3.9B).

To form a barrier, epithelial cells must express proteins of tight junctions and adherens junctions as these structures are responsible for forming the apical junctional complex that links adjacent cells. Figure 3.9C shows wholemount immunostaining of the intracellular scaffolding protein ZO-1 and the sealing proteins occludin and claudin-1. Also depicted is the expression of claudin-2, a pore-forming protein responsible for allowing the paracellular transport of cations between adjacent cells; such expression of claudin-2 suggests that the barrier formed by the Caco-2 epithelium is not completely impermeable, which is necessary to study the transport process. The presence of adherens junctions is shown through immunostaining of E-cadherin and β-catenin which are responsible for cross-linking and maintaining cell-cell contact to the internal actin cytoskeleton respectively (Figure 3.9C). Compressions of top-down Z-stack confocal images demonstrate the clear apical localisation of these tight and adherens junctional proteins above the nuclei of the Caco-2 cells. AJC protein expression outlines adjacent cells, emphasising the polygonal, cobblestone morphology of the cells as well as confirming that the Caco-2 cells form a single monolayer - no multilayers form in the culture.

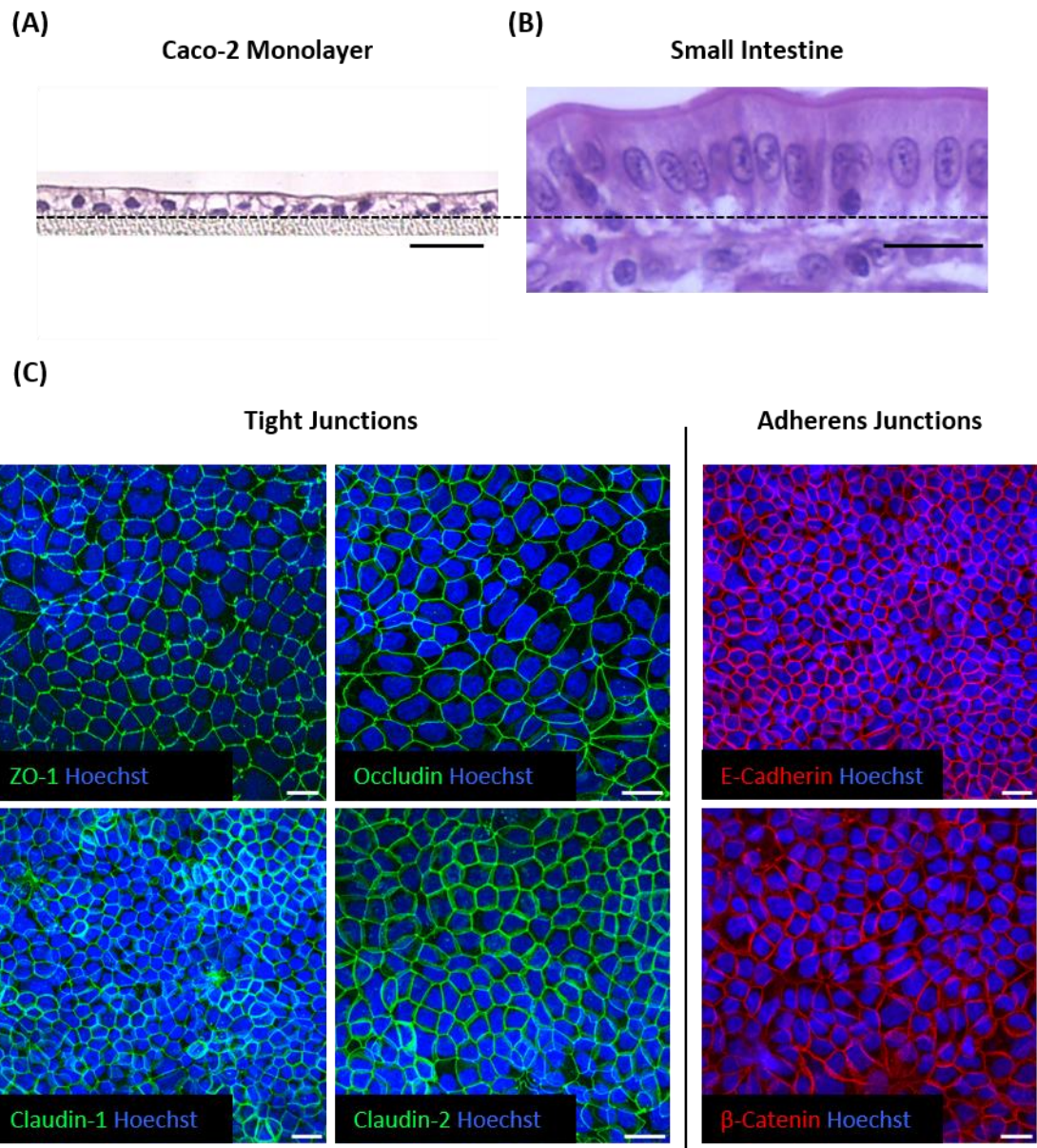


Figure 3.9. Caco-2 cells differentiate into a monolayer of enterocytes when cultured on Transwell® inserts. H&E staining of a 21-day Caco-2 monolayer and of human small intestine (A,B). Caco-2 cells form a monolayer when cultured on Transwell® membranes (A), whereas the epithelial monolayer in human tissue rests on the lamina propria (B). The dashed line indicates the basolateral surface of the epithelium. Top-down compressed Z-stacks of Caco-2 monolayer (C). Models were immunolabeled for the tight junction proteins: ZO-1, occludin, claudin-1 and claudin-2 (green) and for the adherens junction markers E-cadherin and β -catenin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 9 (A) or 3 (B) models. Scale bars: 50 μ m (A), 20 μ m (B,C).

3.4.2.3. Functional characterisation of the Caco-2 monolayer

Barrier function of the Transwell® Caco-2 monolayer was assessed via a number of different methods. The first technique to assess the barrier integrity was through TEER measurements, which were taken every two days of Caco-2 cell culture to monitor cell growth and assess the dynamics of the apical junctional complex over time (Figure 3.10). Analysis of TEER data showed an initial decrease in the resistance values, possibly due to cells clumping together and not having a homogenous distribution across the membrane. However, after day 6 of culture the electrical resistance across the epithelium began to increase until a maximum was reached at day 21 of culture when the Caco-2 cells have fully differentiated into enterocyte-like cells (Figure 3.10A).

To compare the endpoint TEER values of the fully differentiated Caco-2 monolayer to the human intestine, resistance measurements were taken from the literature for both the small and large intestines as very few papers have published TEER values for the *in vivo* tissue (6,245,246). The average resistance measurements of Caco-2 monolayers cultured for 21 days was $3551 (\pm 107.5) \Omega \cdot \text{cm}^2$ while the *in vivo* intestine had an average TEER of $76 (\pm 22.4) \Omega \cdot \text{cm}^2$, at least 46-fold lower than the *in vitro* model (Figure 3.10B). These statistically significant alterations in TEER between *in vitro* models and *in vivo* tissue highlight the differences in barrier tightness and indicate that Caco-2 monolayers are not truly reflective of the human intestine.

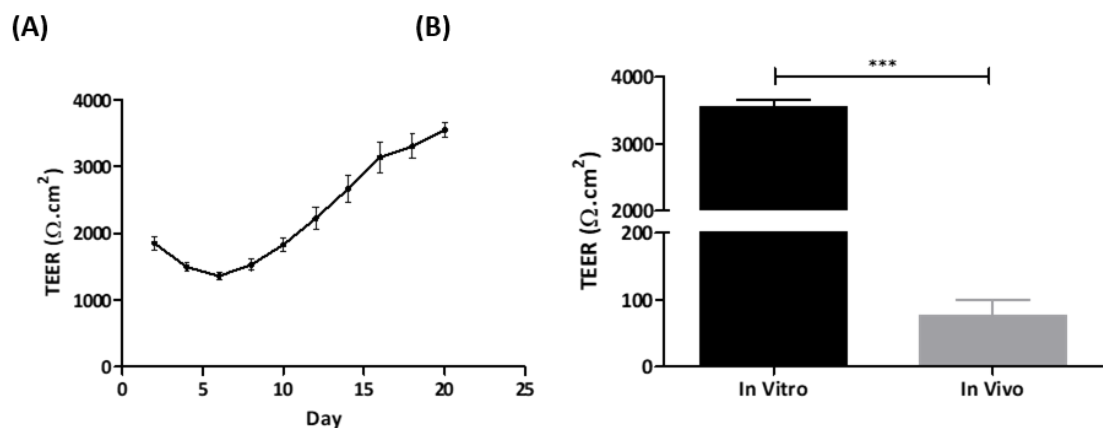


Figure 3.10. Caco-2 TEER is significantly higher than *in vivo*. TEER measurements were taken every two days of epithelial culture (A). Endpoint TEER values were compared with *in vivo* TEER values ($n=7$) reported in the literature (B): Schultz 1979, Balimane and Chong 2005, Sjöberg et al., 2013. Data represent mean $\pm$ SEM, total $n=9$ comprising of 3 independent test occasions each with 3 technical replicates. *** $P \leq 0.0001$ as determined by Student's *t*-test.

The second method to assess the Caco-2 monolayer barrier integrity was through a permeability assay for the fluorescent dye, lucifer yellow (Figure 3.11). Lucifer yellow used in the study is covalently linked to the cation dipotassium salt and due to its hydrophilic nature, is subject to passive paracellular transport, thus can be used to give an indication into how occlusive the tight junctions are between adjacent cells. Lucifer yellow transport was initially assessed as a measure of rejection through the epithelium to determine how impermeable the barrier is. Over 96 % rejection of lucifer yellow was detected in both the absorptive apical-basolateral and secretory basolateral-apical transport directions indicating that an intact barrier had formed across the epithelium (Figure 3.11A).

The apparent permeability coefficient (P_{app}) of lucifer yellow was calculated at $2.2 - 2.3 \times 10^{-8}$ cm/s in both transport orientations (Figure 3.11B), highlighting the low permeability of lucifer yellow and the formation of a strong epithelial barrier. Calculation of the efflux ratio for lucifer yellow was approximately 1, indicating that transport of lucifer yellow showed no directional bias as would be expected from a compound that is not subject to carrier-mediated transport.

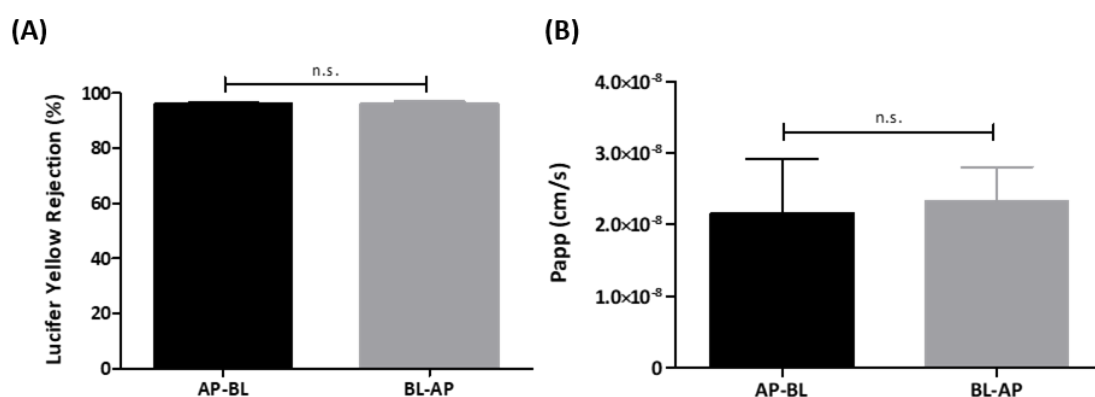


Figure 3.11. Lucifer yellow transport across Caco-2 monolayers. The permeability of lucifer yellow was determined through a rejection assay (A) and through calculation of the apparent permeability coefficient (B). Assays were performed in both the apical-basolateral (AP-BL) and basolateral-apical (BL-AP) directions. Data represent mean $\pm$ SEM total $n=9$ comprising of 3 independent test occasions each with 3 technical replicates. No significant differences as assessed by Student's t -test.

Further functional assessment of the Caco-2 monolayer was performed through a transport assay of rhodamine 123, a chemical dye that is an active substrate of the efflux transporter pump P-glycoprotein (P-gp) (Figure 3.12). Permeability assays were performed in both the apical-basolateral and basolateral-apical directions and revealed that the average P_{app} for rhodamine 123 was 2.74×10^{-7} and 9.41×10^{-7} cm/s respectively, while the average efflux ratio was calculated as $4.68 (\pm 1.12)$ (Figure 3.12A,C). An efflux ratio > 2 suggests that a compound is subject to active efflux as anticipated for rhodamine 123. However, it is known that P-gp is widely overexpressed in Caco-2 monolayers and so to determine if the function of the transporter protein could be diminished, 100 μ M verapamil hydrochloride was added to the transport assay when performed in the basolateral-apical direction. Verapamil hydrochloride is a P-gp inhibitor and its addition to the assay reduced the basolateral-apical transport of rhodamine 123 to 4.88×10^{-7} cm/s, which is more similar to the level transported in the apical-basolateral direction (Figure 3.12B). The efflux ratio was also reduced by 2.2-fold to $2.16 (\pm 0.26)$ indicating that addition of verapamil hydrochloride effectively reduces the level of active efflux of rhodamine 123 (Figure 3.12C).

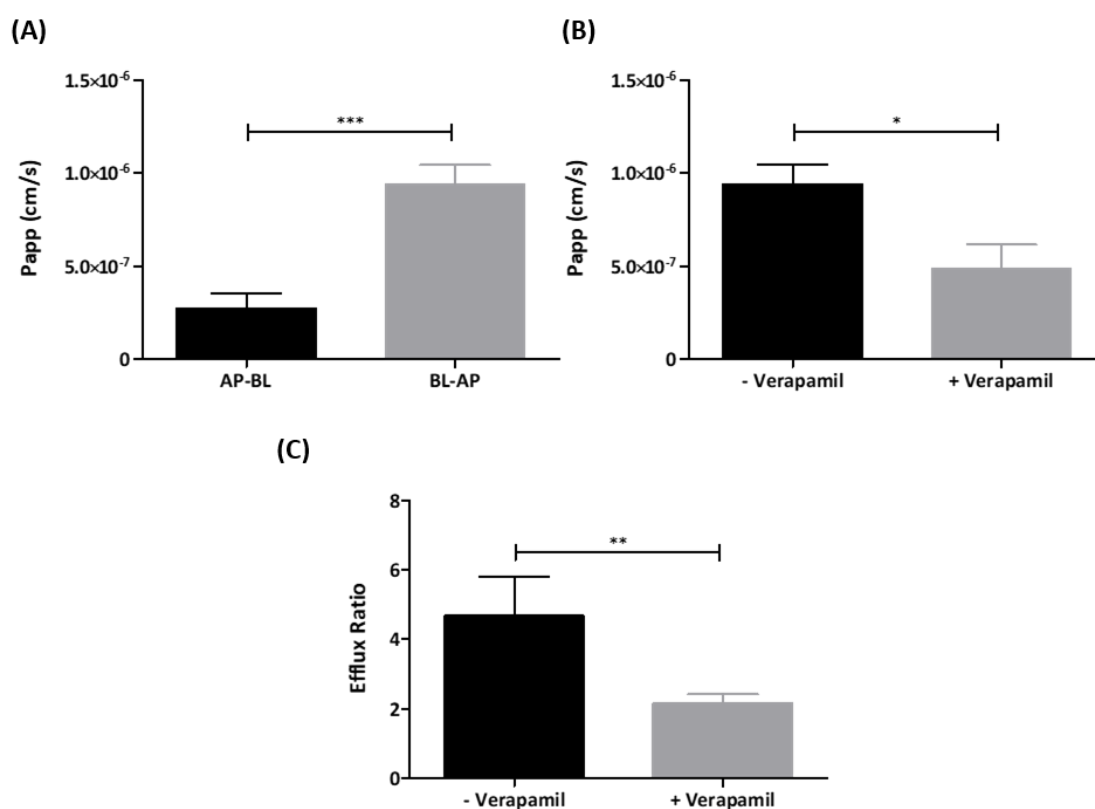


Figure 3.12. Rhodamine 123 transport across the Caco-2 monolayer. The apparent permeability coefficient of the P-gp substrate, Rhodamine 123 was determined over a 2-hour long assay in the AP-BL and BL-AP directions (A). Verapamil hydrochloride, a P-gp inhibitor, was added to the BL-AP assay to determine if Rhodamine 123 was primarily transported via P-gp (B). The efflux ratio of P_{app} in the BL-AP direction to the P_{app} in the AP-BL direction was calculated in the presence or absence of verapamil hydrochloride (C). Data represent mean $\pm$ SEM, total $n=7-9$ comprising of 3 independent test occasions each with 2-3 technical replicates. * $P \leq 0.05$ ** $P \leq 0.001$, *** $P \leq 0.0001$ as determined by Student's t-test.

3.4.2.4. Polaris is an unsuitable alternative cell culture platform to Transwell®

Alvetex® Polaris is an alternative substrate to Transwell® that could potentially be utilised to generate Caco-2 monolayers, though it is not yet commercially available. Unlike Transwell® it is a porous polyHIPE made from polystyrene whereas the Transwell® inserts used in this study are made from polycarbonate. The thickness of the membrane also differs between the two insert types; Transwell® is a very thin substrate of 10 µm with 0.4 µm pores, while Alvetex® Polaris is 200 µm thick with 3-4 µm voids that are linked together by 0.4-0.6 µm interconnecting pores (Table 3.1). SEM analysis of Polaris shows that the surface of Polaris is relatively flat, but that the voids and pores form depressions in the surface (Figure 3.13A). The larger pore sizes and recesses in the surface of Polaris provide a pseudo-3D platform that is an intermediate improvement on the Transwell® culture system which is considered to be a 2D approach to cell culture; therefore Alvetex® Polaris was proposed as an alternative cell culture substrate.

Table 3.1. Substrate properties of Transwell®, Alvetex® Polaris and Alvetex® Scaffold inserts.

	Transwell®	Alvetex® Polaris	Alvetex® Scaffold
Substrate Material	Polycarbonate	Polystyrene	Polystyrene
Membrane Thickness	10 µm	200 µm	200 µm
Surface Area	1.12 cm ²	1.13 cm ²	1.13 cm ²
Pore Size	0.4 µm	3-4 µm voids linked by 0.4-0.6 µm interconnects	36-40 µm voids linked by 15 µm interconnects

H&E staining of Caco-2 cells cultured using Polaris as a scaffold show that the cells grow on the surface of the membrane but also grew on the base of the scaffold, as depicted by the asterisks (Figure 3.13C). Comparison of the model with the Caco-2 Transwell® monolayer shows that a single epithelial layer is formed on Transwell® membranes but that cells form multilayers when cultured on Polaris (Figure 3.13B, C). However, areas where a single monolayer of Caco-2 cells grow on Polaris (arrowheads) have an altered morphology to the Transwell® epithelial layer; cells have a more distinct basal nuclei on Polaris and appear to be more elongated and columnar.

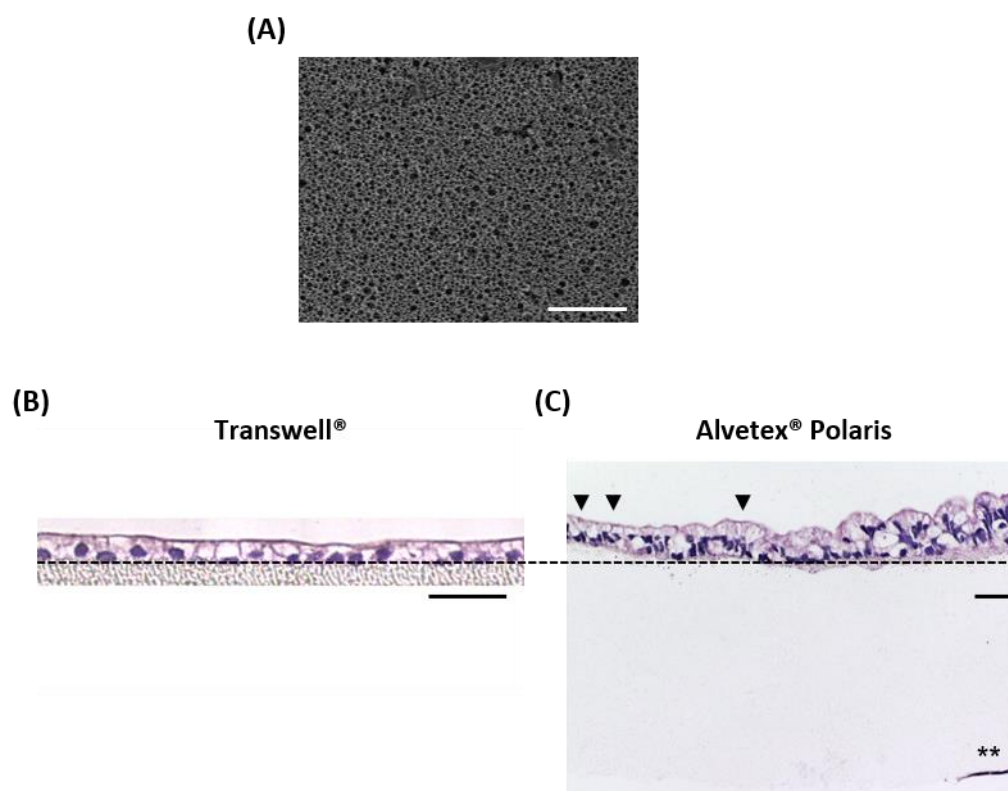


Figure 3.13. Alvetex® Polaris appears to be an unsuitable alternative scaffold to Transwell®. SEM micrograph showing the structure of Alvetex® Polaris (A), image courtesy of Dr Rebecca Quelch. H&E staining of a Caco-2 monolayer cultured on Transwell® (B) and Alvetex® Polaris (C). The dashed line indicates the basolateral surface of the epithelium, arrowheads highlight the formation of a single cell layer, while cells that have fallen through the scaffold are denoted by an asterisk. Images shown are representative of 9 models. Scale bars: 50 μ m.

TEER measurements were also taken every two days of Caco-2 cell culture on Polaris and a general increase in resistance was observed throughout growth, though the TEER was much lower (~37-fold lower) than TEER measurements of the Transwell® monolayer (Figure 3.14A). Comparison of the endpoint TEER to the Caco-2 monolayer and to human intestine demonstrated that even though multilayers were formed on Polaris the resistance values were much more similar to the *in vivo* tissue (Figure 3.14B).

To confirm whether an intact barrier was formed on Polaris, further analysis of barrier function was performed through a lucifer yellow rejection assay. Almost 50 % of the dye was able to pass through the epithelium indicating that the barrier formed was leaky and not suitable to use further in permeability studies (Figure 3.14C). Overall, Polaris appeared to be an unsuitable alternative to Transwell® membranes as cells grew on both sides of the scaffold, multilayers were formed, and the barrier formed between adjacent cells was weak.

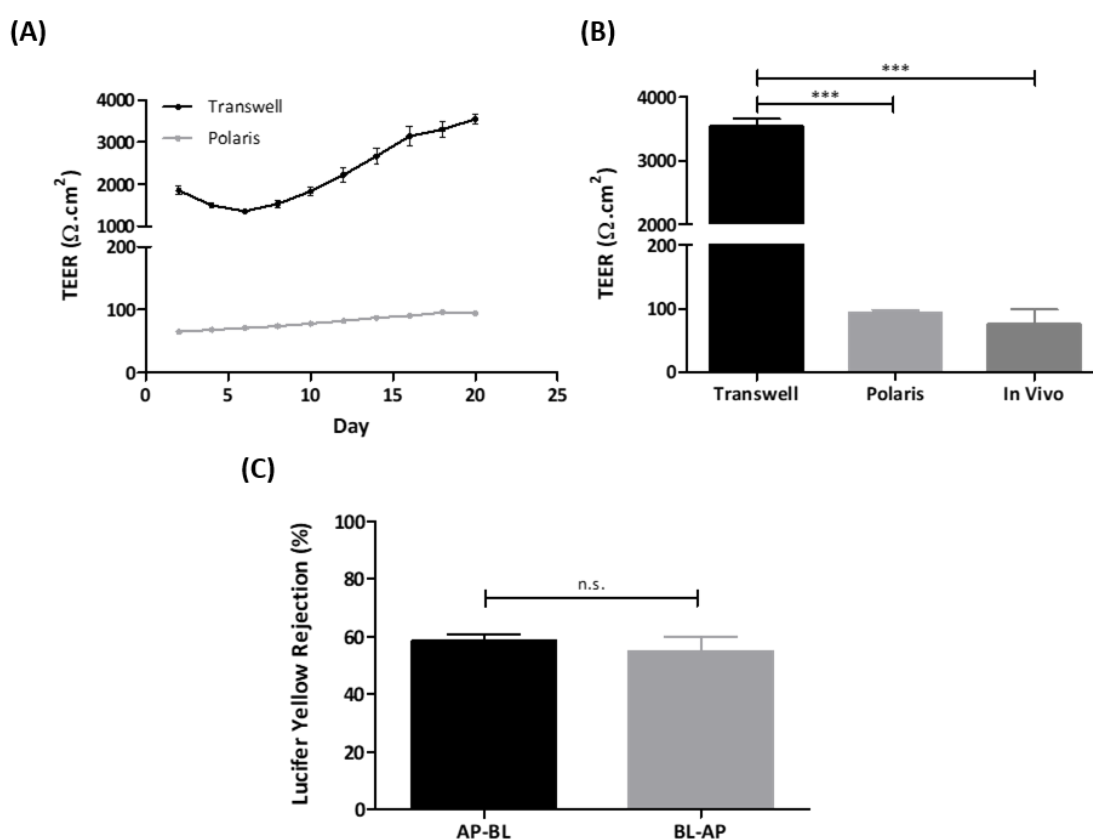


Figure 3.14. Caco-2 monolayers on Alvetex® Polaris have reduced barrier integrity. TEER was measured throughout the 21-day culture period (A) and displayed a lower TEER than the Transwell control throughout culture. Endpoint TEER values were compared with *in vivo* TEER values (*n*=7) reported in the literature (B): Schultz 1979, Balimane and Chong 2005, Sjöberg et al., 2013. The permeability of lucifer yellow was determined through a rejection assay in the apical-basolateral (AP-BL) and basolateral-apical (BL-AP) directions (C). Data represent mean ± SEM, total *n*=9 comprising of 3 independent test occasions each with 3 technical replicates. ****P*≤0.0001 as determined by one-way ANOVA with Tukey's post hoc test to compare the means of individual variables (B).

3.4.3. Optimisation of a Fibroblast Caco-2 Co-Culture Model

3.4.3.1. Use of neonatal human dermal fibroblasts to create a lamina propria equivalent within a 3D cell culture scaffold

To develop a co-culture model consisting of fibroblasts and Caco-2 cells to represent the lamina propria and epithelial compartments of the intestinal mucosa respectively, Alvetex® Scaffold was selected as a substrate for model development as it has previously been used to generate skin equivalents. Skin tissues have a gross anatomy similar to intestine: an epithelium overlying a subepithelial compartment that contains fibroblasts and ECM proteins. Alvetex® Scaffold is another scaffold technology similar to Alvetex® Polaris (discussed in Section 3.4.2.4), the substrate material is polystyrene, and the thickness of the membrane is 200 µm (Table 3.1). However, the voids in Scaffold range from 36-40 µm in size and are linked together by interconnects of approximately 15 µm creating an uneven surface along the membrane (Figure 3.15). The larger pore and void sizes of Scaffold enable cells to grow within the membrane and separate out cells cultured within the scaffold and on the surface.

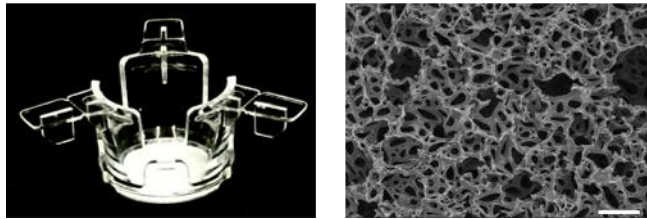


Figure 3.15. Alvetex® Scaffold is a porous polyHIPE. Alvetex® well insert and SEM micrograph showing the structure of the membrane. SEM image courtesy of Dr Rebecca Quelch. Scale bar: 50 µm.

Culture of a full thickness skin equivalent using Alvetex® Scaffold requires the initial formation of a dermal equivalent where fibroblasts are cultured within the membrane before skin keratinocytes are seeded on the surface. The dermal compartment structure is recreated through the culture of neonatal human dermal fibroblasts (HDFn) in Medium 106, a low-serum growth media developed specifically for the culture of human dermal fibroblasts. When cultured in 3D in Alvetex® Scaffold, 5 ng/mL TGF- β 1 and 100 μ g/mL ascorbic acid is supplemented to the media and the cells are maintained for a minimum of 28 days. Keratinocytes are then seeded on the top of the dermal equivalent to form a distinct epidermal compartment and represent the structure of human skin (Figure 3.16A).

To begin the co-culture of an intestinal model, the skin protocol was modified to accommodate the culture of Caco-2 cells on the surface of a dermal equivalent formed using HDFn cells following the same protocol and culture conditions as the skin model. Following the establishment of a dermal equivalent, herein referred to as a lamina propria equivalent to better reflect the intestinal nomenclature, Caco-2 cells were seeded onto the surface and maintained for a further 21 days to allow enterocyte differentiation to occur (Figure 3.16B).

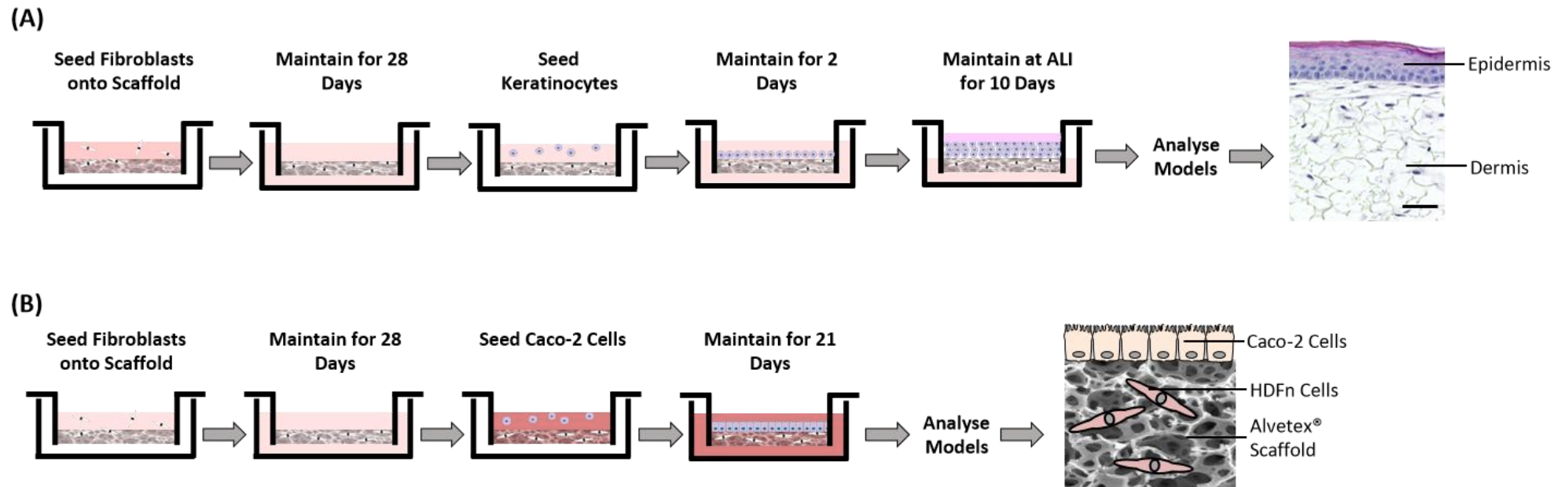


Figure 3.16. Schematic protocol of the culture of mucosal models using Alvetex® Scaffold as a culture support. Schematic detailing the setup of a full-thickness skin equivalent cultured on Alvetex® Scaffold (A). Fibroblasts are cultured for 28 days before the addition of keratinocytes; keratinocytes are maintained in a submerged stage for 2 days before being maintained at the air-liquid interface (ALI) for a minimum of 10 days. H&E staining of the resulting model shows a dermis and overlying epidermis, image courtesy of Dr Lydia Costello. Proposed protocol for the development of an intestinal mucosal model following the initial steps of the skin protocol wherein fibroblasts are seeded into Alvetex® Scaffold and maintained for 28 days. Caco-2 cells will then be seeded on the surface then maintained for a further 21 days to allow cells to differentiate (B). Scale bar: 50 μm .

HDFn cells grown on 2D tissue culture plastic in M106 display a spindle-like morphology with an oval-shaped nucleus characteristic of fibroblasts (Figure 3.17A). They do not grow in distinct colonies and instead spread throughout the entirety of the culture substrate while increasing in confluency. To create a lamina propria equivalent, 0.5×10^6 HDFn cells were seeded onto a 12-well Alvetex® Scaffold and maintained for 28 days in M106 with TGF- β 1 and ascorbic acid supplementation to encourage the production of ECM. H&E staining of the resulting model shows that fibroblasts populate the cell culture support but typically layer on the surface and base of the membranes (Figure 3.17B). Vimentin is a type III intermediate filament protein expressed in mesenchymal cells and was chosen as a marker to highlight the cellular distribution in the scaffold. HDFn cells are vimentin positive cells and the fluorescent staining revealed that cells typically retain a spindle shape morphology when growing within the membrane and demonstrated that the fibroblasts growing on the edges of scaffold are all in the same orientation reflecting their distribution *in vivo* (Figure 3.17C).

One of the primary functions of fibroblasts *in vivo* is the secretion and maintenance of the extracellular matrix (ECM). To examine HDFn production of ECM in 3D cell culture, 28-day lamina propria equivalents cultured in M106 were immunolabelled for the ECM markers type I and III collagen and fibronectin (Figure 3.17C). Fibronectin is an important component of the ECM and forms part of the basement membrane between epithelial tissues and their subepithelial compartments; staining for fibronectin reveals its presence throughout membrane but the majority of positive staining occurred at the edges of the cell culture support where most of the fibroblasts are located. Type I and III collagens are the most abundant and second most abundant collagen in the human body; HDFn cells secreted both collagens I and III in a similar distribution pattern as observed for fibronectin, with the strongest staining occurring at the surface and base of the membrane (Figure 3.17C).

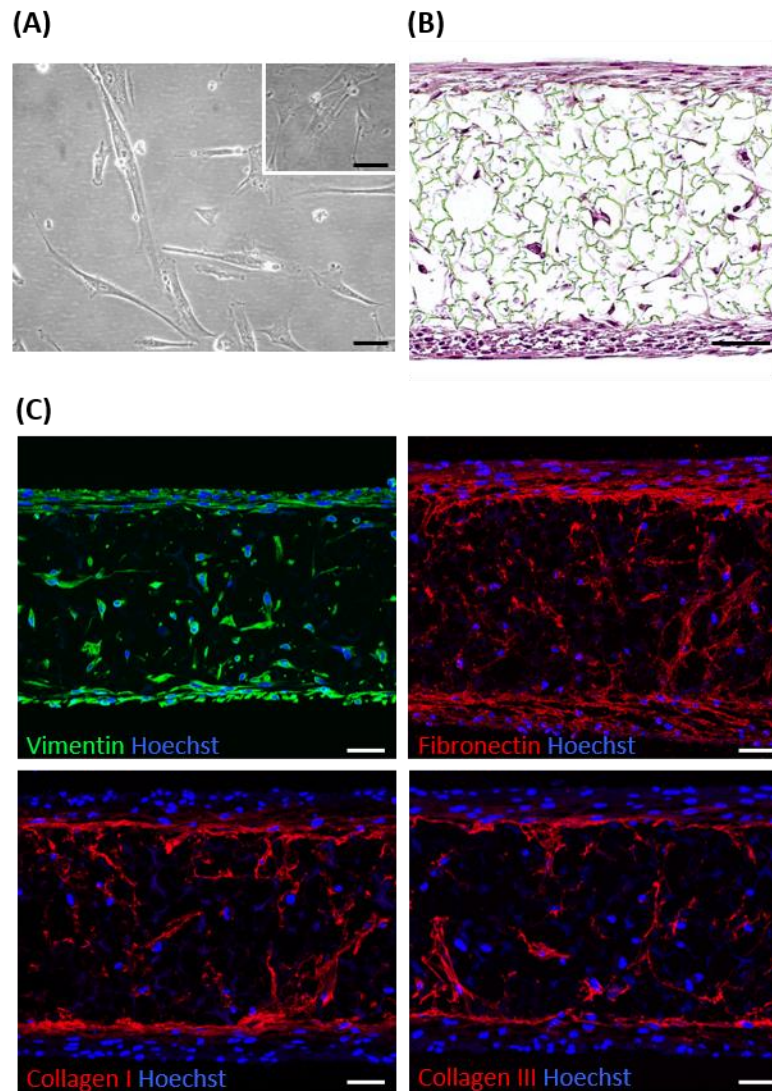


Figure 3.17. Fibroblasts cultured in Alvetex® Scaffold form a lamina propria equivalent. HDFn grow as spindle-shaped cells when cultured traditionally in 2D tissue culture plastic (A). H&E staining of HDFn maintained in Alvetex® Scaffold for 28 days, fibroblasts populate the scaffold and form layers on the membrane base and surface (B). 28-day HDFn cultures immunolabelled for fibroblast markers and ECM proteins (C). Vimentin (green) indicates the distribution of fibroblasts within the membrane. Endogenous ECM proteins: fibronectin, collagen I and collagen III (red) are produced by the fibroblasts and secreted into the voids of Alvetex® Scaffold. Nuclei are counterstained with Hoechst (blue). Images shown are representative of 9 models. Scale bar: 100 μ m (A), 50 μ m (B,C).

Following the development of a HDFn lamina propria equivalent utilising the dermal skin protocol, Caco-2 cells were then seeded on top of the fibroblasts. 0.4×10^6 Caco-2 cells are seeded onto the apical surface of the lamina propria equivalent; from this point of cell culture, models were maintained in complete DMEM growth medium without any additional supplements as per the usual Caco-2 differentiation protocol. Models were cultured for a further 21 days as in the Transwell® model to allow Caco-2 cells to differentiate into epithelial cells resembling small intestinal enterocytes. H&E staining of a full thickness intestinal model with a lamina propria equivalent precultured for 28 days in M106 with TGF- β 1 and ascorbic acid supplements demonstrates that the epithelium formed by the Caco-2 cells was a homogenous single monolayer with highly polarised cells (Figure 3.18). The morphology of the Caco-2 cells was highly columnar; the nuclei were clearly localised to the basolateral side of the cell while the dark staining at the apical cell surface shows evidence of a brush border formation.

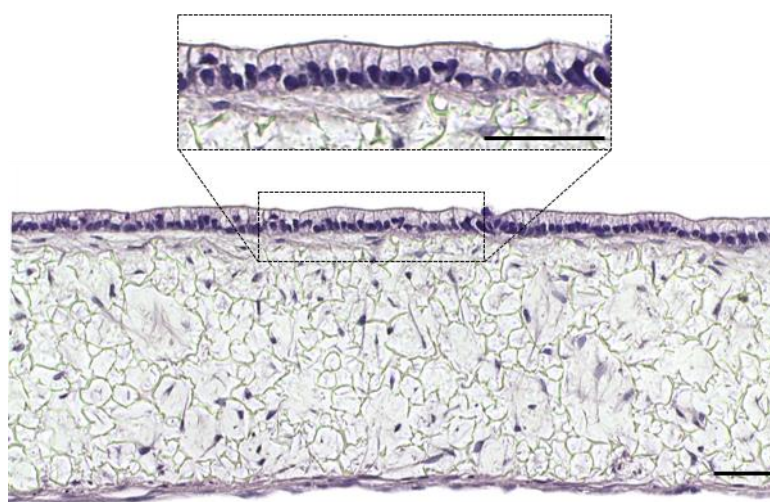


Figure 3.18. Caco-2 form a polarised monolayer when grown on the surface of a lamina propria equivalent. H&E staining of Caco-2 cells cultured for 21 days on the surface of a lamina propria equivalent in which HDFn cells had been pre-cultured in M106 for 28 days. Images shown representative of 9 models. Scale bar: 50 μ m.

Analysis of the electrical resistance across the co-culture epithelium showed an increase in resistance with culture time until a plateau was reached by day 14 and was maintained for the full 21-day Caco-2 cell culture period (Figure 3.19A). The maximal TEER reached was less than 50 $\Omega\cdot\text{cm}^2$, much lower than Transwell® control. Rejection of lucifer yellow remained high, > 90 % of the fluorescent dye did not pass through the epithelium; this indicated that an intact barrier had formed even though the TEER is low and is more reflective of the *in vivo* intestine (Figure 3.19B). Although a statistically significant difference in lucifer yellow rejection was determined between the absorptive and secretory transport directions, it is likely that this effect is not biologically significant due to the overall high levels of rejection.

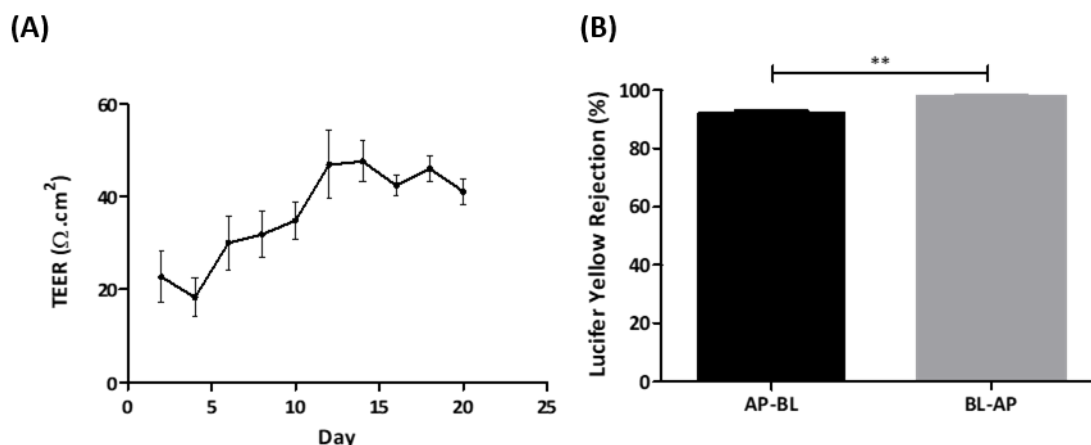


Figure 3.19. Functional properties of the 3D co-culture using fibroblasts precultured in M106. TEER measurements were taken every two days of epithelial cell culture (A). The permeability of lucifer yellow was determined through a rejection assay in the absorptive apical-basolateral (AP-BL) and secretory basolateral-apical (BL-AP) directions (B). Data represent mean $\pm$ SEM, total $n=9$ comprising of 3 independent test occasions each with 3 technical replicates. ** $P\leq 0.001$ as determined by Student's *t*-test.

3.4.3.2. Refining the culture of the lamina propria equivalent by altering the culture media to a more cost-effective alternative

As Caco-2 cells are typically maintained in complete DMEM, it was necessary to determine if the full model culture could be performed in DMEM. Culture of HDFn cells in DMEM would prevent the need to swap media during the culture and would provide a more cost-effective alternative to M106, furthermore it would increase the adaptability for large scale studies. Initial studies were performed to determine how the fibroblasts grew in 2D in M106 and DMEM. Phase contrast images of HDFn cells were taken every two days of cell growth in M106 and DMEM until 100 % confluency was reached; cell morphology did not alter in the different culture media and fibroblasts retained their spindle-cell morphology (Figure 20A). HDFn cultured in DMEM appeared to grow in one orientation when they reached a higher confluency, this was in contrast to maintenance in M106 where cells spread in all directions and seemed to grow on top of one another once they had reached 100 % confluency (Figure 3.20A).

Different growth media have different nutritional contents; M106 is low in serum while DMEM contains 10 % FBS with high levels of growth factors, as well as other supplements such as NEAA that will affect cell growth; therefore, it was important to assess cell growth and viability in the two media types. An MTT assay was performed every two days of HDFn culture and showed little difference in the metabolic activity of the cells at different time points, however at day 6, cells cultured in DMEM exhibited greater metabolic activity which could be used as a proxy to suggest that the cells had greater viability (Figure 3.20B). To further examine this, a cell growth assay was performed wherein cells were trypsinised and counted every two days of culture. HDFn cultured in DMEM consistently had a higher retrieval rate of cells, and this was particularly emphasised at day 6 of culture, reflecting the MTT assay (Figure 3.20C). Calculation of HDFn doubling time revealed that cells cultured in DMEM had a statistically significant faster doubling time of 1.5 days compared to 3.1 days in M106 (Figure 3.20C).

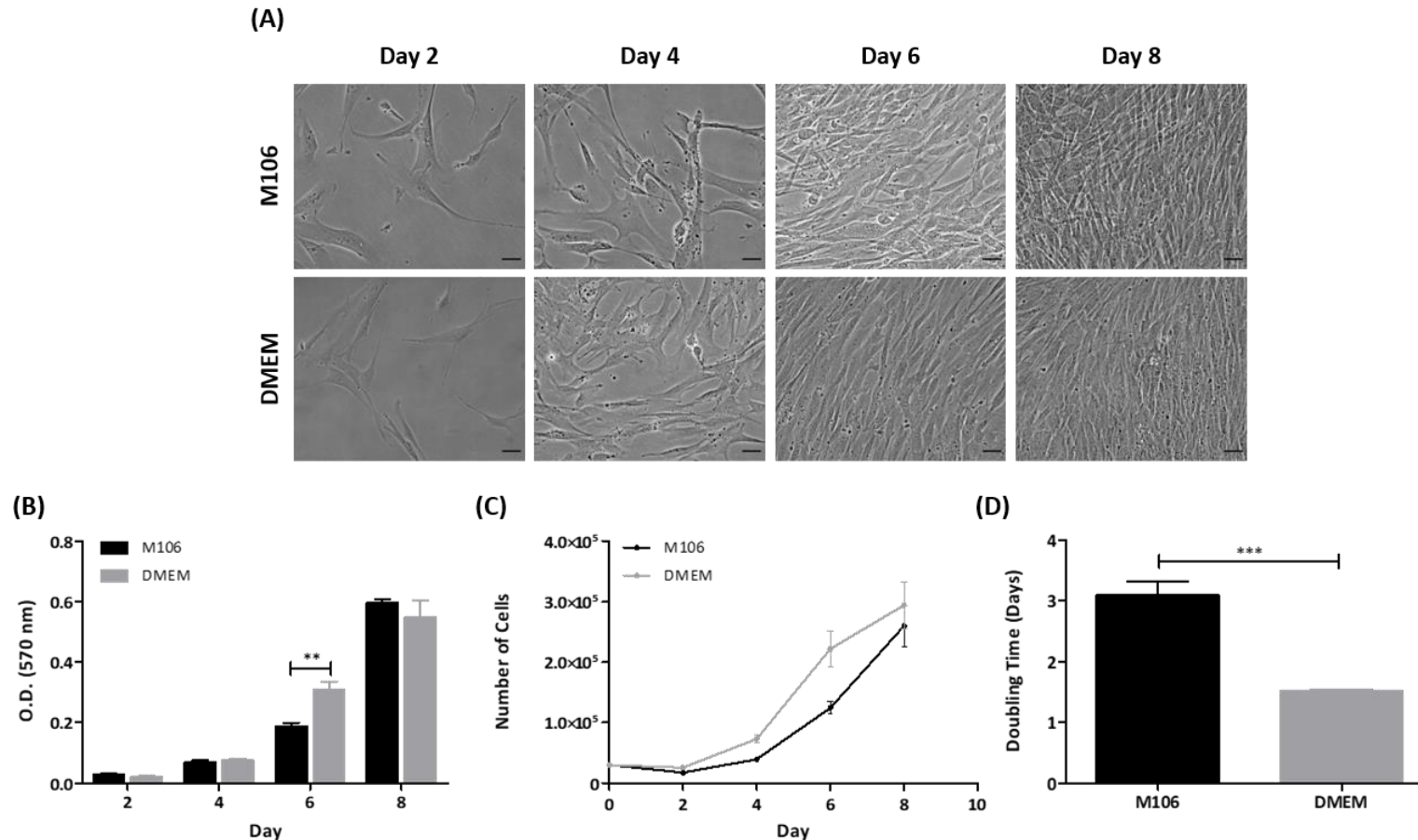


Figure 3.20. Growth characteristics of 2D cultured HDFn cells in M106 and DMEM. Phase contrast images demonstrate the 2D culture of fibroblasts in M106 and DMEM culture medium over time (A). An MTT assay was performed every 2 days of HDFn culture to determine whether culture medium affects the viability of cells over time; the optical density (O.D) was read at 570 nm (B). A trypan blue exclusion assay was performed every 2 days of culture to determine the growth curve (C) and doubling time (D) of the cells in the different growth media. Images shown representative of 9 cultures. Data represent mean $\pm$ SEM, total $n=9$ comprising of 3 independent test occasions, each with 3 technical replicates. ** $P \leq 0.001$ as determined by two-way ANOVA with Bonferroni post-test (B) and *** $P \leq 0.0001$ as determined by Student's t -test (D). Scale bars: 100 μ m.

A quicker growth of 2D cells in DMEM would lead to increased model throughput capacity, and to determine if the cell proliferation capacity had altered, HDFn were cultured in M106 and DMEM for four days and were then stained for the cell cycle proliferation marker Ki67. The pool of Ki67 positive cells was then counted and expressed as a percentage of the total number of cells (Figure 3.21). However, there was no difference in the number of cells expressing Ki67 suggesting that HDFn proliferation capacity is not altered but that the time taken to complete the cell cycle is reduced when cells are maintained in DMEM.

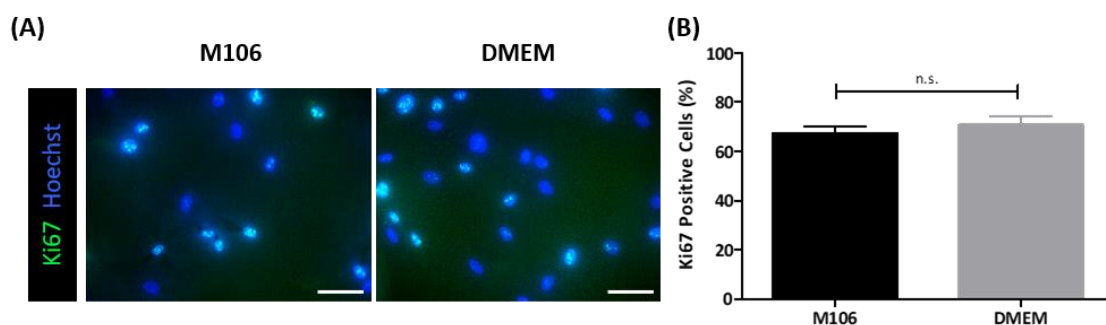


Figure 3.21. Proliferative ability of HDFn cells is unaffected by culture media. Representative Ki67 (green) immunolabelled HDFn cultured in M106 and DMEM for 4 days before fixation in 4 % PFA and staining (A). Cells were counterstained with Hoechst to visualise nuclei (blue). FIJI software was used to calculate the number of Ki67 positive cells to determine if there was any difference in the proliferative capacity of HDFn cultured in different growth media (B). Data represent mean $\pm$ SEM; 90 cells were counted from 3 independent test occasions, i.e., 30 cells counted per experimental repeat. Scale bars: 100 μ m. No significant differences identified by Student's t-test.

Before switching to DMEM culture medium for maintenance of the 3D lamina propria culture, it was necessary to determine if the production of ECM proteins had been altered. Cells were cultured on coverslips for four days before fixation and staining for the fibroblast marker vimentin and the ECM markers collagen I, III and fibronectin (Figure 3.22). Vimentin staining highlights the fibroblast morphology in more detail than phase contrast images and confirmed that HDFn cells retain their spindle-like shape when maintained in DMEM, however the cells do appear to be more spread out and wider in DMEM. The capacity of the cells to produce ECM proteins was not altered between the two media types and cells show evidence of collagen I, III and fibronectin production in M106 and DMEM, though there is little evidence of these proteins being secreted in 2D culture conditions.

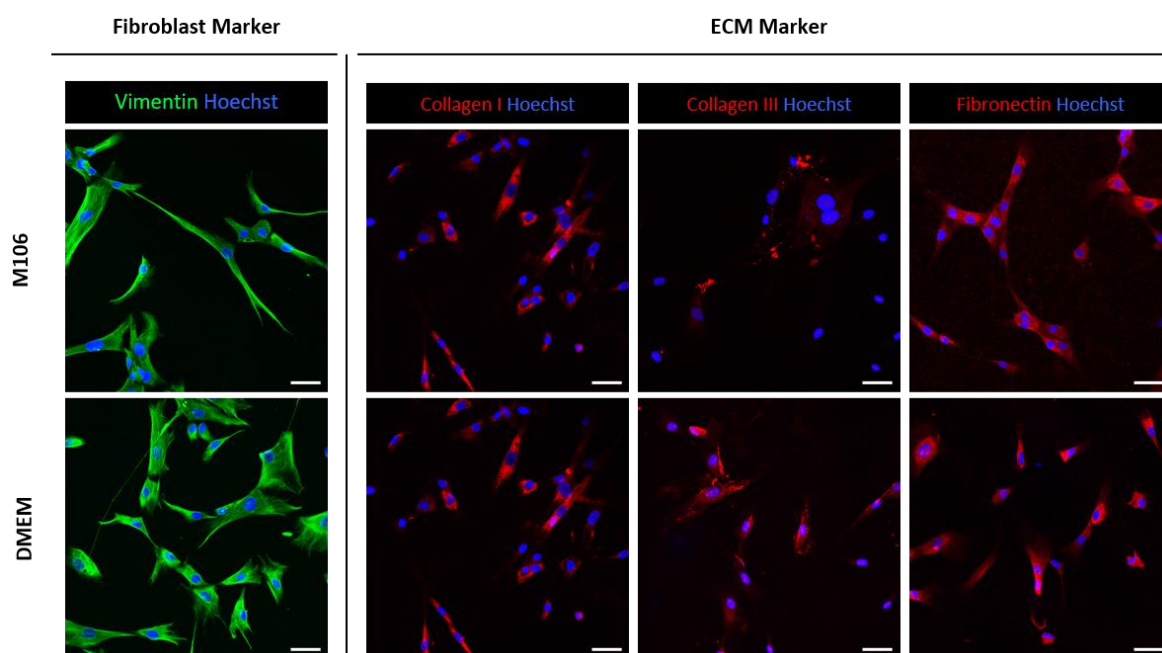


Figure 3.22 Fibroblasts cultured in 2D show evidence of ECM protein production. Fibroblasts cultured on glass coverslips for 4 days were immunolabelled for the cytoskeletal marker, vimentin (green) and the ECM markers collagens I and III and fibronectin (red). Cells were counterstained with Hoechst to visualise nuclei (blue). Images shown are representative of 3 different cultures. Scale bars: 50 μ m.

Following confirmation that HDFn could successfully be maintained in DMEM in 2D, 3D lamina propria equivalents were cultured for 28 days using M106 and DMEM with TGF- β 1 and ascorbic acid supplements. H&E histological staining was used to observe the anatomy of the two lamina propria equivalents in Figure 3.23A; no obvious differences were observed between the two models and the majority of fibroblasts were still found on the edges of Alvetex® Scaffold with some distributed within the membrane. This was further confirmed by staining for vimentin to show HDFn distribution, which also emphasised the greater numbers of cells that resided within the membrane in models maintained in DMEM (Figure 3.23A). Models were also stained for the marker α -smooth muscle actin (α -SMA); a protein expressed in activated fibroblasts and myofibroblasts. Representative images of α -smooth muscle actin staining are shown in Figure 3.23A; no positive staining was detected in models cultured in M106, yet models maintained in DMEM were positive for α -SMA protein expression.

Metabolic activity of HDFn cells was also assessed in 3D culture after 28 days to determine if the fibroblasts remained viable. Both HDFn cultured in M106 and DMEM had evidence of being metabolically active and no difference was detected between the cells when cultured in the different media types confirming that DMEM is also a suitable culture medium to use for the development of a 3D lamina propria equivalent (Figure 3.23B).

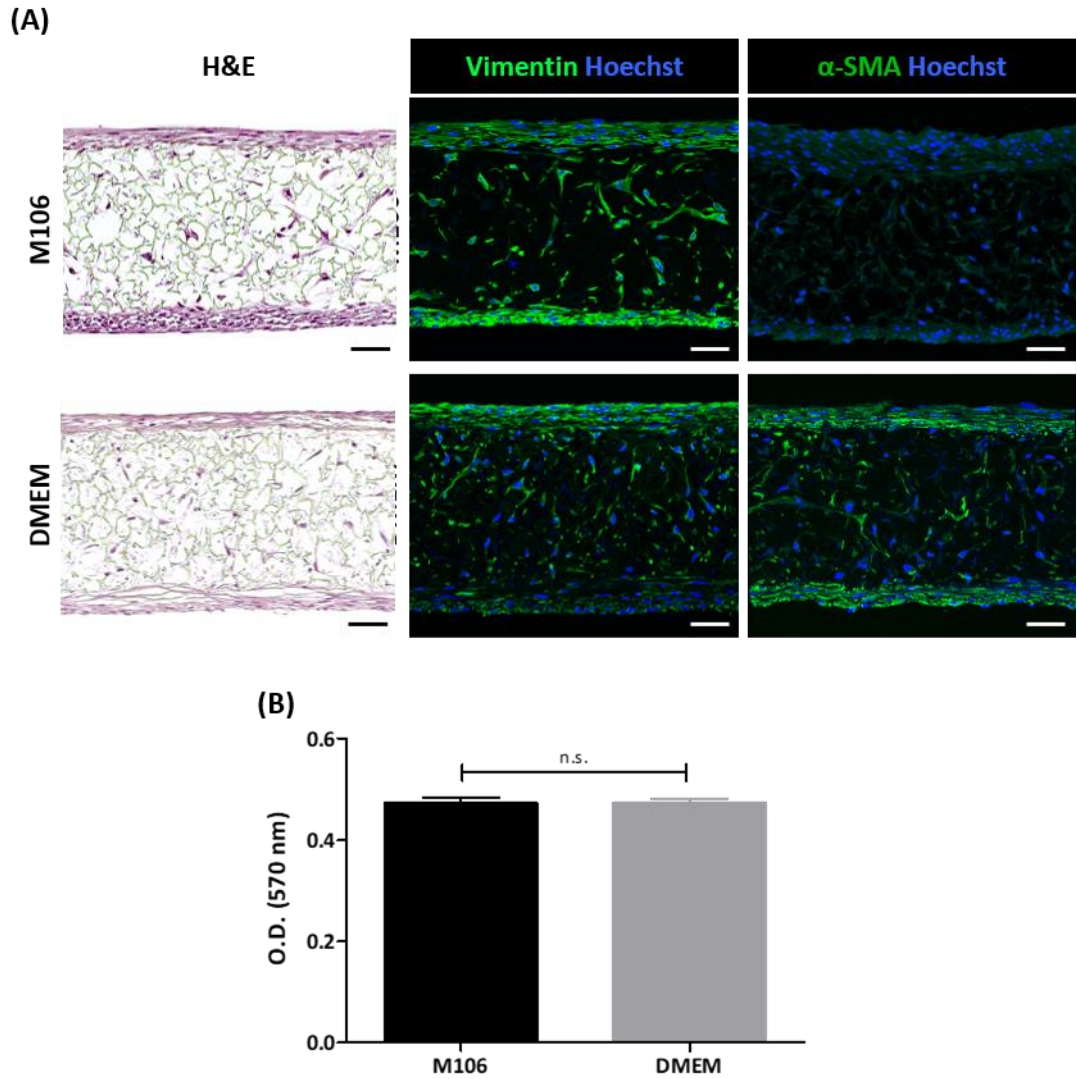


Figure 3.23. Fibroblasts form lamina propria equivalents in both M106 and DMEM. H&E staining and vimentin and α -smooth muscle actin immunolabelling highlight the distribution of fibroblasts (A). Alpha smooth muscle actin staining was not detected in models cultured in M106 but was observed in DMEM samples. Immunolabelled samples were counterstained with Hoechst to visualise nuclei (blue). MTT assay on HDFn equivalents cultured in Alvetex® Scaffold for 28 days in either M106 or DMEM; optical density was read at 570 nm (B). Images shown are representative of 9 models. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions each with 3 technical replicates. Scale bars: 50 μ m. No significant differences identified by Student's t -test.

Further characterisation of the M106 and DMEM lamina propria equivalents, demonstrated that models generated using DMEM had greater deposition of ECM proteins as indicated through staining for the markers collagen I, III and fibronectin (Figure 3.24). ECM was deposited throughout the entirety of the Alvetex® Scaffold membrane when fibroblasts were maintained in DMEM but was only present in the M106 model where there were larger numbers of cells as indicated by the Hoechst staining to visualise nuclei (Figure 3.24). However, as serum is very rich in fibronectin, it is possible that the additional fibronectin deposition is directly from the serum, rather than the fibroblasts themselves.

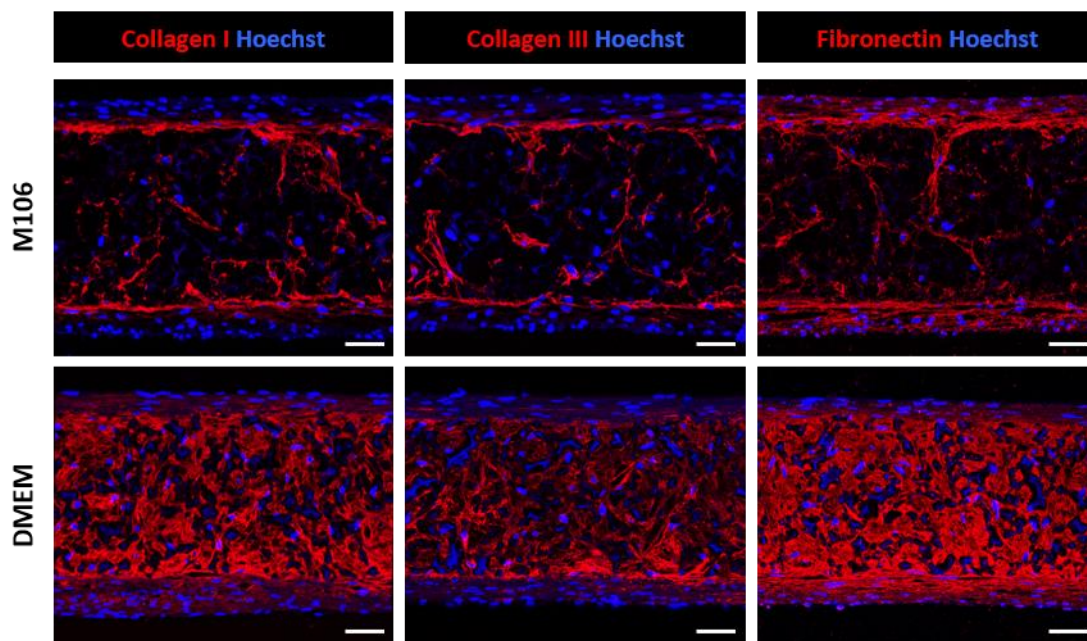


Figure 3.24. 3D cultured fibroblasts secrete endogenous ECM. 28-day lamina propria equivalents secrete endogenous ECM proteins into the membrane. Samples were immunolabelled for collagen I, collagen III, and fibronectin (red) and Hoechst to stain nuclei (blue). Images shown are representative of 9 models. Scale bar: 50 μ m.

Western blot and corresponding densitometry analysis confirmed that fibroblasts cultured in DMEM deposit higher collagen I and III than cells maintained in M106, though this difference was not significant (Figure 3.25). In addition to secreting ECM, HDFn cells also actively remodel the deposited ECM. This was demonstrated through western blot and densitometry analysis for the expression of matrix metalloproteinase-1 (MMP-1); a collagenase that specifically degrades collagens I, III and VII. MMP-1 is secreted as an inactive proMMP-1 which is then subject to proteolytic cleavage to release active MMP-1. Models cultured in DMEM displayed higher levels of proMMP-1, yet the overall active levels of MMP-1 were comparable between the two culture conditions. This suggested that there was no difference in the level of remodelling occurring by MMP-1 in the lamina propria equivalents, as such the statistically significant increase in the expression of proMMP-1 is unlikely to have any significant biological impact.

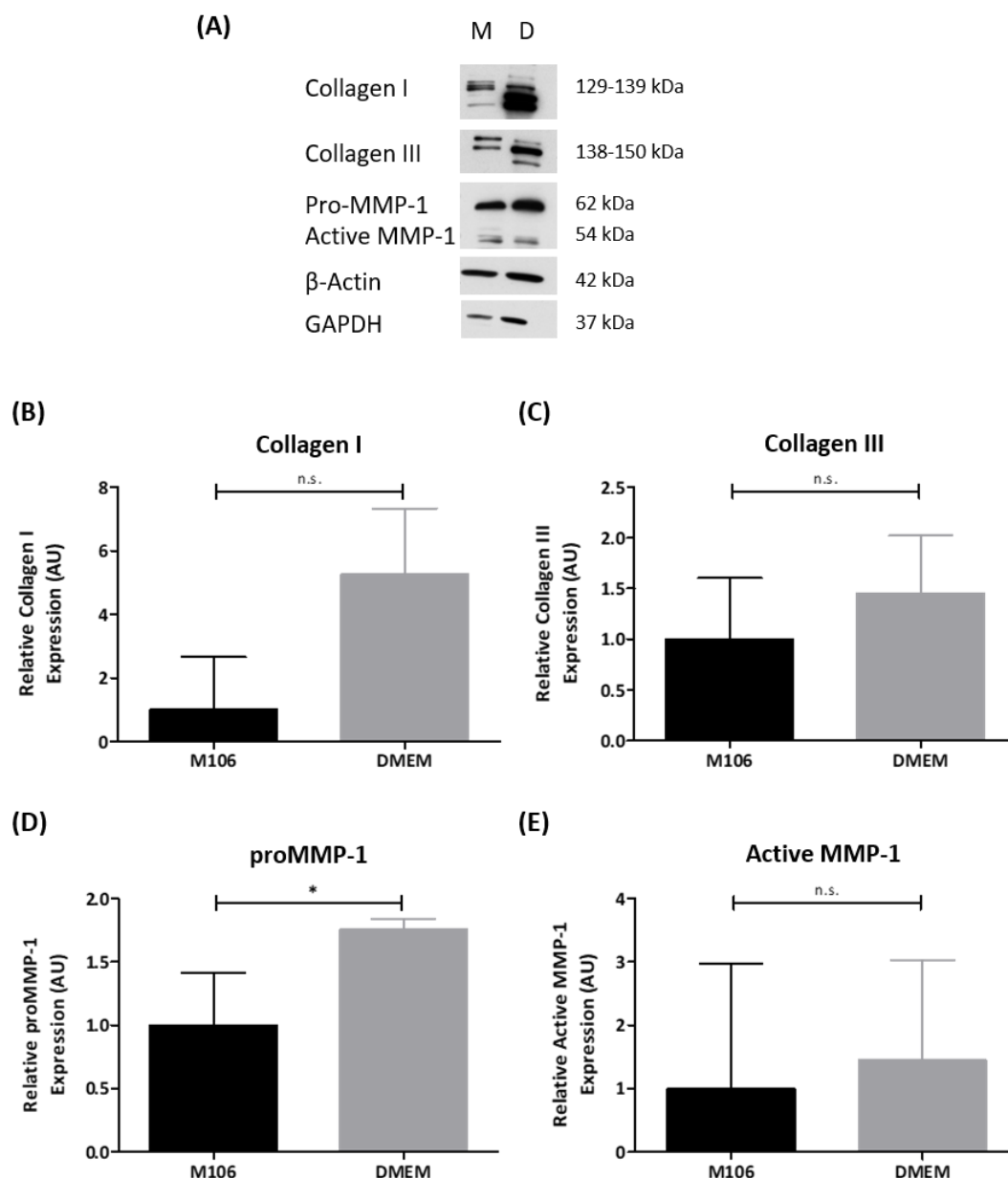


Figure 3.25. Western blot analysis of lamina propria equivalents cultured for 28 days in M106 and DMEM. Dermal fibroblasts were cultured in Alvetex® Scaffold for 28 days in M106 (M) or DMEM (D) media. Models were then harvested for western blot analysis for collagen I, III, MMP-1 and the loading controls GAPDH and β -actin, representative images of 3 gels shown (A). Densitometry analysis was performed using Fiji (B-E). Expression levels of collagen I (B), III (C), proMMP-1 (D) and active MMP-1 (E) were normalised to expression levels of β -actin then to M106. Data represent mean $\pm$ SEM, total $n=3$ from 3 SDS-PAGE gels. * $P \leq 0.05$ as determined by Student's t -test.

Following the establishment of the 28-day lamina propria equivalent in both M106 and DMEM, Caco-2 cells were then seeded onto the surface of the fibroblasts and maintained for a further 21 days. H&E staining demonstrated that a homogenous, polarised epithelial monolayer was formed on the apical surface of the lamina propria equivalents, with no evidence infiltration of Caco-2 cells into the underlying fibroblast layers (Figure 3.26). There was also no difference detected in the structural morphology of the Caco-2 monolayer, as cells exhibited a highly columnar phenotype in both culture conditions.

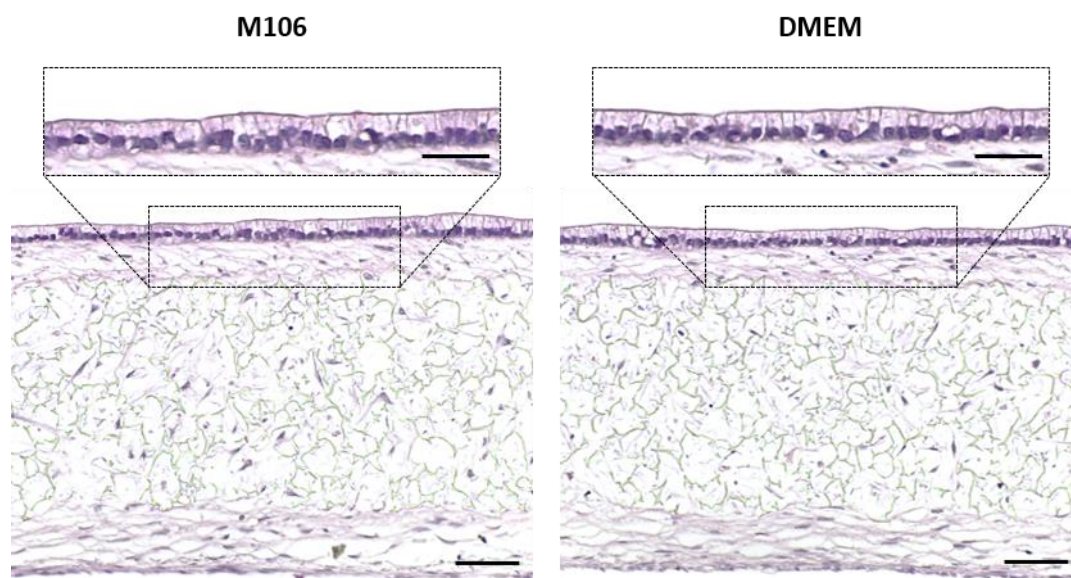


Figure 3.26. Caco-2 cells differentiate into polarised monolayers when cultured on the surface of 28-day lamina propria equivalents cultured in M106 or DMEM. H&E staining of Caco-2 cells grown on the surface of HDFn cells pre-cultured for 28 days in Alvetex® Scaffold in either M106 (left) or DMEM (right). Higher magnification images of the Caco-2 monolayer reveal a polarised, columnar morphology of the epithelium. Images shown are representative of 9 models. Scale bar: 50 μm .

Barrier properties of the resulting co-culture models were assessed through a variety of different techniques. Wholemout immunostaining of the tight junction protein ZO-1 revealed that there was little difference in the expression pattern of the scaffolding protein and confirmed that the apical junctional complex was formed across the epithelium (Figure 3.27A). Further analysis of the barrier integrity was performed through TEER measurements throughout the culture period of the Caco-2 monolayer; TEER increased throughout the growth of the epithelium on fibroblasts precultured in M106 and DMEM with very few differences in the absolute TEER value being observed (Figure 3.27B). Furthermore, the final TEER values taken on the 21st day of Caco-2 culture when they epithelium was fully differentiated revealed no statistically significant differences between the two models; the final TEER for M106 and DMEM were 41 $\Omega\cdot\text{cm}^2$ and 51 $\Omega\cdot\text{cm}^2$ respectively (Figure 3.27C).

The final method of determining whether an intact barrier had formed, was through a lucifer yellow rejection assay. High rejection of lucifer yellow was detected in both models in the apical-basolateral, with no significant differences between the two culture formats (Figure 3.27D), and although a minor difference in lucifer yellow rejection was found between M106 and DMEM models in the secretory direction (Figure 3.27E), it is highly unlikely that this will have any physiological significance, as the overall rejection levels were greater than 98 % for both conditions. Taken together these results demonstrate that DMEM is a suitable culture medium for the growth and maintenance of HDFn cells to form a lamina propria equivalent before the seeding of Caco-2 epithelial cells.

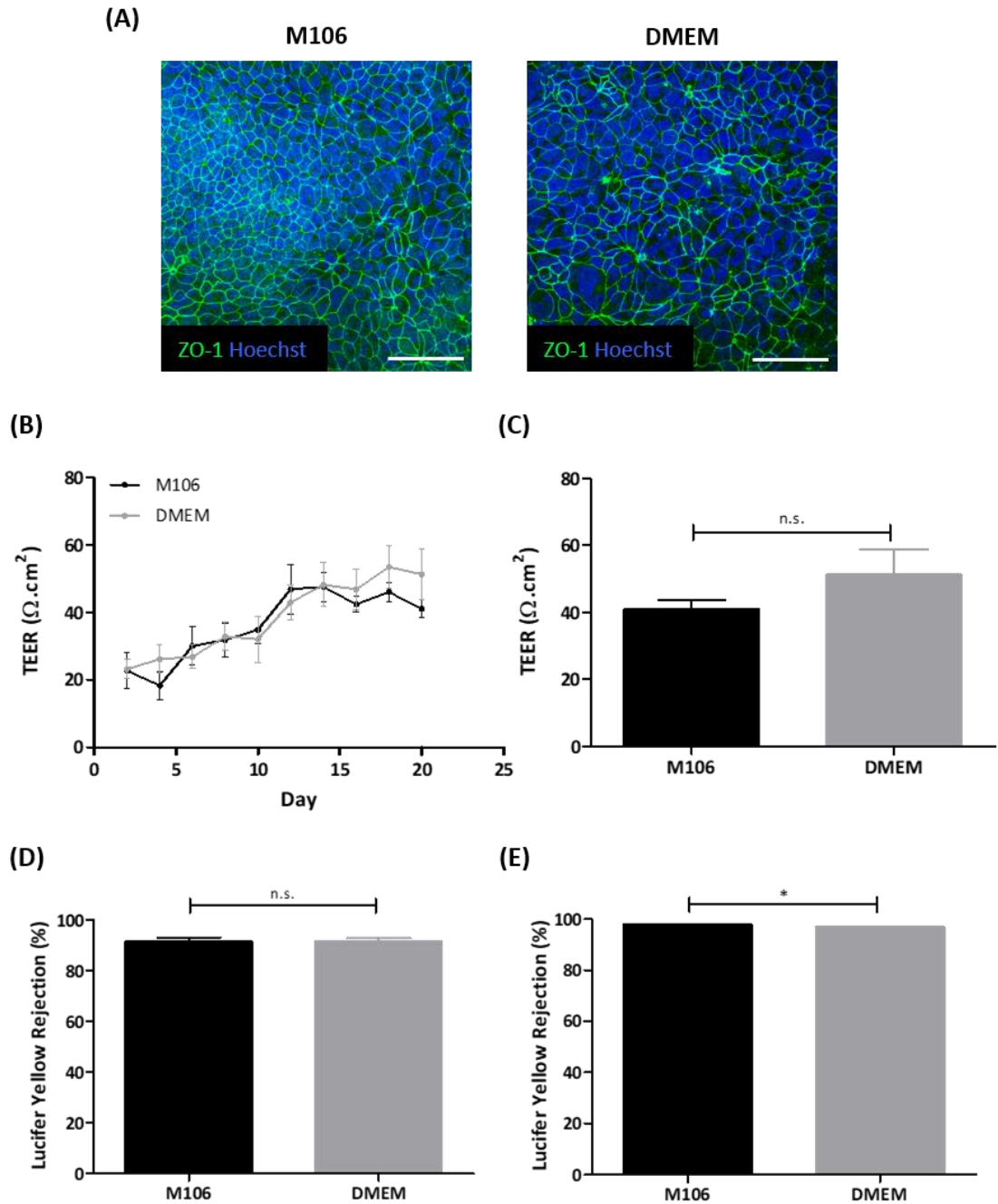


Figure 3.27. Caco-2 cells form an intact barrier when cultured for 21 days on 28-day lamina propria equivalents. Top-down immunolabelling for the tight junction protein ZO-1 (green), nuclei was visualised with Hoechst staining (blue) (A). TEER was measured continuously every two days of Caco-2 growth and displayed a general increase with time (B). Endpoint TEER values did not alter with different culture media (C). A lucifer yellow transport assay was performed on models in the apical-basolateral (D) and basolateral-apical (E), rejection was high when the supporting fibroblasts were cultured in both media types. Images shown are representative of 3 models. Data represent mean $\pm$ SEM, total $n=3-9$ comprising of 3 independent test occasions each with 1-3 technical replicates. * $P \leq 0.05$ as determined by Student's t -test. Scale bars: 50 μm

3.4.3.3. Reduction of HDFn culture time to increase the throughput of model development

Fibroblasts maintained for 28 days in DMEM with additional TGF- β 1, and ascorbic acid supplements have been demonstrated to support the formation of a Caco-2 monolayer. However, the total length of culture time for the co-culture model to reach maturity is 49 days, not including the 2D culture of cells prior to seeding in the model. A reduction in culture time would lead to increased model throughput, should it be adapted to more industrial roles. Fibroblasts were therefore maintained for 7, 14, 21 and 28 days to determine if the growth period could be refined.

As shown by H&E staining in Figure 3.28A, successful lamina propria equivalents were formed at all time points tested. HDFn cells grew within Alvetex® Scaffold and there appeared to be an increased cell density with increasing culture time. Additionally, the layering of fibroblasts on the edges of the membrane also appeared to increase with time. To examine this in greater detail, measurements of the fibroblast thickness were taken along the entirety of the scaffold, by tracing the edges of the membrane and then measuring the layering of the cells (Figure 3.28B). A statistically significant incremental increase in fibroblast thickness was observed with time with HDFn at 7 days having a thickness of $12.24 (\pm 0.53) \mu\text{m}$ while cells maintained for 28 days were $38.95 (\pm 1.82) \mu\text{m}$ thick. Too little fibroblast layering could allow Caco-2 cells to infiltrate into the lamina propria while too thick could prevent nutrient flow and inhibit transport processes.

Metabolic activity of lamina propria equivalents at different time points was also assessed through an MTT assay (Figure 3.28C). Cells were viable at all stages of culture yet a general increase in the metabolic activity of cells was observed with increasing culture time. However, the only significant difference was observed between the 7-day and 28-day time points. The 7-day lamina propria equivalent was much more variable in its metabolic activity than later time points, possibly as a result of cell saturation of the interior membrane at days 14-28.

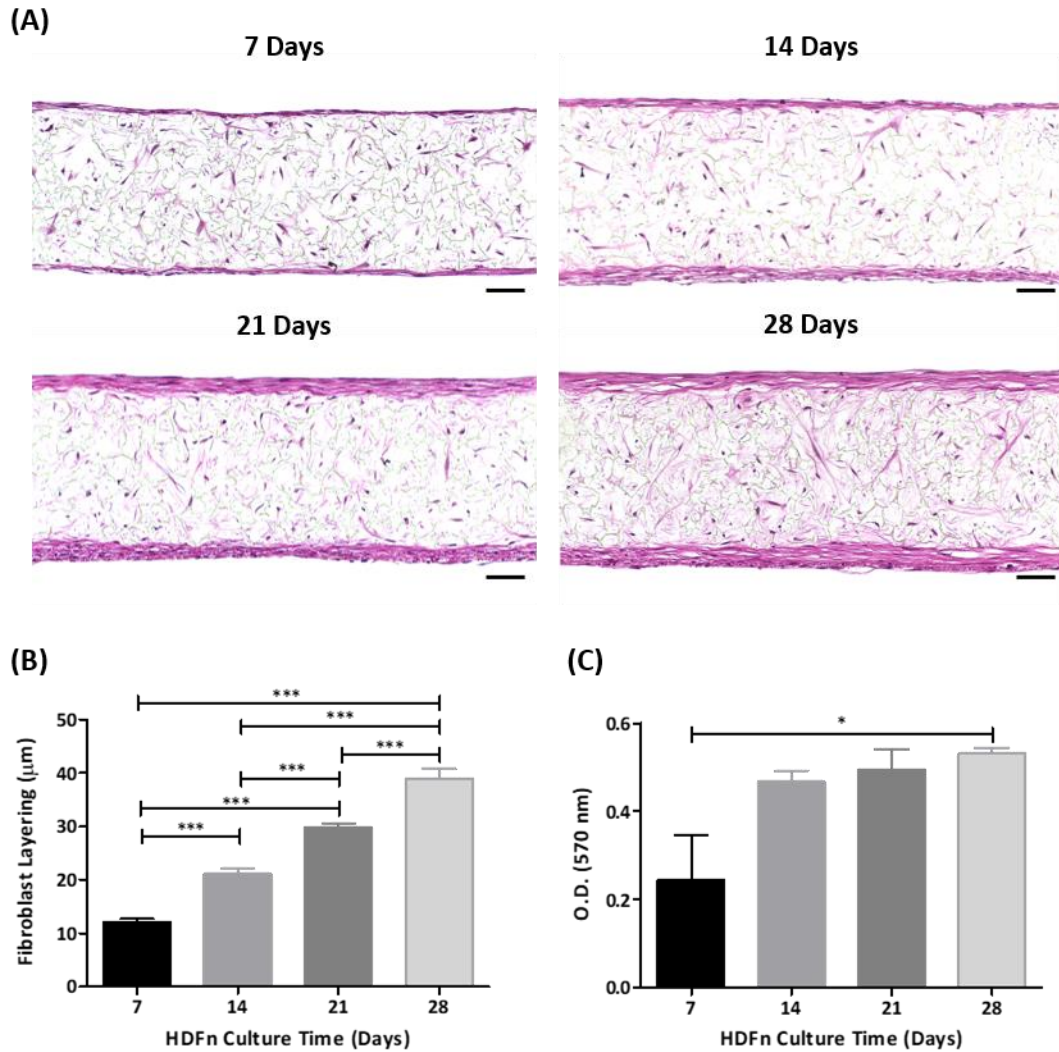


Figure 3.28. Characterisation of lamina propria equivalents cultured for up to 28 days in DMEM. H&E staining of HDFn cultured for 7, 14, 21 and 28 days in Alvetex® Scaffold in DMEM growth medium (A). Quantification of fibroblast layering on the base and surface of the lamina propria equivalents (B). Cell metabolic activity was assessed at day 7, 14, 21 and 28 of HDFn culture via a MTT assay with the absorbance read at 570 nm. Data represent mean $\pm$ SEM, total $n=50$ from 9 models (B), $n=9$ comprising of 3 independent test occasions each with 3 technical repeats (C). * $P \leq 0.05$, *** $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post hoc test. Scale bars: 50 μm .

Assessment of ECM deposition in the 7-28-day lamina propria equivalents was carried out through immunofluorescent staining and western blot analysis. Vimentin staining was again utilised to indicate the distribution of HDFn cells throughout the membrane. Vimentin emphasised how the ratio of cells altered throughout culture; at day 7, few vimentin positive cells are present on the edges of the membrane while most cells appeared to be localised within the scaffold itself. By day 28 of culture cells remained distributed throughout the whole of the membrane but had an increased density of vimentin-positive cells on the surface and base of the scaffold (Figure 3.29). Regarding ECM deposition, an increase was observed in the deposition of collagen I, III and fibronectin from day 7-21, yet no further increase could be detected at day 28 as the membrane appeared to be fully saturated with ECM. Collagen I deposition was greater than that of collagen III at all time points, while strong fibronectin staining was demonstrated at all stages of lamina propria culture, to a greater extent than the collagens (Figure 3.29).

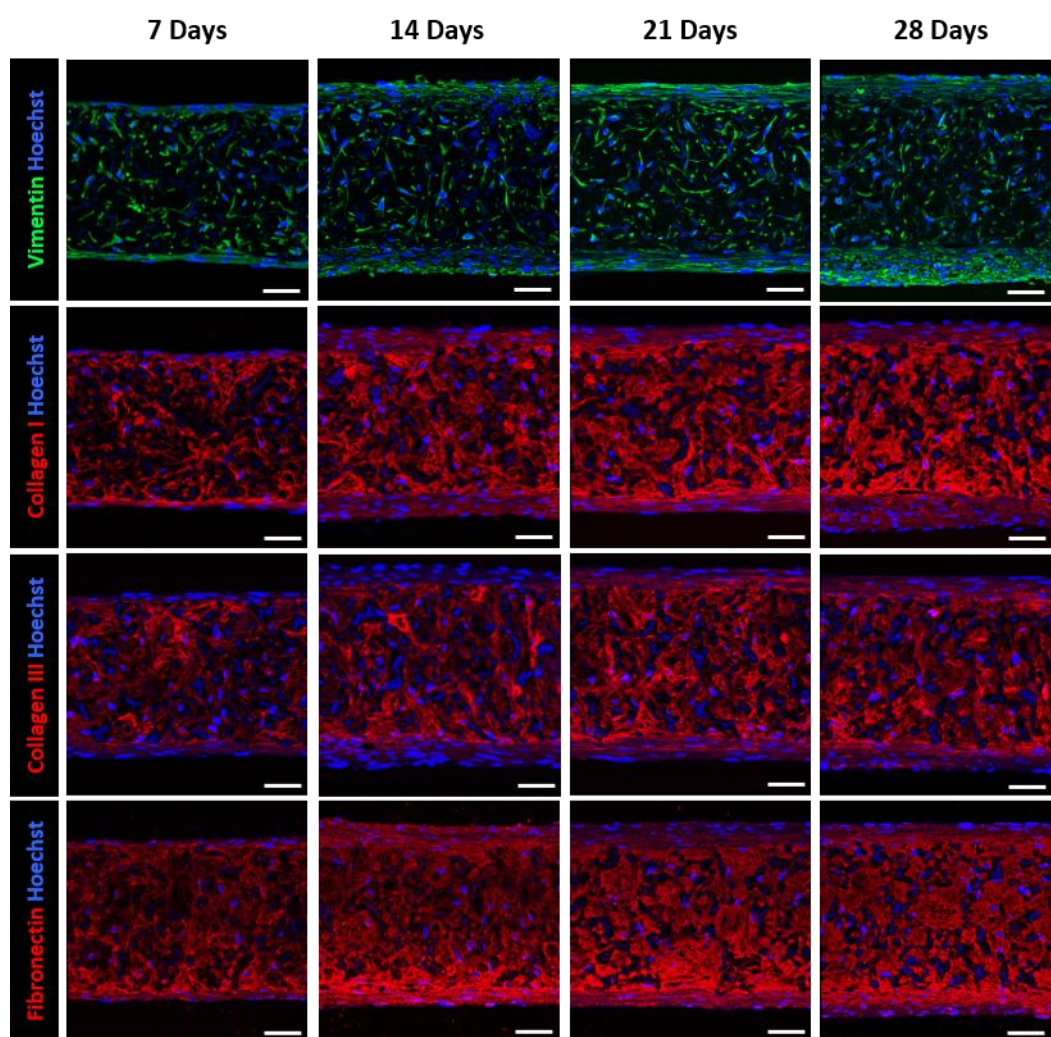


Figure 3.29. Characterisation of ECM deposition by HDFn cultured for up to 28 days in DMEM. Samples were immunolabelled for the fibroblast marker, vimentin (green) and for the ECM markers, collagen I, collagen III, and fibronectin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 9 models. Scale bars: 50 μ m.

Western blot and the corresponding densitometry analysis revealed general trends in the deposition of ECM at the different culture time periods (Figure 3.30). The expression levels of collagens I and III were particularly low in models cultured for 7 days, suggesting that insufficient ECM is being produced and may not successfully support an overlying epithelial layer without Caco-2 cell infiltration into the Alvetex® Scaffold. Expression of collagen I demonstrated a significant incremental increase with culture time, correlating with the immunofluorescent staining for the protein. However, this was not the case with collagen III which had low expression levels at day 7 and highest levels at day 14 then day 21. However, by day 28 a large reduction in the expression of collagen III could be observed. This decline in collagen III correlates with a sharp increase in the expression of active MMP-1 which, although has specificity for collagen I degradation, it preferentially cleaves collagen III. Levels of proMMP-1 did not alter between any of the culture time points but an increased level of active MMP-1 was determined at 28 days as previously mentioned.

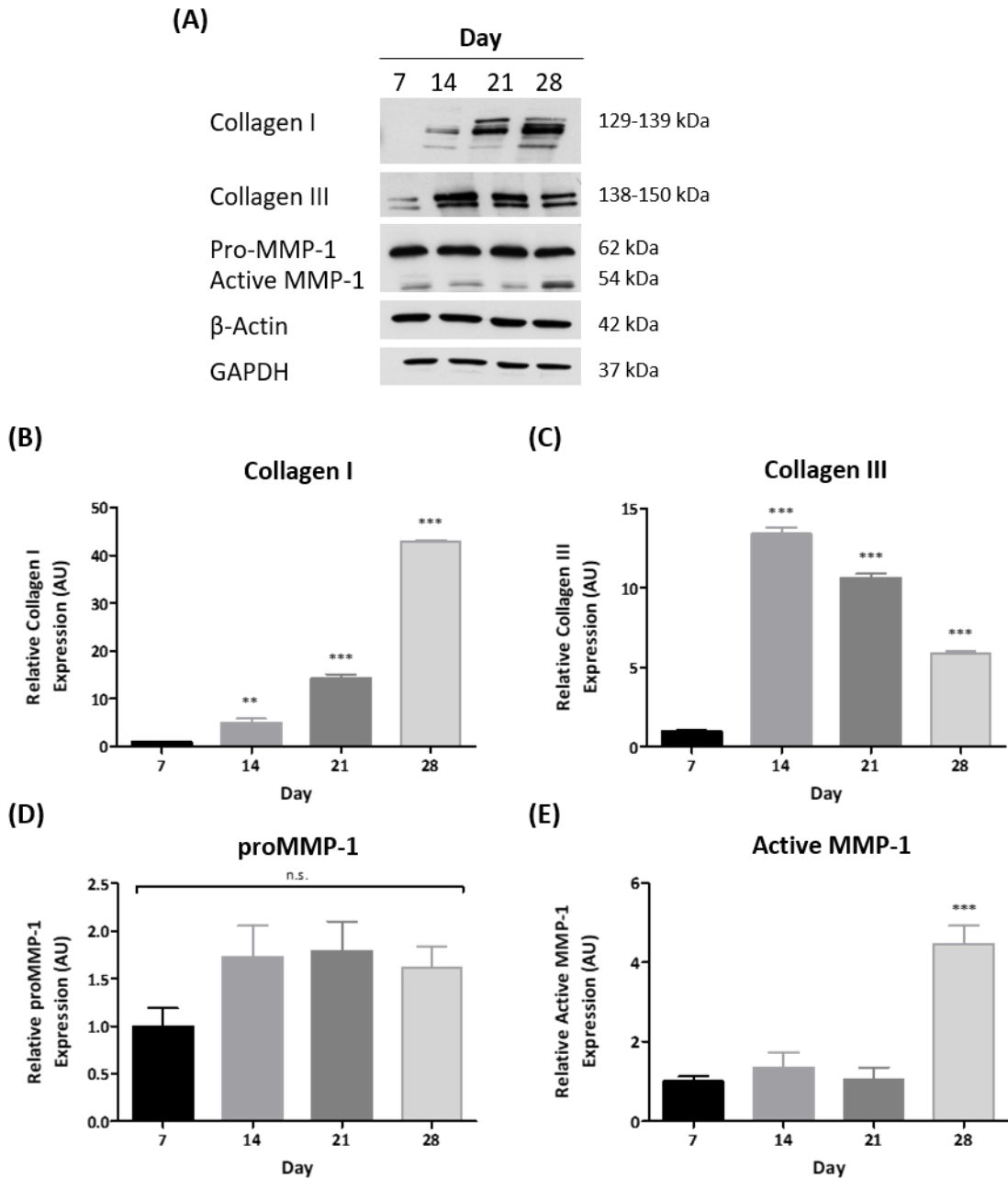


Figure 3.30. Western blot analysis of lamina propria equivalents cultured for 7-28 days in DMEM. Dermal fibroblasts were cultured in Alvetex® Scaffold in complete DMEM with TGF- β 1 and ascorbic acid supplements for 7, 14, 21 and 28 days. Models were then harvested for western blot analysis for collagen I, III, MMP-1 and the loading controls GAPDH and β -actin, representative images of 3 gels shown (A). Densitometry analysis was performed using Fiji (B-E). Expression levels of collagen I (B), III (C), proMMP-1 (D) and active MMP-1 (E) were normalised to the expression levels of β -actin and then to expression at day 7. Data represent mean $\pm$ SEM, total $n=3$ from 3 SDS-PAGE gels. ** $P \leq 0.001$ and *** $P \leq 0.0001$ as determined by one-way ANOVA with Dunnett's post-test with comparison to the 7-day culture period.

Formation of a Caco-2 monolayer was successful on all lamina propria equivalents; with a homogenous, polarised epithelium demonstrating a columnar morphology with evidence of brush border formation (Figure 3.31). No infiltration of Caco-2 cells into the lamina propria equivalents was observed at any of the culture conditions, and no structural differences were noted in any of the models developed.

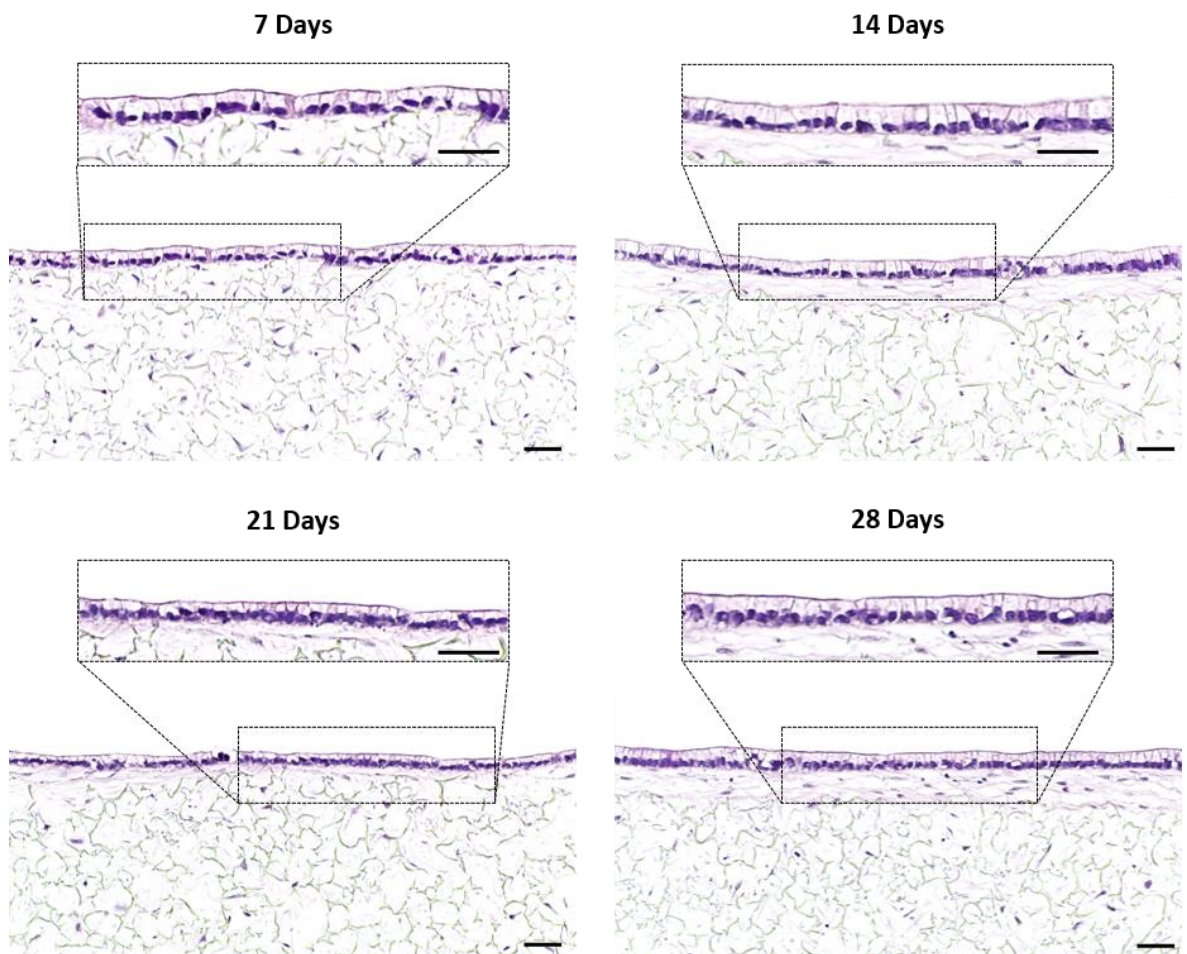


Figure 3.31. Histological analysis of Caco-2 cells cultured on lamina propria equivalents cultured for up to 28 days. H&E staining of Caco-2 monolayers cultured on the surface of HDFn cells precultured for 7, 14, 21 and 28 days in DMEM. Images shown are representative of 9 models. Scale bars: 50 μ m.

Functional characterisation of the barrier properties between the different models was assessed through TEER measurements taken at the final day of Caco-2 maintenance. Resistance ranged from 75-87 $\Omega \cdot \text{cm}^2$ across the different models but no statistically significant differences were determined (Figure 3.32A). Rejection of lucifer yellow was also high across all co-culture conditions but appeared to have higher rejection in the basolateral-apical direction, possibly due to the lamina propria impeding upward flow of the compound (Figure 3.32B, C). A significant reduction in the rejection of lucifer yellow was identified in the secretory transport direction at days 21 and 28, however, the overall difference in lucifer yellow rejection was < 1 % suggesting that it is unlikely to have a physiological impact on the models (Figure 3.32C). Taken together, this data demonstrated that lamina propria culture time can be reduced while still supporting the growth and appropriate function of a Caco-2 epithelial monolayer.

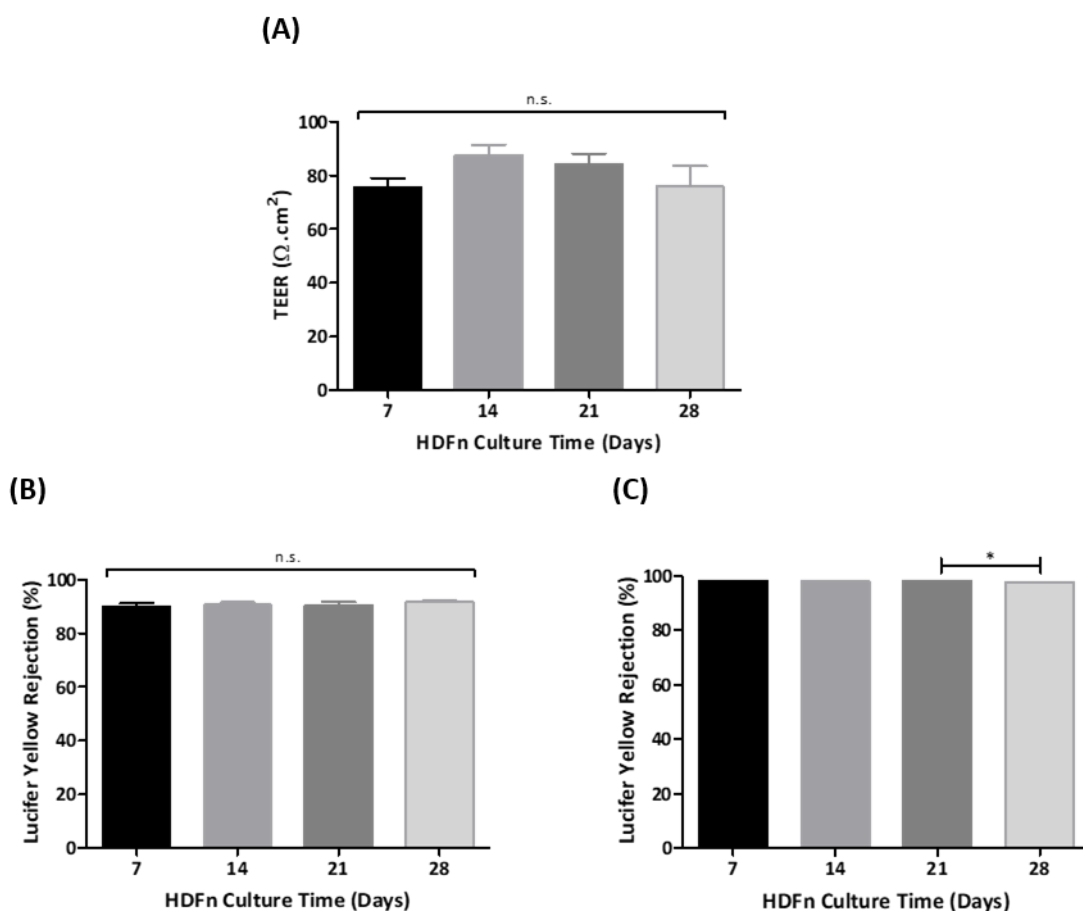


Figure 3.32. Characterisation of the barrier properties of Caco-2 cells cultured on lamina propria equivalents cultured for up to 28 days. Endpoint TEER measurements were taken at day 21 of epithelial cell culture (A). A lucifer yellow rejection assay was performed in the apical-basolateral (B) and basolateral-apical (C) directions. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions each with 3 technical replicates. * $P \leq 0.05$ as determined by one-way ANOVA with Tukey's post hoc test.

3.4.4. Characterisation of the 3D Caco-2 HDFn Co-Culture Model

Although no infiltration was observed of Caco-2 cells into the 7-day lamina propria equivalent, the minimal fibroblast layering along the apical surface of Alvetex® Scaffold indicates that the Caco-2 cells are likely to be resting on the polystyrene surface across some areas of the membrane; the epithelium is not experiencing the more tissue mimetic and physiologically relevant physiochemical properties of the stromal cells. Therefore, the model chosen to take forward was the 14-day lamina propria culture with Caco-2 cells maintained on the apical surface for a further 21 days. These models were highly reproducible, with less than 1 in 50 failing to form an acceptable epithelium as assessed by TEER throughout the differentiation period of the Caco-2 monolayer and confirmed by histology following sample processing.

Histological analysis of the Caco-2 monolayer on the 14-day lamina propria equivalent demonstrated that Caco-2 cells formed a continuous, polarised monolayer when seeded on the surface of the 3D HDFn culture (Figure 3.33A, B). Toluidine blue staining highlighted the extreme columnar phenotype observed along the epithelium and revealed the presence of microvilli at the apical pole of the epithelial cells (Figure 3.33B). TEM micrographs displayed the gross ultrastructural morphology of the Caco-2 epithelium, highlighting the basally located nuclei (Figure 3.33C). Clear evidence of a microvilli brush border and associated glycocalyx, the carbohydrate-rich coating present along the epithelial apical surface of the GI tract, was observed (Figure 3.33D). Electron dense apical junctions were present at the lateral membranes between adjacent Caco-2 cells (Figure 3.33E) along with the presence of electron dense structures identified at the base of the epithelium suggesting the formation of a tri-lamina basement membrane between the epithelium and subjacent fibroblasts (Figure 3.33F).

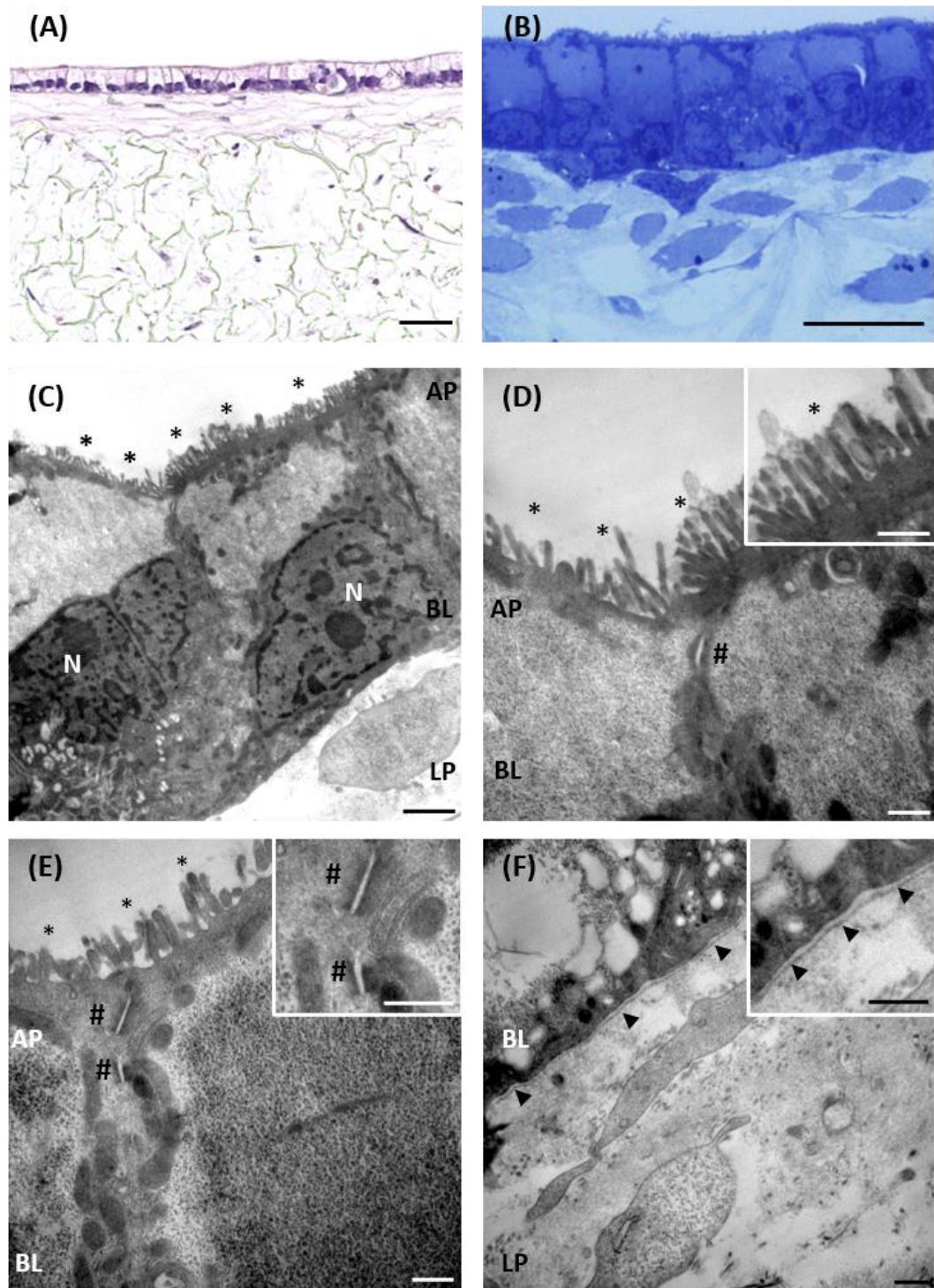


Figure 3.33. In-depth characterisation of the morphology of the co-culture epithelium. H&E staining of Caco-2 cells cultured on the surface of a lamina propria equivalent pre-cultured for 14 days (A). Toluidine blue staining of the co-culture demonstrates the columnar polarisation of the epithelium (B). TEM micrographs of the 3D co-culture model (C,D,E,F) highlight the ultrastructural features of the model including the columnar shape (C), the microvilli brush border (D,D-inset); the formation of junctional complexes between adjacent epithelial cells (D,E, E-inset) and the formation of a basement membrane-like structure (F,F-inset). * denote the microvilli brush border, # indicate the electron dense junctions, and arrowheads highlight the basement membrane-like structure. Images are representative of 3 models. Scale bars: 50 μ m (A), 20 μ m (B), 2 μ m (C), 500 nm (D,D-inset, E, E-inset, F, F-inset).

Immunofluorescent analysis of the lamina propria compartment of the co-culture model demonstrated that the fibroblast distribution and the expression profile of ECM proteins was not altered upon co-culture with Caco-2 cells; endogenous collagen I, III and fibronectin proteins were deposited within the membrane (Figure 3.34). Additional staining was carried out for the expression of collagen IV, the primary collagen component of the basement membrane; although collagen IV was present along the basolateral surface of the epithelium, it was also distributed throughout the membrane potentially due to cross-reactivity of the antibody with other collagens present in the lamina propria.

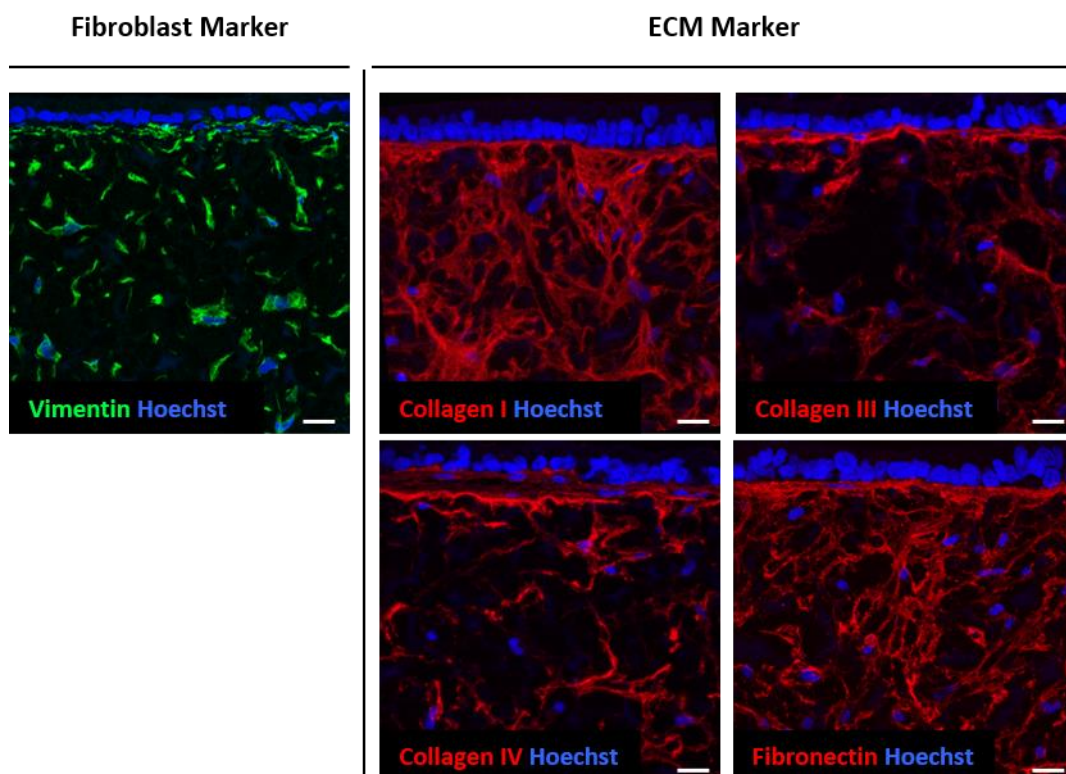


Figure 3.34. Characterisation of the extracellular matrix. Caco-2 cells were cultured for 21 days upon 14-day lamina propria equivalents and were immunolabelled for the fibroblast marker vimentin (green) and the ECM markers; collagen I, III, IV and fibronectin (red). Nuclei were counterstained with Hoechst. Images shown are representative of 9 models. Scale bar: 20 μ m.

Assessment of the apical junctional complex was again performed through immunofluorescent staining. Top-down compressed Z-stacks of co-cultures immunolabelled for tight and adherens junction proteins emphasised the regular cobblestone morphology of the epithelium (Figure 3.35). Both intracellular scaffolding proteins (ZO-1) and intercellular adhesion proteins (occludin, claudin-1 and -2) were observed across the epithelium. The presence of sealing and pore-forming claudin proteins highlights the dynamic nature of the tight junctions and suggests that the barrier is not completely impermeable to the transport of chemical compounds. Additionally, the presence of E-cadherin cell attachments to adjacent cells confirms that adherens junctions were formed and have a role in maintaining the integrity of the tissue.

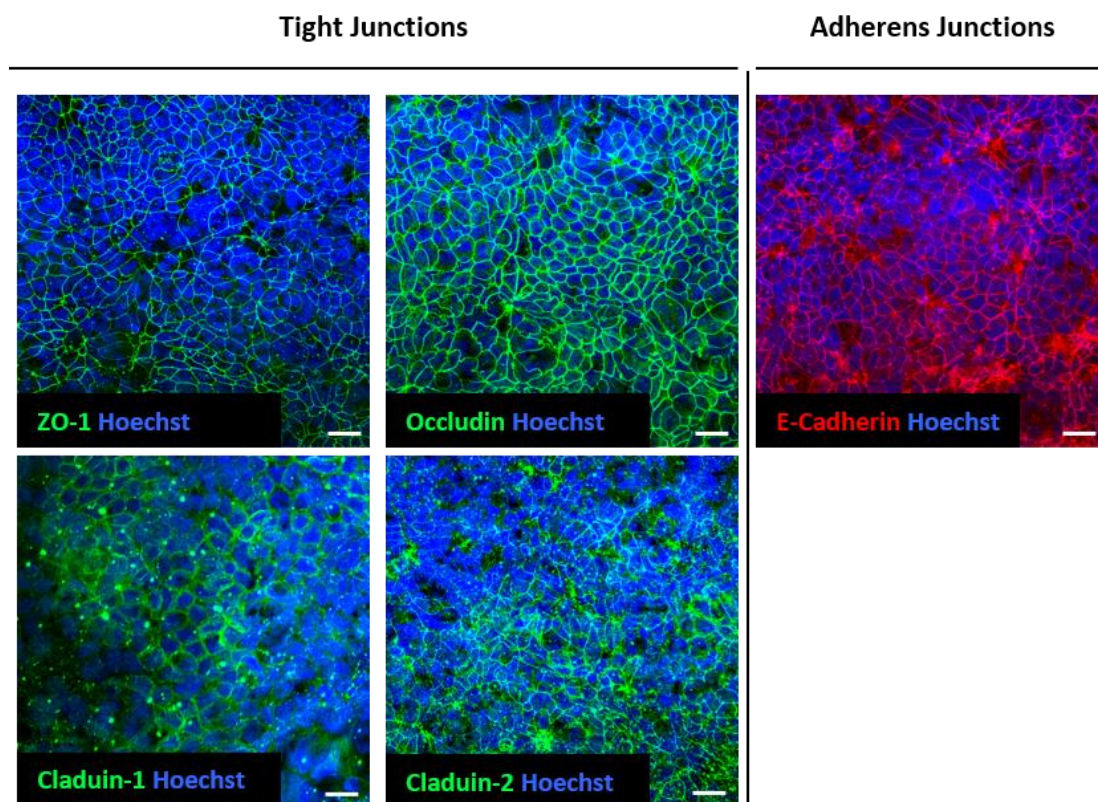


Figure 3.35. Characterisation of the apical junctional complex. Caco-2 cells were cultured for 21 days upon 14-day lamina propria equivalents and were immunolabelled for the tight junction markers (green); ZO-1, occludin, claudin-1 and claudin-2 and the adherens junction marker E-cadherin (red). Nuclei were counterstained with Hoechst. Top-down compressed Z-stacks of the co-cultures. Images shown are representative of 3 models. Scale bar: 20 μ m.

Functional analysis of the optimised Caco-2 HDFn co-culture system was also carried out. TEER analysis demonstrated that resistance increased with Caco-2 cell growth until a plateau of $\sim 65 \Omega \cdot \text{cm}^2$ was reached at day 14 of culture suggesting that by this point the Caco-2 cells had formed a confluent, continuous epithelial layer across the surface of the lamina propria equivalent (Figure 3.36A). Further analysis of lucifer yellow transport indicated that rejection was over 90 % implying an intact barrier was formed, however significantly higher lucifer yellow rejection was observed in the basolateral-apical direction of transport (Figure 3.36B). To confirm that this higher rejection would have biological significance, calculation of the apparent permeability coefficient of lucifer yellow was performed and confirmed that lucifer yellow had significantly greater permeability in the apical-basolateral direction in this culture system (Figure 3.36C).

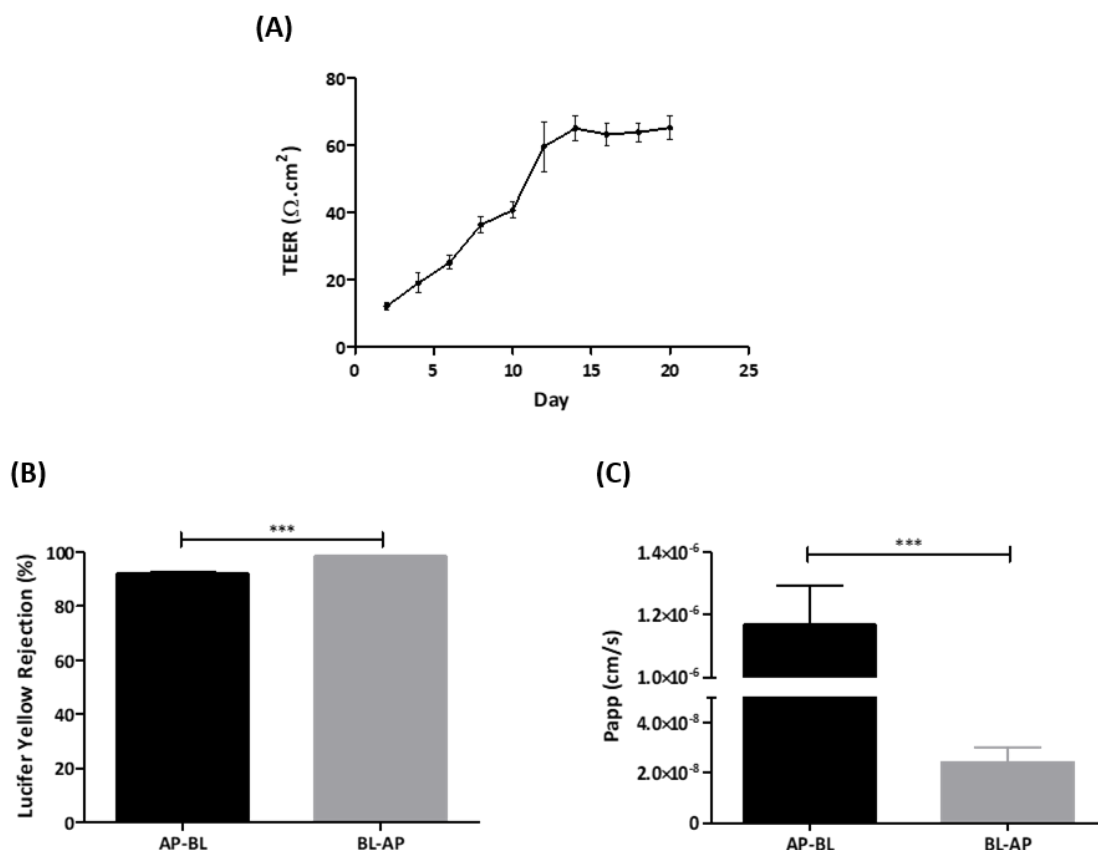


Figure 3.36. Characterisation of the barrier properties of the Caco-2 HDFn co-culture. TEER was measured every two days of Caco-2 culture on the surface of a 14-day lamina propria equivalent (A). The transport characteristics of lucifer yellow was assessed through a rejection assay (B) and through the calculation of the apparent permeability coefficient. Transport experiments were performed in both the apical-basolateral (AP-BL) and basolateral-apical (BL-AP) directions. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. *** $P \leq 0.0001$ as determined by Student's t -test.

Analysis of P-gp function was performed through a transport assay for rhodamine 123. Greater transport of rhodamine 123 was observed in the basolateral-apical direction and the efflux ratio was found to be $2.12 (\pm 0.22)$ suggesting that P-gp is present and actively promoting the efflux of the compound (Figure 3.37A,C). This active transport could be effectively abolished with the addition of the P-gp inhibitor, verapamil hydrochloride which significantly reduced the basolateral-active transport of rhodamine 123 and reduced the efflux ratio to $0.76 (\pm 0.08)$ (Figure 3.37.B,C).

In conclusion, a co-culture model of the intestinal mucosa comprising of an epithelial cell line, Caco-2, and a fibroblast cell line, HDFn, was successfully established. Structural characterisation determined that HDFn cells secrete endogenous ECM proteins that the epithelium rests on. The Caco-2 epithelium was well polarised with evidence of a microvilli brush-border and expressed components of the apical junctional complex. Furthermore, functional characterisation of the 3D co-culture demonstrated that the model had an intact barrier, and the *in vitro* culture could be used in transport assays of passively and actively transported chemical compounds.

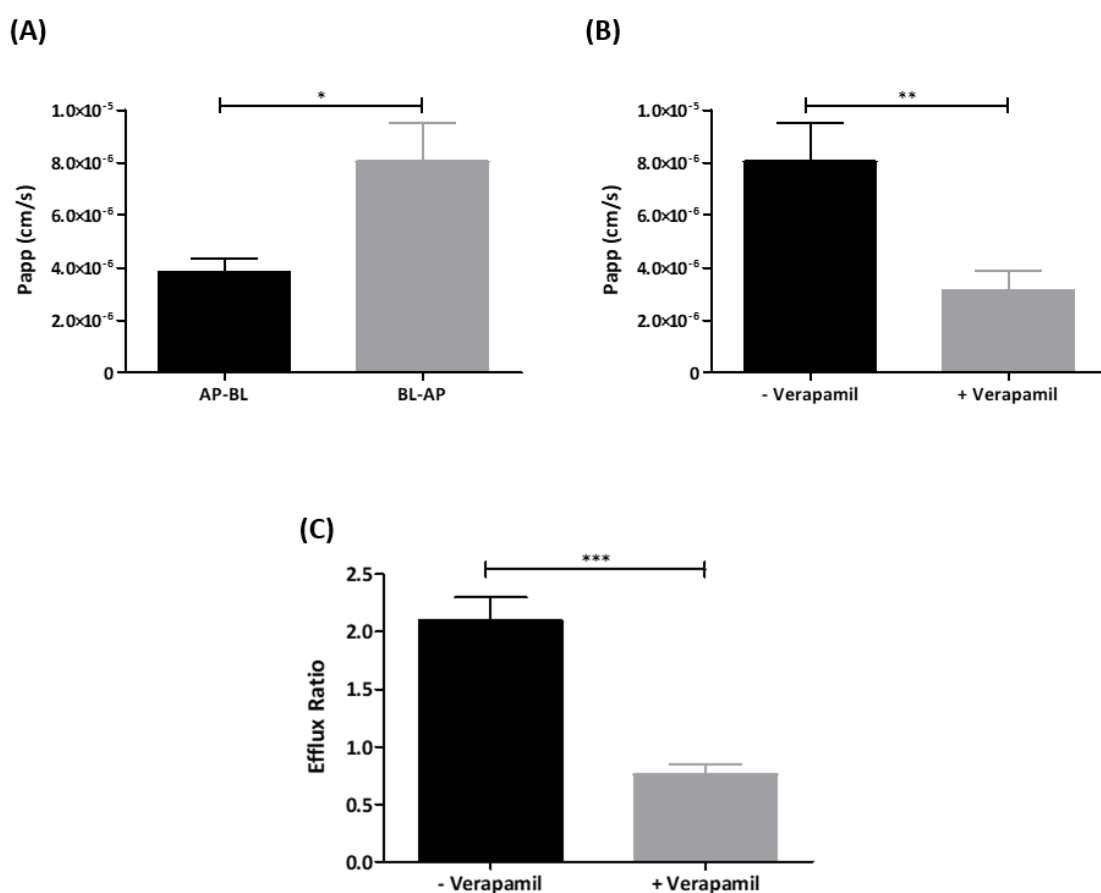


Figure 3.37. Characterisation of Rhodamine 123 transport across the Caco-2 HDFn co-culture model. The apparent permeability coefficient of the P-gp substrate, Rhodamine 123 was determined over a 2-hour long assay in the AP-BL and BL-AP directions (A). Verapamil, a P-gp inhibitor, was added to the BL-AP assay to determine if Rhodamine 123 was primarily transported via P-gp (B). The efflux ratio of Papp in the BL-AP direction to the Papp in the AP-BL direction was calculated in the presence or absence of verapamil (C) Data represent mean $\pm$ SEM, total $n=7-9$ comprised of 3 independent test occasions, each with 2-3 technical replicates. * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$ as determined by Student's t-test.

3.4.4.1. HDFn dermal fibroblasts and CCD-18co intestinal myofibroblasts recreate the structure of the intestine lamina propria when cultured in 3D

In addition to a Caco-2 HDFn co-culture model, a further intestinal model utilising CCD-18co intestinal myofibroblasts has been developed within the research group. Unlike the HDFn lamina propria equivalent, the CCD-18co *in vitro* model required no additional supplements to the growth media but instead had an additional 0.22×10^6 cells seeded at days 7, 9, and 11 of stromal cell culture to ensure sufficient cells were present within the scaffold to support and maintain an above epithelial layer. As this model contained stromal cells of intestinal origin, it was essential to characterise the HDFn model in contrast to the CCD-18co model and human intestine tissue samples to ensure that dermal fibroblasts were suitable for use in the model.

Histological analysis of the structural morphology of the different models in comparison with human small intestinal tissue revealed that like the intestine epithelium, Caco-2 cells cultured on HDFn cells displayed a columnar morphology with their nuclei being located in the basolateral half of the cell cytoplasm. Although Caco-2 cells cultured on the surface of a CCD-18co lamina propria equivalent demonstrated a columnar phenotype, they were not as well polarised as the HDFn model or the *in vivo* tissue. This was strongly indicated through the nuclei localisation, wherein the nuclei cover almost the entire Caco-2 cell, however the overall elongated nuclei shape is more consistent between the CCD-18co co-culture and human tissue. Furthermore, fibroblast layering is minimal in the CCD-18co co-culture system and there is also evidence of epithelial cell infiltration into the lamina propria compartment (Figure 3.38).

Evaluation of the epithelium was carried out through immunofluorescent staining for the junctional proteins occludin and E-cadherin, components of the tight junction and adherens junction, respectively (Figure 3.38). In both Caco-2 monolayers and the intestinal epithelium, occludin was expressed at the apical surface of the cells, reflecting its distribution in the apical junctional complex. E-cadherin, although present in the apical junctional complex is located below the tight junction components, this distribution pattern was reflected in the immunofluorescent analysis where E-cadherin was found along the lateral membranes of the epithelial cells. Immunofluorescence staining confirmed that the Caco-2 epithelium expressed appropriate junctional components that are present in the *in vivo* intestine.

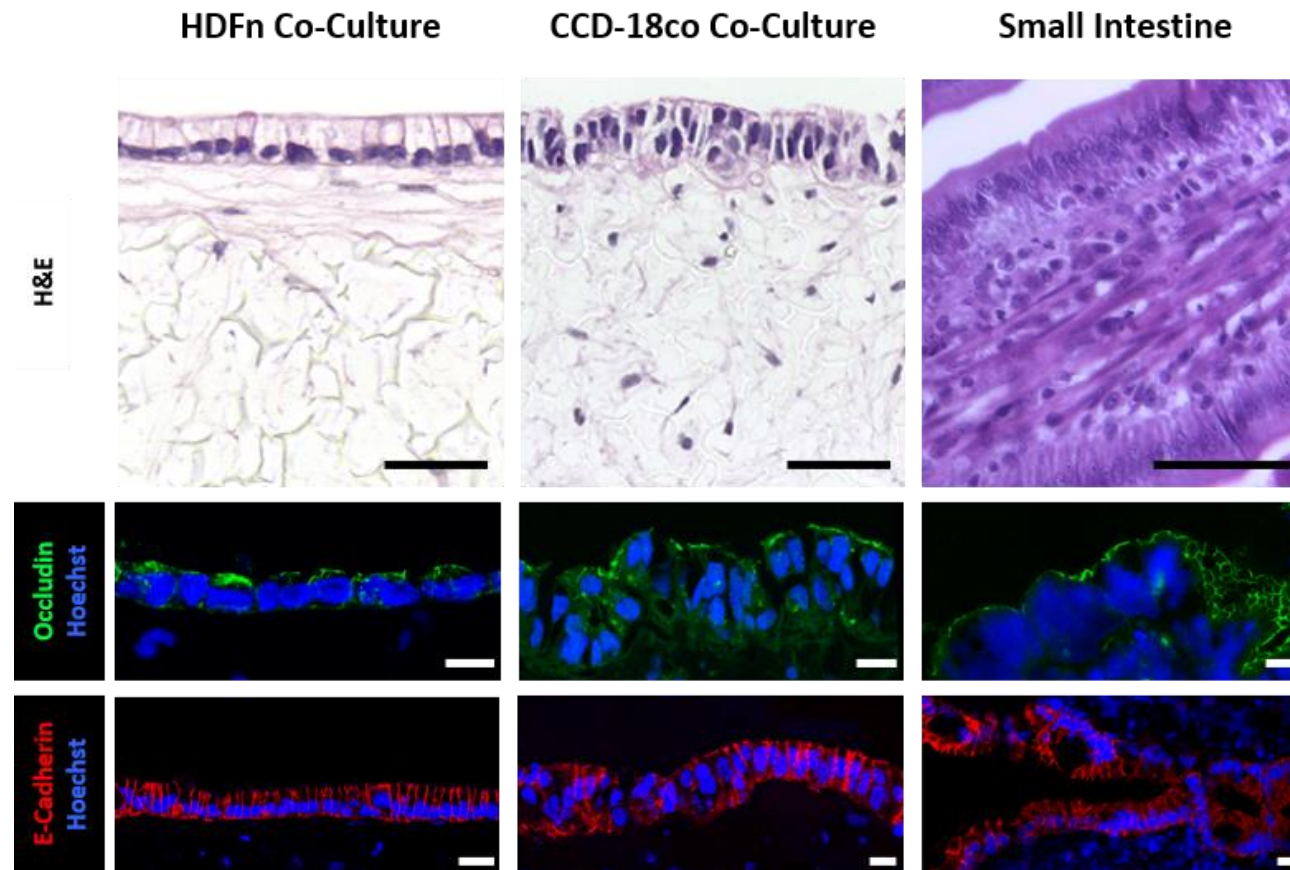


Figure 3.38. Caco-2 cells resemble the intestinal epithelium in HDFn and CCD-18co co-culture models. H&E staining of Caco-2 monolayers cultured on the surface of lamina propria equivalents cultured with HDFn or CCD-18co fibroblasts compared to human small intestine tissue. All models express appropriate tight junctions (occludin, green immunolabelling) and adherens junctions (E-Cadherin, red immunolabelling) that are present in vivo. Immunofluorescent staining of samples was counterstained with Hoechst to visualise nuclei (blue). CCD-18co models cultured and images provided by Dr Matthew Freer. Images shown are representative of 3 models. Scale bar: 50 μ m.

Immunofluorescent staining was also used to further compare the two lamina propria compartments to human tissue (Figure 3.39). Dense vimentin-positive cell staining was observed in the *in vivo* tissue and although present in both models, staining was not as prominent *in vitro*. Furthermore, vimentin staining emphasises the absence of fibroblast layering and epithelial infiltration in the CCD-18co co-culture model. Fibroblasts *in vivo* and *in vitro* in 3D lamina propria equivalents produce abundant endogenous extracellular matrix proteins that provide support to the overlying epithelium. Collagens I, III, IV and fibronectin were all found to be deposited by both dermal and intestinal fibroblasts as well as being observed in human intestinal tissue. Collagen IV could be clearly noted at the basolateral surface of the intestinal epithelium and could also be identified *in vitro*, providing evidence for the formation of a basement membrane in the 3D co-culture models. The presence of ECM proteins *in vitro* that have the same expression pattern and localisation as the human intestine suggests that a physiologically relevant lamina propria-like layer is formed in the co-cultures and emphasises the overall model structure is highly like the human intestine. Importantly, HDFn fibroblasts, although of dermal origin, deposit appropriate ECM proteins for an intestinal model and unlike the CCD-18co fibroblasts, successfully prevent the infiltration of Caco-2 cells into the lamina propria compartment; this resulted in the formation of a polarised monolayer.

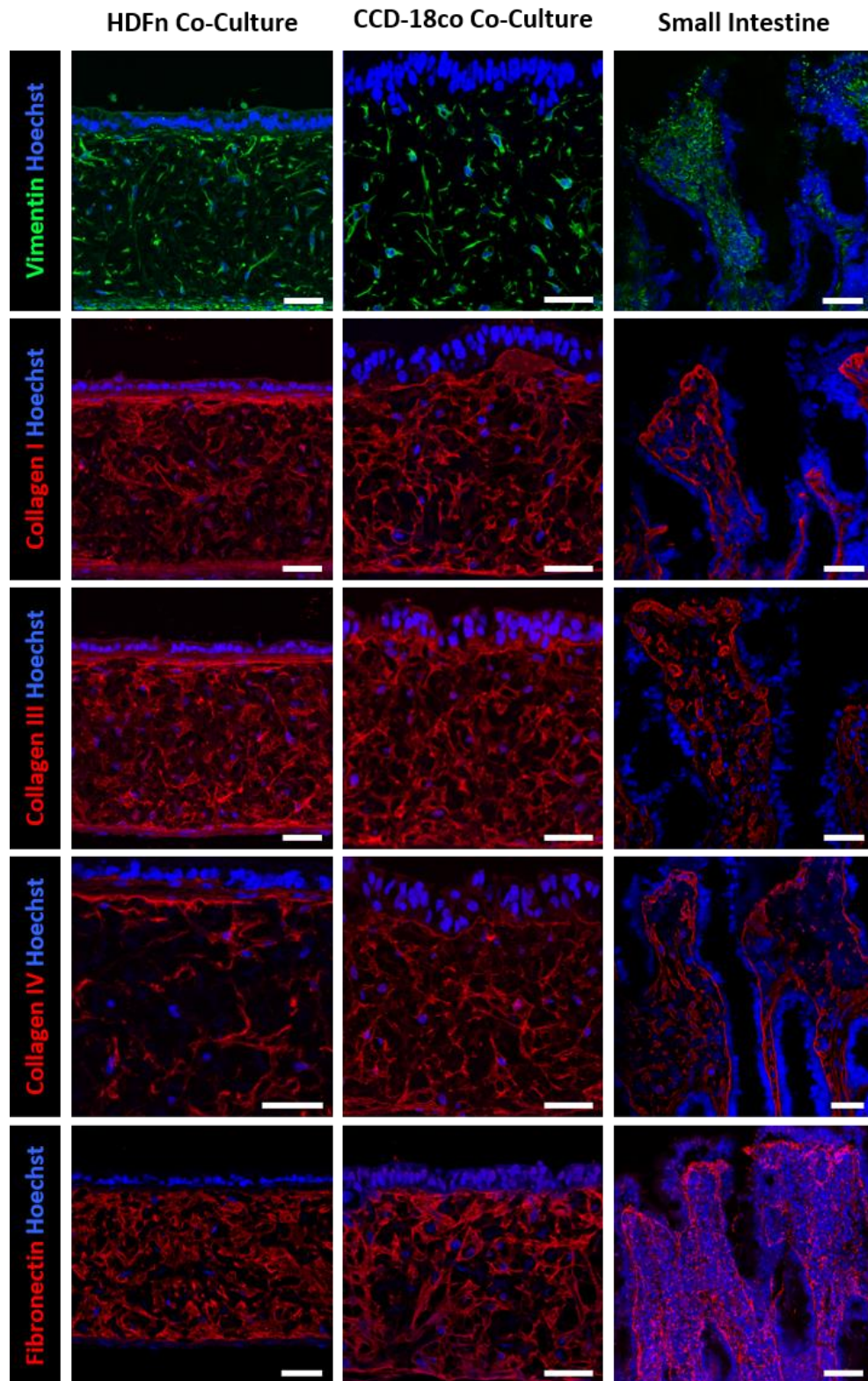


Figure 3.39. HDFn fibroblasts secrete the same ECM profile as intestinal fibroblasts. Caco-2 cells were co-cultured on the surface of HDFn or CCD-18co lamina propria equivalents and immunolabelled for the stromal cell marker; vimentin (green) and the ECM markers; collagens I, III and IV and fibronectin (red) and were compared with human small intestinal samples. Nuclei were counterstained with Hoechst (blue). CCD-18co models cultured and images provided by Dr Matthew Freer. Images shown are representative of 3 models. Scale bars: 50 μ m.

3.4.4.2. 3D HDFn co-cultures have a structural morphology more like the human intestinal mucosa than the Caco-2 monolayer

As the Transwell® Caco-2 monoculture is the current gold standard *in vitro* intestinal model, the 3D HDFn co-culture was also compared against this culture system. H&E staining of the two *in vitro* models generated on Transwell® or Alvetex® Scaffold membranes highlights the differences in Caco-2 cell morphology between the culture conditions. Transwell® Caco-2 control models display a shortened and cuboidal morphology, strikingly different from the 3D co-culture and *in vivo* intestine (Figure 3.40).

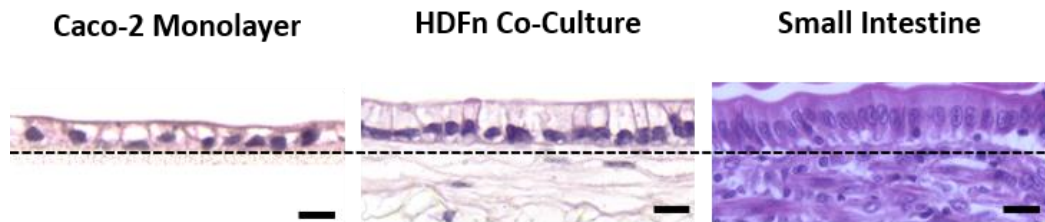


Figure 3.40. Caco-2 monolayers resemble small intestinal tissue. The Transwell® monolayer was compared to the HDFn co-culture system and human small intestinal tissue samples. H&E staining highlights the epithelial cell polarisation. Images depicted are representative of 9 models. Scale bars: 20 μ m.

Immunofluorescent wholemount staining was performed for ZO-1, occludin, claudin-1 and claudin-2 to demonstrate the formation of tight junctions, while E-cadherin was used to visualise adherens junctions. Representative compressed Z-stack images are provided in Figure 3.41 and showed that junctions were clearly located at the apical pole of the cells, however staining is clearer in Transwell® models as they do not have the subjacent fibroblasts that introduce background into the image. In both sets of models, each individual epithelial cell was in direct contact with an average of 6-7 adjacent epithelial cells. Comparison of junction protein expression in the *in vitro* models did not reveal any differences in the protein expression; all tight junction proteins and adherens junction proteins identified in the Transwell® monolayer were also found in the co-culture system with the same staining pattern that highlighted the cobblestone morphology of the epithelium. As all images were taken at the same magnification, it was possible to observe differences in cell morphology; Caco-2 cells within the 3D co-culture exhibited an increased cell density and appeared to have a narrower phenotype than the Transwell® control epithelium.

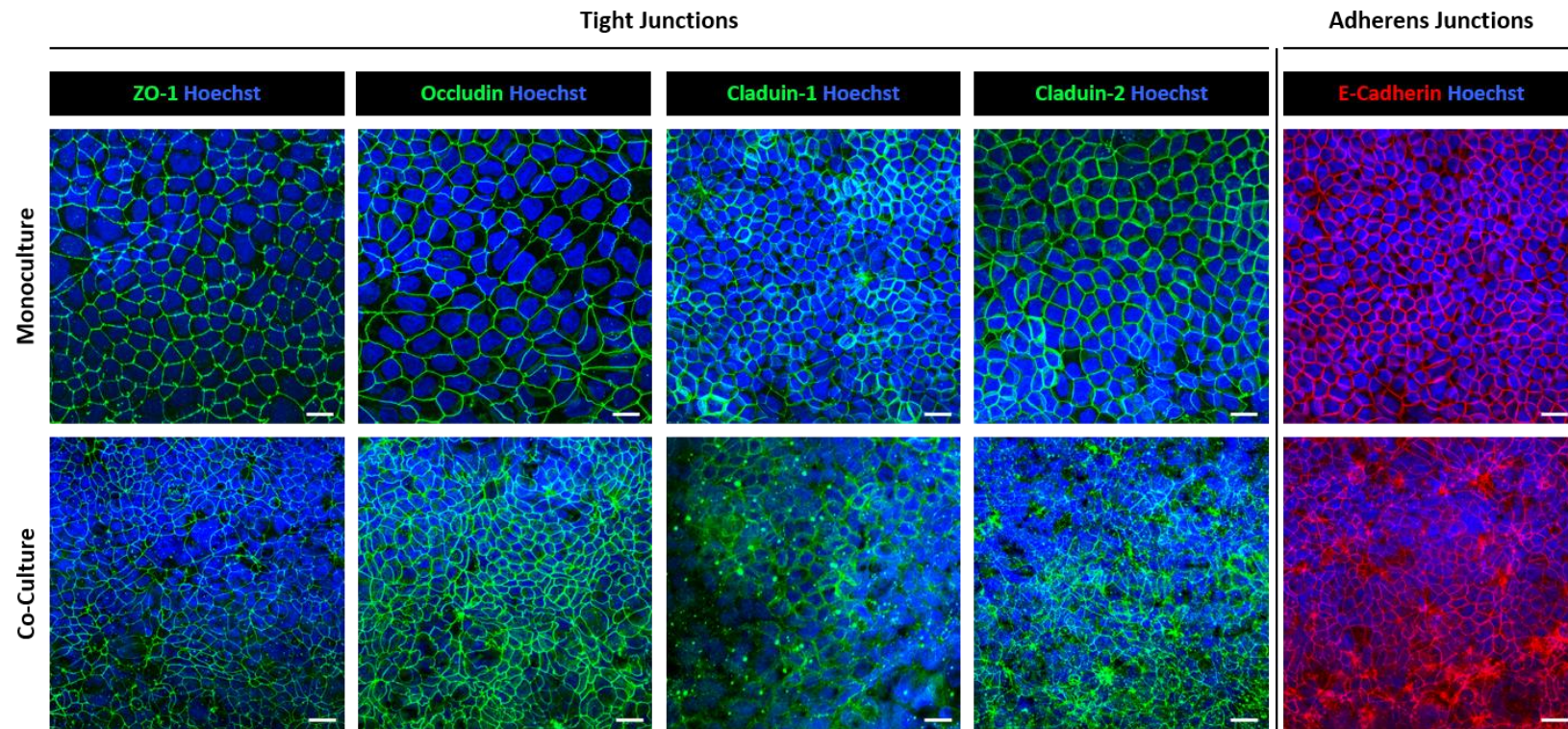


Figure 3.41. Comparison of the apical junctional complex expression in monoculture and HDFn co-culture. Top-down compressed Z-stacks of Caco-2 monolayers cultured on Transwell® membranes or on the surface of a HDFn lamina propria equivalent. Tight junctions (green) were immunolabelled with ZO-1, occludin, claudin-1 and claudin-2; adherens junctions were immunolabelled with E-Cadherin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 3 models. Scale bars: 20 µm.

Close examination of the cellular ultra-structure was performed with TEM analysis. Cell polarisation and microvilli can clearly be observed in TEM micrographs of Caco-2 cells and human tissue samples (Figure 3.42A). Measurements of epithelial cell shape revealed that Caco-2 cell height significantly increased in the 3D co-culture compared to the Transwell® monolayer (Figure 3.42B). Additionally, cell width was altered in the different culture conditions, Transwell® control models exhibited a wide morphology while cells in 3D and *in vivo* had a narrower phenotype (Figure 3.42C). Furthermore, calculation of the ratio of height: width was used to provide an indication into the overall epithelial cell morphology; a ratio close to 1 is suggestive of a square, cuboidal phenotype, while a ratio < 1 is representative of a flattened cell morphology. Conversely, a ratio > 1 is indicative of a columnar phenotype. Transwell® monolayers had a height: width ratio of $0.95 (\pm 0.06)$ and were found to have a more cuboidal and flattened structure. On the other hand, Caco-2 cells in 3D co-culture and epithelial human intestinal cells had a height: width ratio of $3.00 (\pm 0.59)$ and $4.38 (\pm 0.65)$ respectively, demonstrating that both cell types had a columnar morphology (Figure 3.42D).

Additional measurements were taken by carefully tracing the lateral membrane of the TEM images to determine the length. Transwell® cultures were found to have the longest membrane length in contrast to their shorter stature; cells exhibited a highly convoluted and interdigitated membrane, unlike the 3D co-culture or human intestinal epithelium (Figure 3.42D). To directly compare the length of the lateral membrane between the culture conditions, length was normalised to cell height and the epithelium of the 3D co-culture model and the *in vivo* tissue were determined to be very similar suggesting that co-culture with fibroblasts significantly enhanced not only the membrane structure, but also the whole cell shape (Figure 3.42E).

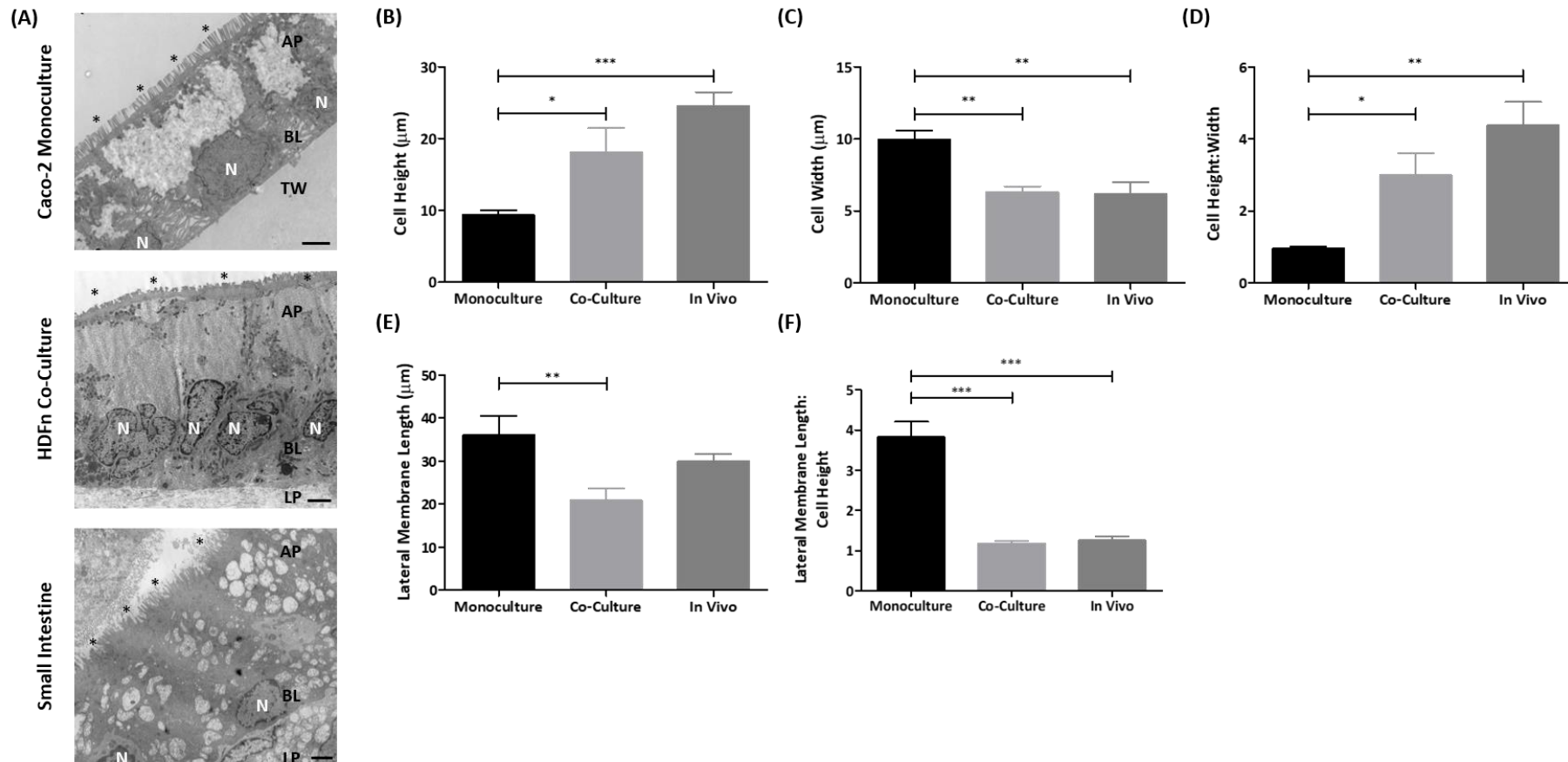


Figure 3.42. Morphometric analysis of epithelial cells in *in vitro* models and intestinal tissue. Representative TEM micrographs of Caco-2 Transwell® control models, Caco-2 HDFn co-culture models and human intestinal tissue (A) * indicates the microvilli; AP and BL denote apical and basolateral surfaces of the epithelial cells respectively; N defines the nuclei; TW indicates the Transwell membrane and LP indicates the lamina propria fibroblast components of the co-culture model and small intestinal tissue. Scale bars: 2 μm . TEM images were used to measure epithelial cell height (B), width (C) and calculate the height: width ratio (D). Images were also used to measure the lateral membrane length (E) and calculate the membrane length: cell height ratio (F). Images of human tissue and the Caco-2 Transwell® monolayer depicted here and used in analysis here kindly provided by Ariana González-Hau. Data represent mean $\pm$ SEM, total $n=7-9$ cells analysed from 3 models and 3 samples human tissue. * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post hoc test.

3.4.4.3. Changes in epithelial structure alter the epithelial barrier properties

The functional characteristics of the Transwell® monolayer were also compared against the 3D HDFn co-culture system. Continuous TEER measurements were taken throughout epithelial growth and differentiation; from the very start of Caco-2 growth the initial TEER in the Transwell® system was higher than the TEER ever reached in the co-culture (Figure 3.43A). Comparison of the fully differentiated TEER values to the human intestine revealed that the monolayer had a significantly higher TEER than both the co-culture and *in vivo* (6,245,246). Furthermore, no significant differences were detected between the TEER values for the co-culture and human tissue suggesting that the 3D model is more physiologically representative of the *in vivo* intestine (Figure 3.43B).

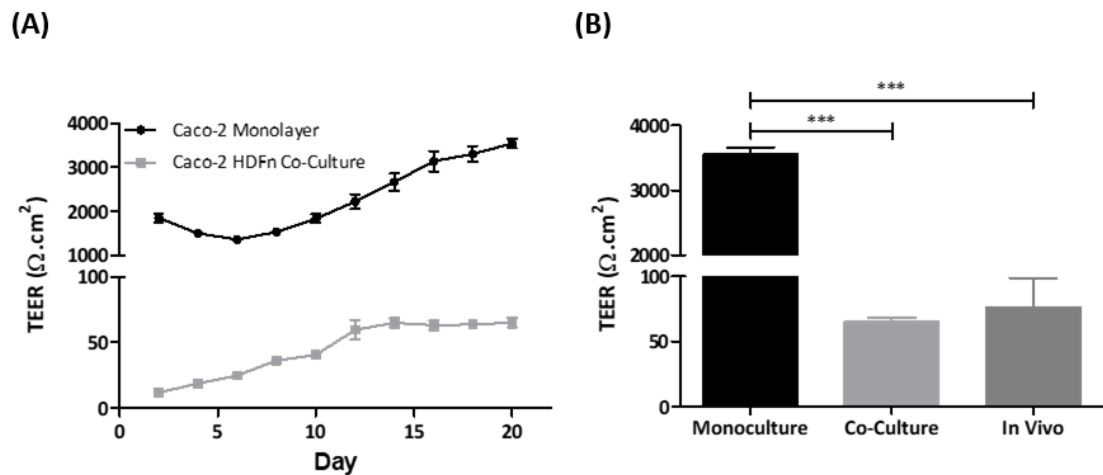


Figure 3.43. Functional assessment of TEER in the Caco-2 monolayer and HDFn co-culture. TEER measurements were taken every two days of Caco-2 cell culture (A). Endpoint TEER values were compared with *in vivo* intestine TEER values ($n=7$) reported in the literature (B): Schultz 1979, Balimane and Chong 2005, Sjöberg et al., 2013. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical repeats. Data previously shown in Figures 3.10 and 3.36 for comparison only. *** $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post hoc test.

A reduction in TEER was suggestive of a more permeable barrier being formed by the Caco-2 epithelium. To confirm whether this reduced TEER had a biologically significant effect, a lucifer yellow transport assay was performed on the Transwell® monolayer and the Alvetex® Scaffold co-culture. High lucifer yellow rejection > 90% was observed in the apical-basolateral and basolateral-apical transport directions in both model systems (Figure 3.44A). Assessment of the apparent permeability coefficient for lucifer yellow demonstrated that although there was no biologically significant increase in the secretion of lucifer yellow, the co-culture model had a significantly higher transport rate in the apical-basolateral direction only (Figure 3.44B). This observed increase in permeability reflects the reduced TEER within the models and suggests that the co-culture induces functional alterations within the Caco-2 monolayer to better resemble the *in vivo* intestine.

The significant increase in the apical-basolateral transport of rhodamine 123 in the co-culture also reflects the changes observed in TEER and suggests that the epithelium is leakier than the Transwell® model (Figure 3.45A). Significantly higher basolateral-apical permeability of rhodamine 123 in the 3D co-culture would indicate that there is an increased expression of P-gp, yet conversely, a significant reduction in the efflux ratio was found in the co-culture model from 4.68 ($\pm$ 1.12) in the Transwell® model to 2.10 ($\pm$ 0.21) in the co-culture, implying that the active efflux of rhodamine 123 was, in fact, lower in the 3D co-culture. This increase in transport yet decrease in efflux ratio could be explained by a reduced expression of P-gp combined with increased overall paracellular permeability in the 3D co-culture; the increase in Papp could therefore be due to a leakier membrane, rather than active transport.

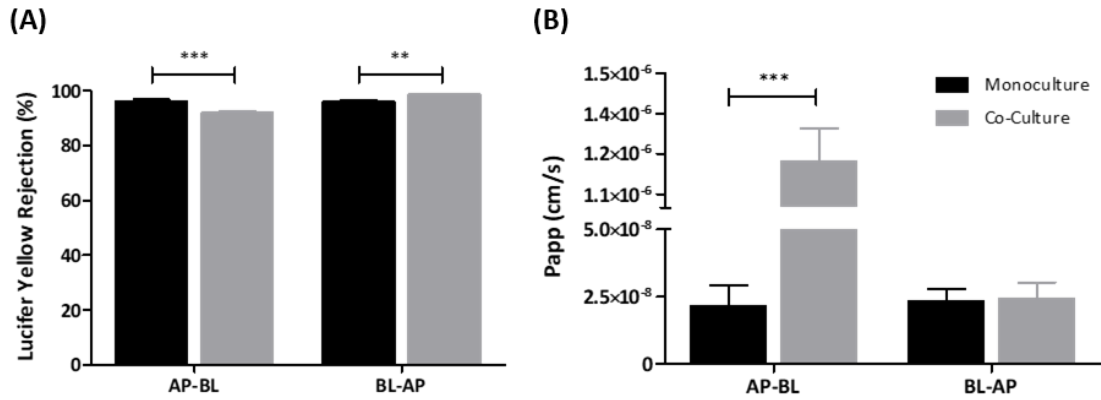


Figure 3.44. Functional assessment of lucifer yellow permeability in the Caco-2 monolayer and HDFn co-culture. Lucifer yellow rejection (A) and apparent permeability coefficients (B) were compared between the Transwell® control model and HDFn co-culture model in both the apical-basolateral (AP-BL) and basolateral-apical (BL-AP) directions. Data represent mean $\pm$ SEM, total n=9 comprised 3 independent test occasions each with 3 technical replicates. Data previously shown in Figures 3.11 and 3.36 for comparison only. ** $P \leq 0.001$ *** $P \leq 0.0001$ as determined by two-way ANOVA with Bonferroni's post-test.

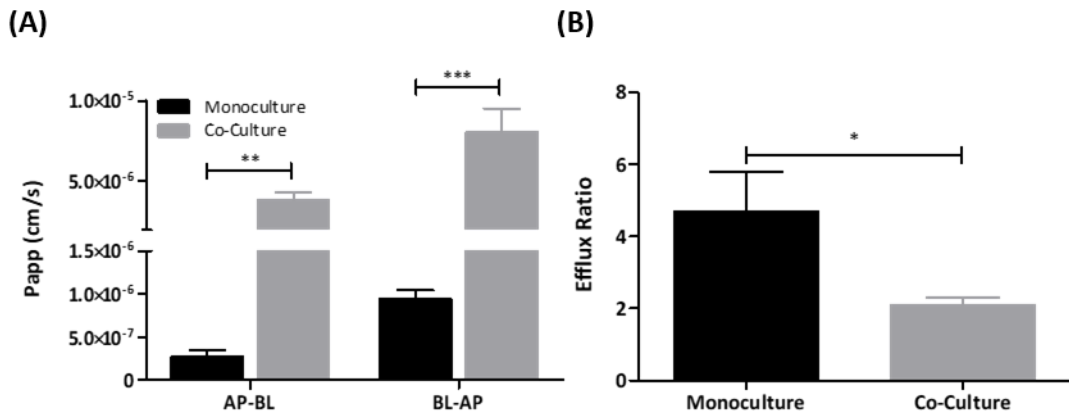


Figure 3.45. Assessment of Rhodamine 123 permeability in the Caco-2 monolayer and HDFn co-culture. Apical-basolateral (AP-BL) and basolateral-apical (BL-AP) transport of the P-gp substrate Rhodamine 123 was compared in the Caco-2 Transwell® control and the co-culture with HDFn cells (A). The efflux ratio of Rhodamine 123 was calculated in the monoculture and co-culture models to determine if Rhodamine 123 was subject to active efflux (B). Data represent mean $\pm$ SEM, total n=9 comprised of 3 independent test occasions each with 3 technical replicates. Data previously depicted in Figures 3.12 and 3.37 for comparison only. ** $P \leq 0.001$, *** $P \leq 0.0001$ as determined by two-way ANOVA with Bonferroni's post-test (A) and * $P \leq 0.05$ as determined by Student's t-test (B).

3.4.5. Summary of Experimental Findings

This chapter aimed to develop a model of the intestinal mucosa incorporating an epithelium and a lamina propria, fibroblast component. This was achieved by employing 3D cell culture techniques through the use of Alvetex® Scaffold technology in addition to using the widely established gold-standard intestinal epithelial cell line, the Caco-2 cell. The main experimental findings of this chapter are summarised below:

- Caco-2 cells differentiate into a polarised monolayer resembling small intestinal enterocytes when cultured on Transwell® membranes but their physical barrier properties are much greater than that of the *in vivo* intestine.
- Human dermal fibroblasts can be cultured within Alvetex® Scaffold following previously established protocols and are able to support the growth of an overlying Caco-2 epithelium.
- Fibroblast culture time could be reduced by utilising DMEM cell culture media; cells retained their ability to secrete endogenous ECM and acted as a foundation for Caco-2 growth and differentiation.
- Caco-2-HDFn co-cultures represent a robust and reproducible model of the human intestinal mucosa and express appropriate components of the apical junctional complex.
- Caco-2-HDFn co-cultures have improved functional properties over that of the Caco-2 monolayer.

3.5. Discussion

Current *in vitro* intestinal models employed in the drug development industry often follow a reductionist approach wherein a monolayer of cells is cultured on a plastic insert using a single cell lineage. These models are used to test the absorption and transport properties of new chemical entities, yet they lack the complex microenvironment that cells reside in *in vivo*, leading to high usage of animals in the drug evaluation process. Therefore, this chapter set out to optimise and characterise a novel model of the human intestinal mucosa using epithelial and stromal cell lines and 3D cell culture techniques to recreate a more physiologically relevant *in vitro* model of the intestine mucosa with the potential to be used as an alternative to previous models and reduce the use of animals.

3.5.1. The Caco-2 Monolayer on Transwell® and Alvetex® Polaris Cell Culture Supports

The epithelial Caco-2 cell line was used in this study due to its well characterised properties of small intestinal enterocytes and its extensive use within the pharmaceutical industry to evaluate the transport properties of newly developed drug compounds. The gold standard culture set up requires Caco-2 cells to be cultured on Transwell® inserts for 21 days to allow differentiation into absorptive epithelial cells. Once cells reach confluence upon the culture inserts, cell-cell attachments form between neighbouring cells and a barrier is formed across the epithelium. However, the overly restrictive nature of the barrier is a major drawback of the Caco-2 monolayer and limits the usefulness of the model in predicting the paracellular permeability of drug compounds. TEER was one of the methods used to assess the barrier properties of the Caco-2 epithelium in this study, and although TEER is known to be highly variable and fluctuates widely due to numerous factors (discussed in more detail in Section 3.1.4), it is a technique that is still employed worldwide. The Caco-2 monolayer herein consistently had a TEER value over 3000 $\Omega\cdot\text{cm}^2$; although this is higher than most values reported in the literature, various studies have found that TEER can reach over 2000 $\Omega\cdot\text{cm}^2$ once the cells are fully differentiated (313,343,367). This high resistance found in Caco-2 monolayers reflects their colonic origin as the colon is known to form a tighter barrier and be less permeable than the small intestine. Furthermore, the permeability of lucifer yellow, a hydrophilic, paracellularly transported compound was low in the monolayer and almost 100 % rejection of lucifer yellow was found in the monolayer highlighting the tightness of the barrier formed across the epithelium. P-gp is recognised as an overexpressed protein within the Caco-2 monolayer and has a major role in limiting the rate of oral absorption of drugs, however the overall level of active P-gp is approximately 10-fold less than the total level P-gp expression (348,368,369). To establish a baseline of P-gp activity within the epithelium as a comparison for later studies, examination of P-gp activity was performed through the transport of its substrate rhodamine 123. Rhodamine 123 was found to be subject to active efflux by the P-gp transporter, yet this active transport could be inhibited by the addition of verapamil hydrochloride.

Transwell® membranes are considered by some to be a 2D cell culture support that provides a very flat surface for the growth of cells which should encourage uniform growth and proliferation, however, it can be challenging to form a homogenous epithelial sheet of Caco-2 cells (227,232,370–372). Alvetex® Polaris was

considered as an alternative cell culture support for the growth of a Caco-2 monolayer due to its similar properties to Transwell® but larger pore sizes that allow for increased nutrient delivery to the basolateral surface of the epithelium. Furthermore, the increased porosity provides a surface that exhibits small recesses that could provide a pseudo-3D microenvironment. Culture of HepG2 cells on Polaris resulted in a densely packed structure resembling the hepatocytes of the liver (Allcock, unpublished data), while culture of the embryonic carcinoma cell line, TERA2.cl.SP12 resulted in an enhanced differentiation potential of the cells and enhanced viability of embryoid bodies (Smith and Quelch, unpublished data). Other pseudo-3D models utilise micropatterned substrates that have been demonstrated to induce apical-basal polarity within cells; a desired phenotype for the Caco-2 monolayer which at present has a flattened cuboidal morphology (371).

Histological analysis demonstrated that not only did Caco-2 cells form multilayers when cultured on the Polaris membranes, they also infiltrated to the basolateral surface of the scaffold and grew on both sides of the membrane (Figure 3.13). The multilayering phenotype suggests a loss of polarity within the cells and although a clearly dense population of Caco-2 cells grew on Polaris, they were found to have a significantly reduced TEER and high permeation of lucifer yellow compared to the Transwell® monolayer; emphasising that an intact barrier had not formed across the epithelium, possibly due to a lack of polarised junctional complexes. Taken together, these results demonstrated that Alvetex® Polaris was not a suitable cell culture support for Caco-2 cells alone.

3.5.2. Use of an Established Fibroblast Cell Line to Optimise the Culture of an Intestinal Mucosal Construct

Changing the substrate for Caco-2 growth did not improve the structure or function of the model, however a key limitation of the Caco-2 monolayer is the lack of other cell lineages within the model and as such there is a need to incorporate more cell types to enhance the complexity. Although many studies have focused on including additional epithelial cell lineages into the culture system, such as the mucus-producing HT29-MTX cell line, fewer studies have attempted to recreate the lamina propria microenvironment in which the epithelial cells reside *in vivo*. The importance of recreating an appropriate microenvironment for *in vitro* cell cultures was highlighted by Gjorevski et al., who defined optimal matrix properties for the culture of intestinal organoids allowing them to undergo long-term propagation and differentiation (373). Further studies have provided accumulating evidence that mesenchymal signals are essential for intestinal epithelial differentiation, morphogenesis and for maintaining homeostasis (374).

Within the intestinal lamina propria itself there are many different cell types, and there is controversy as to the predominating stromal cell lineage. For many years, myofibroblasts were considered to be the predominant cell type yet in-depth analysis has suggested that these cells are in fact activated fibroblasts and no true myofibroblasts are present within the intestine (50,54,55). Although the precise identity and location of myofibroblasts in the intestine is unclear, it is generally accepted that fibroblasts cluster below the epithelium at the villus tip where the majority of absorption occurs (50).

Dermal fibroblasts are used extensively in *in vitro* models of skin tissue and have been used in co-culture with Caco-2 cells (330,358,375–377). Although gene expression patterns of fibroblasts from different anatomical sites have been demonstrated to differ, fibroblasts from different anatomical locations are morphologically similar in structure and the key fibroblast properties - such as ECM secretion and the ability to differentiate into further stromal subtypes - is consistent between sites (378,379). Examination of the effects of paracrine factors from dermal fibroblasts revealed that hepatocyte growth factor, keratinocyte growth factor and TGF- β all enhance the growth and differentiation of intestinal epithelial cells *in vitro* (355,356,380). Within the same studies, hepatocyte growth factor and keratinocyte growth factor were found to also be produced by an intestinal myofibroblast lineage; CCD-18co (355,356). Co-culture of dermal fibroblasts with Caco-2 cells resulted in enhanced differentiation and tight junction protein expression within the epithelium and caused a shift in the expression pattern of carboxylesterase to better resemble the *in vivo* small intestine (358). Therefore, in line with evidence regarding secretion profile and previous studies, human dermal fibroblasts were used within this study to recreate the subepithelial compartment of the *in vitro* intestinal mucosa model.

Alvetex® Scaffold is an inert polystyrene cell culture support that has been reported to sustain the growth of dermal fibroblasts *in vitro* and allows the cells to grow in 3D by introducing a vertical dimension to the culture platform (330). Additionally, keratinocytes can be seeded onto the surface of the 3D cultured fibroblasts creating a full-thickness skin equivalent comprising of a dermis and epidermis, examples of which can be seen in Figure 3.16. This robust skin model accurately recapitulates not only the structure of skin tissues but also the barrier function, which was demonstrated through OECD testing of skin corrosion and skin irritation (330). Other mucosal models have been developed using Alvetex® Scaffold, including a model of the human trachea-bronchial mucosa. This model was constructed using primary cell lines of stromal and epithelial origin to recapitulate the full mucosal structure; it was successfully used to model normal and pathogenic host-microbe interactions with the bacteria, *Haemophilus influenzae* (331). Use of Alvetex® Scaffold negates the need to incorporate a hydrogel-based scaffold to support the fibroblast culture and thereby reduces the use of animal-derived products within the culture; therefore, HDFn cultured within Alvetex® Scaffold was selected as a suitable choice to form the subepithelial compartment of the intestinal model.

Initial optimisation of the HDFn culture followed the protocol previously published by Roger et al., and depicted in Figure 3.16 (330). To refine the culture time and increase model throughput, the culture medium was switched to DMEM. DMEM is a widely used culture medium that is used for the maintenance of Caco-2 cells and is used in the later stages of model growth when the Caco-2 cells are added. Additionally, DMEM has increased nutritional properties as it contains 10 % serum with greater levels of growth factors, rather than the LSGS supplement in M106 medium which only contains 2 % serum. Though the overall cell viability was not altered by the differing nutritional content of the media types, the population doubling time of the fibroblasts was reduced upon maintenance in DMEM, enabling the culture time of the lamina propria equivalent to be reduced while successfully supporting the development of a Caco-2 monolayer. No significant differences were observed between the epithelium that was formed on the surface of lamina

propria equivalents cultured in M106 or DMEM, further emphasising the suitability of DMEM to use for the entirety of model growth and development.

3.5.3. Fibroblasts Produce Endogenous ECM

Existing works that have examined epithelial-stromal interactions using *in vitro* cultures of the intestinal mucosa frequently rely on the use of hydrogels, namely collagen-based gels or Matrigel (313,367,381). Such hydrogels typically have a fibroblast population cultured within them, yet evidence of *de novo* ECM production by fibroblasts embedded within hydrogels is lacking. Additionally, the ECM present within such gels is frequently animal-derived, exhibits batch-batch variability, and their constituents can often be poorly defined. Dermal fibroblasts have previously been shown to produce and secrete endogenous ECM, and the formation of a lamina propria equivalent described herein, further highlights their functional capacity to do this (330,382–384). The predominant ECM protein, collagen I, and the second most common collagen throughout the body, collagen III, were found to be deposited by the dermal fibroblasts cultured within Alvetex® Scaffold. Increasing ECM accumulation was found with increasing the length of culture time, in agreement with a hydroxyproline assay performed by Roger et al., that exhibited increased collagen deposition as their dermal fibroblast construct matured (330). Furthermore, the presence of matrix metalloproteins suggests that the ECM undergoes remodelling throughout the culture (Figures 3.25, 3.30). Additionally, non-collagenous components of the ECM, specifically fibronectin, were also detected. Fibronectin is an ECM component that is usually absent in hydrogel-based models yet has crucial roles in wound healing (385). It has also been implicated in maintaining epithelial attachment and integrity and its binding to epithelial integrins assists in the regulation of epithelial differentiation and polarisation; fibronectin is therefore an essential component to simulate the intestinal lamina propria *in vitro* (385–387). Importantly all ECM proteins examined in the lamina propria equivalent cultured with HDFn cells were found to be present within CCD-18co lamina propria equivalents and the human intestinal mucosa, supporting the suitability of dermal fibroblasts in the model.

To enhance the production of ECM proteins and provide a robust foundation to support epithelial cell growth, HDFn cells were cultured with supplements of TGF- β 1 and ascorbic acid which were then removed upon the addition of Caco-2 cells. Ascorbic acid is a regulator of collagen synthesis, its supplementation to the growth medium increases collagen production (388,389). In addition, ascorbic acid is thought to have growth factor-like properties as it has the ability to increase proliferation of fibroblasts (389). TGF- β 1 also upregulates fibroblast proliferation and the production of collagen both *in vitro* and *in vivo* and has also been shown to increase the expression of fibronectin (390–392). Supplementary to this, TGF- β 1 attenuates the formation of stress fibres in fibroblasts while inducing their expression of α -smooth muscle actin, a molecular marker of activated fibroblasts and their contractile activity (393,394). The presence of TGF- β 1 in the HDFn culture, not only assists in the production of ECM constituents, but also induces the expression of α -smooth muscle actin which is present throughout the lamina propria of *in vivo* intestinal tissue (395). However, positive staining of α -smooth muscle actin was only detected in the DMEM cultures which had a higher serum content (Figure 3.23). This is in contrast to previous findings that serum attenuates the expression of α -smooth muscle actin,

and a reduction in serum content promotes its expression (396,397). Although the reason for detecting α -smooth muscle actin-positive cells is unclear, its presence increases the relevance of the co-culture to the human intestine tissue.

One of the limitations of the use of gel-based scaffolds is the lack of spatio-temporal regulation of ECM deposition and basal lamina formation. Correct orientation of ECM proteins is crucial for tissue genesis and epithelial-mesenchymal crosstalk (386). Alignment of the ECM and fibroblasts is also important for the formation of a basement membrane. Basement membranes are interfaces upon which epithelial-stromal communication occurs and have a major role in supporting the intestine architecture and maintaining immune homeostasis (398,399). They are also crucial for regulating epithelial cell differentiation and attachment to the underlying matrix; therefore their formation in *in vitro* models is essential for determining appropriate cell fate and control of cell behaviour (398–400). A comparison of two intestinal co-cultures using Caco-2 epithelial cells and human intestinal myofibroblast cells cultured either in collagen hydrogels or using microcarrier scaffolds that allowed the cells to secrete endogenous ECM proteins, revealed a greater production of non-collagenous ECM components when cells were enabled to secrete endogenous ECM (401). Furthermore, greater alignment of ECM and cells was observed in the later method and induced the formation of a basement membrane structure; such structures were not detected in the collagen-based model or in control Transwell® monolayers (401). The layering of fibroblasts on the edges of the Alvetex® Scaffold membrane allows the cells to be orientated in the same direction and ECM deposition to be aligned with the fibroblasts; HDFn cells were also shown to form a basement membrane with the above epithelium in Figure 3.33, recreating a more physiological microenvironment to support Caco-2 growth and differentiation.

3.5.4. 3D Co-Culture Enhances the Structure and Alters the Function of Caco-2 Epithelial Cells

The influence of the stromal microenvironment has important consequences for the growth, differentiation, and function of intestinal epithelial cells; as such, studies have sought to recapitulate aspects of this microenvironment through the use of proteinaceous gel-based scaffolds or through incorporation of stromal cells in co-culture with IECs. The importance of the microenvironment has been demonstrated through engraftment studies of Caco-2 cells onto nude mice wherein Caco-2 cells exhibited a wider differentiation profile, and formed all four key intestinal epithelial cells types (enterocytes, goblet cells, enteroendocrine cells and Paneth cells) (402). Further studies have also demonstrated the ability of Caco-2 cells to differentiate into multiple IEC lineages upon co-culture with stromal microenvironment factors; for example, co-culture of Caco-2 cells and intestinal subepithelial myofibroblasts resulted in differentiation towards all four epithelial cell types (401). Although the microenvironment created by the dermal fibroblasts in this study did not induce the full complement of IEC differentiation, a clearly enhanced structure, with greater polarisation and straightened lateral membrane structures, was observed within the Caco-2 monolayer.

Numerous co-cultures of Caco-2 cells and stromal cell lineages have been developed using IECs from humans and fibroblast lineages derived from mice. Zhang et al., employed the use of mouse embryonic 3T3 fibroblasts in a collagen gel with Caco-2 cells seeded on top (367). Similarly, Li and colleagues isolated their own mouse embryonic fibroblasts and cultured them in their intestinal model with Caco-2 cells and the mucus producing HT29-MTX cell line (313). Both models exhibited alterations in the structure and function of the epithelium compared to control Transwell® monolayers; including a reduction in expression of the tight junction protein, ZO-1; a reduced TEER; decreased P-gp activity; increased BCRP activity and an improved correlation with the fraction absorbed in humans (313,367). The results presented in this chapter did not find any differences in ZO-1 expression, however a slight reduction in positive staining for claudin-1 could be observed (Figure 3.41). Claudin-1 is a sealing protein, responsible for maintaining the occlusive nature of tight junctions and the apparent reduction in expression could be responsible for the dramatic decrease in TEER observed within the co-culture model. TEER in all three co-cultures demonstrated an enhanced *in vitro-in vivo* correlation and was accompanied by an increase in the paracellular permeability of model compounds across the epithelium, emphasising the importance of recreating the stromal microenvironment to recapitulate more *in vivo*-like cellular functions.

In contrast to the above models, the HDFn co-culture developed in the chapter initially appeared to have increased function of P-gp with greater efflux of the substrate rhodamine 123 compared to Transwell® monolayers. However, upon closer analysis the efflux ratio in the control monolayer was found to be higher than that of the co-culture, suggesting that the activity of P-gp was reduced in the co-culture model and the observed increase in P-gp efflux may be due to the leakier epithelium (Figure 3.45). Previously established co-cultures of dermal fibroblasts and Caco-2 cells displayed no alteration in the level of transporter protein expression compared to the Caco-2 monolayer, including expression of the efflux pump P-gp. This could suggest that P-gp expression and activity levels are directly influenced by the type of fibroblast cells present within the co-culture and that embryonic fibroblasts have a lesser influence on transporter protein expression. Another possible explanation for the observed differences in transporter protein expression is the animal origin of the embryonic fibroblasts compared to the human origin of the dermal fibroblasts as there are known differences in the expression levels of transporter proteins between the two species (403).

Co-culture of Caco-2 and human dermal fibroblasts has previously been associated with enhanced epithelial cell differentiation and a reduction in the time required to allow Caco-2 cells to differentiate (358). Functionally, the model displayed an increased level of carboxylesterase 2, better resembling the expression profile detected in the human small intestine (358). Also observed was an increase in the mRNA of tight junction proteins, however this is an undesired phenotype as Caco-2 monolayers already exhibit overly restrictive tight junctions (358). Conversely, immunofluorescence analysis of the model developed in this chapter did not demonstrate any significant increases in junctional protein expression and instead highlighted the difference in cell shape between the monolayer and co-culture (Figure 3.41). Caco-2 cells in the co-culture are more densely packed than in the monoculture, emphasising their increased polarity and reduced cell width demonstrated through TEM analysis (Figure 3.45).

Intestinal fibroblasts have been successfully implemented into co-cultures with intestinal epithelial cells. CCD-18co and human intestinal subepithelial myofibroblasts are the most frequently used intestinal stromal cells used *in vitro* and have been used in co-cultures with both Caco-2 and HT29-MTX goblet-like cells (312,336). Such models have demonstrated an increased predictive validity of cultures for the transport of insulin and also have the ability to reduce the oxygen tension to more physiological levels due to the accumulation of mucus (312,336). Furthermore, inclusion of a mucus-producing cell line allows for further experimentation regarding host-bacteria interactions (336).

Fibroblasts from various origins between organs and also within the same tissues, have been shown to have differences in their function, especially regarding the gene expression programmes active within the cells (404). As such, the origin of the fibroblast utilised within the HDFn co-culture model is one of its key limitations. Structural comparisons were therefore made between the model developed with dermal fibroblasts and an alternative model created with intestinal fibroblasts. The CCD-18co co-culture developed within the Przyborski laboratory highlights that these fibroblasts are also able to secrete *de novo* ECM proteins (Figure 3.39) and support the growth of an intestinal epithelial layer, yet the morphology of the Caco-2 cells in this model is not as polarised as in co-culture with HDFn cells (Figure 3.38) (405). Furthermore, the epithelium appears to have some degree of multilayering or pseudostratification in which a single layer of irregularly shaped cells gives the appearance of multilayers due to the nuclei appearing at different depths within the cells. Though the cells in pseudostratified tissues retain their apical-basal polarity, pseudostratification is not representative of the *in vivo* intestine and instead is more reminiscent of the respiratory airways or pre-malignant mucosal polyps in the intestine (406). This suggests that the Caco-2 cells retained a phenotype from their cancerous origin rather than differentiating into small intestinal enterocytes. Furthermore, this pseudostratification has been observed in other co-cultures of CCD-18co and Caco-2 cells where the fibroblasts are encouraged to produce their own ECM, rather than relying on the use of hydrogels (401). Although the ECM profile produced by CCD-18co cells is similar to both the HDFn cells and intestinal tissue, it could imply that slight differences exist and that the interaction between epithelial-stromal cells is promoting a more cancerous phenotype, though more in depth analysis is required to confirm this. Unlike CCD-18co co-cultures, the simple columnar phenotype observed in the HDFn co-culture is more characteristic of the human intestine.

A significant finding regarding the epithelial structure was the extent of lateral membrane interdigitation between adjacent cells. A high degree of lateral membrane interdigitation was observed through TEM analysis of Caco-2 Transwell® monolayers (Figure 3.43). This prominent characteristic was not observed in human intestine tissues and is also known to be limited in mouse intestine tissue samples. Mutations in galectin-3 have been associated with extensive infoldings in mouse models; galectin-3 is in part responsible for maintaining apical-basolateral polarity in cell membranes by transporting non-raft associated glycoproteins to the apical plasma membrane (407). Investigations into the activity of galectin-3 in the different models could provide further insight into the interdigitation phenotype observed and could suggest that that Transwell® monolayers have defective apical-basolateral polarity; this is provisionally supported by

the cuboidal nature of the Caco-2 cells. Moreover, the formation of cellular adhesion complexes between cells is known to be primary to the establishment of apical-basolateral polarity and it is hypothesised that extensive membrane convolution could perturb the formation of appropriate levels of junctional complexes between adjacent cells and alter polarity.

Almost all intestinal epithelial-stromal co-culture models discussed above exhibited a decreased TEER and increased paracellular permeability (312,313,336,367,401). This was also evident in the HDFn co-culture model developed in this chapter where TEER was reduced to levels much more comparable to the human intestine and the apical-basolateral permeability of lucifer yellow was increased, suggesting that the changes observed in the epithelial cell structure had biologically significant effects. A major drawback of the Caco-2 monolayer emanates from its overly restrictive barrier resulting in an inability to accurately predict the paracellular permeability of compounds. One finding that could be linked to the excessively occlusive barrier in the Transwell® system extreme interdigitation of adjacent cell membranes. The numerous infoldings and protrusions were significantly reduced upon co-culture with fibroblasts, and it is possible that straightening of lateral membranes and increased columnar phenotype could increase the permeability of paracellularly transported compounds as they would have a shorter retention time and route between the membranes of adjacent cells. This lateral membrane phenotype has also been demonstrated in direct co-culture with CCD-18co fibroblasts, yet treatment of Caco-2 Transwell® monolayers with paracrine factors secreted from both fibroblast lineages did not induce a significant change in the extent of lateral membrane convolution observed between adjacent cells or in the paracellular permeability; emphasising the need for direct contact between epithelial and stromal cells in intestinal co-cultures (405). ECM is a critical regulator of epithelial morphogenesis and polarity; inclusion of an ECM component within these models could induce structural changes in the lateral membranes and be partially responsible for the observed increase in paracellular permeability within these models. ECM specifically regulates cell polarity through integrin-mediated signals that in turn affect the actin dynamics of cells and expression of junctional complex proteins such as E-cadherin (408,409). Further examination of these interactions within monoculture and co-culture models could provide invaluable insights into structural and functional differences observed between them.

3.6. Conclusions

This chapter aimed to develop an *in vitro* co-culture model of the intestinal mucosa using established cell lines and simple 3D cell culture techniques. Existing *in vitro* models often follow a reductionist approach that do not accommodate stromal cells even though epithelial-mesenchymal interactions are crucial for the proper control of cell behaviour, fate, structure, and function. It was hypothesised that inclusion of a stromal cell line would improve the structure and function of the current gold standard Caco-2 monolayer.

Fibroblasts were successfully employed to establish a subepithelial compartment using Alvetex® Scaffold as an inert cell culture support to encourage the growth of cells in 3D. Fibroblasts produced *de novo* ECM; the constituents of which were demonstrated to be expressed in intestinal tissues and produced by fibroblast lineages isolated from the intestine. Although the fibroblasts used within the culture were not of intestinal origin, the 3D fibroblast construct was termed the lamina propria equivalent as it effectively formed a robust and reproducible foundation on which to support the growth of an epithelial monolayer.

Culture of the extensively utilised Caco-2 cell line upon the surface of the lamina propria equivalent resulted in the formation of an epithelial monolayer of cells. The epithelium was clearly demonstrated to have strong apical-basolateral polarity and a columnar phenotype. Cells expressed appropriate junctional complexes between adjacent cells and exhibited a microvilli brush border at the apical surface. Furthermore, a basement membrane was formed between the epithelium and subjacent fibroblasts which is hypothesised to have fundamental roles in the establishment of cell polarity and the improved lateral membrane architecture observed within the co-cultures. Further investigations into the role of the epithelial-stromal crosstalk are required to gain a broader understanding of the changes in epithelial morphology and polarity, including the regulation of actin dynamics and expression of adhesion molecules.

Co-culture of Caco-2 and HDFn cells not only resulted in structural changes within the epithelium but also impacted the function. TEER was reduced to more *in vivo*-like levels in the co-culture when compared to the Transwell® Caco-2 monoculture. The concurrent reduction in TEER and straightened lateral membrane phenotype are hypothesised to be responsible for the apparent increase in the permeability of paracellularly transported compounds. Additional transport experiments using a range of compounds of varying molecular weights could be used to confirm this. Transport assays of passive transcellularly transported compounds would prove to be a useful control in this scenario.

Overall, this chapter has successfully recapitulated the structure of the intestinal mucosa where the absorption process occurs. However, the model still lacks the incorporation of additional epithelial cell lineages, thus, the following chapter will seek to incorporate a further relevant cell line into the co-culture to improve the likeness to native intestinal tissue.

Chapter 4: Bioengineering a Mucus-Secreting Human Intestinal Mucosa

4.1. Introduction

Intestinal epithelial cultures predominantly focus on the role of absorptive enterocyte cells to model drug transport. However, the role of goblet cells and the mucus layers that they secrete to cover the epithelial surface of the intestine can have potentially rate limiting effects regarding absorption. It is therefore important that goblet cells are included in *in vitro* models to improve their accuracy in predicting permeability.

4.1.1. The Roles of Mucus *In Vivo*

Goblet cells are the second most dominant cell type within the intestinal epithelium after the absorptive enterocytes (28). The abundance of goblet cells increases distally throughout the small intestine and colon and their primary function is to secrete mucins; highly glycosylated glycoproteins that constitute part of the mucus-gel overlying the epithelial surface (28). Goblet cells are also responsible for the secretion of trefoil peptides that enhance epithelial migration and restitution following injury and have roles in the stabilisation and reinforcement of the mucus-gel (29,410).

4.1.1.1. The composition of mucus

Mucus is a complex hydrogel that is continuously produced and secreted by goblet cells as it is subject to constant enzymatic degradation and continuous physical erosion. Mucus is synthesised in the rough endoplasmic reticulum of the goblet cells and is exocytosed from the apical surface (411). Over 10 L of mucus is produced throughout the gastrointestinal tract every day and up to 9.8 L of that is then reabsorbed (412). The primary component of mucus is water which constitutes up to 95 % of the hydrogel, 2-3 % of the hydrogel is comprised of mucins while the remaining components include proteins, lipids, sloughed off epithelial cells, and bacteria (413,414). Additionally mucus can also contain DNA in some diseased states (413,414).

Mucins are a heterogenous meshwork of high molecular weight glycoproteins that are responsible for the gel formation and allow for efficient nutrient exchange. They are comprised of a polypeptide backbone with numerous hydrophilic oligosaccharide side chains that account for 50-80 % of the weight of the glycoprotein.

The five main sugars forming the oligosaccharide side chains are: galactose, fucose, sialic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine (415–417). However, there are also hydrophobic domains devoid of side chains and these naked protein segments are susceptible to proteolysis (411,418). There are two classes of mucins present in the intestinal epithelium; membrane bound mucins that have an additional hydrophobic domain to anchor the mucin to the plasma membrane, and secreted mucins that perform the classical gel-forming function. MUC 1, 3, 4, 12, 13, 16 and 17 are the predominant membrane bound mucins while MUC 2, 5AC, 5B, 6 and 7 are the principal secreted mucins with MUC 2, 3 and 5AC being the main mucins present in the intestine (28,411,419).

4.1.1.2. The properties of mucus

Mucins are responsible gel formation and are the primary determinant of the rheological properties (i.e. flow) of the mucus gel (415). Mucus is a non-Newtonian fluid whose viscosity changes with an applied force; application of high shear stress to mucus decreases its viscosity thereby increasing the rate of flow (415,420,421). This is crucial for the viscoelastic properties of the mucus layer that provide lubrication to aid the passage of luminal contents over the epithelial lining thereby limiting potential damage from erosion and shear stress.

Traditionally mucus was considered to be an unstirred layer that prevented the diffusion of acidic hydrogen ions, present in secretions from the stomach, to the epithelium (422). However, studies have demonstrated that acidic contents would permeate adherent mucus layers 100-200 μm thick within minutes *in vivo*, leading to the hypothesis that mucus has a more active role in providing protection from acid and does not only act as a diffusion barrier (423,424). Additional experiments have shown that as well as forming barrier to movement, the mucus layer creates a pH gradient from the lumen, across the mucus to the epithelial lining, with a pH of 2-3 in the lumen to near neutral at the epithelial surface (425–427). Chyme entering the intestine has a very acidic pH due to the secretion of hydrochloric acid from the epithelial parietal cells of the stomach; this stimulates the secretion of bicarbonate from the surface of enterocyte cells within the duodenum creating an alkaline tide to buffer and neutralise the acid and prevent corrosion of the epithelial lining (422). The cystic fibrosis transmembrane conductance regulator (CFTR) protein was established as a crucial factor in controlling this release of bicarbonate from the epithelial surface; biopsies from cystic fibrosis patients exhibited reduced bicarbonate secretion compared to normal healthy controls (428,429).

In addition to forming a neutralising buffer to acid, the meshwork of the mucin side chains also acts as a barrier to the diffusion of pepsin and other digestive enzymes. Pepsin is the principle protease enzyme of the stomach acid and is highly active in low pH conditions including those of the intestinal lumen (430). It has been implicated in the formation of haemorrhagic gastric ulcers leading to upper gastrointestinal bleeding and is also considered to be the principle factor in gastroesophageal reflux damage (422,431). It is therefore essential that the mucus gel forms a sufficient barrier to the pepsin to prevent degradation of the epithelial lining. Although there is evidence that pepsin can hydrolyse the mucus gel, this is mitigated by the presence of other secretions (422,423). Furthermore, pepsin cannot effectively permeate the mucus barrier within

physiologically meaningful time scales, taking hours to diffuse through even a thin layer, in part due to the stable, unstirred properties of the gel and the heavily glycosylated structure of mucins (422,432).

4.1.1.3. Mucus as a barrier to absorption

Not only does mucus act as a barrier to the diffusion of digestive enzymes, but it is also the first absorption barrier of orally administered drugs irrespective of the final transport route across the cells (transcellular, paracellular etc.) and thus is an important consideration when developing new chemical entities.

Mucus can adversely affect the absorption of drug compounds via three different barrier mechanisms: dynamic, steric, and interactive. Continuous turnover of mucus in combination with the proximal-distal flow due to peristaltic contraction constitutes the dynamic barrier; drug molecules must therefore be able to quickly penetrate the mucus layer before removal from the intestine lumen (411). Steric exclusion of drugs by the meshwork formed by mucins prevents the diffusion of larger particles across the mucus layer (Figure 4.1) (411,415,433). Additionally, the viscosity and its function as an unstirred water layer has an impact on the steric barrier; increased viscosity and thickness of the layer reduces the rate of diffusion (411). However, size filtering has very little effect on drug absorption *in vivo* as the majority of drug compounds are at least 100X smaller than the mesh size of the mucus layer (415). The final type of barrier formed by the mucus layer is based on the principle of interactive filtering wherein drugs form multiple low-affinity interactions with components of mucus, preventing their diffusion to the epithelial surface (Figure 4.1) (433). The interactive barrier is governed by the surface properties of drugs; lipophilic and hydrophobic interactions can occur between drug particles and the naked protein core of mucins, along with ionic and electrostatic interactions (411,415). Additionally, hydrogen bonds can form between drug particles and the oligosaccharide side chains of mucins, preventing diffusion. Binding of drugs to mucus glycoproteins has been shown to be non-specific, however they are of low magnitude (434). Lipophilic drugs displayed higher affinity for mucus binding and exhibited slower diffusion than hydrophilic drugs through pig intestinal mucus (435,436).

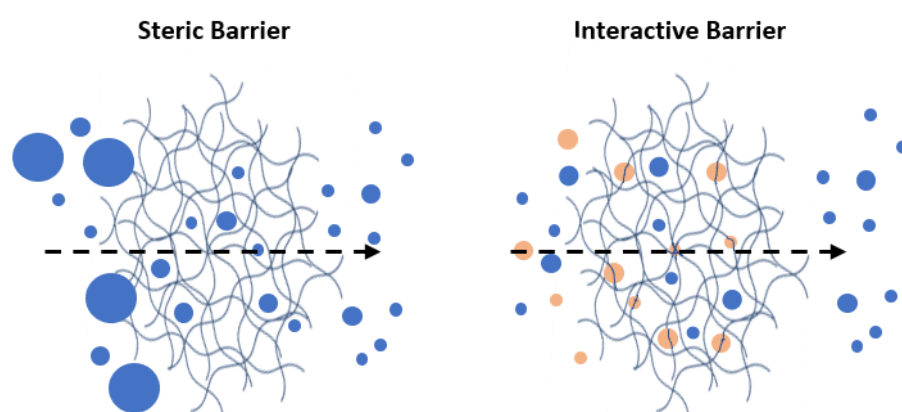


Figure 4.1. The filtering properties of mucus. Two primary mechanisms exist to prevent the diffusion of molecules across the mucus gel layer. Steric barrier properties arise from size filtering of particles rejecting the passage of larger compounds and retarding the diffusion rate of intermediate size compounds (left). Interactive barrier properties stem from the surface properties of the particles themselves interacting with the mucus layer (right); orange compounds interact with the mucus layer while blue particles have weak interaction and thus can diffuse through the mucus barrier. Arrows indicate the direction of transport. Adapted from Lieleg and Ribbeck 2011. Figure created, in part, with BioRender.com.

Although the mucus layer acts as a barrier to the passage of molecules, it is not completely impenetrable and can even be exploited to enhance absorption through the development of new drug delivery systems. Nanoparticle carriers are being utilised as novel drug delivery carriers; biocompatible polymers made out of uncharged, hydrophilic molecules. For example, polyethylene glycol and hydroxypropyl methacrylamide have been proposed as suitable polymers for this purpose; such polymers minimise the number of interactions forming with the mucus and enhance the diffusion potential of the particles. Testing of nanoparticle carriers *in vivo* have shown promising results by increasing the oral bioavailability of drug compounds (437–439).

4.1.1.4. Mucus as a barrier to pathogenic infection

Mucus exists as a double layer in the intestinal lumen with an inner adherent layer, though the presence of this layer in the small intestine is debated, and an outer loosely adherent layer (419,440). The inner layer is generally devoid of bacteria and pathogens under normal homeostatic processes due to the presence of defensins, secreted by the Paneth cells, and IgA, produced by mature B cells which decrease the levels of bacteria that can adhere to the mucus (109,110).

Although a key role of mucus is to provide a niche for commensal bacteria such as species of *Bifidobacterium* and *Lactobacillus*, it also acts as a barrier to the external environment to prevent pathogenic infection. The branched network of oligosaccharides of the mucin glycoproteins form a mesh that can trap pathogens which can then be cleared by the distal flow of mucus (441). One of the first demonstrations of this trapping mechanism was performed with enterotoxigenic *Escherichia coli* (*E. coli*); a pathogenic species with specialised pili structures that confer virulence by allowing adherence to enterocyte cells (442). These pathogenic strains of *E. coli* had greater attachment to mucus in comparison to non-pathogenic strains which did not exhibit any adherence properties (442). Experiments performed with *Yersinia enterocolitica*, also found that binding to intestinal epithelial cells was inhibited in the presence of mucus (443). Taken together, these results demonstrate that mucus contains interactive sites that can bind to bacterial adherence proteins, thus inhibiting their ability to attach to the epithelium and prevent infection.

Mucins have been demonstrated to influence the expression of bacterial genes involved in colonisation and pathogenicity, often inadvertently increasing their pathogenicity; for example, the presence of MUC2 upregulates the expression of virulence genes in *Campylobacter jejuni* (444). Expression of the biofilm component, *Vibrio* polysaccharide, in *Vibrio cholerae* is downregulated in the presence of mucus, however this has the adverse effect of promoting bacterial motility (445). Therefore, the dynamic barrier function of the mucus gel layer, i.e. continuous secretion and resorption, is pivotal in limiting the risk of pathogen invasion and the presence of *Vibrio cholerae* increases mucin synthesis to enhance this process (446).

4.1.2. *In Vitro* Intestinal Models with Goblet Cell Integration

Due to the significant role of mucus as a barrier to absorption, it is necessary to incorporate mucus-producing cells into intestinal cultures. Organoids (discussed in more detail in Section 1.5.2.3) have the unique ability to

contain all four key intestinal epithelial cell types, including goblet cells. Their unique properties allow for in depth studies regarding intestinal development and host microbe interactions, however the apical pole of the epithelial cells is orientated to towards the lumen of the organoid, rendering them almost inaccessible for studies of absorption. Consequently, alternative cell lines that mimic goblet cell function are frequently employed to study the role of mucus in absorption; namely the HT29 cell line.

HT29 cells were first isolated from a colorectal adenocarcinoma from a Caucasian female. They belong to the type 2 class of human intestinal epithelial cell lines as they are capable of differentiating into intestinal enterocyte cells with brush border hydrolase expression but require the addition of galactose to the culture medium (239,266). Further supplements to the culture medium can induce HT29 cells to differentiate into mucus-producing goblet-like cells.

4.1.2.1. The HT29-H subclone

The HT29-H subclone was selectively cultured from the parental cell line in galactose supplemented media in the absence of glucose. Culture of HT29-H cells on Transwell® membranes resulted in the formation of a polarised monolayer wherein over 80 % of the cells differentiated into mature goblet cells that secreted mucin molecules and formed a layer of mucus over the apical surface of the epithelium (267). This model had distinct advantages over the Caco-2 monolayer as it had reduced TEER levels and increased paracellular permeability (267). Furthermore, use of this model highlighted the role of mucus as a barrier to lipophilic drugs, namely testosterone. The permeability of testosterone was retarded in HT29-H monolayers with a mucus layer compared to models where the mucus had been removed (267,447). Subsequent studies of HT29-H were performed in co-culture with Caco-2 cells as it was thought that the permeability of such models would be closer to the fraction absorbed in humans. Initial co-cultures utilised a seeding density that would represent the level of goblet cells in the colon, rather than the small intestine and HT29-H cells were found to cluster in small groups amongst the Caco-2 cells (448). Although the permeability of the co-cultures increased compared with Caco-2 monolayers, tighter monolayers were found than initially expected with only a 5-fold increase in permeability in the co-culture; this was significantly lower than the 50-fold increase in paracellular permeability in the HT29-H monocultures (448,449). While the co-culture exhibited an improvement on traditional Caco-2 monoculture, the HT29-H subclone was found to be phenotypically unstable in later experiments and formed multilayers in which only the outermost layer of cells differentiated into mucus-producing goblet-like cells (450).

4.1.2.2. The HT29-MTX subclone

An alternative mucus-producing subclone of HT29 cells was selected by the stepwise addition of the anti-cancer drug, methotrexate, up to 10^{-6} M to the culture medium that induced metabolic changes within the cells. This subclone was termed HT29-MTX and has also been used in monocultures to recreate the goblet cell component of the intestinal epithelium (268). Monocultures of HT29-MTX cells have been further subcloned and two isolations, HT29-MTX-D1 and HT29-MTX-E12, were found to form confluent monolayers with tight junction protein expression and the presence of a thick mucus layer on the apical surface of the cells (451). Monolayers were demonstrated to be more reproducible than HT29-H cultures and were useful for studying the adherence and invasion of *Salmonella* species; significantly higher adherence of *Salmonella* occurred on HT29-MTX monolayers compared to HT29 or Caco-2 monolayers due to the presence of the mucus layer (452). Furthermore HT29-MTX monolayers exhibited an increase in the invasion of clinically relevant *Salmonella* strains, highlighting the ability of bacteria to subvert mucus to enhance pathogenicity and virulence and emphasising the suitability of HT29-MTX monolayers to study pathogen interactions with cells (452).

Like HT29-H monolayers, HT29-MTX cultures displayed a decrease in the apparent permeability of lipophilic drugs when compared to the Caco-2 monolayer (267,451). Testosterone, the most lipophilic drug tested exhibited a 43 % decrease in permeability adding weight to previous hypotheses that mucus forms a diffusion barrier (451). Conversely to this, Pointer and colleagues determined that there were similar permeabilities of both lipophilic and hydrophilic drug compounds in HT29-MTX monolayers and Caco-2 monolayers suggesting that mucus did not form a barrier to diffusion (453). However, this experiment was only conducted with low molecular weight molecules and further testing of larger compounds is required to confirm this. Nevertheless, HT29-MTX monolayers have been demonstrated to be suitable alternatives to study intestinal absorption, especially owing to their lack of P-gp expression. This allows for initial paracellular permeability to be measured for P-gp substrates such as cimetidine and sumatriptan to be determined irrespective of their affinity for P-gp, potentially facilitating optimisation of physiochemical properties of drug compounds (453). This is especially useful considering the gold standard Caco-2 monolayer overexpresses P-gp and cannot be used to accurately predict absorption of such drugs (453). Furthermore, Caco-2 monolayers have diminished expression of CYP3A enzymes and HT29-MTX monolayers have been shown to express these proteins, allowing first-pass metabolism to be examined (454).

4.1.2.3. Co-Culture of Caco-2 and HT29-MTX cell lines

HT29-MTX cells have also been employed in numerous co-cultures with Caco-2 cells to mimic the interaction of goblet cells and absorptive enterocytes, respectively, *in vitro*. Varying ratios of Caco-2:HT29-MTX cells have been proposed including 90:10, 70:30 and 50:50, however this has since been refined by Taguchi design which stipulated that 90:10 was the optimal ratio to use and is also the most physiologically relevant ratio of enterocytes to goblet cells within the small intestine (274,455).

Co-culture of Caco-2 and HT29-MTX cells displayed an increase in the passive permeability of compounds compared to the Caco-2 monolayer; this increase was most prominent in paracellular transport while the enhanced transport of highly permeable compounds was less obvious (274). Co-culture models have been demonstrated to be a suitable model to predict intestinal permeability exhibiting good correlation to fraction absorbed in humans. They can also be used to classify high and lowly permeable drugs according to the Biopharmaceutical Classification System (BCS), thus could potentially replace Caco-2 monocultures, though these are simpler to culture and maintain (273,456). Moreover P-gp substrates exhibited an increase in the apical-basolateral transport and a concurrent decrease in the basolateral-apical transport reflecting the lack of P-gp expressed by HT29-MTX cells (274). On the other hand, transport through carrier-mediated influx proteins was found to be lower than that of Caco-2 monolayers which is already known to be lower than the *in vivo* intestine transport rate (274).

4.1.2.4. Enhancing the complexity of Caco-2 HT29-MTX co-cultures

Advancements on the co-culture of Caco-2 and HT29-MTX cells have been made through the incorporation of Raji B cells. Raji B cells were isolated from human Burkitt's lymphoma and can induce an M cell phenotype in Caco-2 cells wherein Caco-2 cells exhibit a sparse and irregular brush border and have enhanced transcytosis of nanoparticles (277,278). When co-cultured with Caco-2 and HT29-MTX cells, Raji B cells are grown on the base of the well that house the Transwell® inserts with the Caco-2 and HT29-MTX cells and the co-culture contains cells resembling enterocytes, goblet cells and M cells, thus increases the physiological relevance of the model to the native human intestine structure (275,279,280). Functionally, the triple co-culture had an increased transport of insulin encapsulated nanoparticles and is a further suitable alternative to the Caco-2 system regarding classification of drug compounds according to the BCS criteria (275,280,456).

Alternative culture systems have been developed that not only incorporate absorptive and goblet cell phenotypes but also combine the use of stromal cells to provide a foundation on which the cells can grow. Initial techniques involved mouse embryonic fibroblasts embedded in a collagen gel on Transwell® membranes with the epithelial cells cultured on the surface; this model exhibited enhanced *in vitro-in vivo* correlation regarding the transport of highly permeable drugs, but did not significantly improve over the Caco-2 monolayer with respect to the transport of lowly permeable drugs (313). Furthermore, this model demonstrated an improved correlation of transporter protein expression with the human intestine which increases its physiological relevance over the Caco-2 model (313). Similar studies using CCD-18co fibroblasts embedded in Matrigel on Transwell® scaffolds with Caco-2 and HT29-MTX cells cultured on top demonstrated a reduced TEER and an efficient prediction in the permeability of insulin (312).

A further recent culture incorporating Caco-2 and HT29-MTX cells on a collagen gel with human intestinal fibroblasts has also been developed (314). Few studies have demonstrated the ability of fibroblasts to produce *de novo* ECM when embedded within hydrogels, however in this model fibroblasts were shown to secrete laminin and fibronectin ECM proteins, in addition to remodelling the collagen hydrogel (314). Furthermore, this culture system exhibited functional properties more representative of the *in vivo* intestine,

with a reduction in TEER compared to Caco-2 control monolayers and increased permeability of fluorescent dextran compounds (314).

Chen et al., have also sought to develop a triple co-culture incorporating Caco-2, HT29-MTX and stromal cells (336). Unlike previous models, they did not take advantage of the Transwell® culture set up and instead developed a novel porous protein scaffold system to replicate the architecture of the intestinal lumen. Human intestinal myofibroblasts were cultured in the bulk-space of the scaffold embedded in collagen gels with Caco-2 and HT29-MTX cells lining the lumen (336). Although no functional transport data was performed on this model, a decrease in luminal oxygen tension was detected increasing the physiological relevance of this model and has been proposed as a culture system to study host-commensal and host-bacteria interactions (336).

4.1.3. Conclusions

Mucus is an important feature of the intestinal mucosa that acts not only as a barrier to absorption but also as a barrier to pathogenic invasion. Its protective nature is conferred by the mucin proteins that are continuously replenished, in addition to secreted antimicrobial peptides and IgA from the underlying epithelial and immune cells. Considering the varying roles of mucus in limiting the process of absorption, it is a factor that should be considered when designing new chemical entities. It is therefore necessary to incorporate a mucus-producing cell line into intestinal epithelial models to recapitulate the structure and function of the mucosa more accurately. Numerous models have incorporated goblet-like cell lineages, predominantly derived from the HT29 cell line, such as HT29-H cells and HT29-MTX cells. Thus HT29-MTX cells were selected for incorporation into the co-culture of Caco-2 and HDFn cells developed in Chapter 3 to develop a more physiologically representative model.

4.2. Hypotheses and Aims

This chapter aims to develop an epithelial co-culture model of the intestinal epithelium utilising the Caco-2 cell line to represent absorptive enterocytes and the HT29-MTX cell line as the mucus-producing goblet cells. This co-culture will then be adapted to incorporate the stromal cell component optimised in Chapter 3. It is hypothesised that addition of the HT29-MTX cell line will enhance the physiological relevance of the model by producing a mucus layer over the surface of the epithelium which will in turn improve the *in vitro-in vivo* correlation of the model regarding function.

4.2.1. Objectives

- Optimise the co-culture of Caco-2 and HT29-MTX cells initially using Transwell® membranes as cell culture supports.
- Determine the effects of dermal fibroblast conditioned media on the structure and function of the epithelial models.
- Adapt the epithelial co-cultures to incorporate the subepithelial compartment developed in Chapter 3 and characterise the resulting triple co-culture structure.
- Assess the functional properties of the triple co-culture through TEER and transport assays of lucifer yellow, fluorescent dextrans, and rhodamine 123.

4.3. Experimental Design

The primary aim of this chapter was to develop a 3D triple co-culture model of the human intestinal mucosa that incorporated a lamina propria component in addition to the two primary epithelial lineages within the intestine; the absorptive enterocytes and the mucus-producing goblet cells. As in Chapter 3, a Transwell®-based monolayer of the goblet-like HT29-MTX-E12 cells (Section 2.3.2) was initially established to confirm that the cells successfully differentiated into a monolayer, with evidence of *de novo* mucin production, and to utilise as a control model for later comparison studies. Co-culture models of Caco-2 and HT29-MTX-E12 cells were then generated to establish the optimal seeding ratio of the two cell lineages for future 3D studies (Section 2.3.3). A total of 9 models, that were established over 3 different experimental occasions were subject to epithelial barrier assessment before being processed for histological analysis.

Prior to establishing a triple co-culture of the different cell lineages, the media of dermal fibroblasts was collected and utilised in conditioned media models of the Transwell® co-cultures (Section 2.3.4) to determine whether the fibroblast secretome induced any structural changes or adverse effects within the epithelial mono- and co-cultures. Models that were cultured using conditioned media were again analysed in the same manner as described for control models and a total of 9 models were generated from 3 separate experimental set ups, each with 3 technical replicates.

Finally, co-cultures were established using the lamina propria platform developed in the previous chapter (Section 2.4.1). Epithelial monolayers on the surface were generated as outlined in Sections 2.5.3 and 2.5.4, in an attempt to recreate the structure and function of the intestinal mucosa in a more holistic manner. Like, the previous Transwell® based models, 9 cultures set up over 3 separate days were analysed for their epithelial barrier properties prior to histological analysis. In addition, TEM images were generated of models ($n=3$) to characterise the ultrastructure of the models. Western blot and qPCR analysis were also performed on lysates generated from 3 independent experimental occasion to assess the apical junctional complex and confirm the presence and levels of intestinal transporter proteins. Within each independent experiment, lysates 3 technical replicates were pooled together, and the analytical techniques were performed 3 separate times.

4.4. Results

4.4.1. Characterisation of the HT29-MTX-E12 Monolayer

The HT29 cell line was first isolated from a 44-year-old Caucasian female in the same study in which Caco-2 cells were isolated. They can be differentiated with increasing concentrations of methotrexate to induce a mucus-producing phenotype of the cells and further subclones of these HT29-MTX cells have been generated. The specific subclone used within this study is the HT29-MTX-E12 cell line which have previously been demonstrated to form confluent monolayers with the expression of tight junctions and mucus secretion (451).

4.4.1.1. Morphological characterisation of 2D cultured HT29-MTX-E12 cells

Phase contrast images of HT29-MTX-E12 were obtained following traditional cell culture of cells on tissue culture plastic to show the morphological characteristics of the cells in 2D (Figure 4.2A). HT29-MTX-E12 cells are an adherent epithelial cell line that grows in discrete colonies or patches, they exhibit a polygonal shape that appears as a cobblestone morphology. At higher cell densities the colony sizes increase and merge with one another, displaying evidence of mucin granule production at the apical pole of the cells as indicated by asterisks. The ability of these cells to form junctional complexes with adjacent cells is highlighted by the expression of E-cadherin, which can be observed to clearly outline the membranes of the cells (Figure 4.2B). Furthermore, the localisation of E-cadherin indicates that cells grow as monolayers and do not form multilayers under 2D culture conditions.

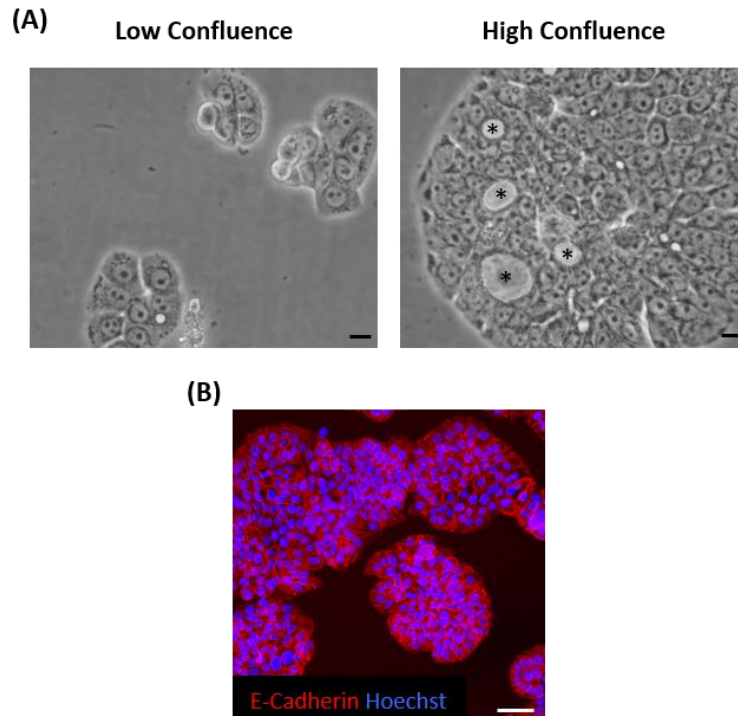


Figure 4.2. HT29-MTX-E12 cells cultured in 2D display cobblestone morphology. Phase contrast images of HT29-MTX-E12 cells cultured on traditional tissue culture plastics at low (left) and high (right) confluency (A). Cells grow in distinct colonies with evidence of mucin granule accumulation (denotated by *) at higher confluency. Confocal images of HT29-MTX-E12 cells cultured on glass coverslips for 4 days (B); cells were stained for the adherens junction marker E-cadherin (red) and nuclei were counterstained by Hoechst. Images shown are representative of 3 cultures. Scale bars: 50 μ m.

4.4.1.2. Structural characterisation of the HT29-MTX-E12 monolayer

To create a model system in which HT29-MTX-E12 cells could be used to mimic the mucus-secreting phenotype of the intestinal goblet cells, HT29-MTX-E12 cells were cultured upon a Transwell® membrane for a minimum of 21 days. Previous studies utilising HT29-MTX cells demonstrated that 21 days was sufficient time for a confluent monolayer to form and goblet cell differentiation to occur (451,453,454) and as this timepoint was in agreement with the differentiation protocol for Caco-2 cells, 21 days was selected for the length of culture of the HT29-MTX-E12 monolayer.

Histological analysis of wax embedded sections revealed strong columnar polarisation of the HT29-MTX-E12 epithelium (Figure 4.3). H&E staining highlights the basolateral localisation of the nuclei and a bulbous apical cytoplasmic region, with apparent evidence of multilayering or pseudostratification of the cells (Figure 4.3A). Periodic acid Schiff (PAS)/Alcian blue staining was used to detect the presence of mucosubstances (Figure 4.3B). Mucin granules were shown to be stored within the swollen apical region of the epithelial cells. The strong blue staining of these areas is a result of Alcian blue binding to the acidic mucins. PAS staining typically defines neutral mucins and colours them magenta; there is little evidence of neutral mucin staining in the apical region of the HT29-MTX-E12 cells where the mucin granules are stored and instead, the basolateral part of the cells have a magenta colouring. The histological analysis clearly demonstrates that HT29-MTX-E12 cells have differentiated into a polarised epithelium resembling the goblet cells of the human intestinal mucosa.

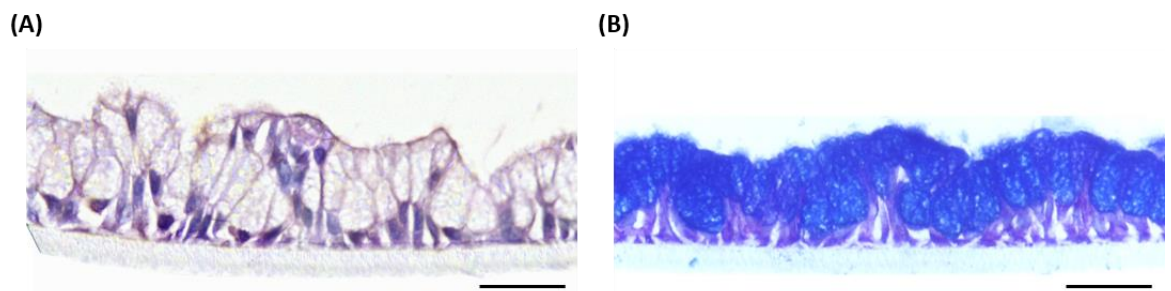


Figure 4.3. HT29-MTX-E12 cells differentiate into polarised goblet-like cells when cultured on Transwell® inserts. Histological staining of HT29-MTX-E12 cells cultured for 21 days on Transwell® membranes. H&E staining highlights the cellular morphology (A) and PAS/Alcian Blue staining indicates the presence of mucin granules (B). Images shown are representative of 9 models. Scale bars: 50 μ m.

Wholemount immunofluorescent staining techniques were carried out on the goblet-like cell monolayers to visualise components of the apical junctional complex (Figure 4.4). Although there was additional background staining due to the presence of mucus, clear apical localisation of the tight and adherens junctional markers can be observed indicating that appropriate AJC localisation is found within the monolayers. Staining revealed that the HT29-MTX-E12 cells typically form AJC contacts with 5-7 adjacent cells. Expression of the sealing tight junction proteins occludin and claudin-1 suggests that the cells have formed a barrier that could prevent the paracellular diffusion of chemical compounds, while the presence of claudin-2 suggests that the barrier formed is not completely impermeable. Additional immunostaining for the intracellular scaffolding protein, ZO-1 suggests that cells are forming appropriate connections with the intracellular cytoskeleton while the adherens junction proteins E-cadherin and β -catenin are responsible for maintaining the monolayer integrity. Expression of these AJC proteins emphasises the cobblestone morphology of the cells and suggests that the epithelium has formed a functional barrier.

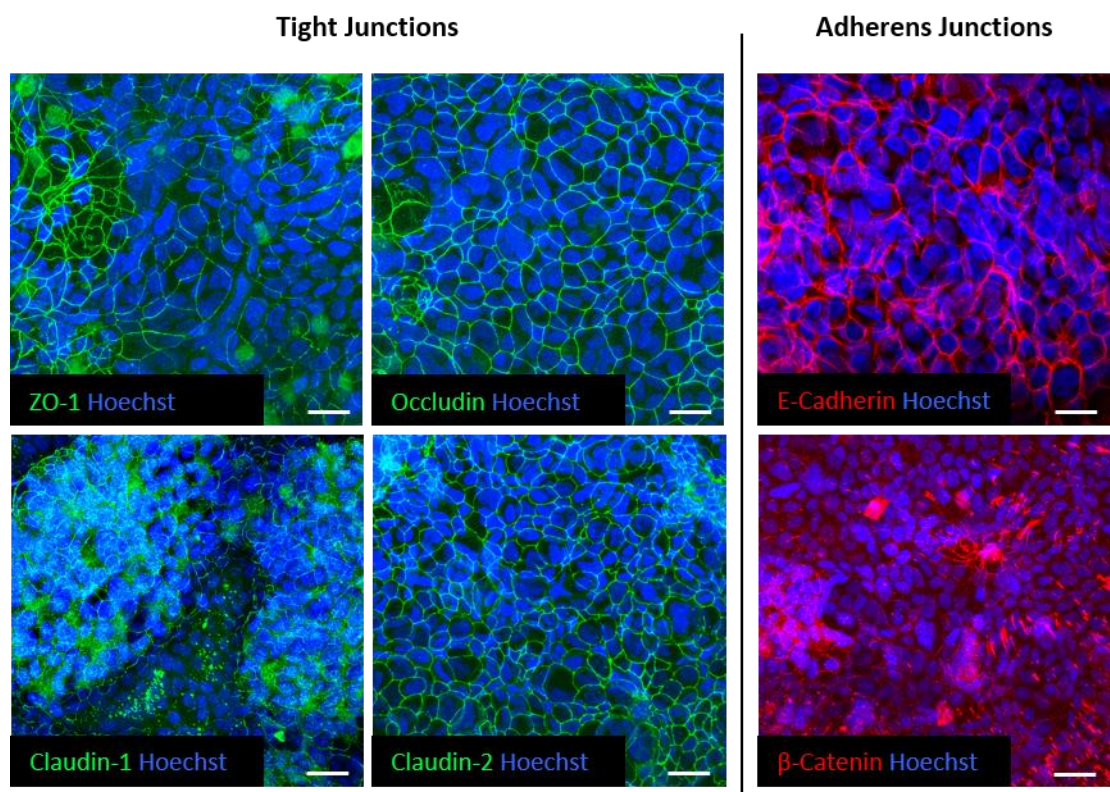


Figure 4.4. HT29-MTX-E12 monolayers express tight and adherens junctional proteins. Top-down compressed Z-stacks of HT29-MTX-E12 monolayers. Models were immunolabelled for the tight junction proteins ZO-1, occludin, claudin-1 and claudin-2 (green) and for the adherens junction markers E-cadherin and β -catenin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 3 models. Scale bars: 20 μ m.

4.4.1.3. Functional characterisation of the HT29-MTX-E12 monolayer

Initial characterisation of the barrier function of the HT29-MTX-E12 monolayer was performed through analysis of TEER. As in the previous chapter, TEER measurements were taken every two days of epithelial cell culture to monitor cell growth and assess the AJC dynamics over time (Figure 4.5). Similar to the observed TEER increase in culture of Caco-2 cells on Transwell®, TEER of the HT29-MTX-E12 culture increased with time as the cells formed a confluent layer and differentiated into their mucus-producing phenotype (Figure 4.5A). A maximal TEER level of $455.88 (\pm 65.81) \Omega \cdot \text{cm}^2$ was reached at the end of the 21-day culture period, significantly higher (~6-fold) than the TEER values found in the *in vivo* human intestinal tissue (Figure 4.5B).

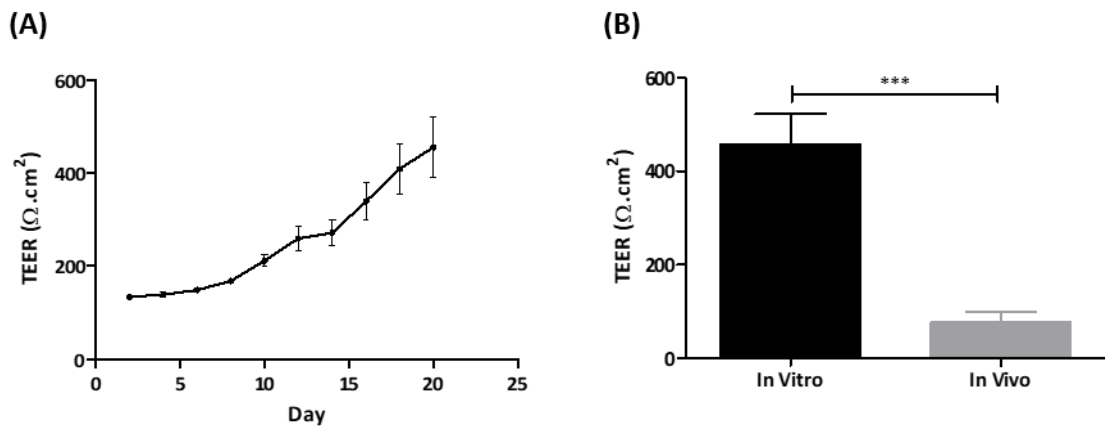


Figure 4.5. HT29-MTX-E12 TEER increases with time. TEER measurements were taken every two days of epithelial cell culture and exhibit an increase with culture time (A). Endpoint TEER values were compared with *in vivo* TEER values ($n=7$) reported in the literature (B): Schultz 1979, Balimane and Chong 2005, Sjöberg et al., 2013. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. *** $P \leq 0.0001$ as determined by Student's *t*-test.

An additional technique to assess the barrier integrity of the HT29-MTX-E12 monolayer was through a lucifer yellow transport assay. As discussed previously, lucifer yellow is a hydrophilic dye that is subject to passive diffusion through the paracellular transport pathway. Two different methods were used to assess the transport characteristics of lucifer yellow. The initial technique was to evaluate the ability of the monolayer to exclude lucifer yellow, over 95 % rejection was observed in both the apical-basolateral and basolateral-apical directions indicating that an intact barrier was formed across the epithelium (Figure 4.6A). The alternative method to assess lucifer yellow transport was through quantification of the apparent permeability coefficient. The apical-basolateral and basolateral-apical Papp was calculated at 3.73×10^{-8} and 3.51×10^{-8} respectively and displayed no directional transport bias as would be expected by a compound subject to passive diffusion (Figure 4.6B).

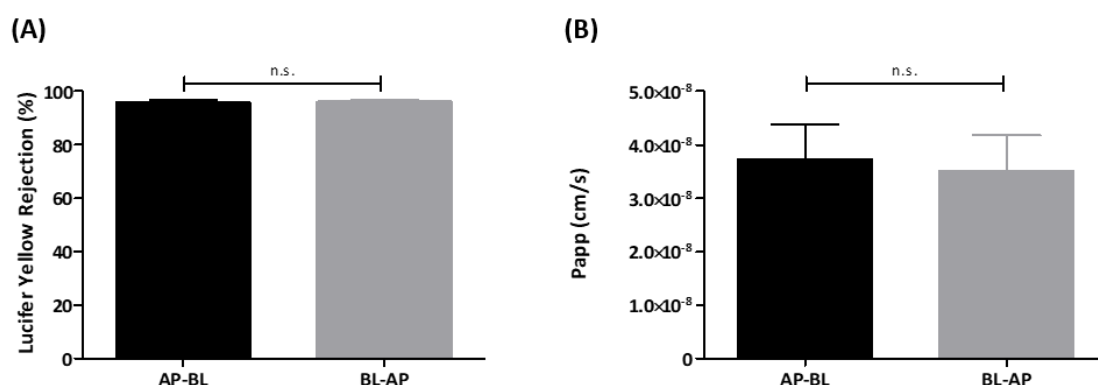


Figure 4.6. Lucifer yellow transport across HT29-MTX-E12 monolayers. The permeability of lucifer yellow was determined through a rejection assay (A) and through calculation of the apparent permeability coefficient (B). Assays were performed in both the apical-basolateral (AP-BL) and basolateral-apical (BL-AP) directions. Data represent mean $\pm$ SEM, total $n=9$ comprised 3 independent test occasions, each with 3 technical replicates. No significant differences identified by Student's *t*-test.

Additional functional characterisation of the HT29-MTX-E12 monolayer was carried out through a transport assay for rhodamine 123, a P-gp substrate. Monolayers exhibited no significant alterations in the Papp of rhodamine 123 between the apical-basolateral or basolateral-apical directions (Figure 4.7A). Furthermore, although the basolateral-apical transport of rhodamine 123 was reduced upon the addition of verapamil hydrochloride to the transport assay, the difference was not significant (Figure 4.7B). However, upon comparison of the efflux ratio between control assays and those in the presence of verapamil hydrochloride, a significant reduction in the level of rhodamine transport activity was determined, reducing from an efflux ratio of 1.86 to a ratio of 0.65 (Figure 4.7C). Nevertheless, the efflux ratio remained under 2 within both transport assays indicating that there is no active efflux of rhodamine 123 within the monolayer and therefore suggests that no biologically significant effect is incurred by this reduction in efflux ratio. The lack of efflux activity reflects the little to no P-gp transporter expression within HT29-MTX-E12 and is therefore consistent with the characteristics of the cell line.

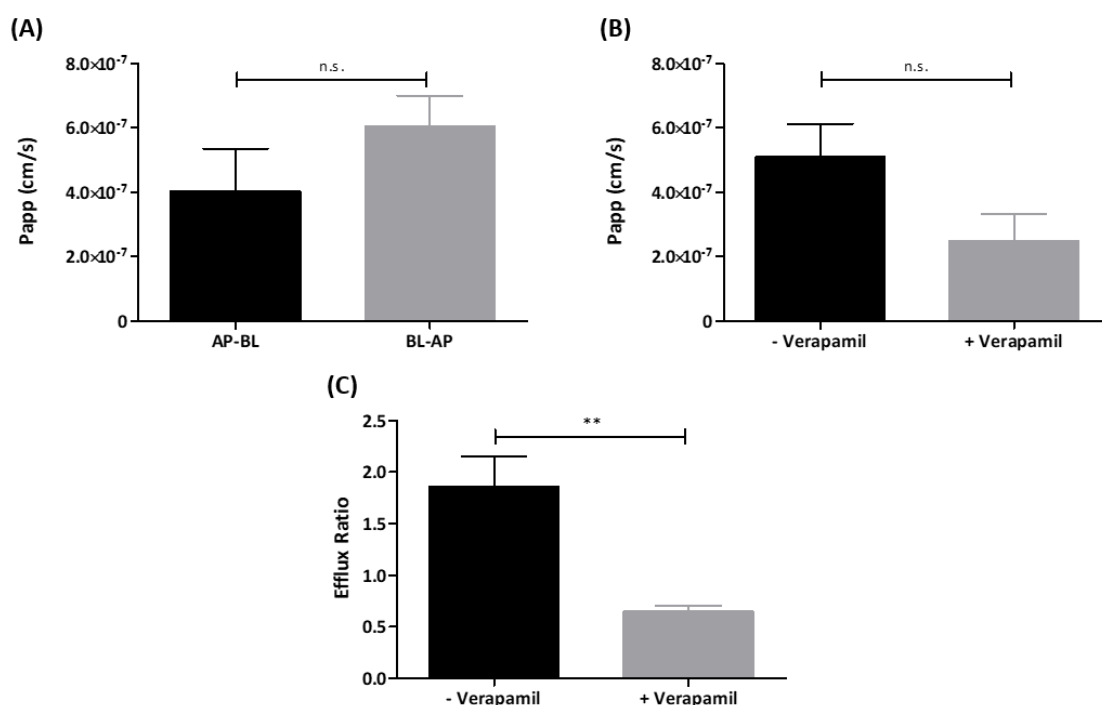


Figure 4.7. Rhodamine 123 transport across the HT29-MTX-E12 monolayer. The apparent permeability coefficient of the P-gp substrate, Rhodamine 123 was determined over a 2-hour long assay in the AP-BL and BL-AP directions (A). Verapamil hydrochloride, a P-gp inhibitor was also added to the BL-AP assay to determine if the primary transport mechanism of Rhodamine 123 was controlled by P-gp (B). The efflux ratio of Papp in the BL-AP transport direction to the Papp in the AP-BL direction was calculated in the presence and absence of verapamil (C). Data represent mean $\pm$ SEM, total n=7-9 comprised of 3 independent test occasions, each with 2-3 technical replicates.

4.4.2. Optimisation of an Epithelial Co-Culture Comprising of Enterocytes and Goblet-Like Cells

In order to more accurately recapitulate the structure of the intestinal epithelium, co-cultures incorporating Caco-2 and HT29-MTX-E12 cells were developed to simulate the absorptive and mucus-secreting components, respectively. The following ratios of Caco-2:HT29-MTX-E12 were initially utilised in this study: 100:0, 90:10, 70:30 and 0:100. Within the small intestine goblet cells constitute ~ 10 % of the total epithelial cells and this increases to ~ 24 % in the distal colon. Thus, different areas of the intestinal mucosa could be mimicked with the selected ratios of cells.

4.4.2.1. Characteristics of epithelial cell growth in 2D

Prior to co-culture of Caco-2 and HT29-MTX-E12 cells, the growth profile of the two cell lineages was assessed under traditional 2D maintenance conditions on plastic surfaces. Cells were seeded into 25 cm² culture flasks at 200 000 cells / flask and were trypsinised and counted every day for the subsequent 4 days. By day 4 of culture both cell types were at approximately 100 % confluence, therefore no further counting experiments were undertaken to maintain high viability of the cells.

Figure 4.8A demonstrates the growth profile of Caco-2 and HT29-MTX-E12 cells. Following initial seeding into flasks, cells required time to adhere to the tissue culture plastic and as a result, very little proliferation was detected at day 1 of cell counting. Both lineages then underwent a growth phase and similar cell numbers were retrieved from the flasks at days 2 and 3, though there was greater retrieval of HT29-MTX-E12 cells than Caco-2. However, by day 4, the number of HT29-MTX-E12 cells was much greater than Caco-2 cells. This was also evident in the doubling time of the cells (Figure 4.8B) where HT29-MTX-E12 cells exhibited a small but significantly faster doubling time than the Caco-2 cells. It was therefore necessary to be mindful of the varying doubling times and growth profiles of the two cell lineages when undertaking co-culture experiments.

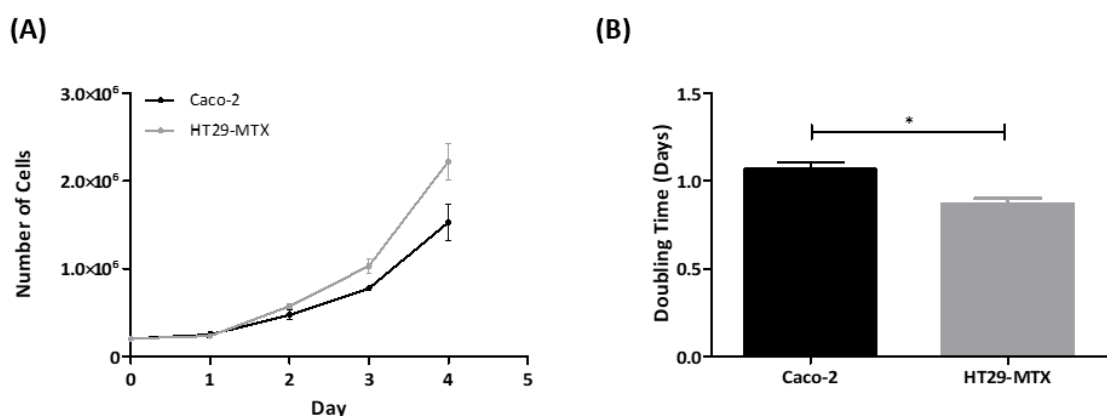


Figure 4.8. HT29-MTX-E12 cells have a faster doubling time than Caco-2 cells. A trypan blue exclusion assay was performed every day of epithelial cell growth (A) and was used to calculate the doubling time of the cells (B). Data represent mean $\pm$ SEM, total $n=3$ from 3 independent test occasions. * $P \leq 0.05$ as determined by Student's t -test.

4.4.2.2. Refinement of the Caco-2:HT29-MTX-E12 seeding ratio

Caco-2 and HT29-MTX-E12 cells were seeded onto Transwell® membranes and were maintained in culture for a minimum of 21 days to allow confluent monolayers to form and complete differentiation to occur. Caco-2 monolayers and HT29-MTX-E12 monolayers were used as controls to represent the enterocyte and goblet cell components respectively. Seeding of epithelial cells at 90:10 Caco-2:HT29-MTX-E12 was used to represent the ratio of cells in the small intestine while 70:30 reflected more colonic tissue.

To assess the overall structural morphology of the monolayers, models were wax embedded prior to sectioning and histological staining. H&E staining revealed that Caco-2 cells in monoculture display a flattened cuboidal phenotype. In co-culture with HT29-MTX-E12 cells, however, the Caco-2 cells exhibit an enhanced polarisation with an increased basal localisation of the nuclei (Figure 4.9). This structural change was particularly evident at ratios of 90:10 Caco-2:HT29-MTX-E12. Within the co-cultures, the different cell populations appeared in distinct islands with little evidence of individual cells interspaced in areas of the opposing cell lineage. Models cultured using the 70:30 seeding density had an appearance closer to HT29-MTX-E12 monocultures than to co-cultures with respect to cellular composition; clusters of Caco-2 cells within the goblet-like cells were sparse. Apical mucus buds were visible within the HT29-MTX-E12 cells in all culture conditions that contained a mucus-producing cell constituent, though the extreme columnar polarisation observed in monoculture models appeared slightly diminished in the co-cultures.

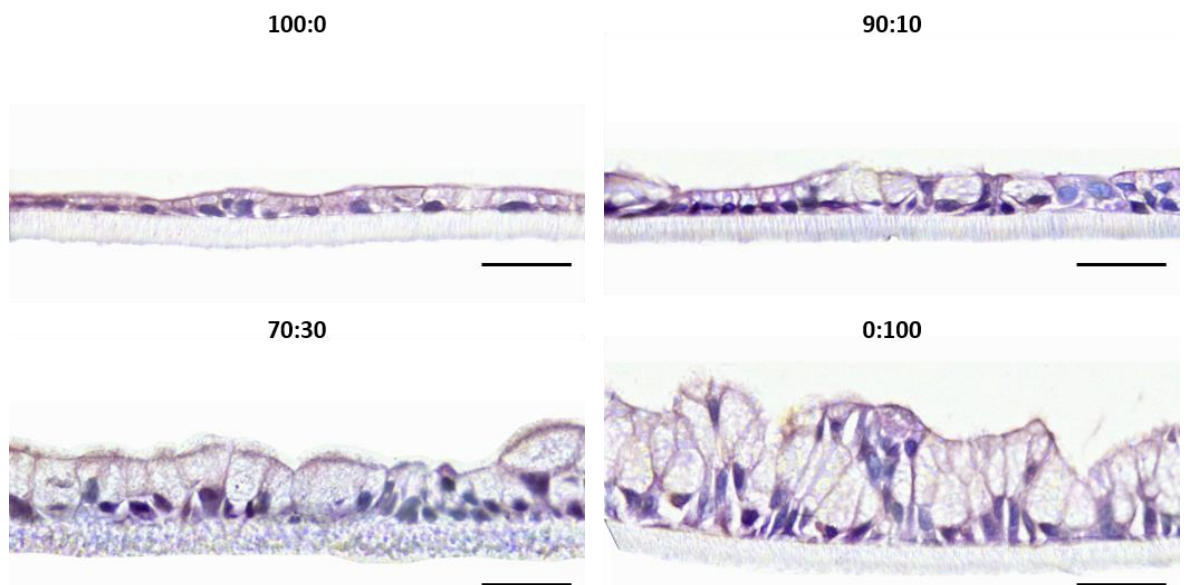


Figure 4.9. H&E staining of intestinal epithelial in vitro models. H&E staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Transwell® inserts. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, 70:30 and 0:100. Images shown are representative of 9 models. Scale bars: 50 μ m.

Supplementary to H&E staining, PAS/Alcian blue staining was also performed on the varying epithelial models (Figure 4.10). As discussed in the previous chapter and Section 4.4.1.2. Alcian blue has a pH of 2.5 and stains acidic mucins while PAS stains neutral mucins magenta. Staining of the Caco-2 only monolayer resulted in the complete absence of blue acidic mucin staining, however there appeared to be magenta staining indicative of the presence of neutral mucins. Since there is a lack of mucus-producing cells in these models, it is possible that this staining comes from the presence of glycocalyx at the apical surface of the cells which has a neutral pH of ~ 7.3 (457). Strong blue staining is present on the apical mucus buds of HT29-MTX-E12 cells in the 70:30 and 0:100 Caco-2:HT29-MTX-E12 models indicating a high content of acidic mucins. However, in the 90:10 culture system, the blue staining has a weaker colour intensity suggesting that the mucin content is not as acidic and more neutral mucins are present. Furthermore, there appears to be weak blue staining on the Caco-2 cells which may imply that a secreted mucus layer had been present over the entire surface but had been mostly removed during washing steps and ethanol dehydrations prior to wax embedding. PAS/Alcian blue staining also highlights the spatial distribution of the two cell lineages in co-culture, emphasising the distinct clusters formed by differing populations (Figure 4.10).

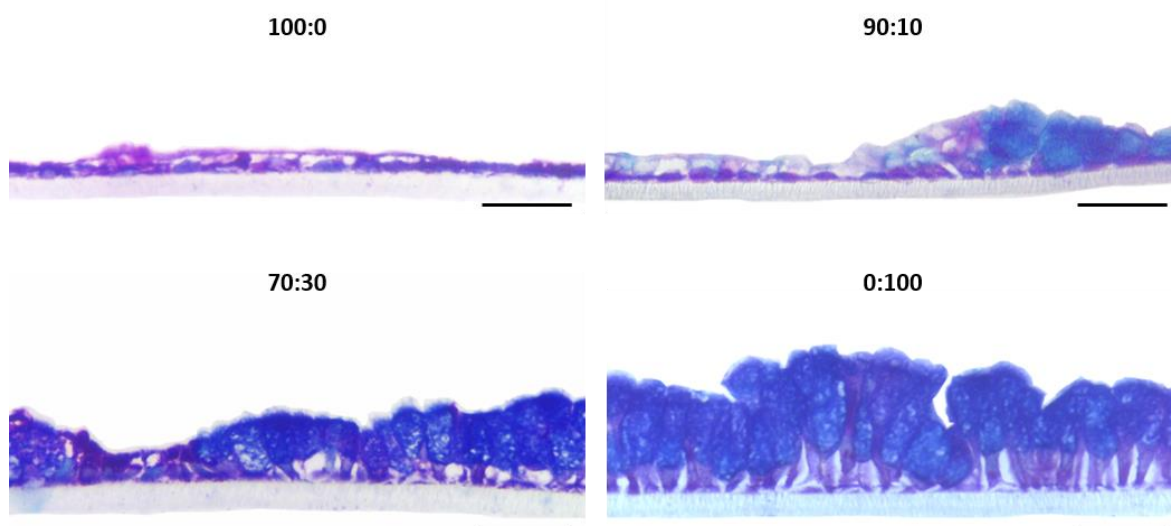


Figure 4.10. Mucin staining of intestinal epithelial in vitro models. PAS/Alcian blue staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Transwell® inserts. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, 70:30 and 0:100. Images shown are representative of 9 models. Scale bars: 50 μm .

In addition to histological analysis, wholemount immunostaining of epithelial models was performed to assess the ability of cells to form cell-cell junctional contacts. Compressed Z-stacks of the immunofluorescent images could then be generated to visualise the connections forming across different planes of focus. Initial immunostaining focused on two tight junction proteins, ZO-1 localised intracellularly and occludin which forms intercellular connections between adjacent cells, as well as E-cadherin to visualise adherens junctions (Figure 4.11). Such AJC proteins were present within all models and it was possible to observe where the two cell populations merge in the co-cultures as the pattern of expression has distinct areas of wider (HT29-MTX-E12) and narrower (Caco-2) staining that outlines individual cells reflecting the more swollen apical region of the goblet-like cells. Additionally, the expression of the AJC components was found on different planes of view in the co-cultures due to the significant height differences of the two cell populations; junctional staining surrounding the HT29-MTX-E12 cells has a fainter appearance due to the enhanced columnar polarisation and heightened morphology compared to Caco-2 cells.

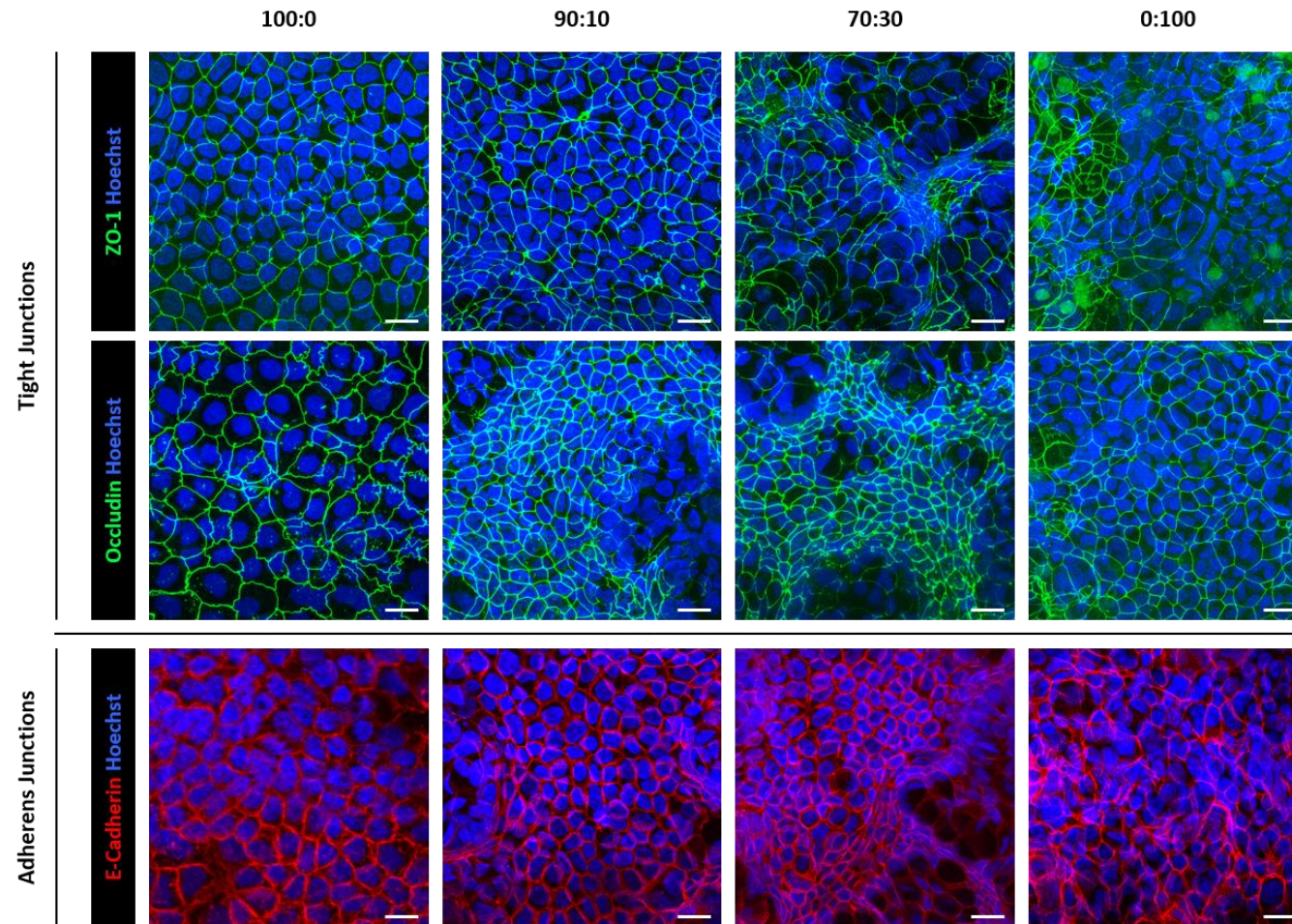


Figure 4.11. Expression of apical junctional components in in vitro intestinal epithelial co-cultures. Top-down compressed Z-stacks of staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Transwell® inserts. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, 70:30 and 0:100. Models were immunolabelled for the tight junction proteins ZO-1, occludin (green) and for the adherens junction marker E-cadherin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 3 models. Scale bars: 20 µm.

Due to the ability of HT29-MTX-E12 cells to outcompete and overpopulate the Caco-2 cells in the 70:30 Caco-2:HT29-MTX-E12 co-culture as observed in Figures 4.9-4.11, this ratio was excluded from future experiments. This ratio was highly variable and often had very few visible Caco-2 cells within the culture. Moreover, this ratio is representative of the colonic epithelium while Caco-2 cells differentiate into small intestinal-like enterocytes. Thus, it was deemed more appropriate to continue experiments with a ratio of 90:10 Caco-2:HT29-MTX-E12 cells to accurately resemble the small intestine cellular composition.

4.4.2.3. In-depth structural characterisation of three epithelial models

Further wholemount immunostaining analysis of the 90:10 co-culture in comparison to the monocultures was performed to investigate the localisation of claudins -1 and -2 as these have important consequences on the permeability characteristics of the models (Figure 4.12). Strong expression of both claudins was identified in the Caco-2 monolayer suggesting that an intact but permeable barrier was formed across the epithelium. Contrastingly, the HT29-MTX-E12 monolayer exhibited weak claudin-1 expression but strong levels of claudin-2. Though the reduced appearance of claudin-1 could be due to background staining of mucin granules stored within the cells, it is possible that the reduced staining could contribute to weaker barrier properties of the monolayer. Similar claudin-1 staining was observed in the epithelial co-culture which displayed weak staining in areas populated by HT29-MTX-E12 cells and strong staining in areas where Caco-2 cells were present, which again could alter the sealing barrier properties of the monolayer. Claudin-2 staining within the co-culture was present throughout the model, however it did not display a uniform distribution of expression across the monolayer.

Additional staining of the adherens junction protein β -catenin was carried out to assess the cellular localisation of the protein (Figure 4.12). Within the Caco-2 monolayer and the co-culture of Caco-2 and HT29-MTX-E12 cells, β -catenin was mostly present at the apical pole of the cells surrounding individual cells highlighting its important role in the stabilisation and maintenance of cell-cell junctions. In regard to the HT29-MTX-E12 monolayer, β -catenin also displayed evidence of AJC localisation, yet intracellular staining was also observed. This could either be due to the additional function of β -catenin as a transcription factor or may be due to rapid turnover of adherens junctions within the model.

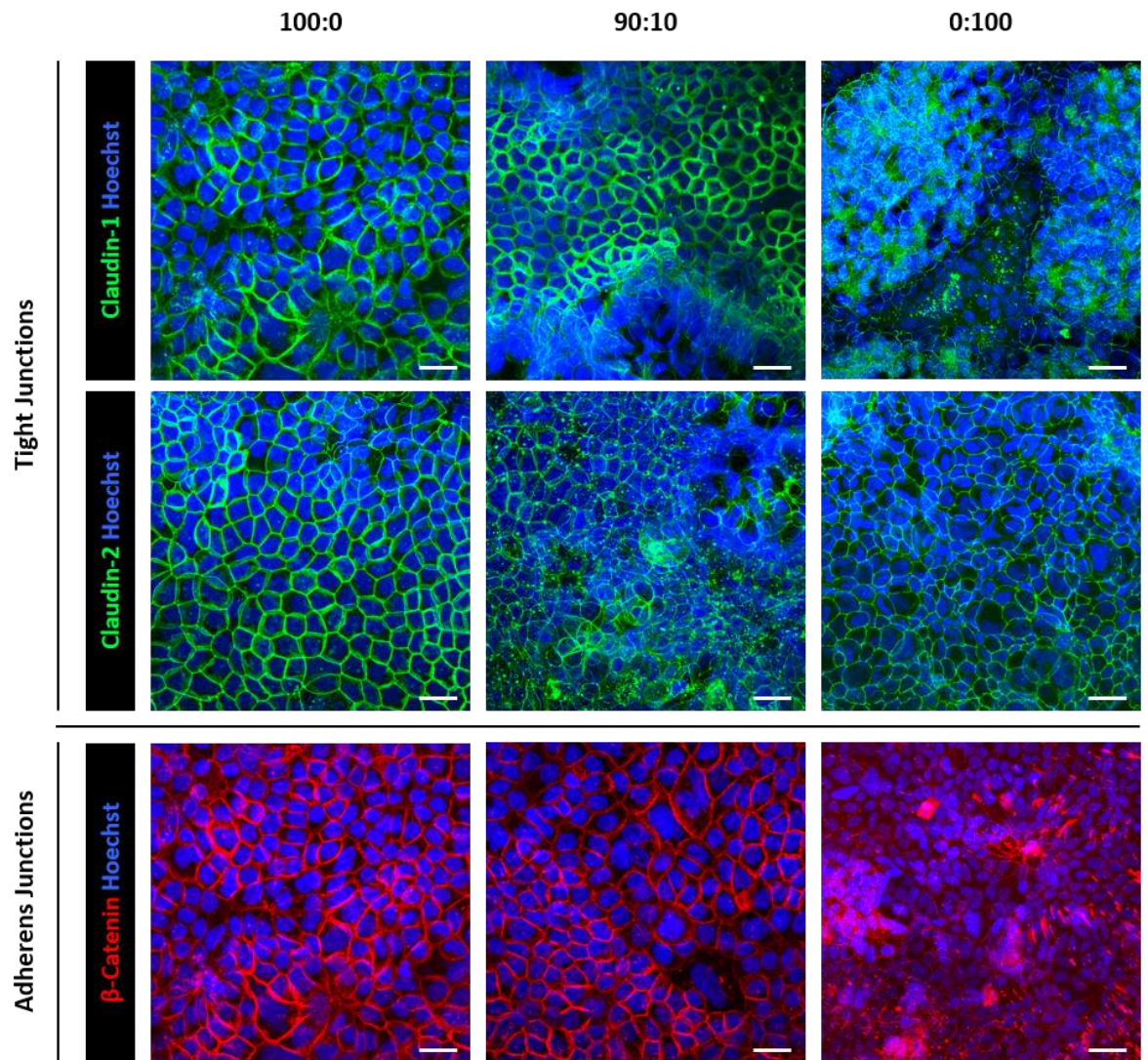


Figure 4.12. Further characterisation of apical junctional components in in vitro intestinal epithelial co-cultures. Top-down compressed Z-stacks of staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Transwell® inserts. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. Models were immunolabelled for the tight junction proteins claudin-1, claudin-2 (green) and for the adherens junction marker β -catenin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 3 models. Scale bars: 20 μ m.

Along with wholemount immunostaining, in profile staining of wax embedded sections was also completed to visualise the villin, the core protein backbone of the microvilli brush border (Figure 4.13). Presence of the villin protein indicates that the cells have differentiated into mature intestinal epithelial cells. Within the Caco-2 monoculture model, villin is localised at the apical surface, directly adjacent to the nuclei, while in the HT29-MTX-E12 monoculture, villin is present at a higher apical location highlighting the cuboidal nature of Caco-2 cells and the columnar phenotype of the goblet-like cells. Within the co-culture, enhanced structural polarisation and of the Caco-2 cells is evident as villin is not localised directly against the nuclei and appears at a higher focal plane more like the HT29-MTX-E12 cellular expression pattern.

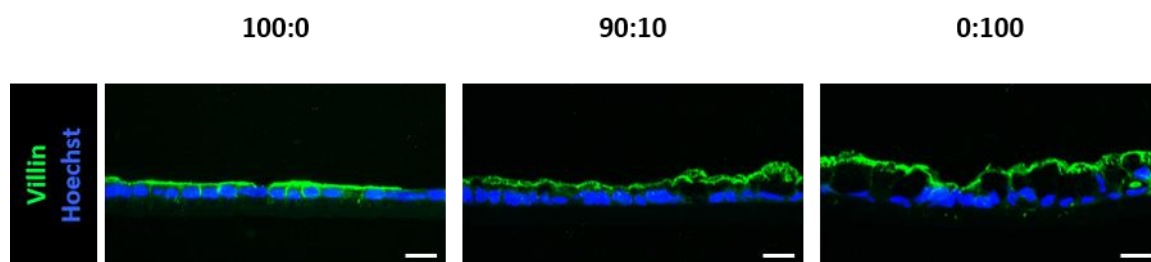


Figure 4.13. Intestinal epithelial cultures exhibit evidence of a microvilli brush border. In profile compressed Z-stacks of staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Transwell® inserts Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. Models were fixed in 4 % PFA and embedded in wax before sectioning. Samples were immunolabelled for the cytoskeletal brush border protein, villin (green). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 9 models. Scale bars: 20 μ m.

4.4.2.4. Characterisation of the functional properties of mono- and co-cultures of the intestinal epithelium

As in previous experiments, TEER was used to assess the barrier properties of the three epithelial models. TEER increased with culture time as the cells formed confluent monolayers and differentiated into enterocytes and goblet-like cells (Figure 4.14A). Models of Caco-2 monoculture and co-culture with HT29-MTX-E12, showed an increase in TEER until day 12-14 then experienced a plateau before a small drop off is observed by day 21 of culture. Unlike the other models, HT29-MTX-E12 monocultures exhibited a small but steady increase in TEER throughout their culture period with no plateau being measured. HT29-MTX-E12 monolayers exhibited a final TEER of $455.88 (\pm 65.81) \Omega \cdot \text{cm}^2$, almost 6-fold lower than the Caco-2 monolayer which has a TEER of $2771.13 (\pm 91.03) \Omega \cdot \text{cm}^2$ (Figure 4.14B). Inclusion of the goblet-like cells in the co-culture significantly reduced the TEER of the Caco-2 cells by ~ 3-fold to $956.40 (\pm 24.64) \Omega \cdot \text{cm}^2$. Although these levels are still higher than the *in vivo* intestinal TEER, incorporation of HT29-MTX-E12 cells into a co-culture with Caco-2 cells results in a significant improvement over Caco-2 monoculture and creates a more *in vivo*-like intestinal epithelial model with greater TEER reproducibility.

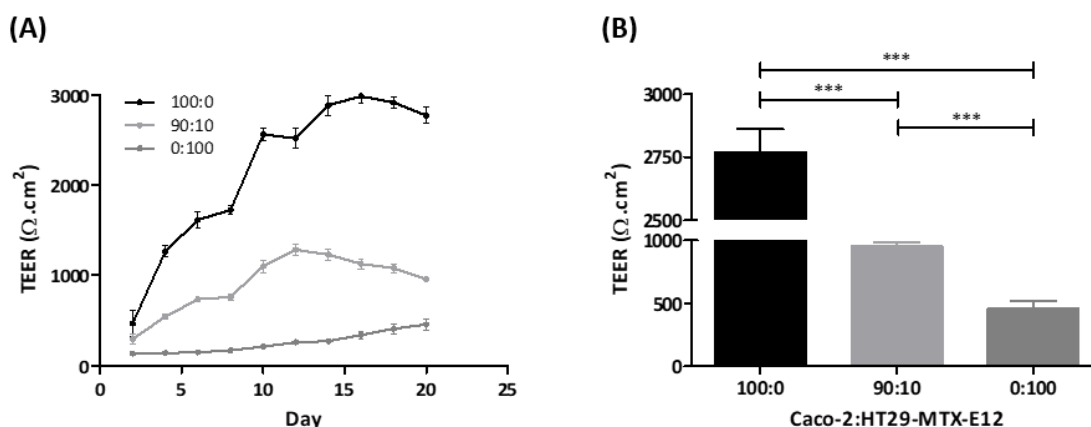


Figure 4.14. Inclusion of HT29-MTX-E12 cells in culture reduces TEER. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. TEER measurements were taken every two days of epithelial cell culture and exhibit an increase with culture time (A). Endpoint TEER values were compared across the three culture systems (B). Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical repeats. 0:100 Caco-2:HT29-MTX-E12 data previously shown in Figure 4.5. *** $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post hoc test to compare the means of individual variables.

Characterisation of lucifer yellow transport revealed high rejection levels over 95 % in all culture systems in both transport directions with no significant differences between the models (Figure 4.15A). Additionally, the apparent permeability coefficient displayed no significant differences between the three different *in vitro* culture systems, however an increasing trend was observed regarding the increased Papp of lucifer yellow with the inclusion of goblet cells in the culture system (Figure 4.15B). This perceived trend is reflective of the lower TEER found in cultures with greater levels of HT29-MTX-E12 cell incorporation.

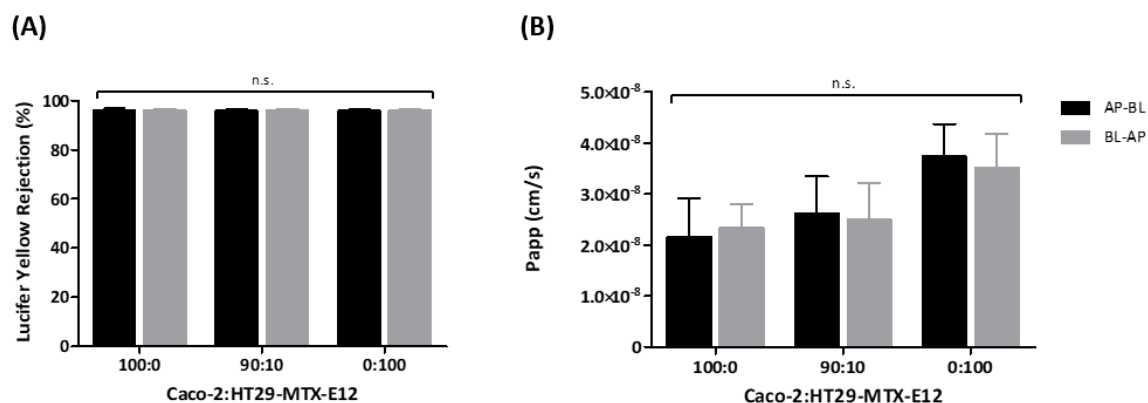


Figure 4.15. Lucifer yellow transport characteristics across epithelial cultures. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. Between days 21-29 of culture, the permeability of lucifer yellow was determined through a rejection assay in the absorptive AP-BL and secretory BL-AP (A) directions and through calculation of the apparent permeability coefficient in the AP-BL and BL-AP (B) directions. 0:100 Caco-2:HT29-MTX-E12 model data previously depicted in Figure 4.6. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. Data was found to be not significant by one-way ANOVA.

Additional functional characterisation was carried out through rhodamine 123 transport assays to determine how the efflux activity of P-gp changes in the varying culture conditions. Assays were performed in the absorptive apical-basolateral and secretory basolateral-apical directions allowing for the efflux ratio to be calculated to determine whether rhodamine 123 was subjected to active efflux in the differing models (Figure 4.16A). The Caco-2 monoculture exhibited a significantly higher rhodamine 123 transport in the basolateral-apical direction (9.4×10^{-7} cm/s) when compared to the apical-basolateral direction (2.74×10^{-7} cm/s). Like the Caco-2 monolayer, the 90:10 co-culture of Caco-2 and HT29-MTX-E12 cells also had significantly higher transport in the basolateral to apical direction. This is in contrast to the HT29-MTX-E12 monoculture which had no significant differences in the directional transport of rhodamine 123. To confirm whether the significantly higher secretory transport of rhodamine 123 was biologically significant, the efflux ratio was calculated and determined to be 4.68, 3.58 and 1.86 for models seeded at 100:0, 90:10 and 0:100 Caco-2:HT29-MTX-E12 respectively (Figure 4.16B). This indicated that the effect of the higher basolateral-apical transport rate did have a physiological impact as the efflux ratios were greater than 2 and active efflux was occurring. Within the later model, rhodamine 123 is not subject to active efflux as the ratio is < 2 . Addition of HT29-MTX-E12 cells to Caco-2 culture reduces the active efflux of P-gp due to the lack of P-gp expression in the goblet-like cells, creating a more physiologically relevant model.

Introduction of verapamil hydrochloride, a P-gp inhibitor, to the transport assays reduces the basolateral-apical transport of rhodamine 123, however this was not found to be significant amongst any of the models (Figure 4.16C). The resultant efflux ratios comparing the apical-basolateral and basolateral-apical apparent permeability coefficients in the presence of verapamil hydrochloride were found to only be significantly altered in the Caco-2 monolayer wherein the efflux ratio was reduced almost 2-fold (Figure 4.16D). The inhibitory effect of verapamil hydrochloride was still observed in the co-culture and goblet cell monoculture but to a lesser extent than in the Caco-2 epithelium.

Overall, inclusion of HT29-MTX-E12 goblet-like cells into enterocyte culture induced physiologically relevant changes within the epithelium. Enhanced Caco-2 polarisation was observed in co-culture in addition to the presence of mucus production by the HT29-MTX-E12 cells. Functionally, incorporation of goblet-like cells reduces the abnormally high TEER and activity of P-gp which is known to be overexpressed and a limitation of the Caco-2 monolayer.

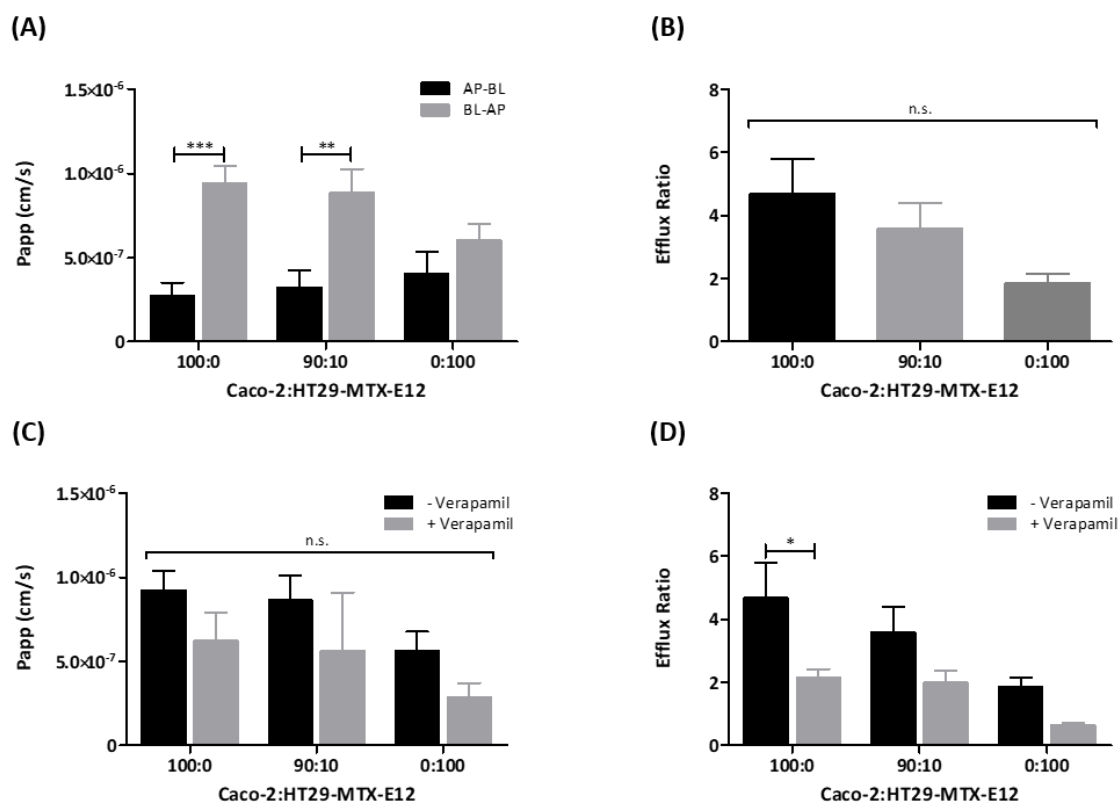


Figure 4.16. Rhodamine 123 transport characteristics across the epithelial cultures. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. Between days 21-29 of culture, the apparent permeability coefficient of the P-gp substrate, Rhodamine 123 was determined over a 2-hour long assay in the absorptive AP-BL and secretory BL-AP directions (A). The efflux ratio of Papp in the BL-AP transport direction to the Papp in the AP-BL direction was calculated (B). Verapamil hydrochloride, a P-gp inhibitor was also added to the BL-AP assay to determine if the primary transport mechanism of Rhodamine 123 was controlled by P-gp (C). The efflux ratio calculated in the presence and absence of verapamil (D). Data represent mean $\pm$ SEM, total $n=7-9$ comprised of 3 independent test occasions, each with 2-3 technical replicates. 0:100 Caco-2:HT29-MTX-E12 model data previously depicted in Figure 4.7. * $P \leq 0.05$, ** $P \leq 0.001$, and *** $P \leq 0.0001$ as determined by two-way ANOVA with Bonferroni post-test (A,C) and by one-way ANOVA with Tukey's post hoc test to compare the means of individual variables (B).

4.4.3. Investigating the Effect of Fibroblast Conditioned Medium on the Differentiation and Function of Epithelial Cultures

Before creating a triple co-culture comprising of stromal cells, enterocytes and goblet cells, the paracrine effects of HDFn cells on the epithelial cultures were investigated. This was performed through collection of fibroblast conditioned media and application of this media to the Transwell® models as described in Section 4.3.3.

4.4.3.1. The effect of HDFn conditioned media on the growth profile of epithelial cells cultured under 2D conditions

To determine whether HDFn conditioned media had differential effects on the growth of epithelial cells, Caco-2 and HT29-MTX-E12 were maintained in conventional 2D culture conditions and grown in either complete DMEM or HDFn conditioned DMEM mixed 1:1 with fresh complete DMEM (herein referred to as conditioned media). Application of this conditioned media did not affect the number of cells retrieved during a trypan blue exclusion assay performed over the course of 4 days of epithelial cell growth, however, both cell lineages were demonstrated to have slightly lower cell retrieval at day 4 of growth when cultured in the presence of conditioned media (Figure 4.17A,B).

Immunostaining for E-cadherin was also performed on epithelial cells grown on glass coverslips for 4 days in the two different media conditions (Figure 4.17C). The ability of the cells to form the initial cell-cell contacts required for cell differentiation and tissue integrity remained unaltered in the presence of the fibroblast secreted factors. This could imply that cells would retain the capacity to form epithelial sheets if cultured on Transwell® membranes. Both cell lineages grew as a monolayer of polygonal adherent cells in patches irrespective of culture media. However, the cells exhibit varying morphological characteristics; the patches of Caco-2 cells resemble sheets while the HT29-MTX-E12 cells typically grow in rounded colonies that merge together as the confluency of the cells increases.

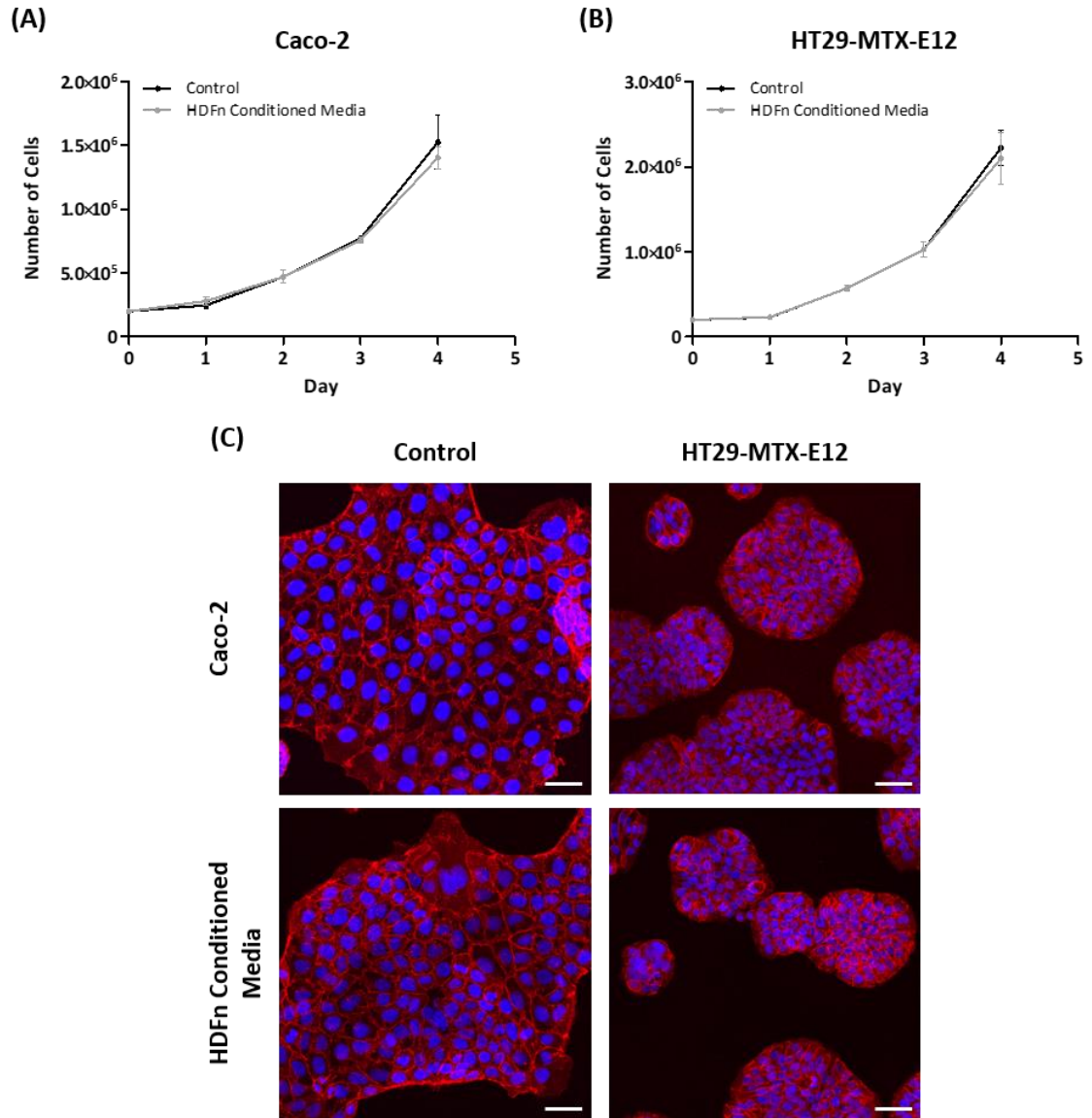


Figure 4.17. Conditioned media from stromal cells does not affect the 2D growth capability of Caco-2 and HT29-MTX-E12 cells. A trypan blue exclusion assay was performed on both Caco-2 (A) and HT29-MTX (B) cells cultured with or without fibroblast conditioned media every day for 4 days of growth. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent experimental occasions each with 3 technical replicates. Epithelial cells were also seeded onto glass coverslips and maintained in fresh DMEM or HDFn conditioned media mixed 1:1 with fresh media for 4 days on glass coverslips. Cells were then fixed in 10 % formalin and immunolabelled for E-Cadherin (red) with Hoechst (blue) counterstaining to visualise nuclei (C). Images shown are representative of 3 cultures. Scale bars: 50 μ m.

4.4.3.2. HDFn conditioned media exerts differential effects on the differentiation of Caco-2 and HT29-MTX-E12 epithelial cells

Culture of Caco-2 and HT29-MTX-E12 on Transwell® membranes in the presence or absence of fibroblast conditioned media proceeded with the optimised ratios outlined in Section 4.4.2.2. (i.e., 100:0, 90:10 and 0:100 Caco-2:HT29-MTX-E12). Histological analysis performed through H&E staining revealed that in the presence of conditioned media, Caco-2 monolayers exhibited an enhanced polarisation with an increased columnar shape, strong basolateral distribution of nuclei and a strongly stained apical membrane indicative of the formation of a brush border (Figure 4.18). This increased polarity of Caco-2 cells was evident in both the Caco-2 monoculture and in the 90:10 co-culture with goblet-like cells. However, HT29-MTX-E12 cells demonstrated differential morphological changes depending on the *in vitro* epithelial model being examined. When in co-culture with Caco-2 cells, HT29-MTX-E12 cells retain their goblet-like cell phenotype with evidence of apical mucus buds in both control samples and in models treated with fibroblast conditioned media. However, when cultured alone in the presence of conditioned media, HT29-MTX-E12 cells appear to lose their goblet cell phenotype and display a highly flattened, aberrant structure and demonstrate no signs of apical-basolateral polarity. Furthermore, the HT29-MTX-E12 cells had no detectable mucin granules suggesting they had lost their ability to differentiate into mucus-producing cells.

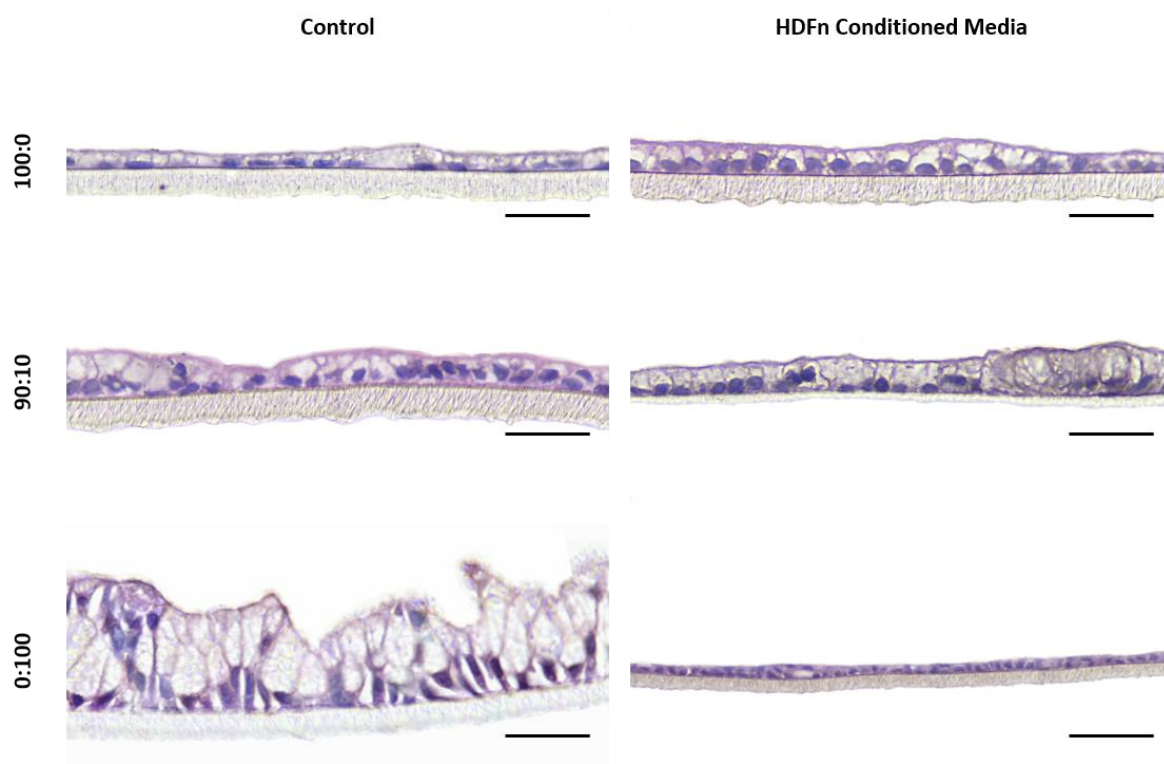


Figure 4.18. Morphological changes in epithelial cell cultures are induced by fibroblast conditioned media. H&E staining of Caco-2 and HT29-MTX-E12 cell cultures on Transwell® cultured in complete DMEM or HDFn conditioned DMEM. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12: 100:0, 90:10 and 0:100. Images shown are representative of 9 models. Scale bars: 50 μ m.

To confirm that the *in vitro* models retained their ability to form a barrier across the epithelium, wholemount immunostaining was performed for the apical junctional complex proteins occludin and E-cadherin (Figure 4.19). In all conditions, occludin expression remained unaltered and highlighted the cobblestone morphology of the epithelium. Additionally, it was possible to observe the regularity of the epithelial sheets formed in the monoculture conditions and the distinctive islands of the differing cell populations within the co-culture. E-cadherin displayed a similar expression profile to occludin. However, in regard to the HT29-MTX-E12 monoculture cultured with the addition of HDFn conditioned media, E-cadherin had a slightly altered distribution in which it was not found to be distinctly outlining all cells. There also appeared to be some evidence of overlapping cells suggestive of the formation of multilayers. As correct adherens junction formation is required for appropriate cellular polarisation, it is possible that the altered distribution pattern of E-cadherin in the conditioned media treated goblet-like cells is partially responsible for the lack of distinct polarity within the models. Overall, although the HT29-MTX-E12 monocultures maintained in the presence of fibroblast conditioned media displayed poor morphological structure, they retained their ability to form appropriate cell-cell junctions with adjacent cells.

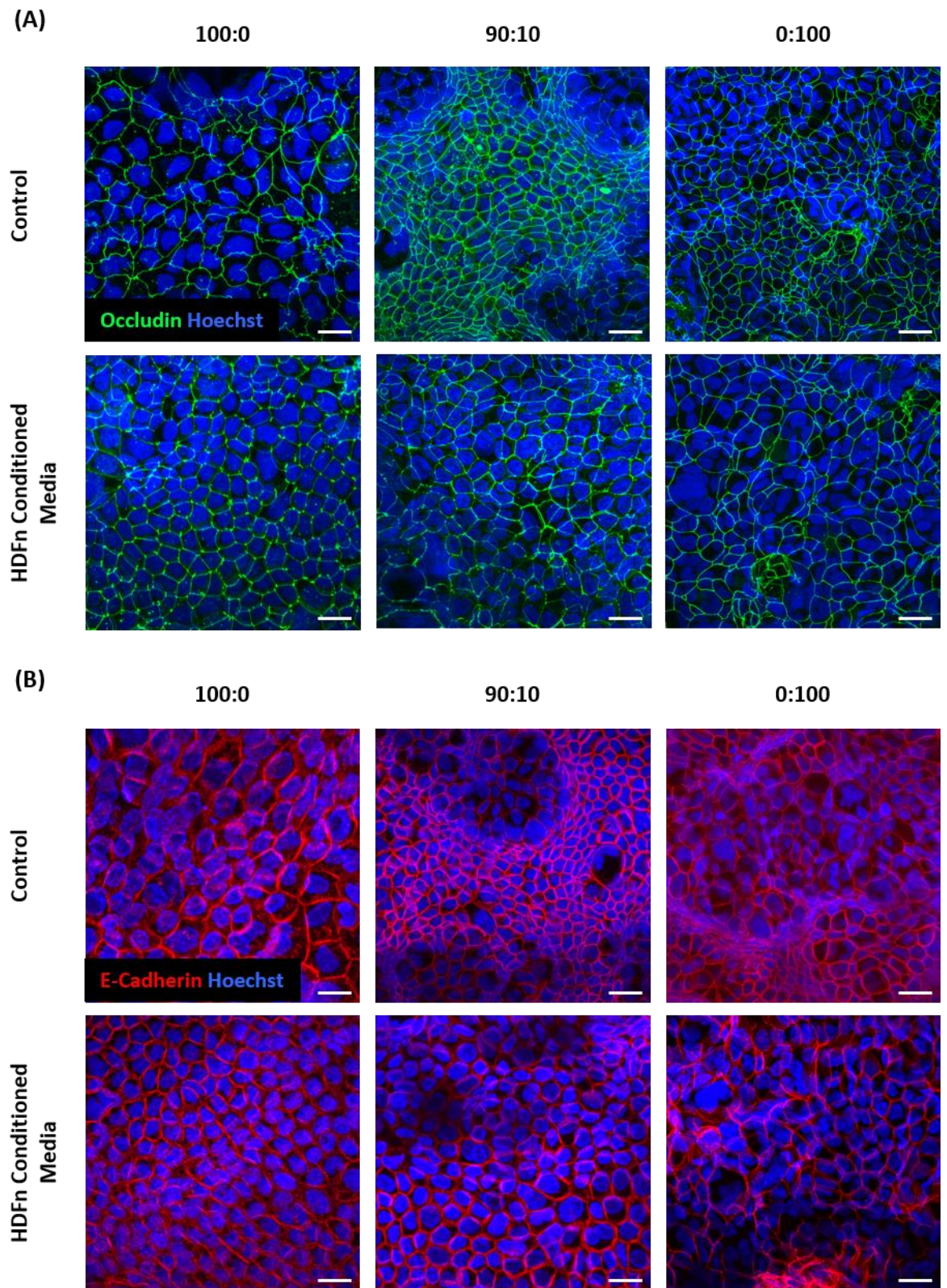


Figure 4.19. Expression of apical junctional complex components is not altered by the addition of fibroblast conditioned medium. Top-down compressed Z-stacks of staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Transwell® inserts. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100 and were maintained in either complete DMEM or HDFn conditioned media. Models were immunolabelled for the tight junction protein occludin (green) and for the adherens junction marker E-cadherin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 3 models. Scale bars: 20 μ m.

4.4.3.3. Characterisation of functional properties of epithelial models treated with fibroblast conditioned media

Functional properties of epithelial models were compared against one another when cultured in HDFn conditioned media and against their control samples to determine the effects of the fibroblast secretome on barrier integrity and P-gp activity.

Assessment of TEER demonstrated that when models are grown in fibroblast conditioned media, electrical resistance continued to increase over time (Figure 4.20A). However, the increase in TEER of the Caco-2 monolayer and the 90:10 co-culture was much more variable with peaks and troughs occurring throughout the 21-day differentiation period rather than a general increase and plateau as observed previously in control models. Final TEER values showed a significant 1.2-fold reduction in Caco-2 TEER with the addition of goblet-like cells under conditioned media culture conditions from 2420 ($\pm$ 58.90) to 1980.12 ($\pm$ 73.71) Ω .cm². HT29-MTX-E12 endpoint TEER was determined to be the lowest of all models when cultured with conditioned media displaying a value of 900.23 ($\pm$ 37.33) Ω .cm² (Figure 4.20B).

Conditioned media exerted differential effects on the electrical resistance of the varying epithelial models compared to controls. The Caco-2 monolayer maintained in conditioned media initially had a higher TEER than the control samples, however by day 4, TEER had dropped to levels below the control and remained that way for the remainder of the culture period (Figure 4.20C). Addition of the fibroblast conditioned media to these Caco-2 monocultures resulted in a significant reduction in endpoint TEER, suggesting that secreted factors from HDFn cells induce functional changes within the epithelial culture to better resemble the *in vivo* intestine (Figure 4.20F).

Unlike the Caco-2 monolayer, fibroblast conditioned media induced a significant increase in TEER in both the 90:10 and 0:100 Caco-2:HT29-MTX-E12 cultures (Figure 4.20D,E,F). It is hypothesised that this is due to effects exerted on the HT29-MTX-E12 cells as it has previously been demonstrated that conditioned media can induce changes in the morphology of these cells (Section 4.4.3.2) and reduces the TEER of Caco-2 cells. However, the endpoint TEER of these two epithelial models remains lower than the Caco-2 monolayer (Figure 4.20F).

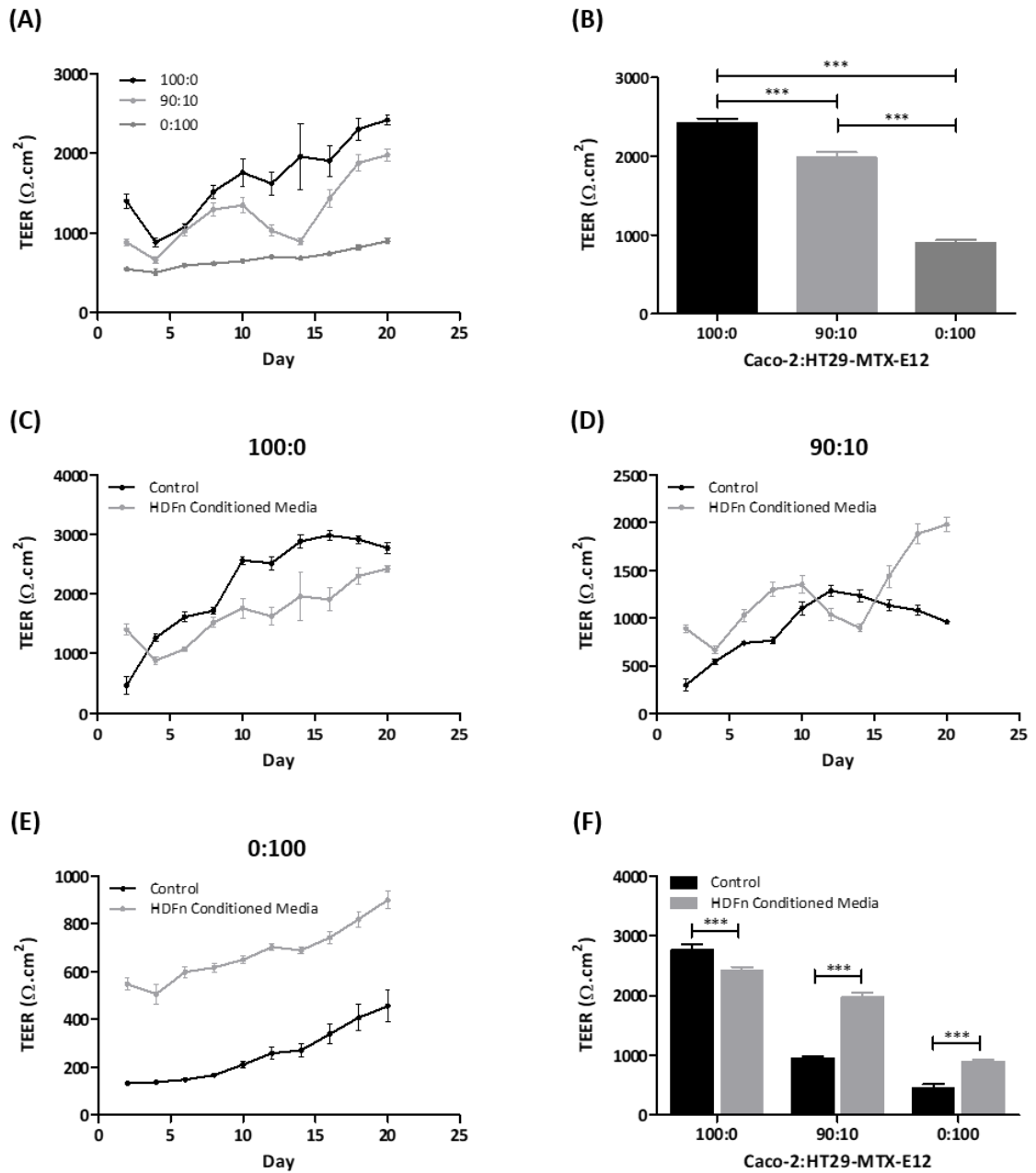


Figure 4.20. Epithelial models cultured with fibroblast conditioned medium display an increase in TEER over time. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100 and were maintained in complete DMEM (control) or HDFn conditioned DMEM. TEER measurements were taken every two days of epithelial cell culture in HDFn conditioned media and exhibit an increase with culture time (A). Endpoint TEER values were compared across the three culture systems when maintained in conditioned media (B). TEER measurements were taken every two days of epithelial cell culture in both media conditions for epithelial cells seeded at 100:0 (C), 90:10 (D) and 0:100 (E) Caco-2:HT29-MTX-E12. Endpoint TEER values were compared across the three culture systems maintained in either control or HDFn conditioned media (F). Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions each with 3 technical repeats. *** $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post hoc test to compare the means of individual variables (B) and by two-way ANOVA with Bonferroni post-test (F).

Assessment of lucifer yellow rejection revealed that the models cultured in fibroblast conditioned media had a strong and intact barrier with the levels of dye excluded being over 95 % in both the absorptive apical-basolateral and secretory basolateral-apical directions (Figure 4.21A). However, a significant reduction in barrier integrity was detected in the basolateral-apical direction of transport in all paracrine culture conditions, though this is unlikely to have any biological impact due to the overall high levels of lucifer yellow rejection in all conditions. There were no significant differences in the rejection of lucifer yellow between control and conditioned media treated models in either transport direction (Figure 4.21B,C).

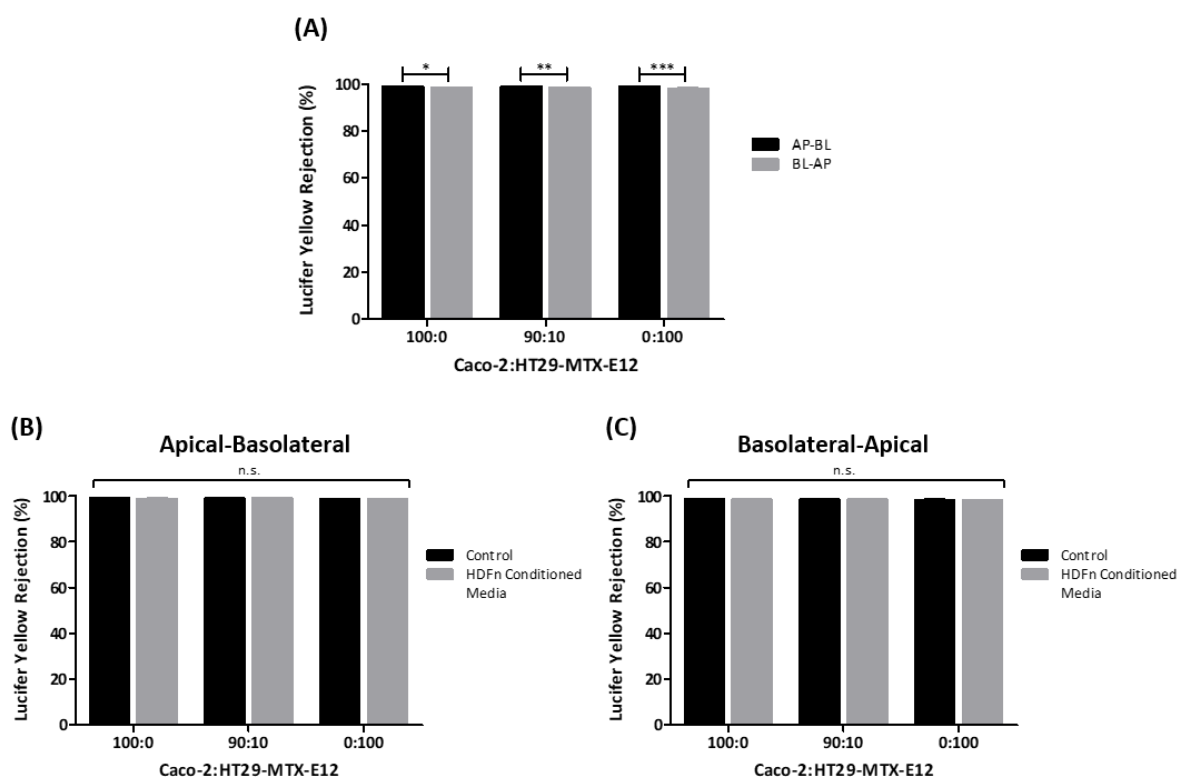


Figure 4.21. Epithelial models cultured in control and fibroblast conditioned DMEM display high barrier integrity. Cells were seeded at the following ratios of *Caco-2*:*HT29*-*MTX*-*E12* cells: 100:0, 90:10, and 0:100. A lucifer yellow rejection assay was performed on models maintained in HDFn conditioned media in the absorptive AP-BL direction and the secretory BL-AP direction (A). Lucifer yellow rejection was also assessed in control and HDFn conditioned media models in the AP-BL (B) and BL-AP (C) directions. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical repeats. * $P \leq 0.05$, ** $P \leq 0.001$, and *** $P \leq 0.0001$ as determined by two-way ANOVA with Bonferroni post-test.

To quantify the transport of lucifer yellow the Transwell® monolayers more accurately, the apparent permeability coefficient was determined. Models cultured in fibroblast conditioned media demonstrated an increased basolateral-apical permeability of lucifer yellow in all *in vitro* monolayers, though the only significant increase occurred in the HT29-MTX-E12 monolayer (Figure 4.22A). Comparison of lucifer yellow permeability between models cultured in fresh complete DMEM and fibroblast conditioned media displayed no significant differences in transport in the apical-basolateral direction (Figure 4.22B). Regarding basolateral-apical transport, significantly increased lucifer yellow permeability was detected in the 90:10 and 0:100 Caco-2:HT29-MTX-E12 monolayers maintained in conditioned media in comparison to control models (Figure 4.22C), inconsistent with the higher TEER in the former cultures.

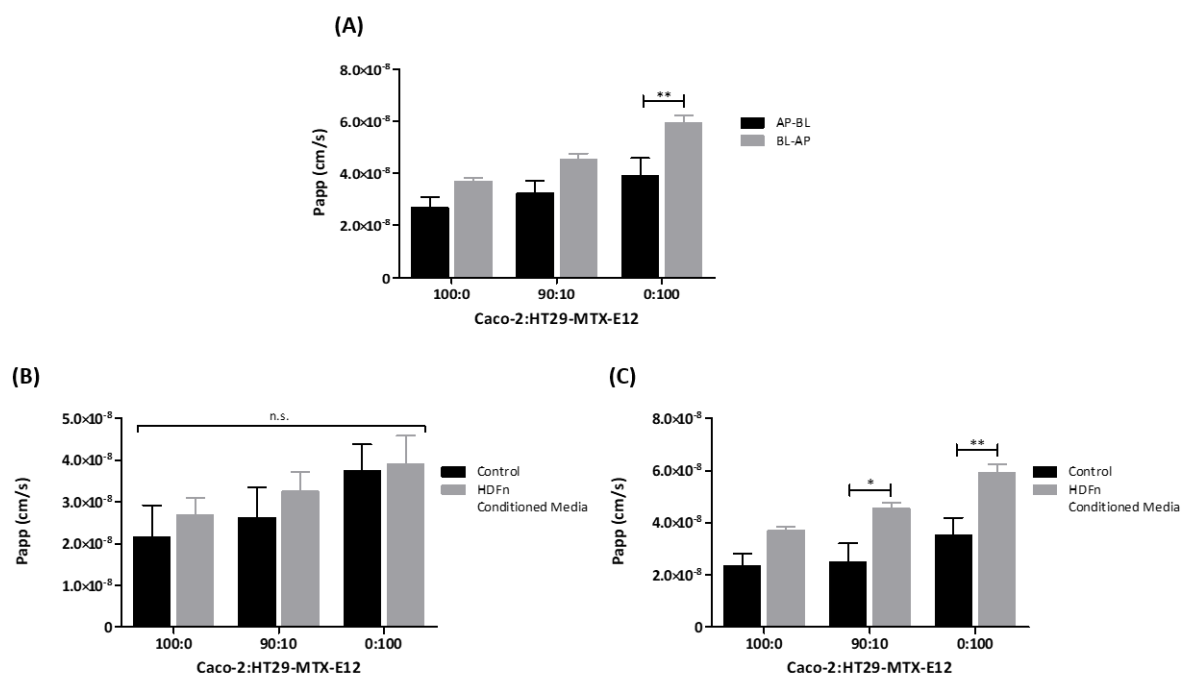


Figure 4.22. Lucifer yellow transport characteristics across control and fibroblast conditioned media treated epithelial models. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. The transport of lucifer yellow was determined through the calculation of the apparent permeability coefficient of models maintained in HDFn conditioned media in the AP-BL direction and the BL-AP direction (A). The Papp of lucifer yellow also assessed in control and HDFn conditioned media models in the AP-BL (B) and BL-AP (C) directions. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. * $P \leq 0.05$ and ** $P \leq 0.001$ as determined by two-way ANOVA with Bonferroni post-test.

The activity of P-gp was also assessed in models treated with conditioned media through a rhodamine 123 transport assay. In all three models, the transport of rhodamine 123 in the apical-basolateral direction increased upon treatment with conditioned media, though the only significant difference was detected within the Caco-2 monolayer (Figure 4.23A). A similar trend was also observed in the basolateral-apical transport direction, with an increase in permeability in all models cultured with fibroblast conditioned media. However, no statistically significant changes were found (Figure 4.23B). Comparison of the efflux ratios between the varying models and culture conditions also did not reveal any statistically significant changes, although a reduced efflux ratio was evident upon culture with conditioned media in all monolayers (Figure 4.23C). In both the 100:0 and 90:10 Caco-2:HT29-MTX-E12 monolayers, the efflux ratio remained greater than 2 during both culture conditions, showing that active efflux of rhodamine 123 was occurring and suggesting that activity of the P-gp transporter protein was retained in the models when introduced to the fibroblast secretome. Investigation of the effects of verapamil hydrochloride on the varying cultures was also performed, but no differences in the in the basolateral-apical transport of rhodamine were found in models cultured in the presence of fibroblast conditioned media (Figure 4.23D,E,F).

Taken together, these results demonstrate that factors from the fibroblast secretome exert varying effects on both the structure and function of epithelial cells. HT29-MTX-E12 cells lost their goblet-like cell morphology and instead resembled the structure of Caco-2 cells, however this only occurred in the HT29-MTX-E12 monolayer and not when the cells were in co-culture with Caco-2 cells. Furthermore, fibroblast conditioned media favoured an increased permeability in all models for both lucifer yellow and rhodamine 123, yet conversely, the TEER of the 90:10 and 0:100 Caco-2:HT29-MTX-E12 monolayers was greater than controls.

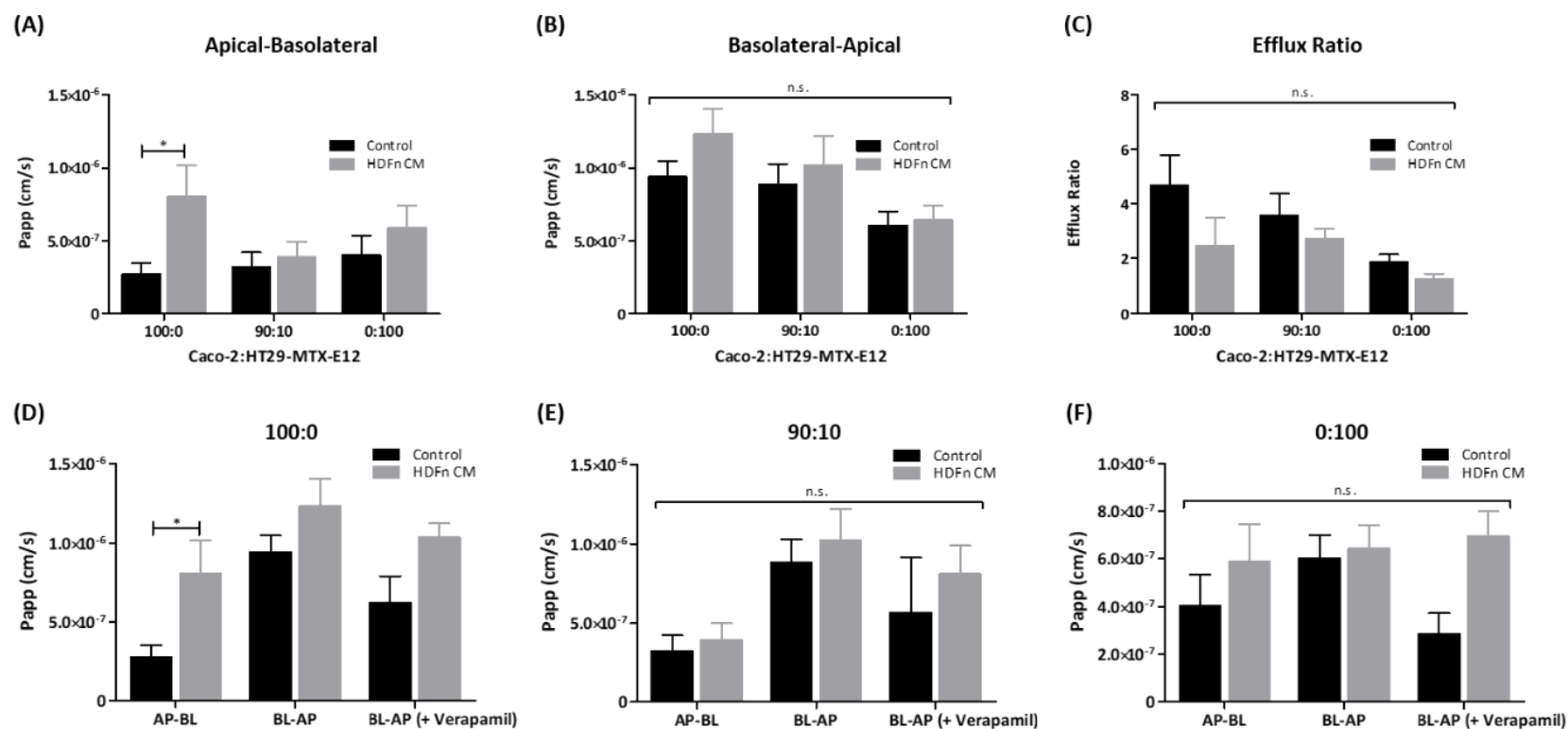


Figure 4.23. Rhodamine 123 transport characteristics across epithelial models treated with fibroblast conditioned media. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100 and were maintained in HDFn conditioned media. Between days 21-29 of culture, the apparent permeability coefficient of the P-gp substrate, Rhodamine 123 was determined over a 2-hour long assay in the absorptive AP-BL (A) and secretory BL-AP (B) directions. The efflux ratio of Papp in the BL-AP transport direction to the Papp in the AP-BL direction was then calculated and was also compared to models cultured in control complete DMEM (C). Verapamil hydrochloride was added to the transport assay and the resulting apparent permeability coefficients were calculated in the 100:0 (D), 90:10 (E) and 0:100 (F) maintained in either control or fibroblast conditioned medium. Data represent mean $\pm$ SEM, total n=7-9 comprised of 3 independent test occasions, each with 2-3 technical replicates. * $P \leq 0.05$ as determined by two-way ANOVA with Bonferroni post-test.

4.4.4. Development and Characterisation of a Triple Co-Culture to Represent the Human Intestinal Mucosa

To accurately recapitulate the structure of the intestinal mucosa, epithelial cells were again seeded at the following ratios of Caco-2:HT29-MTX-E12: 100:0, 90:10 and 0:100 onto 14-day HDFn lamina propria equivalents developed in Chapter 3. Over 90 % of co-cultures successfully formed an epithelial monolayer as assessed by TEER throughout the Caco-2 culture period and by histological analysis.

4.4.4.1. Histological analysis of epithelial cell differentiation

Epithelial cell culture on the surface of lamina propria equivalents was analysed through H&E staining as seen in Figure 4.24. In all models, fibroblasts can be observed to populate throughout the Alvetex® Scaffold and formed well orientated layers along the edges of the Alvetex® membranes where a greater density of fibroblasts are located. Epithelial monocultures generally had homogeneous growth and differentiation along the entire lamina propria equivalent with high apical-basolateral polarity emphasised by the columnar cell shape and basal nuclei. Apical mucus buds were found within HT29-MTX-E12 cells in both mono- and co-culture suggesting that the goblet-like cell phenotype of the cells was preserved. Additionally, in co-culture models with Caco-2 cells, goblet-like cells did not distribute evenly throughout the epithelium and instead were found interspersed in Caco-2 cells as islands. Strong apical membrane staining of the epithelial cells in all three models is indicative of microvilli brush border formation, emphasising the cellular differentiation into intestinal cells. The H&E staining profile of the different cell lineages strongly resembles staining of human intestinal tissue samples (Figure 3.3) regarding the almost transparent appearance of the mucus producing cells, the highly polarised morphology of the epithelium and the identification of a brush border along the apical surface of the epithelium.

Models were also stained with PAS/Alcian blue to visualise the production of mucins by the goblet-like cells (Figure 4.25). No positive staining for mucus was detected in the Caco-2 epithelium when seeded onto the surface of fibroblasts, however positive blue staining was identified in both the Caco-2:HT29-MTX-E12 co-culture and HT29-MTX epithelium on the lamina propria compartment. Within the co-culture model in which both epithelial cell types were present, cells that were stained blue confirmed that they were the mucus producing goblet-like cells. Such cells appeared in distinct patches, as previously discussed, this was in contrast to the HT29-MTX-E12 epithelium where a homogenous layer of positively stained cells could be seen across the entire monolayer. When comparing the mucus production to human tissue (Figures 3.5 and 3.6), the 90:10 model appeared to be morphologically more similar to small intestinal tissue with fewer goblet cells than the large intestine, while the 0:100 model was phenotypically more like the colonic tissue which had a high number of goblet cells across the epithelial surface. Although mucin-positive cells could be detected, there was no evidence of a mucus layer lining the surface of the epithelium, potentially due to the fixation and dehydration processes that the models were subjected to.

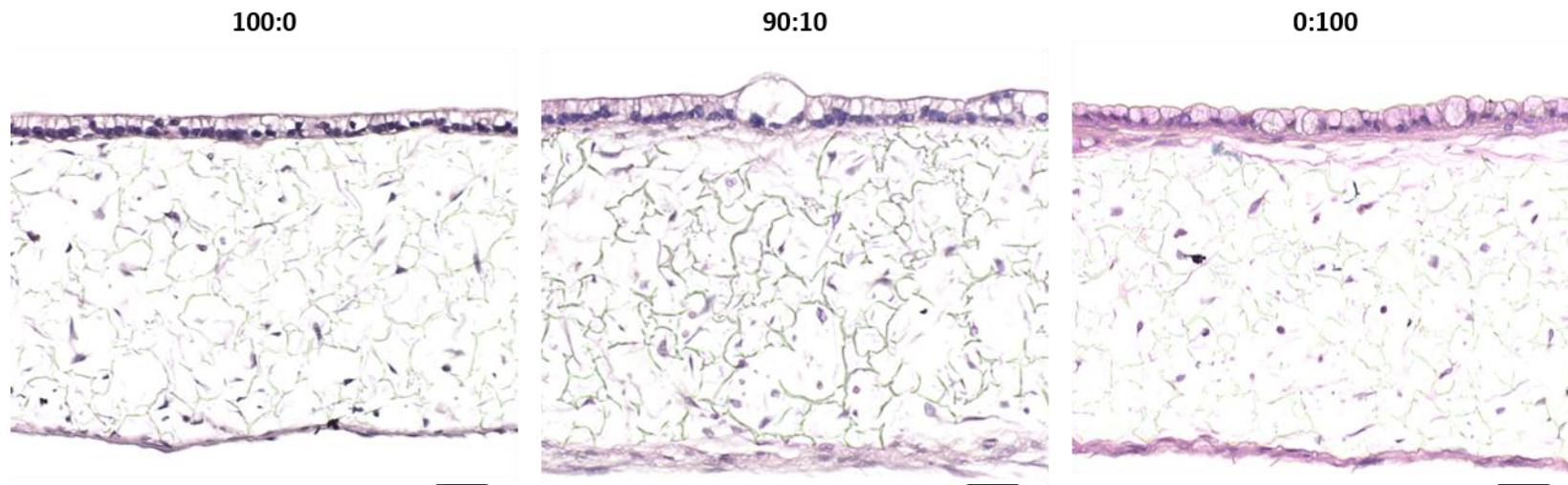


Figure 4.24. H&E staining of in vitro intestinal mucosa models. H&E staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Alvetex® Scaffold containing fibroblasts grown for 14 days prior to epithelial cell seeding. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10 and 0:100. Images shown are representative of 9 models. Scale bars: 50 μm.

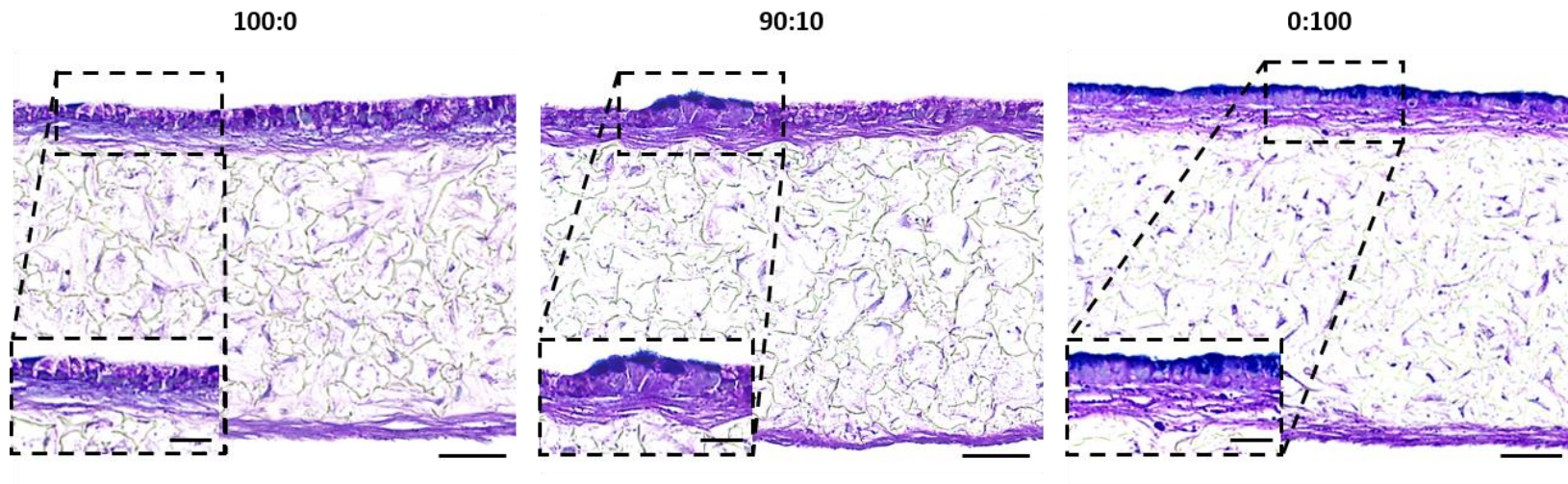


Figure 4.25. PAS/Alcian blue staining for evidence of mucus production within in vitro intestinal mucosa models. PAS/Alcian blue staining of Caco-2 and HT29-MTX-E12 epithelial cell cultures on Alvetex® Scaffold containing fibroblasts grown for 14 days prior to epithelial cell seeding. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10 and 0:100. Positive blue staining denotes the presence of acidic mucins. Images shown are representative of 9 models. Scale bars: 50 μ m and 25 μ m – inset images.

4.4.4.2. In-depth characterisation of epithelial cell structure upon culture with fibroblasts

Supplementary to histological staining techniques, TEM images were acquired of the three different epithelial monolayers cultured on the surface of lamina propria equivalents. The Caco-2 epithelium is perceived as a monolayer of polarised epithelial cells on the surface of the fibroblast compartment of the model (Figure 4.30A). Finger-like, microvilli projections are evident lining the apical surface of the cells forming a brush border across along the epithelium (Figure 4.26A,B,C). Furthermore, electron-dense staining is apparent between adjacent cells. This staining is typically located at the apical surface, immediately below the microvilli brush border and provides evidence that junctional complexes have formed between cells. Mitochondria were also present throughout the cell cytoplasm but were more prevalent along the lateral membranes between cells and at the apical and basolateral membranes (Figures 4.26B,C,D). Near the basolaterally-located mitochondria, lipid-like droplets were also identified suprajacent to the basolateral membrane (Figure 4.30D).

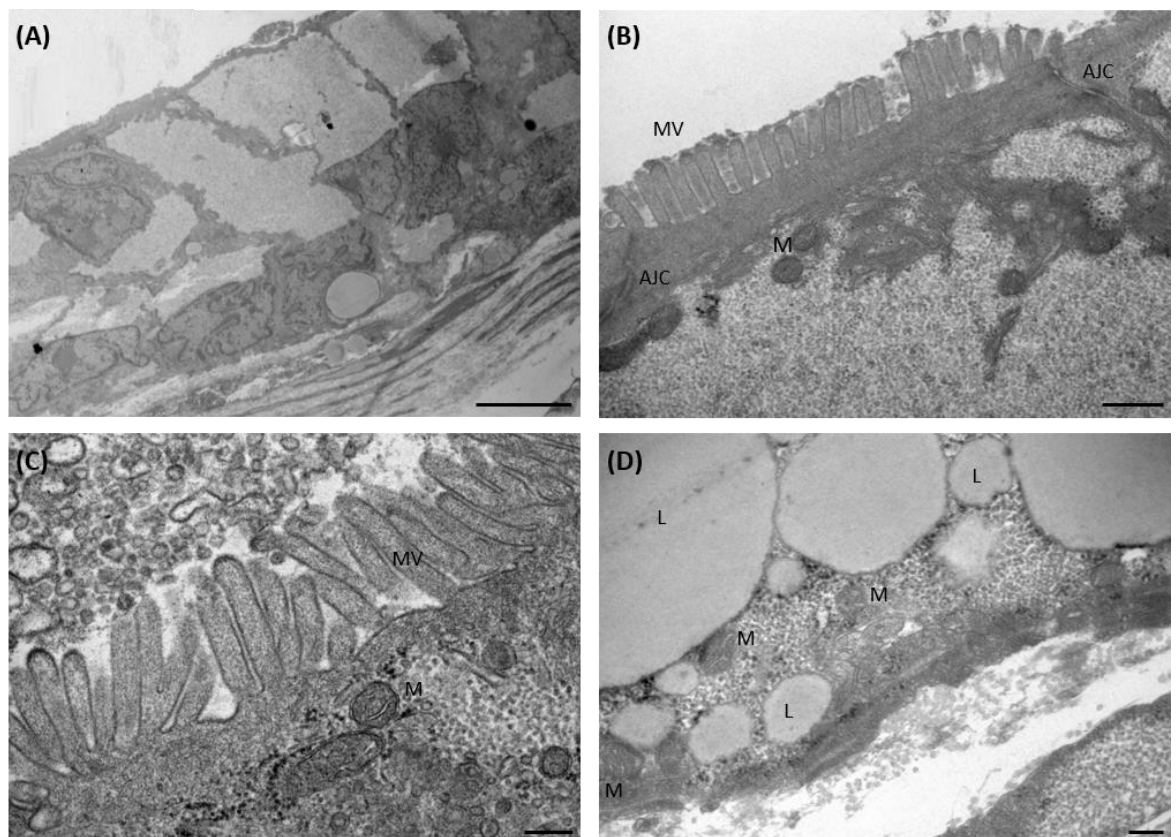


Figure 4.26. Characterisation of the Caco-2 epithelium ultrastructure in 3D co-culture with the lamina propria compartment. Samples were fixed and processed for TEM analysis. The Caco-2 epithelium presented a monolayer of polarised epithelial cells, with a microvilli brush border, lying on top of a fibroblast component (A). TEM micrographs highlight the structure of the microvilli brush border at the apical surface of the epithelium and show electron dense staining between adjacent cells suggestive of a junctional complex between the cells (B,C). At the base of the cells, large lipid droplets are present and typically cluster near the basolateral membrane (D). MV=microvilli, AJC=apical junctional complex, M=mitochondria and L=lipid droplet. Images shown are representative of 3 models. Scale bars: 5 μ m (A), 500 nm (B), 200 nm (C,D).

Examination of the 90:10 Caco-2:HT29-MTX-E12 epithelium provided greater insight into the structure of the goblet-like cells and their attachment to the enterocytes (Figure 4.31). Goblet-like cells could be identified by the presence of mucins contained within the many vesicles located in the apical part of the cell cytoplasm (Figure 4.27A). These goblet-like cells exhibited sparse microvilli along their apical surface, in contrast to the Caco-2 cells which displayed a dense apical brush border. Like the Caco-2 epithelium, mitochondria were present along the lateral membranes of the cells within the triple co-culture. Electron-dense junctional complexes were detected between adjacent Caco-2-HT29-MTX-E12 cells (Figure 4.27B), adjacent Caco-2 cells (Figure 4.27B) and adjacent goblet-like cells (Figure 4.27C). Closer examination of the electron dense staining showed multiple junctional complexes down the length of the cells' lateral membranes; the most basally located of which were typically found near the equator of the cell and are postulated to be desmosomes (Figure 4.27D). Junctions located towards the apical surface of the cells were more likely to be tight and adherens junctions (Figure 4.27E,F).

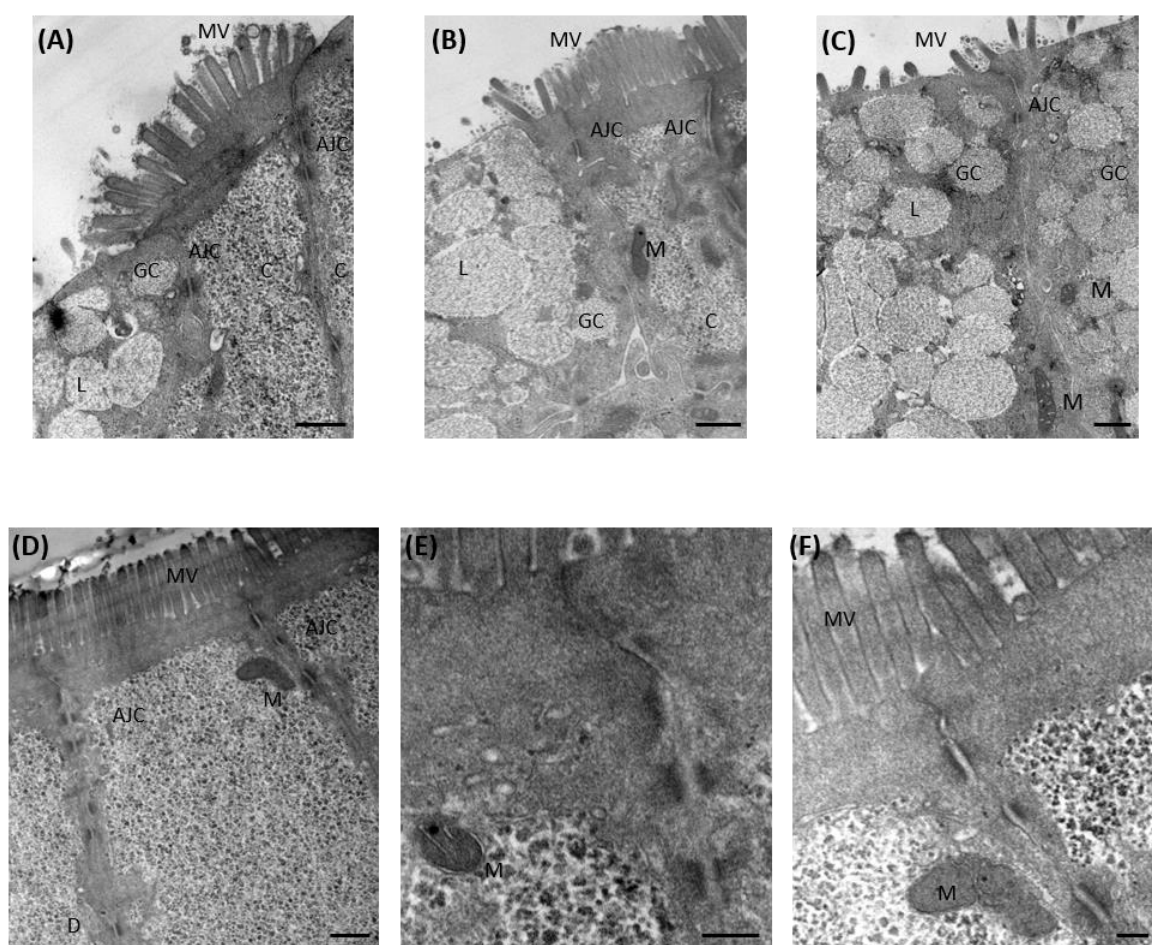


Figure 4.27. Characterisation of the epithelial ultrastructure in 3D co-culture models with Caco-2 and HT29-MTX-E12 epithelial cells and the lamina propria compartment. Samples were fixed and processed for TEM analysis. TEM micrographs show adjacent Caco-2 and HT29-MTX-E12 cells within the epithelium (A,B,C). Goblet-like cells can be identified by the presence of many lipidic vesicles within the apical cytoplasm of the cell. Apical junctional complexes were also found between adjacent epithelial cells (D,E,F). C=Caco-2 cell, GC=goblet-like cell, MV=microvilli, AJC=apical junctional complex, D=desmosome, M=mitochondria and L=lipid droplet. Images shown are representative of 3 models. Scale bars: 500 nm (A,B,C,D), 200 nm (E,F).

Finally, the ultrastructure of the HT29-MTX-E12 co-culture was examined. Lipid vesicles were present in all cells along the entire epithelium and microvilli were identified along the apical surface of the cells, yet this was not as obvious as in the Caco-2 epithelium (Figure 4.28). Abundant Golgi apparatus was found within the cells and was surrounded by darkly stained mitochondria (Figure 4.28A). Extensive folds, known as cristae, were seen within these mitochondria (Figure 4.28A-inset).

Unlike the Caco-2 monolayer wherein the epithelial cells were packed close together, the goblet-like cells displayed a less “tight” morphology and gaps could be observed between the lateral membranes of adjacent cells (Figure 4.28A). Despite the apparent distance between the cells, junctional complexes were still found at the apical surface of the cells (Figure 4.28B,C).

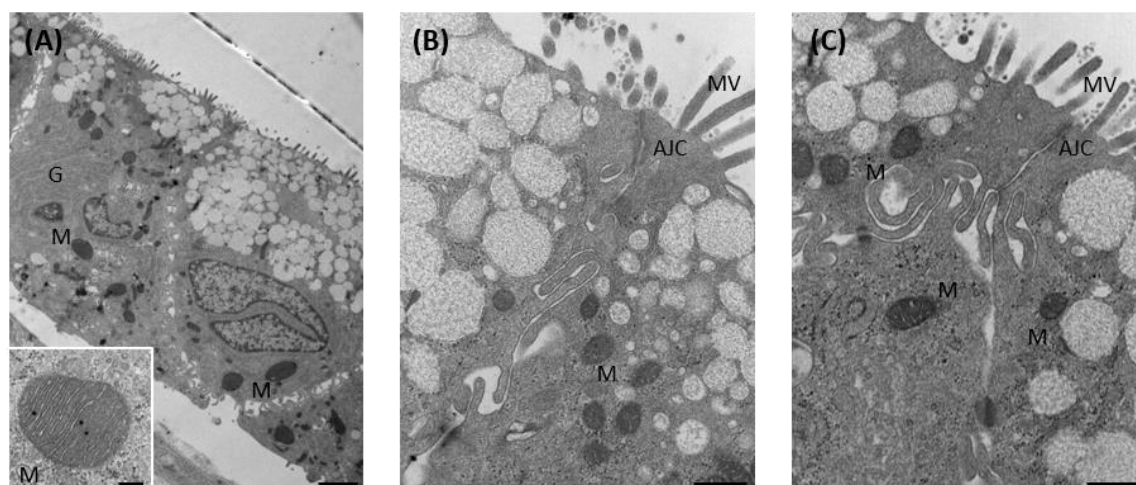


Figure 4.28. Characterisation of the HT29-MTX-E12 epithelium ultrastructure in 3D co-culture with the lamina propria compartment. Samples were fixed and processed for TEM analysis. Goblet-like cells, identified by the presence of dense vesicles found apically in the cell cytoplasm, form a monolayer on top of the lamina propria compartment and form apical junctional complexes between adjacent cells (A,B,C). Abundant mitochondria are present in the HT29-MTX-E12 epithelium and have possess the cristae structures (A-inset). MV=microvilli, AJC=apical junctional complex, M=mitochondria and G=Golgi. Images shown are representative of 3 models. Scale bars: 2 μ m (A), 200 nm (A-inset), 500 nm (B,C).

4.4.4.3. Analysis of the apical junctional complex

Analysis of the components apical junctional complex in the three different culture systems was performed through immunostaining. Imaging of HT29-MTX-E12 cultures with HDFn cells proved to have large levels of background noise due to the presence of mucus and underlying fibroblasts. Figure 4.29 shows compressed top-down Z-stacks of wholemount immunostaining for the tight junction proteins ZO-1 and occludin. ZO-1 staining outlines individual epithelial cells emphasising their polygonal shape and the ability of the cells to form a monolayer sheet across the surface of the lamina propria equivalent. As ZO-1 is an intracellular tight junction scaffold protein, its presence demonstrates that the appropriate internal tight junction structures have formed and suggests that connections are being made to the actin cytoskeleton. Unlike ZO-1, occludin is a transmembrane protein, thought to have roles in creating a tight barrier connection to adjacent epithelial cells. All models also express occludin though staining appears to be stronger in the 100:0 and 90:10 Caco-2:HT29-MTX-E12 co-cultures than in the 0:100-fibroblast co-culture.

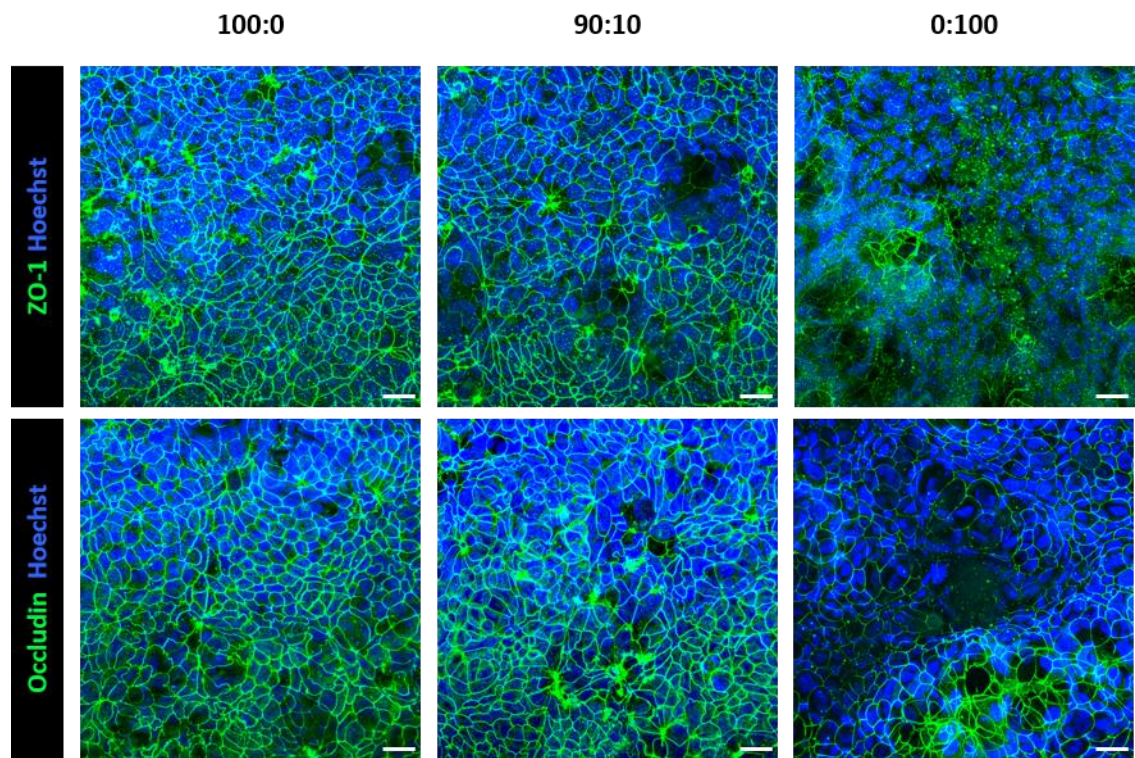


Figure 4.29. Expression of ZO-1 and occludin tight junction proteins in *in vitro* intestinal mucosa models. Top-down compressed Z-stacks of staining of Caco-2 and HT29-MTX-E12 cultures on Alvetex® Scaffold containing fibroblasts grown for 14 days prior to epithelial cell seeding. Cells were seeded at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10 and 0:100. Samples were immunolabelled for the tight junction proteins ZO-1 and occludin (green) and nuclei were counterstained with Hoechst (blue). Images shown are representative of 3 models. Scale bars: 20 μ m.

Assessment of claudin protein distribution and expression levels was also examined. Claudin-1 was selected to study the expression of a sealing claudin with a role in restricting diffusion across the epithelium, while claudin-2 was selected as it has a role in the formation of pores to allow the movement of compounds, specifically cations. Wholemount immunostaining can be observed in Figure 4.30A and side-on images of claudin-1 only are depicted in Figure 3.30B. Claudin-1 is present in all models but appears to have weaker expression in the 0:100 model in both wholemount and in profile immunostained images. Additionally, the staining did not appear to localise to the apical half of the lateral membrane when examining the stain from side-on. This could suggest that the ability of HT29-MTX-E12 cells to form a tight, intact barrier when cultured on lamina propria equivalents is diminished, however other sealing claudins may perform compensatory roles. Unlike claudin-1, claudin-2 appears to have equivalent levels of staining amongst all three models, however within the individual models there were patches of stronger and weaker staining.

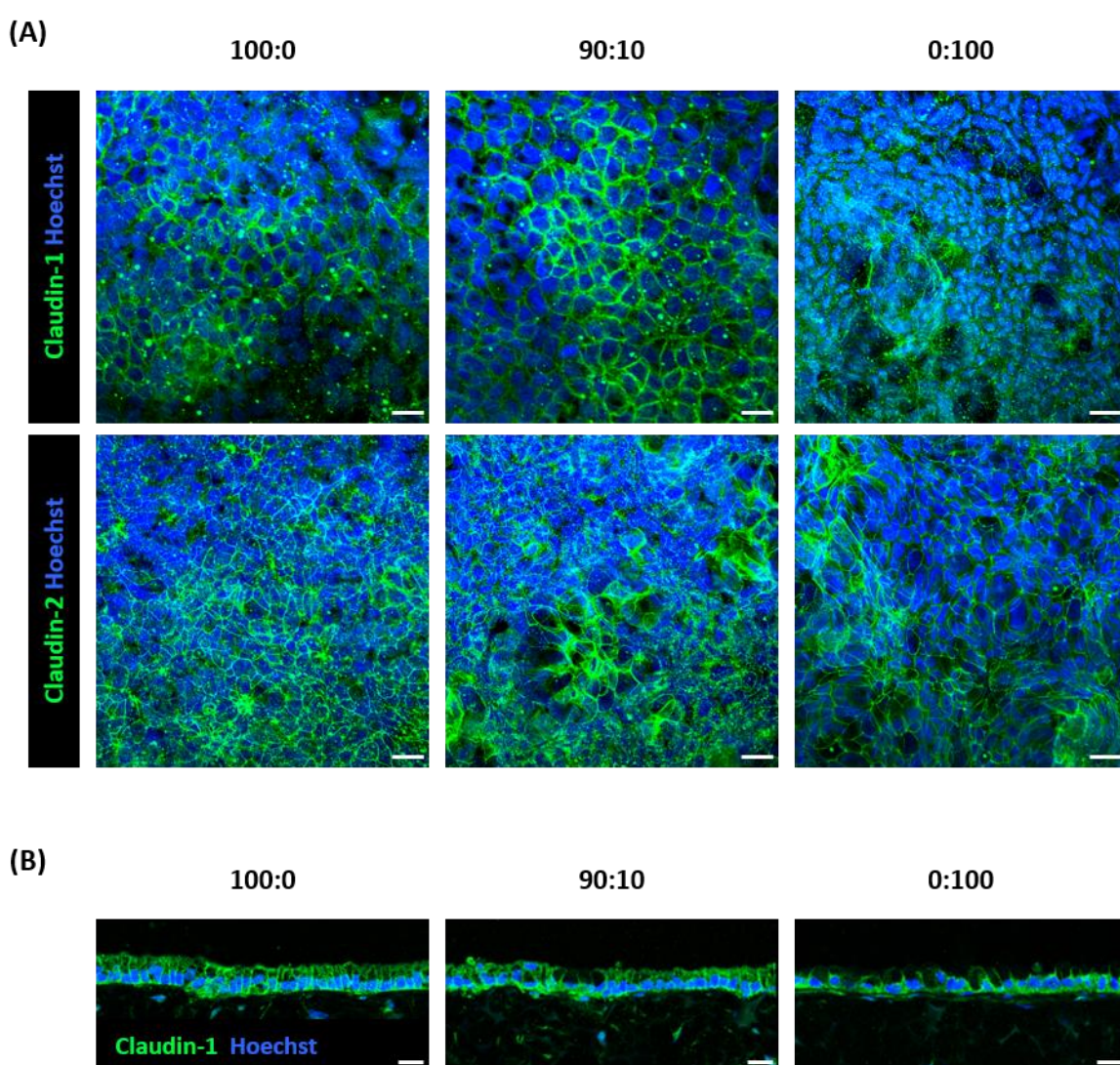


Figure 4.30. Claudin expression in *in vitro* intestinal mucosa models. Epithelial cells were seeded onto lamina propria equivalents at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10 and 0:100. Models were immunolabelled for the tight junction proteins claudin-1 and claudin-2 (green) and nuclei were counterstained with Hoechst (blue). Samples were then imaged as top-down compressed Z-stacks (A) or in profile for claudin-1 (B). Images shown are representative of 3 (A) or 9 (B) models. Scale bars: 20 μm.

In addition to immunofluorescent staining, claudin-1 and claudin-2 were also examined by qPCR analysis to quantify the presence of mRNA within the three *in vitro* mucosal models. All PCR data was normalised to GAPDH and then to the Caco-2 only model. Statistical significance was not determined due to the overall number of replicates being low; only 3 separate qPCR reactions were performed, each with 3 technical replicates to confirm there were no intra-assay outliers.

Claudin-1 (*CLDN1*) mRNA expression was high in the 100:0 model and expression decreased with increased numbers of HT29-MTX-E12 cells suggesting that models without or with fewer goblet-like cells would have a lower paracellular permeability than those with goblet-like cells due to a reduced level of sealing tight junction components (Figure 4.31A). However, the overall fold changes were less than 1, as such the biological impact of the change in gene expression is likely to be minimal. Conversely, claudin-2 (*CLDN2*) mRNA expression was found to increase with higher levels of HT29-MTX-E12 cells, with greater fold changes than *CLDN1*, suggesting that a more permeable barrier is present in monolayers that contain goblet-like cells (Figure 4.31B).

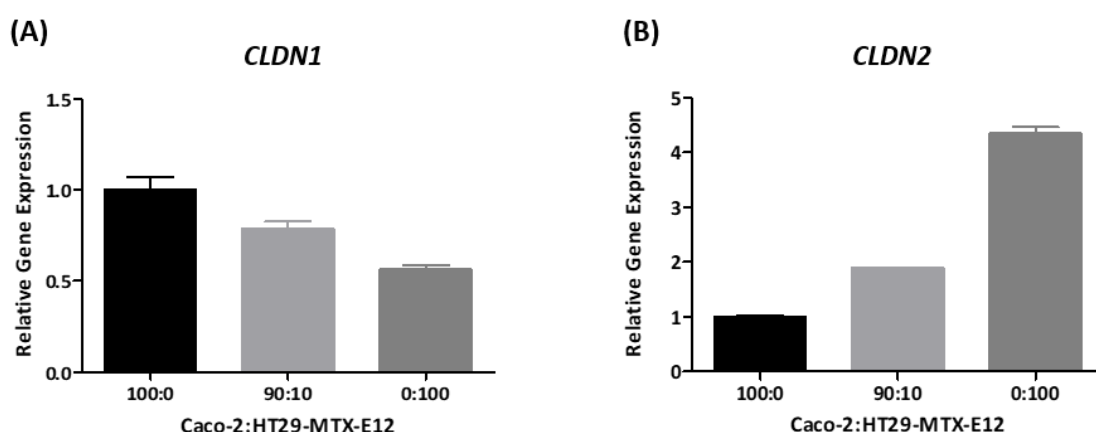


Figure 4.31. Assessment of claudin gene expression in intestinal mucosal models. 3D cell models with Caco-2:HT29-MTX-E12 cells cultured on lamina propria equivalents at 100:0, 90:10 and 0:100 were subject to qPCR analysis. RNA was extracted from the three models, converted to cDNA and the expression levels of claudin-1 (*CLDN1*) (A) and claudin-2 (*CLDN2*) (B) genes were quantified. Expression was first normalised against GAPDH expression levels and compared against the 100:0 model. Data represent mean $\pm$ SEM. 3 independent qPCR reactions were performed on lysates generated from a total of 3 independent experimental occasions, each with a single lysate collated from 3 pooled technical repeated models per experiment. Within each PCR plate, 3 technical replicate wells were included within each PCR run providing a total $n=9$.

The final protein examined in the *in vitro* models with a stromal compartment was E-cadherin. As discussed previously E-cadherin is a transmembrane component of adherens junctions. E-cadherin strongly defines the outline of individual epithelial cells and is present along the lateral membrane of both Caco-2 and HT29-MTX-E12 cells (Figure 4.32). Wholemout immunostaining for E-cadherin clearly demonstrates the differences in cell density and structure between the two epithelial cell types. Caco-2 cells appear to be densely packed, contrastingly HT29-MTX-E12 cells seem less compacted, possibly due to the distended apical surface where the mucus buds are located (Figure 4.32A). Figure 4.32B highlights the narrow, elongated phenotype of the Caco-2 cells and provides a potential explanation as to why Caco-2 cells appear highly compact when visualising the monolayer from the top-down.

Quantification of the levels of E-cadherin was performed through western blot and corresponding densitometry analysis (Figure 4.33). No significant differences were found in E-cadherin expression between the three models, however, models with only HT29-MTX-E12 cells in their epithelium displayed a reduced expression level when compared to the other culture systems.

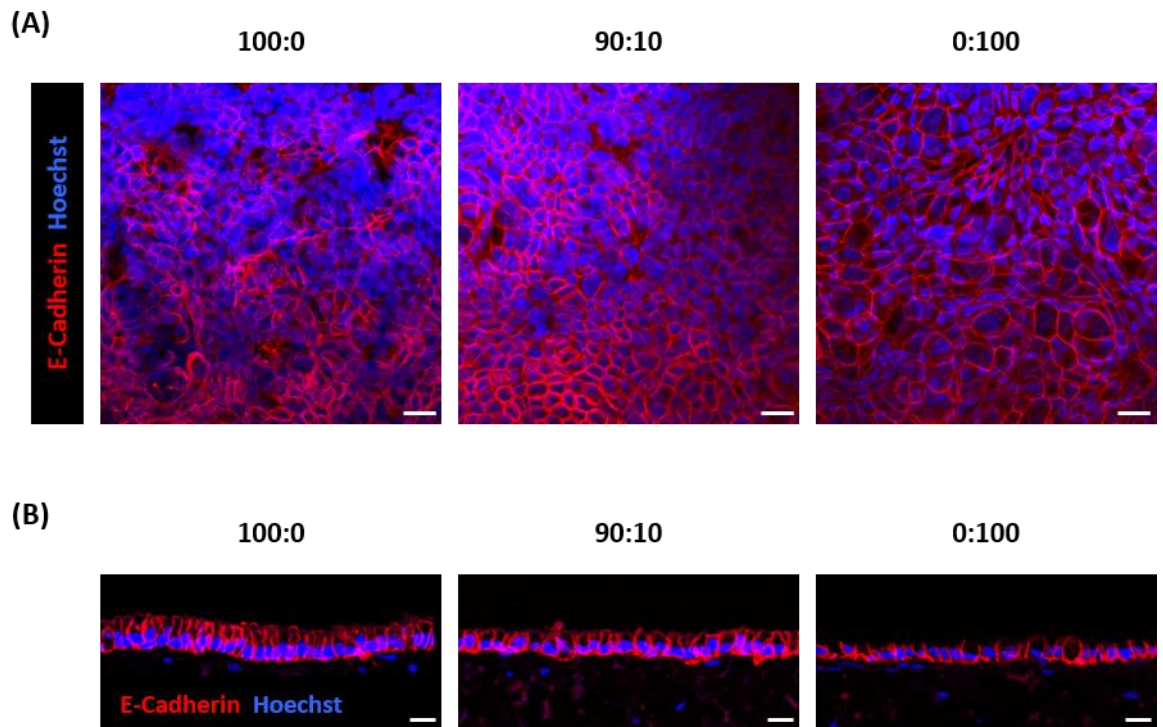


Figure 4.32. Expression of E-cadherin in *in vitro* intestinal mucosa models. Epithelial cells were seeded onto lamina propria equivalents at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10 and 0:100. Samples were immunolabelled for the adherens junction protein E-cadherin (red) and nuclei were counterstained with Hoechst (blue). Samples were then imaged as top-down compressed Z-stacks (A) or in profile (B). Images shown are representative of 3 (A) or 9 (B) models. Scale bars: 20 μ m.

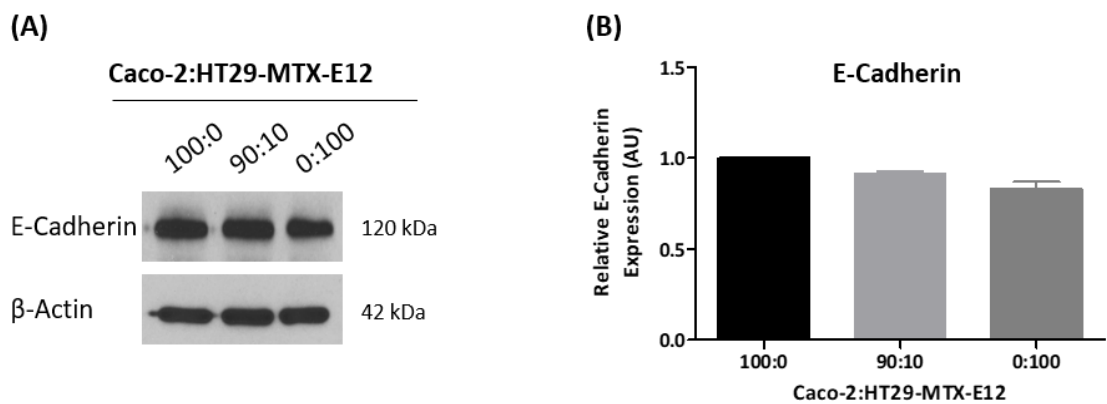


Figure 4.33. Western blot analysis of E-cadherin expression levels in the intestinal mucosa models. Epithelial cells were seeded onto 14-day lamina propria equivalents at 100:0, 90:10 and 0:100 Caco-2:HT29-MTX-E12. Following 21 days of epithelial cell culture models were processed for western blot analysis of E-cadherin and GAPDH (A), representative images of 3 SDS-PAGE gels shown. E-cadherin expression levels were then normalised to GAPDH and compared to the 100:0 model (B). Data represent mean $\pm$ SEM, total $n=3$ from 3 western blot gels.

4.4.4.4. Assessment of transporter protein gene expression within the epithelial monolayers

Further assessment of the three epithelial monolayers on the surface of lamina propria equivalents, was conducted to provide an insight into the expression level of three epithelial apical transporter proteins; this was performed through qPCR analysis and as before no statistical analysis was performed. The P-gp transporter, multidrug resistance 1 (MDR1) is encoded by the *ABCB1* gene. The highest mRNA expression levels were detected in the Caco-2 epithelium with decreases found in the other *in vitro* models; over a 2-fold reduction in MDR1 mRNA was observed in the epithelial co-culture, while almost no mRNA was present in the goblet-like cell epithelium (Figure 4.34A). A similar trend was observed in the mRNA expression levels of the multidrug resistance protein 2 (MRP2), encoded by the *ABCC2* gene. The enterocyte only epithelium had the highest mRNA expression levels of MRP2 and a slight decrease in the mRNA levels was present in the co-culture epithelium. A further reduction in mRNA levels were then found in the goblet-like cell epithelium (Figure 4.34B). Finally, the mRNA expression levels of the breast cancer resistance protein, encoded by the gene *ABCG2*, were assessed. Unlike the previous apical transporter proteins, expression of BCRP mRNA was highest in the HT29-MTX-E12 epithelium with over a 7-fold increase compared to the Caco-2 only co-culture, suggesting that this change is likely to have a physiological impact; little difference was observed between the other two models (Figure 4.34C).

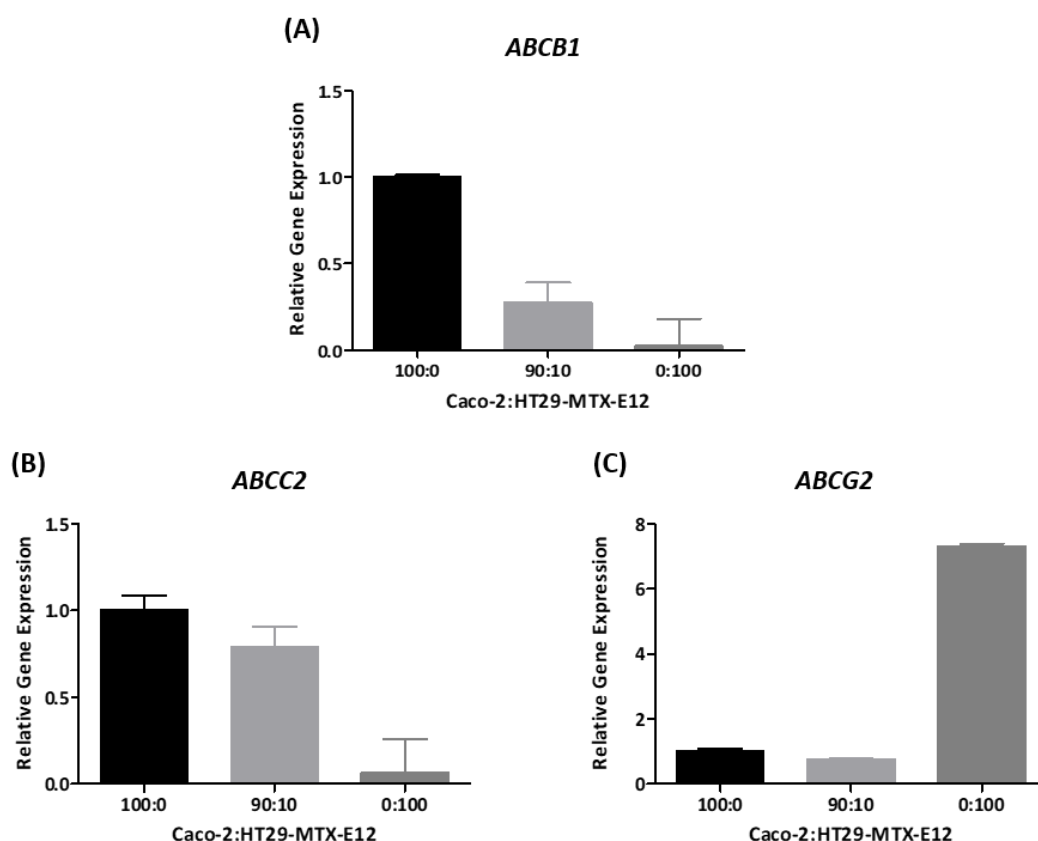


Figure 4.34. Assessment of transporter protein gene expression in intestinal mucosal models. 3D cell models with Caco-2:HT29-MTX-E12 cells cultured on lamina propria equivalents at 100:0, 90:10 and 0:100 were subject to qPCR analysis. RNA was extracted from the three models, converted to cDNA and the expression levels of MDR1 (*ABCB1*) (A), and MRP2 (*ABCC2*) (B) and BCRP (*ABCG2*) (C) genes were quantified. Expression was normalised against GAPDH expression levels and compared against the 100:0 model. Data represent mean $\pm$ SEM. 3 independent qPCR reactions were performed on lysates generated from a total of 3 independent experimental occasions, each with a single lysate collated from 3 pooled technical repeated models per experiment. Within each PCR plate, 3 technical replicate wells were included within each PCR run providing a total $n=9$.

4.4.4.5. Characterisation of the lamina propria compartment when fibroblasts are cultured with different epithelial cells

Along with characterising the epithelial structure in the intestinal mucosal models, the fibroblast lamina propria compartments were also structurally assessed. Characterisation of the cellular component of the lamina propria was performed through TEM analysis. The area of focus for this ultrastructural characterisation was immediately subjacent to the epithelium, as these are the fibroblasts most likely to directly interact with the epithelial cells. Figures 4.35A and B demonstrate that the stromal cells display an elongated morphology and appear compacted together. Interestingly, there was evidence of cellular junctions forming between some of the adjacent fibroblasts, possibly JAM or cadherin protein junctional components, as seen by the electron dense staining and denoted by the asterisk in Figure 4.39A (458,459). Abundant rough endoplasmic reticulum is present in the cytoplasm of the fibroblasts, which appears as continuous flattened sacs studded with ribosomes (Figure 4.35B).

In addition to observing the fibroblasts of the lamina propria, evidence of ECM production, specifically collagen, could also be seen (Figure 4.35C-F). Transverse collagen is depicted in Figures 4.35C and D and appears as rounded circular structures, while longitudinal collagen, filament/strand-like structures, is shown in Figures 4.35E and F. This suggests that collagen is primarily orientated in the same direction along the surface of the Alvetex® membrane and the direction visualised is dependent on the plane of sectioning.

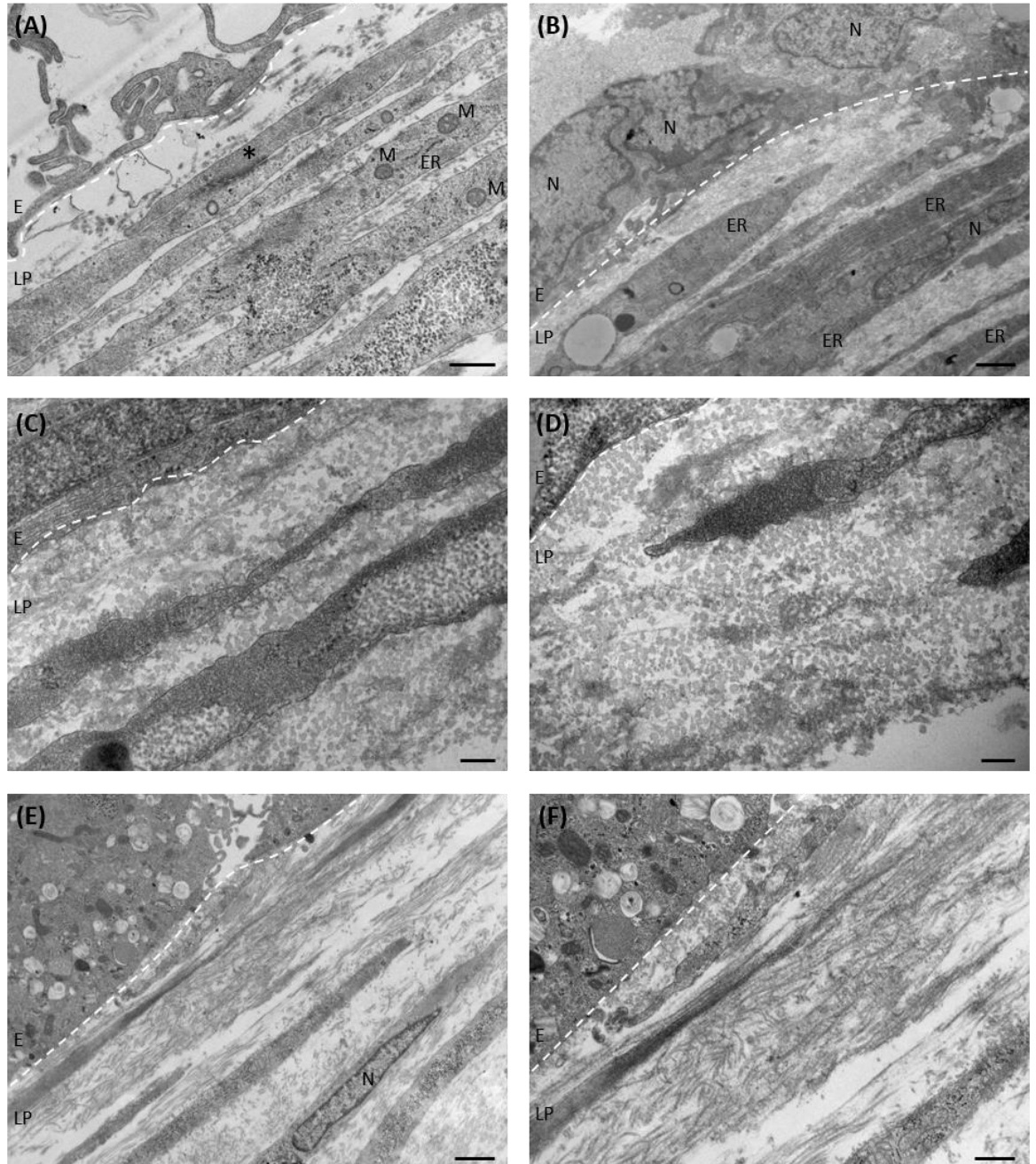


Figure 4.35. Characterisation of the lamina propria equivalent ultrastructure. Representative TEM micrographs of the lamina propria compartment of all three 3D co-culture models. Fibroblast cells (A,B), transverse collagen (C,D) and longitudinal collagen (E,F) are depicted within the micrographs. Dashed white lines provide an approximate separation of the epithelium and underlying lamina propria. E=epithelium, LP=lamina propria, N=nucleus, M=mitochondria, ER=endoplasmic reticulum and *=electron dense connections between adjacent fibroblasts. Images shown are representative of 3 models. Scale bars: 500 nm (A,F), 1 μ m (B,E) and 200 nm (C,D).

Collagens I and III were then further investigated by immunofluorescent staining as they are the most abundant and second most abundant collagens within the body (Figure 4.36). Both collagens were present in all culture conditions with the strongest staining localised immediately subjacent to the epithelial monolayers. Collagens were distributed throughout Alvetex® Scaffold but also exhibited strong staining at the bottom part of the scaffold where the fibroblasts also layer. There was evidence of less collagen staining in the HT29-MTX-E12 monoculture on the lamina propria equivalent, therefore the expression levels of pro- and active MMP-1 were assessed through western blot analysis. No changes in the expression of either pro- or active MMP-1 were evident between the three models, however, the relative expression of proMMP-1 within the 0:100 culture system widely varied between individual experiments (Figure 4.37)

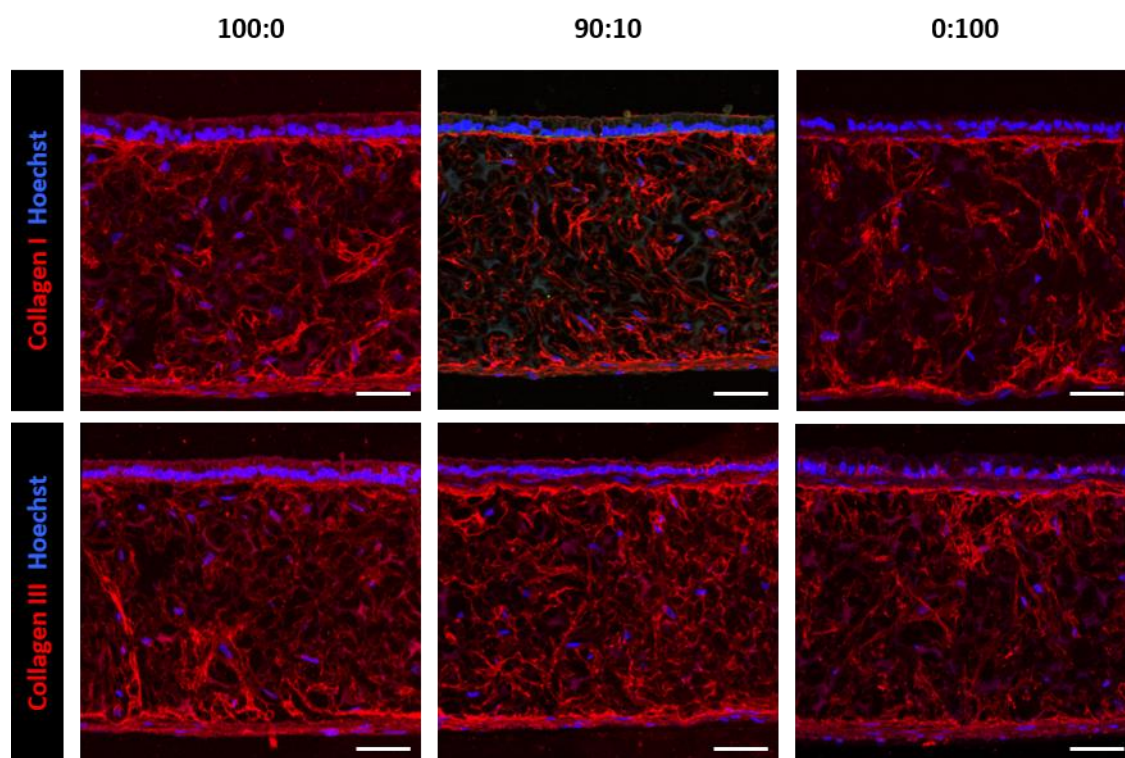


Figure 4.36. ECM production by fibroblasts is not altered by co-culture with different epithelial cell lineages. Epithelial cells were seeded onto lamina propria equivalents at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10 and 0:100. Samples were stained for the ECM components; collagen I, and collagen III (red). Hoechst counterstaining was used to visualise nuclei (blue). Images shown are representative of 9 models. Scale bars: 50 μ m.

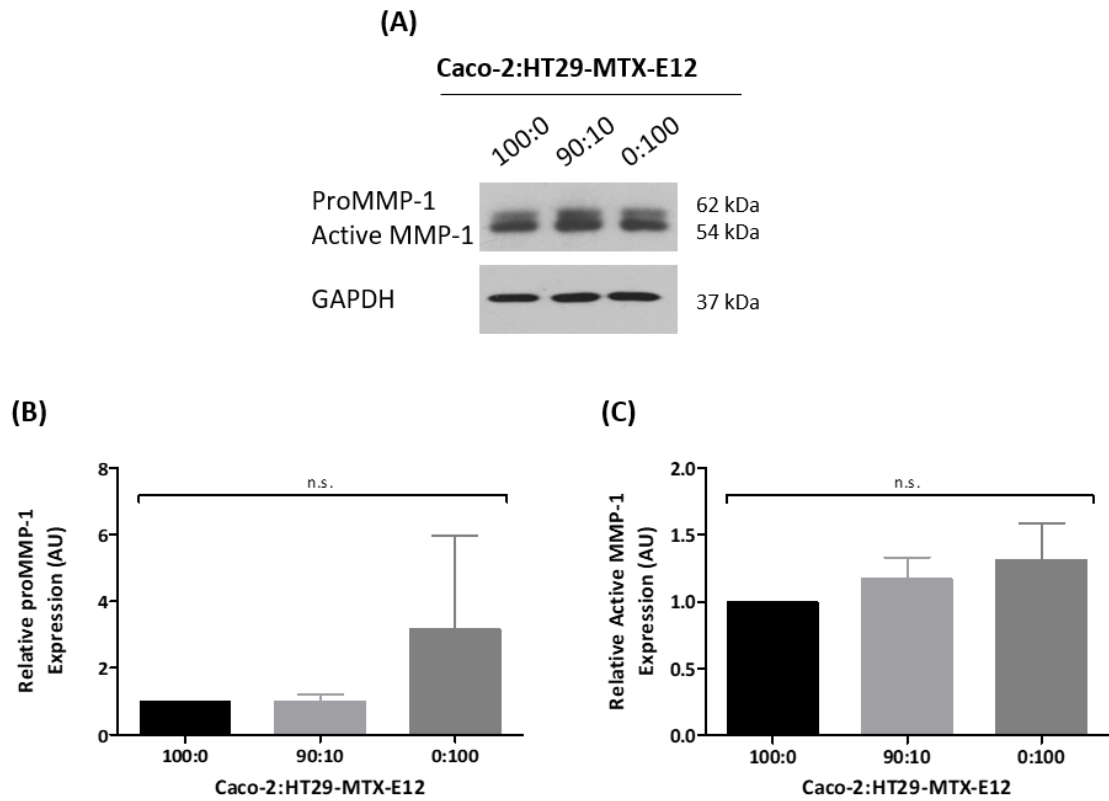


Figure 4.37. Western blot analysis of MMP-1 expression levels in the intestinal mucosa models. Epithelial cells were seeded onto 14-day lamina propria equivalents at 100:0, 90:10 and 0:100 Caco-2:HT29-MTX-E12. Following 21 days of epithelial cell culture models were processed for western blot analysis of proMMP-1, active MMP-1, and β -Actin (A), representative images of 3 western blot gels shown. Expression levels of MMP-1 were then normalised to β -Actin and compared to the 100:0 model (B). Data represent mean $\pm$ SEM, total $n=3$ from 3 independent SDS-PAGE gels. No significant differences were found by one-way ANOVA with Tukey's post-test.

4.4.4.6. Determining the functional properties of intestinal mucosa models

TEER was monitored throughout the 21-day epithelial culture period when the cells were maintained on lamina propria equivalents. A steady increase in TEER was observed over the first two weeks of culture before models experienced a drop off followed by a plateau; this drop off was particularly evident in the 90:10 and 0:100 Caco-2:HT29-MTX-E12 models (Figure 4.38A). Unlike previous models generated using Transwell® membranes, the TEER across all three models was very similar throughout the culture period with no obvious differences. Endpoint TEER values were determined to be as follows: 65.12 ($\pm$ 3.60), 65.50 ($\pm$ 3.48) and 47.04 ($\pm$ 3.14) Ω .cm² for the models seeded at 100:0, 90:10 and 0:100 respectively (Figure 4.38B). HT29-MTX-E12 epithelial monocultures on HDFn cells had a significantly lower TEER than the other two models. Furthermore, it was determined that co-culture of epithelial cells with dermal fibroblasts has a greater impact on TEER than simple epithelial co-culture, with a 10 to 43-fold decrease observed in the various culture models when HDFn cells were present (Figure 4.38C-F). Fibroblasts also improved the reproducibility of TEER than culture with epithelial cells alone and highlights the importance of including a stromal cell lineage to increase the relevance of models to the human intestine function.

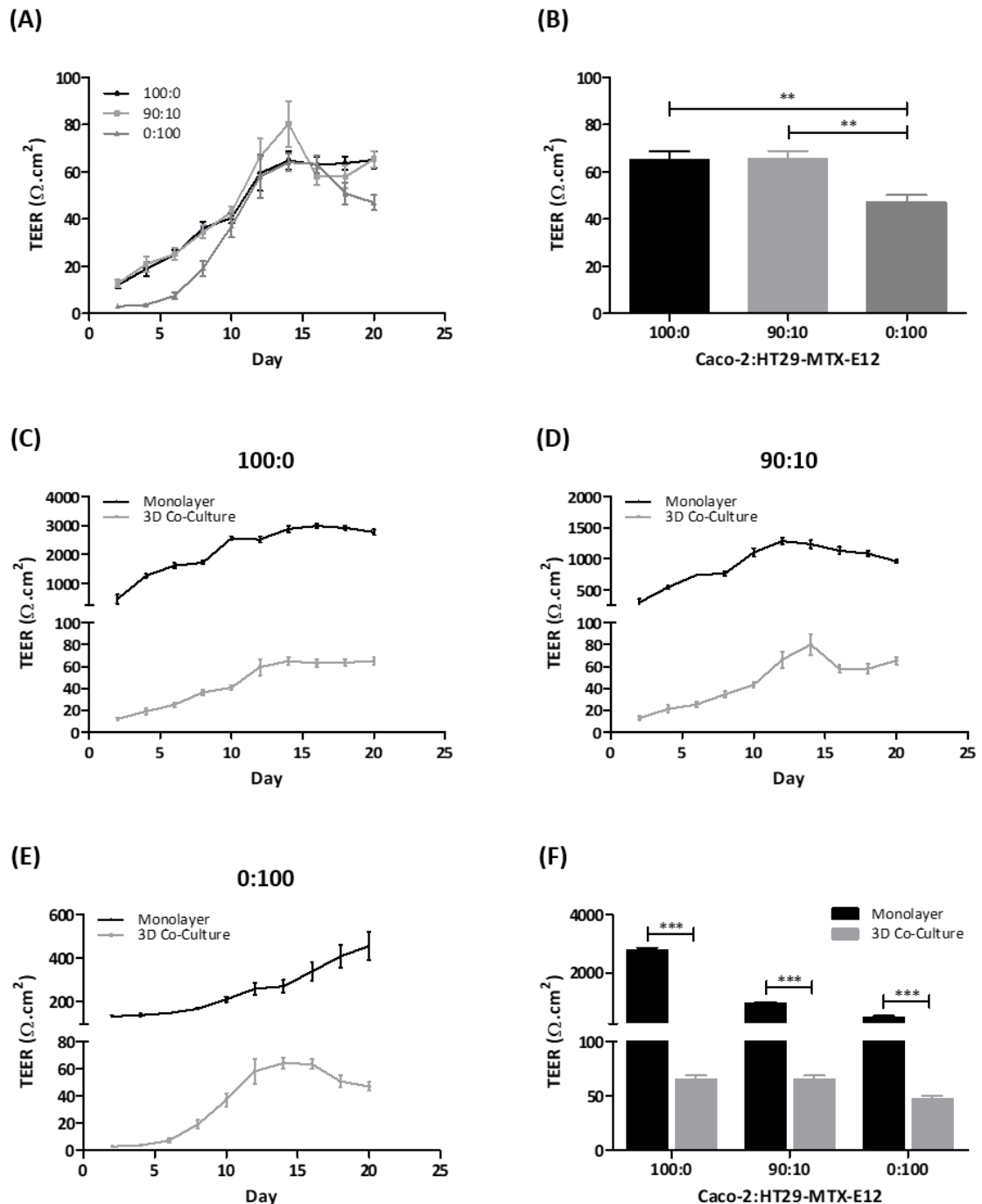


Figure 4.38. Analysis of *in vitro* intestinal mucosal model TEER properties. Epithelial cells were seeded onto lamina propria equivalents at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. TEER measurements were taken every two days of epithelial cell culture and exhibit an increase with culture time (A). Endpoint TEER values were compared across the three culture systems (B). TEER was then compared to the corresponding epithelial monolayers on Transwell® membranes over the 21-day epithelial culture period (C,D,E) and at the culmination of epithelial cell culture. Monolayer data previously shown in Section 4.4.2. Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. ** $P \leq 0.001$ as determined by one-way ANOVA with Tukey's post hoc test to compare the means of individual variables (B) and *** $P \leq 0.0001$ as determined by two-way ANOVA with Bonferroni post-test (F).

Analysis of lucifer yellow rejection revealed that the epithelial monolayers formed intact barriers when co-cultured with fibroblasts as all cultures excluded over 95 % of lucifer yellow irrespective of the direction of transport (Figure 4.39A), however the rejection of lucifer yellow in the absorptive apical-basolateral direction was significantly lower in all three models compared to transport in the secretory basolateral-apical direction. Calculation of the apparent permeability coefficient revealed a significantly higher permeability for lucifer yellow in the 0:100 Caco-2:HT29-MTX-E12 model in both directions than the 100:0 and 90:10 models, correlating with the lower TEER value (Figure 4.39B). Furthermore, no significant differences were found in the permeability of the 100:0 and 90:10 cultures. The enhanced permeability in the absence of Caco-2 cells and the lack of difference in permeability between the 100:0 and 90:10 co-cultures was not unexpected as lucifer yellow is a hydrophilic molecule and as such would be expected to cross the mucus barrier quickly. Concurrent with the lucifer yellow rejection assay, an increased permeability was observed in the apical-basolateral direction, possibly due to fibroblasts impeding flow in the opposite direction, suggesting a biologically significant difference.

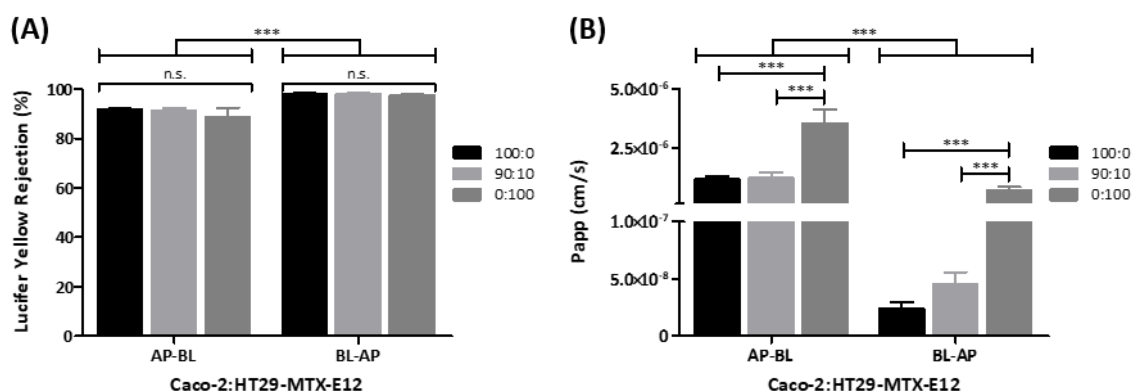


Figure 4.39. Lucifer yellow transport characteristics across in vitro intestinal mucosal models. Epithelial cells were seeded onto lamina propria equivalents at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. Between days 21-29 of culture, the permeability of lucifer yellow was determined through a rejection assay in the AP-BL and BL-AP directions (A) and through calculation of the apparent permeability coefficient in the AP-BL and BL-AP directions (B). Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. *** $P \leq 0.0001$ as determined by two-way ANOVA with Bonferroni post-test.

Additional transport assays were conducted for fluorescein isothiocyanate-dextran (FD) molecules of two different molecular weights; 4000 and 20 000 kDa, herein named FD4 and FD20, respectively (Figure 4.40). Transport assays were performed in the absorptive apical-basolateral direction only. Like lucifer yellow, dextrans are also hydrophilic in nature, thus transport can sometimes be underrepresented in Caco-2 monolayers but are more likely to have enhanced permeability through mucus-gel layers. As expected, FD4 had greater transport than FD20 in all cultures due to its smaller size. Models with only Caco-2 cells present in their epithelium exhibited the lowest permeability of both dextrans tested. Incorporating 10 % HT29-MTX-E12 cells into the Caco-2 epithelium resulted in a slight increase in permeability of both molecules while the greatest level of permeability was found in the 0:100 3D culture.

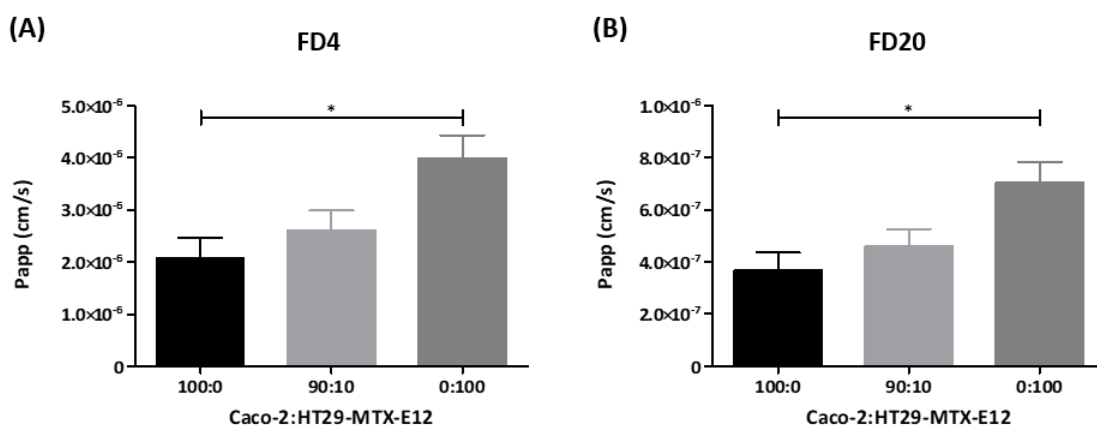


Figure 4.40. Permeability assessment of FD4 and FD20 in the 3D intestinal mucosal models. Epithelial cells were seeded onto lamina propria equivalents at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. Between days 21-29 of culture, the permeability of lucifer yellow was determined through a rejection assay in the AP-BL and BL-AP directions (A) and through calculation of the apparent permeability coefficient in the AP-BL and BL-AP directions (B). Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with technical replicates. * $P \leq 0.05$ as determined by one-way ANOVA with Tukey's post hoc test to compare the means of individual variables.

Studies of rhodamine 123 transport demonstrated models with Caco-2 cells present in their epithelium had significantly higher efflux of rhodamine 123 than the HT29-MTX-E12 monolayer without Caco-2 cells (Figure 4.41A). This was expected due to the presence of P-gp in Caco-2 cells. However, secretory transport was greater than absorptive transport, in all mucosal models. Determination of the efflux ratio showed that in models with 100:0 Caco-2:HT29-MTX-E12 cells in combination with HDFn cells had an active efflux of rhodamine 123 with an efflux ratio of 2.10. However, the ratio was found to be 1.92 in the 90:10 model and 0.86 in the 0:100 set up, indicating that P-gp is not actively inducing efflux of rhodamine 123 as the efflux ratio is below 2 (Figure 4.41B). Although Caco-2 cells overexpress P-gp, the reduction of active efflux in the 90:10 co-culture almost stops its activity and thus limits the usefulness of the model for studies that require P-gp substrates to be identified. When exposed to verapamil hydrochloride treatment, P-gp activity in all models is effectively abolished with a reduction in both basolateral-apical permeability and the efflux ratio, to 0.4-0.7 depending on the model, being observed (Figure 4.41C, D).

Comparing the overall function of the three intestinal mucosa models, reveals that the 90:10 Caco-2:HT29-MTX-E12 3D co-culture has an intermediary function between the pure enterocyte and pure goblet-like cell epithelia. This was true in all three functionality tests: TEER, lucifer yellow and rhodamine 123 transport assays. Furthermore, the triple co-culture has a structure and cellular composition more similar to the *in vivo* intestine than the other model systems.

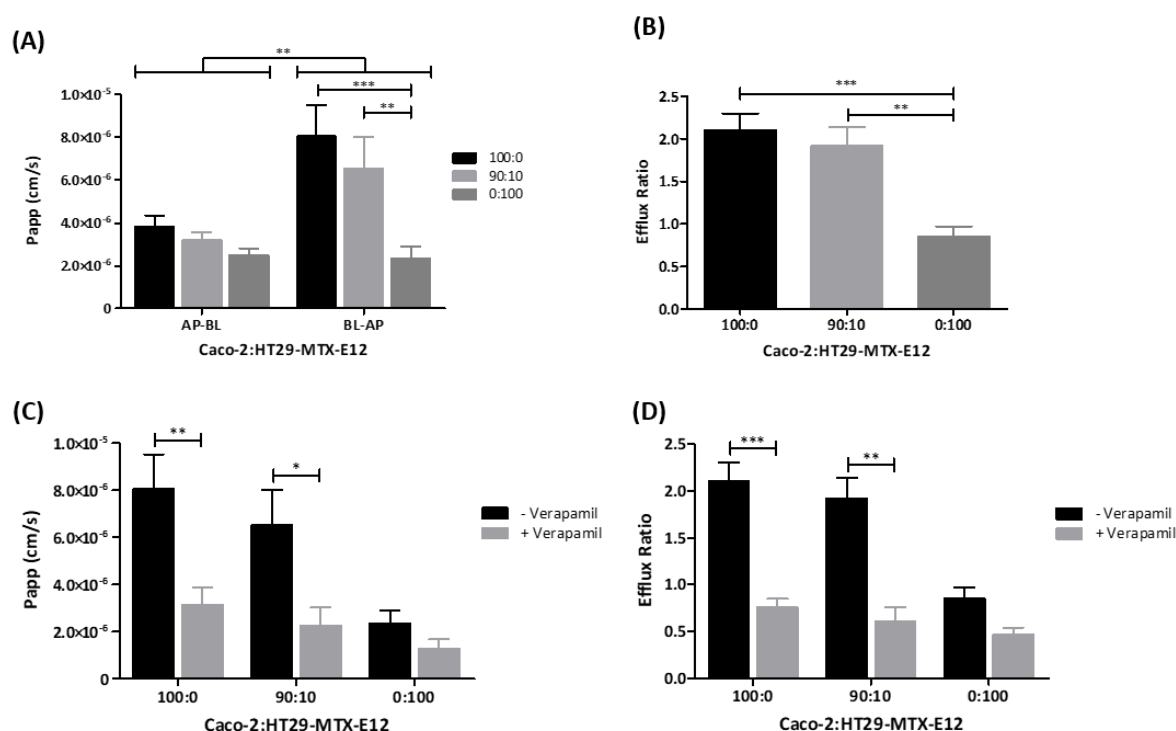


Figure 4.41. Rhodamine 123 transport characteristics across *in vitro* intestinal mucosal models. Epithelial cells were seeded onto lamina propria equivalents at the following ratios of Caco-2:HT29-MTX-E12 cells: 100:0, 90:10, and 0:100. Between days 21-29 of culture, the apparent permeability coefficient of the P-gp substrate, Rhodamine 123 was determined over a 2-hour long assay in the absorptive AP-BL and secretory BL-AP directions (A). The efflux ratio of Papp in the BL-AP transport direction to the Papp in the AP-BL direction was calculated (B). Verapamil hydrochloride, a P-gp inhibitor was also added to the BL-AP assay to determine if the primary transport mechanism of Rhodamine 123 was controlled by P-gp (C). The efflux ratio calculated in the presence and absence of verapamil (D). Data represent mean $\pm$ SEM, total n=7-9 comprised of 3 independent test occasions, each with 2-3 technical replicates. * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$ as determined by two-way ANOVA with Bonferroni post-test.

4.4.5. Summary of Experimental Findings

The primary focus of this chapter was to develop a co-culture model of the intestine incorporating a goblet-like mucus-producing cell line. HT29-MTX-E12 cells were selected for this purpose due to their ability to secrete mucus upon differentiation. Experiments initially focused on the development of a Caco-2-HT29-MTX-E12 co-culture before concentrating on the lamina propria. Structural and functional analysis of the resulting models was performed, and the key findings are summarised below:

- HT29-MTX-E12 cells can be successfully differentiated into a monoculture of mucus-producing goblet-like cells when cultured utilising the Transwell® cell culture platform.
- The optimal ratio of Caco-2:HT29-MTX-E12 co-culture was determined to be 90:10, a ratio representative of the small intestine epithelium.
- Fibroblast conditioned media exerts differential effects on Caco-2 and HT29-MTX-E12 cell lineages.
 - Caco-2 cells exhibited enhanced polarisation and reduced TEER with fibroblast conditioned media.
 - HT29-MTX-E12 cells appeared to lose their mucus-producing phenotype and instead resembled normal intestinal epithelial cells.
- A Caco-2:HT29-MTX-E12 epithelium was successfully established on the fibroblast lamina propria compartment and exhibited a minor increase in permeability compared to Caco-2-HDFn control models.

4.5. Discussion

The tissue construct developed in the previous chapter utilises a co-culture of absorptive epithelial cells and supportive stromal cells. However, within the intestinal epithelium there are numerous cell lineages, the second most abundant of which is the goblet cell population. Goblet cells have fundamental roles in the production and secretion of a mucus gel lining the epithelium that in turn functions as a lubricant in addition to its roles as a barrier against infection and absorption. Therefore, this chapter set out to optimise the development of a triple co-culture model of the intestinal mucosa incorporating fibroblasts, absorptive enterocytes and mucus-producing goblet cells and evaluate the tissue construct with regard to structure and function.

4.5.1. The HT29-MTX Cell Line as a Model to Study the Effects of Mucus on Absorption and Transport

HT29 cells are a parental cell line with subclone derivatives that differentiate into mucus producing goblet-like cells of intestine. The two primary subclones; HT29-H and HT29-MTX are induced into goblet-like cells by the addition of supplements to their growth media as discussed in Section 4.1.2. Due to the HT29-H clone exhibiting an unstable phenotype upon extended culture, the HT29-MTX cell line was selected for use in this study to recapitulate the goblet cell component of the intestinal epithelium (450). The specific subclone utilised herein is the HT29-MTX-E12 isolation as it is readily available commercially, and has been demonstrated to form confluent monolayers with a secreted mucus layer at the apical surface (451). Histological analysis of the monolayers formed by HT29-MTX-E12 cells within this chapter also demonstrated the formation of a monolayer with distended mucus buds at the apical surface of the cells as seen in Figure 4.3. Mucins within the HT29-MTX-E12 monolayer presented with blue staining in PAS/Alcian blue histology indicating that they were acidic mucins; as Alcian blue has a pH of approximately 2.5, this stains acidic mucins blue. This reflects the colonic nature of the HT29 cells where the majority of mucins are acidic, while the mucins in the ileum and jejunum are mostly neutral (460–462). However, little evidence of extracellular mucus production lining the epithelium was visible on wax embedded sections possibly due to the use of organic solvents such as ethanol which result in the shrinking and dehydration of the mucus gel layer (422).

Additionally, monolayers of HT29-MTX-E12 cells displayed evidence of apical junctional complex formation including the presence of both tight junction components and adherens junctions. TEER steadily increased throughout the 21-day culture period and as TEER can be used as a proxy for tight junction dynamics, this implies that junctions form tighter complexes with time resulting in the development of a barrier. As such, the ability of the monolayer to form a barrier to diffusion was assessed through a transport assay for the paracellular marker lucifer yellow. No directional differences in the transport of lucifer yellow were identified confirming that the production of mucus within these monolayers did not adversely affect the apical-basolateral transport of this specific compound.

In agreement with previous studies was the non-directional bias in the transport of P-gp substrates, in this instance rhodamine 123, confirming the lack of P-gp expression within the HT29-MTX cell population (453). Further demonstrations of this were shown through the inability of verapamil hydrochloride, a P-gp, inhibitor to exert any significant effects on the basolateral-apical transport of rhodamine 123. This could allow for the absorption properties of newly identified P-gp substrates to be assessed before moving onto a model with expression of P-gp to examine the efflux of the compound.

4.5.2. The Use of Epithelial Co-Culture to Recreate a More Physiologically Relevant Intestinal Model

Co-cultures of Caco-2 and HT29-MTX cells have used a variety of different seeding ratios of cells including, 90:10, 80:20, 75:25, 70:30 and 50:50 (274,455,463). Physiologically up to 90 % of intestinal epithelial cells are enterocytes with goblet cells constituting up to 10 % of the epithelial cell population of the small intestine. The number of goblet cells increases distally with up to 24 % of the total IEC population within the distal large intestine being goblet cells (274). Therefore, two ratios were selected for the optimisation of epithelial/goblet cell co-cultures using Transwell® membranes to represent the small and large intestines: 90:10 and 70:30 Caco-2 to HT29-MTX-E12.

Staining for the production of mucins was carried out through PAS/Alcian blue staining (Figure 4.10). Caco-2 epithelia did not show any evidence of mucin production, however positive mucin staining was present in all other models. In agreement with Hilgendorf, who developed a series of co-cultures of Caco-2 and HT29-MTX cells, the initial seeding ratio of the two cell lineages resulted in qualitative differences in the mucin staining (274); Alcian blue staining of acidic mucins appeared to have a greater intensity in monolayers with higher levels of HT29-MTX cells.

Histological analysis and immunofluorescent staining techniques also revealed that within the 70:30 co-culture, HT29-MTX-E12 cells tended to overpopulate the cell culture supports resulting in monolayers that more closely resembled a pure HT29-MTX culture rather than a co-culture in regard to the cellular composition of the model. This was not entirely unexpected due to the quicker doubling time of the HT29-MTX-E12 as demonstrated in this study and others, confirming that the lower population doubling time of the goblet-like cells had a biologically significant effect. This, in combination with the fact that Caco-2 cells differentiate into cells resembling small intestinal enterocytes, and the study by Chen and colleagues that demonstrated that the seeding ratio of cells has little impact on the functionality of the model, led to the 90:10 ratio being selected for all further epithelial co-culture experiments (464,465). Conversely, some studies determined that Caco-2 cells were the lineage that tended to take over the monolayer, resulting in the researchers switching to heat inactivated FBS to minimise this issue (274). The precise HT29 subclone utilised in the above study was not specified, thus the apparent differences between the monolayers developed within could be due to a varied subclone growth rate. Therefore, further studies with different subclones cultured under different media conditions would be needed to confirm this hypothesis.

Tight junctions are known to restrict the paracellular passage of small hydrophilic molecules (451); as such immunofluorescent labelling of AJC components was performed. Claudin-1 appeared diminished in the 0:100 model compared to the other monolayers, while claudin-2 had strong positive staining. The differences between sealing and pore-forming claudins could potentially explain the observed TEER and permeability changes across the three models. TEER was attenuated in monolayers with HT29-MTX-E12 cells included in the epithelium and was lowest overall in the 0:100 monolayer. This was found in conjunction with a small increase in lucifer yellow, FD4, FD20, and rhodamine 123 passive permeability within the 90:10 monolayer and was further amplified in the 0:100 epithelium indicating there was a correlation between electrical resistance and permeability of the membranes. Although a change in permeability was observed, it was not found to be significant. This was in contrast to previous studies in which HT29-MTX monolayers were found to be up to 30X more permeable to lipophilic drugs and 15X more permeable to hydrophilic compounds than their Caco-2 counterparts (274,451).

Interestingly, co-culturing HT29-MTX cells with Caco-2 cells reduced the activity of the P-gp efflux transporter protein regarding basolateral-apical transport of rhodamine 123, in agreement with other experiments (466). Researchers identified that not only does inclusion of HT29-MTX cells into Caco-2 cultures reduce the secretory function of P-gp, but the day that HT29-MTX cells were seeded into the culture also had a significant impact on the proteins' activity, with an earlier seeding day resulting in a greater P-gp activity. This study then went on to demonstrate that HT29-MTX cells do not express the P-gp protein and as such any permeability in the secretory basolateral-apical direction observed in the pure HT29-MTX cultures within resulted from passive transport of rhodamine 123 (466). The reduction of P-gp efflux in co-cultures indicates that they could potentially be used as an alternative to Caco-2 monocultures which drastically over express the exporter protein.

4.5.3. The Effect of Fibroblast Conditioned Media on the Development of Epithelial Tissue Culture Constructs

Chapter 3 demonstrated that co-culture of Caco-2 cells with HDFn resulted in significant morphological and functional alterations to the Caco-2 epithelium compared to Caco-2 culture alone. Therefore, part of this chapter sought to investigate whether these effects were induced by the fibroblast secretome or direct contact with fibroblasts in epithelial monolayers of Caco-2 and HT29-MTX cells seeded at 100:0, 90:10 and 0:100, respectively. To assess the fibroblast secreted factors, conditioned medium from 2D cultured fibroblasts was collected and applied to Transwell® monolayers in equal parts with fresh media. This method was selected rather than culturing the fibroblasts at the base of the well in which the Transwell® inserts were sitting, as in direct 3D co-culture, epithelial cells are in the presence of fibroblasts for the full 21-days of differentiation. Thus, it would not have been possible to keep the monolayers in the same wells as the fibroblasts for the full culture period due to fibroblasts becoming contact inhibited and potentially undergoing necrosis and apoptosis during the prolonged culture that would have released additional factors into the media. Furthermore, such factors could have negatively impacted epithelial cell growth and viability.

Conditioned media collected from dermal fibroblasts enhances epithelial cell attachment and proliferation of keratinocyte epithelial cells and studies with bronchial fibroblast conditioned medium have also demonstrated similar results regarding the proliferation of bronchial epithelial cells (467,468). However, no such enhancement of proliferation or growth rate was found in either the 2D Caco-2 or HT29-MTX-E12 cells grown in fibroblast conditioned media presented here (Figure 4.17).

Fibroblast conditioned media exerted differential effects on the Caco-2 and HT29-MTX cells. Caco-2 cells displayed an enhanced polarisation in both the mono- and co-culture. On the other hand, differential effects were induced in the HT29-MTX cell population when comparing their morphology in co-culture and monoculture. When subjected to paracrine factors from fibroblasts in monoculture, the HT29-MTX-E12 monolayer lost the apically located mucus present in the cells. The cells essentially lost their goblet-like cell phenotype, yet this was not the case in co-culture, suggesting that the epithelial cells also have direct and paracrine signalling events occurring between them and influencing their structure and morphology.

Functionally, models cultured in the presence of conditioned media also demonstrated differing effects. TEER was reduced only in the Caco-2 monolayer and displayed a corresponding increase in the apical-basolateral paracellular permeability through rhodamine 123. A similar decrease in TEER and increase in permeability was also observed when Caco-2 epithelia were treated with CCD18-co intestinal fibroblast conditioned media (405). Conversely, the 90:10 and 0:100 monolayers displayed an amplified electrical resistance in the presence of conditioned media but did not present with a decrease in permeability.

Potential future studies could include collection of conditioned media from fibroblasts cultured in 3D within Alvetex® Scaffold membranes to determine if there were any changes in epithelial structure and function when compared to 2D conditioned media, though controlling the cell numbers would prove to be difficult. It would then prove interesting to compare the exact paracrine secreted factors and their concentrations and directly apply specific molecules to the epithelial cultures to attempt to recapitulate some of the observed phenotypes. Possible molecules of interest include hepatocyte growth factor, keratinocyte growth factor and TGF- β which have all previously been shown to affect the proliferation and differentiation of different intestinal epithelial cells including Caco-2 and T84 cells (355,356,380).

4.5.4. Direct Co-Culture with Stromal Cells Exhibits Greater Effects on Epithelial Structure and Function than Conditioned Media or Co-Culture with Additional Epithelial Lineages Alone

Direct 3D co-culture with fibroblasts induced greater structural and functional changes overall than co-culture with two epithelial lineages or treatment of the monolayers with conditioned media. This was especially evident when examining the structure of the epithelial cells; Caco-2 cells displayed enhanced polarisation in both the 100:0 and 90:10 3D co-cultures when compared to both sets of Transwell® counterparts, while HT29-MTX cells retained their goblet-like cellular appearance that was previously lost in conditioned media studies. TEM micrographs confirmed the presence of apically clustered mucin vesicles within these cells in 3D cell

culture in both the 90:10 and 0:100 models. Furthermore, characterisation of the functional characteristics of the epithelia where paracellular permeability was significantly increased in all three intestinal mucosa models over both normal and conditioned media treated epithelial models, suggested that it is the direct contact of epithelial and stromal cells/ECM and the resultant physical support and juxtracrine signalling processes, that induced the biggest changes in the epithelial cells.

TEER was reduced 40-fold in the 100:0 3D co-culture compared to its corresponding monolayer, while 90:10 and 0:100 3D co-cultures saw 14-fold and 10-fold reductions, respectively. This corresponded with a drastic increase in the overall epithelial permeability in 3D co-cultures of both lucifer yellow and rhodamine 123 in comparison with monolayer cultures. Similar 3D co-cultures of Caco-2, HT29-MTX and stromal cells have also identified a reduced TEER and an increase in lucifer yellow paracellular permeability when compared to Transwell® counterparts (312,313). Inclusion of goblet-like cells into the 3D enterocyte model induced a slight increase in the paracellular permeability of the hydrophilic compound, lucifer yellow. This could be associated with a concurrent decrease in sealing claudin-1 tight junction mRNA - though the overall fold change in the expression of claudin-1 was less than 1 suggesting that the biological impact of this change may be minimal – and an increase in pore-forming claudin-2 mRNA when greater numbers of goblet-like cells were present in the epithelium (Figure 4.31). Claudin-2 has been demonstrated to be increased in intestinal epithelial monolayers in the presence ECM components, namely collagen (469). Moreover, a comparison of the ultrastructure of Caco-2 and HT29-MTX cells revealed that Caco-2 cells form tightly compacted monolayers with long interdigitating lateral membranes, while large gaps can be observed between adjacent HT29-MTX cells (Figures 4.25-4.27) (405,451). This was not, however, found between adjacent Caco-2 and HT29-MTX cells. The greater distance between the lateral membranes of HT29-MTX cells could potentially be responsible for some of the observed differences in paracellular permeability and TEER between the three monolayers.

Alternatively, the paracellular transport of rhodamine 123 in the absorptive direction did not demonstrate any increase in permeability when goblet cells were included in the model and instead, displayed a slight decrease in permeability. This was expected as rhodamine 123 is a lipophilic drug and as such would not be expected to easily pass through any mucus barrier present. Transport of rhodamine 123 in the secretory direction also demonstrated expected results with the 100:0 3D co-culture having the highest level of transport, the 0:100 3D co-culture exhibiting no active transport and the 90:10 3D co-culture having an intermediary level of transport. This was also reflected in the qPCR data wherein the same trend was found in the mRNA expression levels of MDR1, the P-gp transporter protein. Almost no MDR1 mRNA expression was identified in the 0:100 3D model, in agreement with other studies that demonstrated that P-gp was not expressed in HT29 cells (466). This could indicate that the 3D co-culture model with both enterocytes and goblet-like cells present in their epithelium could be used as a more *in vivo*-like alternative model to the current gold standard, which greatly overexpresses P-gp (470).

MRP2 is an apically expressed excretory transporter protein that primarily functions to secrete organic ions, heavy metals, and bile salts from the intestinal epithelium. Two different subclones of Caco-2 cells have previously been shown to have greater mRNA expression levels than the human small intestine and high

expression levels were retained in the 3D culture when compared to the 90:10 and 0:100 3D models (470). Similar to MDR1, HT29-MTX epithelial cells expressed very little MRP2 mRNA and the 90:10 model had an intermediate level of expression, again supporting its use as an alternative to the Caco-2 monolayer.

Unlike MDR1 and MRP2, BCRP mRNA expression levels were over 7-fold higher in the 0:100 culture system than the other models. BCRP is another efflux transporter protein that shares substrate specificity overlap with both P-gp and MRP2 (83). One of the model substrates of BCRP is methotrexate (471,472), and as differentiation of HT29-MTX cells requires continuous application of methotrexate, it is hypothesised that the high levels of BCRP mRNA could be due to this treatment, as cells would be required to systematically export methotrexate to prevent cytotoxic levels accumulating within the cell. Another MTX-differentiated cell line, MCF7/MX has been shown to exhibit resistance to methotrexate drugs due to an overexpression of the BCRP transporter (472,473). It would therefore be interesting to test this hypothesis, by comparing the expression levels of BCRP in HT29 cells that have and have not been differentiated with methotrexate. Although HT29-MTX 3D models had high levels of BCRP, this was not carried over to the 90:10 co-culture system which retained low levels of mRNA expression and was more comparable to the 100:0 3D model, possibly due to the low numbers of HT29-MTX cells in the epithelium. Other 3D intestinal models consisting of Caco-2, HT29-MTX cultured on the surface of stromal cells and immunocytes embedded in a hydrogel matrix found a significant increase in BCRP mRNA and activity in comparison to Caco-2 Transwell® control monolayers, yet no comparison was conducted on co-culture of either the epithelial cells on Transwell® alone or the with a pure Caco-2 monolayer on the hydrogel containing stromal and immune cells (313).

4.6. Conclusions

This chapter aimed to build on the monoculture and co-culture models described in the previous chapter wherein Caco-2 cells were used in models to represent the absorptive component of the intestinal epithelium. Use of HT29 cells differentiated with methotrexate was therefore employed to represent the goblet cell element of the mucosa.

Initial experiments confirmed that HT29-MTX cells could successfully form a monolayer on their own or in co-culture with Caco-2 cells. Two different ratios of Caco-2:HT29-MTX cells were utilised in preliminary epithelial studies, 90:10 and 70:30 to represent the small and large intestines, respectively. However, the 70:30 monolayer had few noticeable Caco-2 cells remaining after the 21-day differentiation period. This, in combination with the fact that Caco-2 cells best represent small intestinal enterocytes, rather than colonocytes, led to the 90:10 ratio being carried forward in all further studies.

Conditioned media increased the similarity of the Caco-2 monolayer to the *in vivo* intestine yet did not induce physiologically relevant changes within the 90:10 and 0:100 epithelial models. On the other hand, direct co-culture with fibroblasts did cause physiologically relevant changes regarding both epithelial cell structure and permeability. Co-culture with fibroblasts resulted in enhanced polarisation of Caco-2 cells, while HT29-MTX cells successfully produced mucin granules and mimicked intestinal goblet cells. Paracellular permeability of both lucifer yellow and rhodamine 123 was higher in all 3D co-cultures than their Transwell® counterparts, more like the *in vivo* intestine. Additional studies to investigate the function of various transporters and further characterisation of paracellular transport are required to fully understand the relevance of the three intestinal mucosal models compared to the human intestine.

Overall, this chapter has successfully recreated a 3D triple co-culture of the intestinal mucosa comprising of an absorptive cell lineage, a mucus-producing cell line and a stromal compartment of both fibroblasts and ECM components. However, the cellular composition of the intestine is particularly complex as described in Chapter 1, with immune cells having a predominant role in maintaining homeostasis and altering epithelial barrier function during active inflammation. Thus, Chapter 5 will seek to optimise a 3D co-culture that incorporates immune cells into the stromal compartment that could then be used to generate inflammatory responses and mimic aspects of inflammatory bowel diseases.

Chapter 5: Optimisation of an Immune-Competent Intestinal Mucosa Model

5.1. Introduction

Incorporation of components of the intestinal immune system in *in vitro* models allows for investigations into epithelial transport, inflammatory signalling pathways and aspects of diseased intestinal tissue. Two main strategies have been employed to recreate aspects of the intestinal immune system *in vitro* cell culture models: recreation of the M cell antigen sampling cell lineage or, inclusion of a specific immune cell type such as a macrophage or dendritic cell lineage, which can be used to study nanoparticle transport or aspects of the immune response, respectively. This chapter will be focused on the development of immune-competent *in vitro* models, while their applications in relation to health and disease will be the focus of Chapter 6.

5.1.1. Recreating the M Cell Phenotype *In Vitro*

The intestinal mucosa contains numerous lymphoid follicles that can be found individually or arranged in clusters to form distinct structures such as Peyer's patches. Such lymphoid follicles are separated from the lumen by a specialised epithelial monolayer known as the follicle-associated epithelium (FAE). Within the FAE, there are specialised intestinal epithelial cells known as M cells that perform antigen sampling roles.

5.1.1.1. M cell structure and function

M cells are a crucial cell lineage involved in communication between the intestinal epithelium and the immune system. They account for less than 1 % of cells across the entire epithelial surface (278,474). Phenotypically, they have a poorly organised brush border with short, irregular microvilli much unlike the highly organised enterocyte brush border wherein the microvilli are uniform, densely packed structures protruding from the apical surface of the cells (41,276,475). Additionally, M cells lack alkaline phosphatase and sucrase-isomaltase typically present on the apical membrane of enterocytes (475,476).

A key morphological distinction between enterocytes and M cells is the presence of intraepithelial invaginations, termed “pockets” on the basolateral surface of M cells (Figure 5.1) (477). These pockets contain a variety of immune cells including B and T cells in addition to macrophages and dendritic cells, allowing the M cells to function efficiently as antigen sampling cells that acquire and transport luminal antigens across the epithelial barrier to mucosal lymphoid tissues where antigen presentation and immune response initiation occurs (475,478). The mechanism by which antigens are transported by M cells is through transcytosis – a rapid temperature dependent transcellular endocytosis whereby antigens become enclosed within vesicles. M cells are not covered by the thick mucus layer that covers the rest of the intestinal epithelium and are instead associated with only a thin layer of glycocalyx; as such they are often exploited by pathogenic microorganisms as a route of host invasion to gain entry into the underlying internal tissues (39).

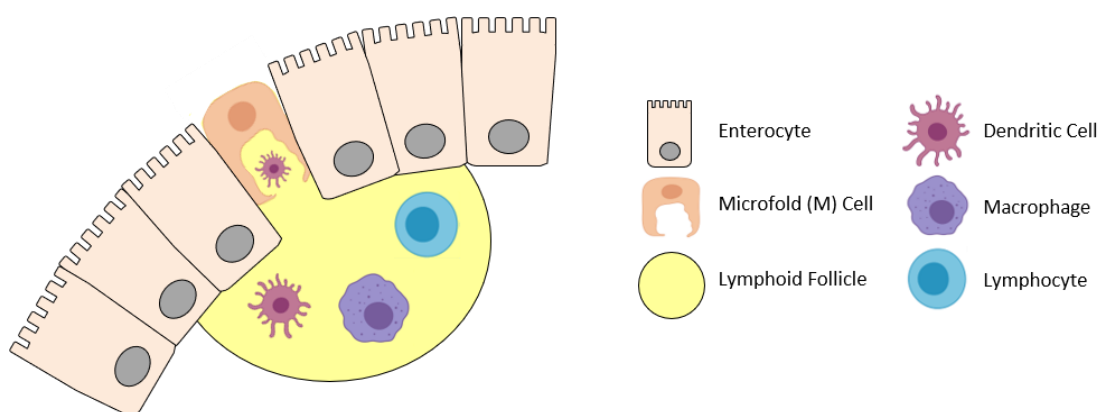


Figure 5.1. M cells in the follicle associated epithelium. M cells are specialised epithelial cells present within the lymphoid follicles of the intestinal mucosa. Structurally, they are defined by pocket-like invaginations at their basolateral surface wherein a variety of immune cells reside. Image not to scale. Figure created, in part, with BioRender.com.

5.1.1.2. Modelling M cell function *in vitro*

Pioneering work to reproduce characteristics of the FAE was carried out by Kerneis using the Transwell® Caco-2 monolayer with the addition of murine lymphocytes isolated from Peyer’s patches. The group found that the lymphocytes settled into the epithelial monolayer and induced reorganisation of the enterocyte brush border (276). The expression of villin and sucrase-isomaltase was redistributed from the apical membrane and downregulated respectively, while TEER remained unaffected. A temperature-dependent transport of particulate matter was observed in co-cultures while no alteration was found in monocultures confirming the function of M cells within the co-culture model. The mechanism of nanoparticle transport was later determined to be micropinocytosis, a form of actin-dependent endocytosis typically involving the engulfment of soluble extracellular material (278,479). Additionally, injection of isolated lymphocytes into the duodenal mucosa of murine models induced the formation of Peyer’s patch-like structures. It was therefore hypothesised that lymphocytes played a major role in the induction of the FAE and M cells (276). Further studies determined that B lymphocytes, specifically, were responsible for the differentiation of cells into these phenotypes. Raji B cells are a cell line isolated from a human Burkitt’s lymphoma and are representative of B lymphocytes when cultured *in vitro*. Co-culture of Caco-2 and Raji B cells induced a M cell morphology

and structure of 15-30 % of cells within the Caco-2 epithelium, yet the same did not occur with Jurkat T cells (276–278). Results from these trans-differentiation experiments supported the hypothesis that M cells are not their own lineage and are instead differentiated enterocytes that have changed their phenotype after M cell induction by B cells. However, it is important to note that Caco-2 cells are cancerous in origin and therefore do not accurately represent the gene or protein expression profiles observed in native intestinal enterocytes, as such the fluid phenotypes observed in the *in vitro* models may not occur within the human intestine.

To increase model complexity, numerous groups have introduced Raji B cells to the basolateral compartment of a Caco-2, HT29-MTX co-culture model (275,279,280,456,480). Typically models utilise Caco-2 and HT29-MTX cells at a ratio of 90:10 respectively (275,279,456), however some models have employed a ratio of 70:30 to produce a more even mucus layer over the epithelial surface (280). A reduced TEER, closer to *in vivo* levels, was detected in triple co-cultures compared to monocultures or co-cultures with only two cell lines present (279). Triple co-cultures also exhibited strong correlation with permeability coefficients from *ex vivo* porcine intestinal mucosa (280). Furthermore, an increased permeability of the transport of insulin encapsulated in nanoparticles was observed in the triple co-cultures with comparable absorption levels to *ex vivo* rat intestinal tissue (275). However, there was little difference in the permeability of “free”, unencapsulated insulin between the models suggesting that triple co-cultures could prove useful in testing the transport rates of nanoparticles but have no improved ability to assess the transport of unencapsulated drugs over the gold standard Caco-2 monolayer. Supplementary to this when assessing the transport of 12 model drugs across the different cultures systems, Lozoya-Agullo et al., identified that all models were successful in classifying compounds according to the Biopharmaceutical Classification System (BCS), however there was no functional advantage of using co-cultures over the Caco-2 monoculture for assessment of passive diffusion (456). They proposed that, for simplicity, the Caco-2 monolayer should be used during the initial screening stages of the drug development process but recommended the use of more complex models to study the transport mechanisms of specific drugs as the models will have greater physiological relevance with regards to intestinal structure. For example, as M cells have high transcytotic capabilities, a model involving Raji B cell induction of the M cell phenotype within Caco-2 cells could be a putative model for assessing the oral delivery of nano-encapsulated therapeutic compounds and could also prove to be of use in the testing of novel encapsulation polymers toxicity and permeability.

5.1.2. Immune-Competent Epithelial Co-Cultures

Although *in vitro* models containing M cells have been developed, these cannot be used to study the immune response and inflammatory pathways resulting from exogenous stimuli. Therefore, researchers have sought to develop models whereby innate and/or adaptive immune cells are incorporated into the culture creating immune-competent co-cultures. Both primary cells and cell lines have been used to represent the immune cell component and the resulting models will be discussed in Sections 5.1.2.1. and 5.1.2.2. respectively.

5.1.2.1. The use of peripheral blood mononuclear cells in *in vitro* co-cultures of the intestinal epithelium

Peripheral blood mononuclear cells (PBMC) are defined as any peripheral blood cell that possesses a round nucleus. Cells are isolated from the peripheral blood of human donors and contain a variety of different cell types including cells of the innate immune system such as monocytes, dendritic and natural killer cells, in addition to lymphocytes of the adaptive immune system. PBMCs are an attractive option for *in vitro* studies as they are an easily accessible source of human immune cells and they can also be collected from both healthy and diseased patients allowing studies of normal and diseased function (222). However, the precise composition of the PBMCs will be donor dependent resulting in high experimental variation when PBMCs from different donors are used; models developed with PBMCs therefore lack reproducibility compared to *in vitro* models with immune cell lines (222,481). Furthermore, the composition of immune cells in the blood and intestinal mucosa differs significantly. For example, populations of various cell types including innate lymphoid cells and B1 B cells do not exist in the blood but are present in the intestinal mucosa, yet the ratio of CD4⁺:CD8⁺ T cells is similar between the mucosa and blood with CD4⁺ T lymphocytes being the most predominant (222,482–484). Further subsets of specific cell lineages, such as dendritic cells and macrophages can be isolated from PBMCs and have been used in intestinal co-cultures (485,486).

Co-cultures of intestinal epithelial cells and PBMCs tend to follow a similar protocol of model development. IECs are seeded onto Transwell® inserts and a PBMC suspension is introduced to the basolateral compartment during epithelial culture. Caco-2 and HT29 cells have been utilised in these models along with the inclusion of endothelial cells to create a more holistic view of the intestinal mucosa (484,487). PBMC co-cultures are frequently used to investigate the establishment of tolerance and bacterial interactions at the mucosal surface and examine the differing inflammatory responses induced by pathogenic and non-pathogenic bacteria (484,487–491). In contrast, few studies have employed these models that contain a wide array of immune cells, to investigate aspects of inflammatory bowel diseases. However, investigations of PBMC co-cultures in which the PBMC components are further purified to give a specific cell lineage such as macrophages, have been used to examine the signalling interactions of IEC and immune cells during an inflammatory response (485,486). Furthermore whole PBMC isolates from patients with IBD and not used in co-culture systems have revealed reduced activity of enzymes involved in the production of intracellular antioxidants resulting in diminished antioxidant activity and increased levels of oxidative stress and damage in the diseased mucosa (492). PBMCs can also be used to identify which signalling pathways and mechanisms are active in IBD patients, for example patients with active IBD have been shown to have amplified IFN- γ production in CD8⁺ lymphocytes in both active and inactive states of the disease when compared to healthy patient controls (493). Additionally, PBMCs have been proposed to be used to produce gene expression arrays that can provide molecular biomarkers in the diagnoses of IBD (494).

5.1.2.2. Immune-competent co-cultures developed using established cell lines to represent the intestinal immune system

Several *in vitro* models of the intestinal epithelium have incorporated immune cell lines enabling the study of the immune response and signalling cascade in IECs and immune cells. Generally, IECs are seeded onto a Transwell® membrane and, during epithelial differentiation, they are introduced to a well containing differentiated immune cells adhered to the base of the well (Figure 5.2). Several different cell lines representing immune cells have been utilised for this purpose including U937, THP-1 and RAW 264.7 cells.

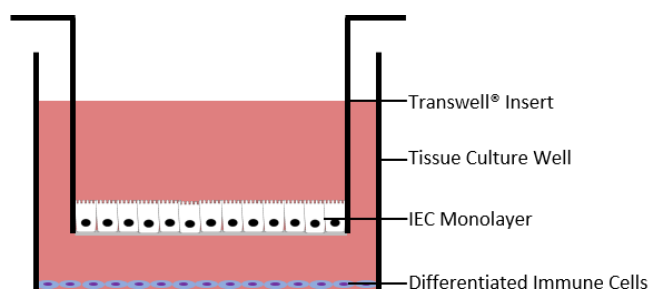


Figure 5.2. Schematic detailing the typical set up of an immune-competent *in vitro* model of the intestine. Intestinal epithelial cells (IEC) are cultured on a Transwell® membrane. During epithelial culture, Transwell® inserts are transferred to a well containing differentiated immune cells adhered to the basolateral surface of the well.

5.1.2.2.1. RAW 264.7 macrophages

RAW 264.7 are a murine cell line that can be used to model mouse macrophages. They have been successfully co-cultured with human Caco-2 cells as outlined in Figure 5.2 (495). Resultant models have been used to assess the anti-inflammatory effects of food factors when models were treated with lipopolysaccharide (LPS), a component found in the membrane of Gram-negative bacteria, as a proinflammatory stimulus. Cinnamon, fucoidan (polysaccharides found in brown algae) and Maillard reaction products (produced by a chemical reaction between reducing sugars and amino acids) have all been demonstrated to have anti-inflammatory effects using this *in vitro* model, causing a reduction in proinflammatory cytokine secretion and decrease in nitric oxide levels produced when models were treated with LPS (495–497). Caco-2 RAW 264.7 co-cultures have additionally been utilised to identify novel probiotic lactic acid bacteria strains of *Weissella* that could also reduce the proinflammatory response induced by LPS (498).

5.1.2.2.2. THP-1 and U937 cell lines

THP-1 and U937 cells are both human pro-monocytic cell lines with similar characteristics and properties, that can be differentiated into either macrophages or dendritic cells. THP-1 cells were isolated from the blood of a patient with acute monocytic leukaemia, while U937 cells were isolated from a tissue biopsy from a patient with histiocytic lymphoma (499,500). These cell lines are advantageous to use of RAW264.7 cells as they are of human origin, additionally the cells have a homogenous genetic background therefore the variability of THP-1 and U937 cells is much lower than that of PBMC (222,501).

Both THP-1 and U937 cells can be differentiated into macrophages through treatment with phorbol-12-myristate-13-acetate (PMA). Though the exact incubation time and concentration of PMA treatment varies from study to study, 48 hours appears to be the most common for both cell lineages (502–508). Other factors have also been shown to induce macrophage differentiation including retinoic acid and 1,25-dihydroxyvitamin D₃ (509). Resultant macrophages are termed M0 macrophages as they are not activated, however they can be further polarised into M1 classical proinflammatory or M2 alternative anti-inflammatory subsets through additional treatments. Alternatively, both lineages can be differentiated into dendritic cells, though this is more common for the THP-1 cell line; differentiation requires a mixture of both pro- and anti-inflammatory cytokines including TNF- α and IL-4 (222). However, when used in *in vitro* co-cultures with intestinal epithelial cells, THP-1 and U937 cells are frequently differentiated with PMA into macrophages. Such co-cultures have been used to investigate the effects of immune cells on the IEC structure and barrier function and can be used to understand aspects of inflammatory bowel diseases. The applications and experimental findings from these models will be discussed in detail in Chapter 6.

Although studies investigating the role of immune cells in the intestinal mucosa normally rely on the *in vitro* epithelial-immune cell co-cultures discussed above, some researchers have begun developing triple co-culture models that include epithelial cells, immune cells, and other stromal cells; namely fibroblasts or endothelial cells. Inclusion of these cells into such models increases the physiological relevance and allows for further cellular and ECM interactions to be investigated (313,487).

5.1.3. Conclusions

Multiple *in vitro* models of the intestine mucosa have been developed to include an immune cell lineage in the culture systems to either induce an alternate phenotype in the epithelium or to investigate pro- and anti-inflammatory signalling pathways in response to various stimuli. The most frequently employed co-culture utilises macrophages differentiated from either the THP-1 or U937 cell line. Although there is a wealth of literature focusing on Caco-2 THP-1 cultures, fewer intestinal models have focused on the U937 cell line even though it is considered to be the most widely used cell line to represent macrophages overall (222). In addition, very few co-cultures involving epithelial, fibroblast and immune cell components have been developed. Therefore, the approach taken in this chapter was to take a macrophage lineage differentiated from U937 cells and include that in an established epithelial-stromal *in vitro* model of the intestine mucosa.

5.2. Hypotheses and Aims

This chapter aims to develop an immune-competent triple co-culture model of the intestinal mucosa. This will involve adapting the lamina propria and epithelial-stromal co-cultures developed in Chapter 3 to incorporate macrophages differentiated from the U937 cell lineage. It is hypothesised that the macrophages in the co-culture will remain in an inactive M0 state and will not alter the functional properties of the epithelial barrier without the addition of external inflammatory stimuli. The resultant triple co-culture will then be utilised in inflammation studies in Chapter 6.

5.2.1. Objectives

- Optimise the protocol used to differentiate U937 cells into M0 macrophages.
- Develop an indirect co-culture model of differentiated U937 macrophages and the Caco-2 epithelium and characterise the effects of co-culture on the epithelial structure and function.
- Adapt the lamina propria compartment developed in Chapter 3 to incorporate U937 macrophages.
- Develop a 3D triple co-culture where HDFn and differentiated U937 cells in a lamina propria culture support the growth and differentiation of an intact Caco-2 epithelial monolayer.
- Characterise the structure and function of the immune-competent triple co-culture, with respect to the Caco-2 HDFn intestinal mucosa model.

5.3. Experimental Design

The principal aim of this chapter was to develop an immune-competent co-culture of the human intestinal mucosa to be used in Chapter 6 to create an inflammatory response. U937 cells were selected as the immune cell lineage to be incorporated into the model due to their reproducibility when compared to PBMC derived cells, and their ability to be differentiated into macrophage-like cells. Initial experiments focused optimising the differentiation protocol of the U937 cells (Section 2.1.3). To confirm cells had differentiated, flow cytometry and immunostaining was performed on cells for macrophage-specific cluster of differentiation (CD) marker expression ($n=3$).

Once immune cells had been successfully differentiated, they were indirectly co-cultured with Caco-2 Transwell® monolayers (Section 2.3.5) to create an intermediary model prior to generation of a direct epithelial-stromal-immune cell co-culture system. Assessment of epithelial barrier function and monolayer structure was conducted to ensure there were no adverse effects on the Caco-2 epithelium. A total of 9 models set up over 3 separate days were assessed in this manner.

Differentiated U937 cells were then seeded onto Alvetex® membranes to ensure that they could be cultured within the polystyrene inserts, before co-cultures with HDFn and U937 macrophage-like cells were established (Section 2.4.3). Following the development of an immune-competent lamina propria compartment, Caco-2 cells were seeded onto the apical surface and allowed to differentiate into an enterocyte monolayer (Section 2.5.2). Models were again assessed histologically and for the epithelial barrier function in comparison to the Caco-2-HDFn co-culture system ($n=9$).

5.4. Results

5.4.1. Optimising the Differentiation Protocol for U937 Cells

U937 cells were derived from malignant cells of a pleural effusion from a 37-year-old Caucasian male with diffuse histiocytic lymphoma; a non-Hodgkin lymphoma with abnormal lymphoid cells that typically arises in the GI tract or the skin (500). They are a suspension cell line that exhibit properties of myeloid cells that can be differentiated into cells resembling macrophages.

5.4.1.1. Differentiation of U937 with PMA

Prior to differentiation, U937 cells are maintained in RPMI cell culture media in suspension. Figure 5.3 shows phase contrast images of U937 cells maintained under conventional 2D culture. Cells can be observed at different focal planes within the images due to their nature of growth as suspension cells; they appear as rounded cells that grow either as single free-floating cells or as small clumps of cells at higher densities.

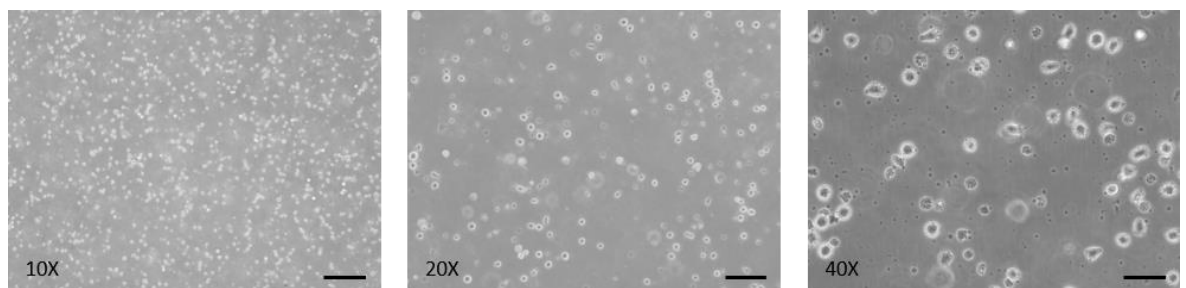


Figure 5.3. Conventional 2D cell culture of U937 cells. Phase contrast images of U937 suspension cells cultured in RPMI at increasing magnifications from left to right. Images shown are representative of 3 cultures. Scale bars: 200 μm (10X), 100 μm (20X) and 50 μm (40X).

Typically, U937 cells are differentiated into macrophages upon exposure to phorbol 12-myristate 13-acetate (PMA) for 48 hours. However, the exact concentration of PMA used varies from study-to-study; the two most common concentrations utilised are 10 ng/mL and 100 ng/mL, thus these two concentrations were selected to begin the optimisation of U937 cell differentiation. Upon treatment with PMA, U937 cells gain properties of adherent cells and form attachments to the underlying tissue culture plastic, irrespective of the PMA concentration used (Figure 5.4A). Cells retain their rounded morphology, but small protrusions can be seen at the cell surface, indicative of monocytic cells.

In order to assess the general health of the cells upon PMA exposure, an MTT assay was performed on cells treated with 10 and 100 ng/mL PMA. Cells exhibited similar levels metabolic activity following differentiation and no significant differences could be detected between the two PMA concentrations (Figure 5.4B). As an MTT assay could not be performed on the undifferentiated suspension cells, viability was also assessed via a trypan blue exclusion assay. Cells were over 98 % viable in all conditions indicating the treatment with PMA did not induce cell death and was suitable as a differentiation agent (Figure 5.4C). In addition, to assessing cell viability, the trypan blue exclusion assay was used after passaging to quantify the cell yield to ensure that enough cells were retrieved and could be seeded into intestinal models (Figure 5.4D). When seeded at the same density of 1×10^6 cells/well in 6-well plates, U937s that were not exposed to PMA treatment returned a significantly higher cell yield after 36 hours of growth than the differentiated cells, almost 4X that of the initial seeding density. Cells treated with PMA had a cell yield of just over 1×10^6 cells/well, the same as the initial seeding density. This was due to PMA inhibiting proliferation by stimulating the differentiation of cells over a 48-hour treatment period followed by a 24-hour rest period prior to performing the trypan blue exclusion assay. Although a slightly higher cell yield was found in the cells exposed to 100 ng/mL PMA, this was not significantly higher than cells treated with 10 ng/mL PMA.

A final measure of macrophage function in the differentiated cells was performed through a Vybrant™ phagocytosis assay kit to quantify the internalisation of a foreign particle. The foreign particles used within this kit are dead *E. coli* bacteria that have been fluorescently labelled with fluorescein. Though not significant, higher fluorescence levels were detected within U937 cells differentiated with 100 ng/mL PMA compared to those treated with 10 ng/mL PMA suggesting that the former concentration induced a greater macrophage-like phenotype within the cells (Figure 5.4E).

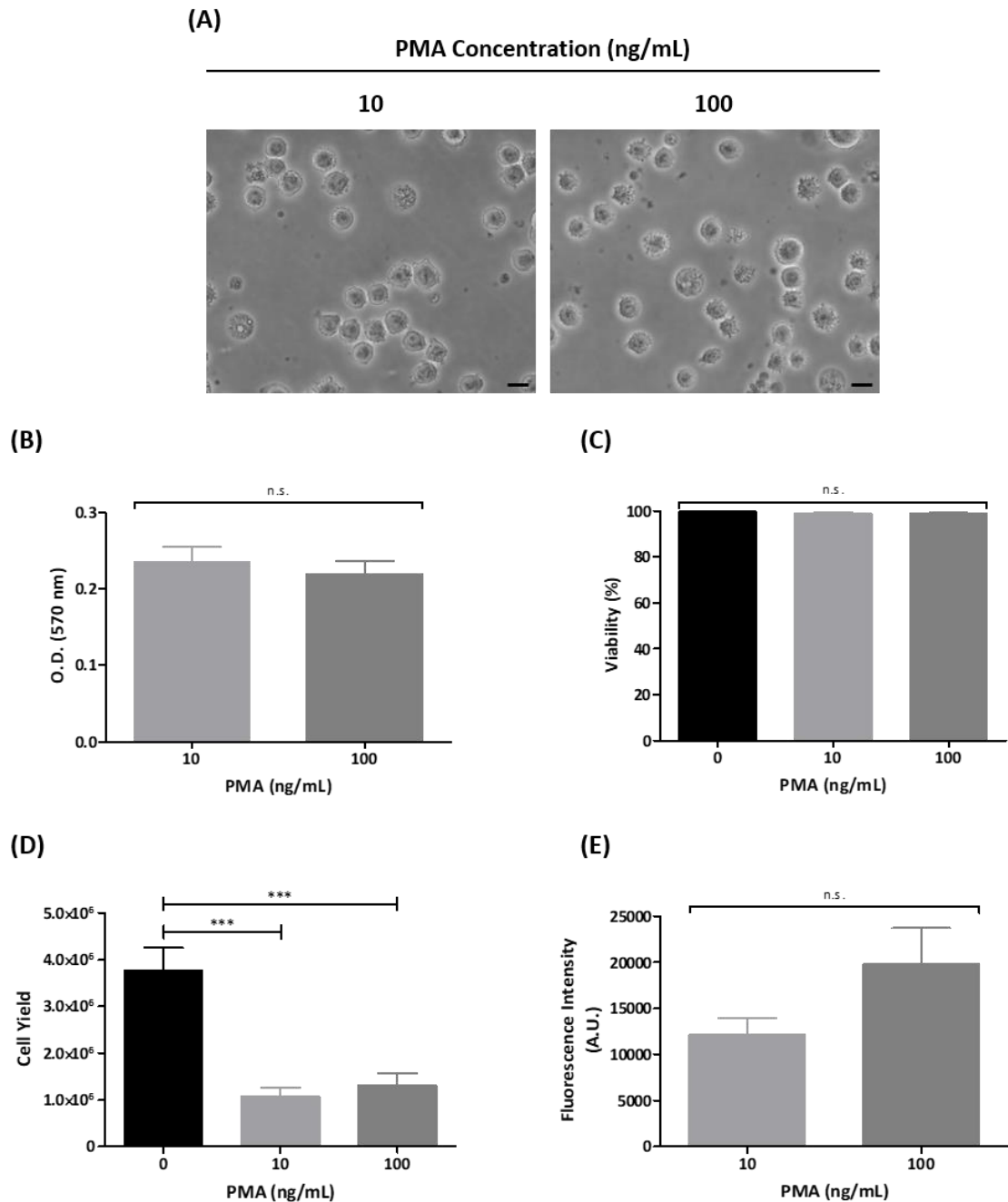


Figure 5.4. Optimisation of U937 differentiation in RPMI. U937 cells were maintained in RPMI and treated with either 10 or 100 ng/mL PMA for 48 hours with a 24-hour recovery period to induce differentiation. Phase contrast images were taken after the rest period (A). Images shown are representative of 9 cultures. An MTT assay was also performed after the recovery period on cells treated with PMA, untreated cells remained in suspension and so an MTT could not be performed (B). The viability of cells was calculated following a trypan blue exclusion assay to quantify the number of live/dead cells (C). The total cell yield was assessed after the differentiation period through passaging of the cells and counting via a trypan blue exclusion assay (D). Additionally, assessment of how the cells differentiated with different PMA concentrations, phagocytosed fluorescent particles was performed (E). Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. *** $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post hoc test. Scale bars: 50 μ m.

Assessment of differentiation was performed through flow cytometry analysis for several cluster of differentiation (CD) macrophage markers (Figure 5.5). CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein receptor that acts as a co-receptor for various Toll-like receptors (TLRs), specifically working with TLR4 to facilitate cellular responses to lipopolysaccharide (LPS) (510). Over 50 % of undifferentiated cells are positive for CD14 and this increases upon exposure to PMA. Supplementary to this the fluorescence intensity of CD14 also increases upon differentiation suggesting the cells become more macrophage-like in nature. Along with CD14, expression of the pan-macrophage marker CD11b was also examined. CD11b, also known as integrin α M, is an integrin that mediates cell adhesion and has roles in the initiation of phagocytosis (511). Evidence of cellular adhesion was demonstrated in phase contrast images of U937 cells in Figure 5.6A. An increase in both the expression levels and fluorescence intensity of CD11b was found upon PMA treatment when compared to untreated cells, confirming the acquisition of adherence properties within the cells. The number of cells positive for the expression of a further pan-macrophage marker, CD68, also increased upon exposure to PMA in addition to the fluorescence intensity. Furthermore, the expression levels and fluorescence intensity of CD15, a protein thought to be involved in phagocytosis, similarly increased with PMA treatment. Although clear differences could be observed between undifferentiated and differentiated cells, the expression levels and fluorescence intensity were not found to differ between the two concentrations of PMA, suggesting they were both suitable to use to initiate the differentiation process in U937 cells.

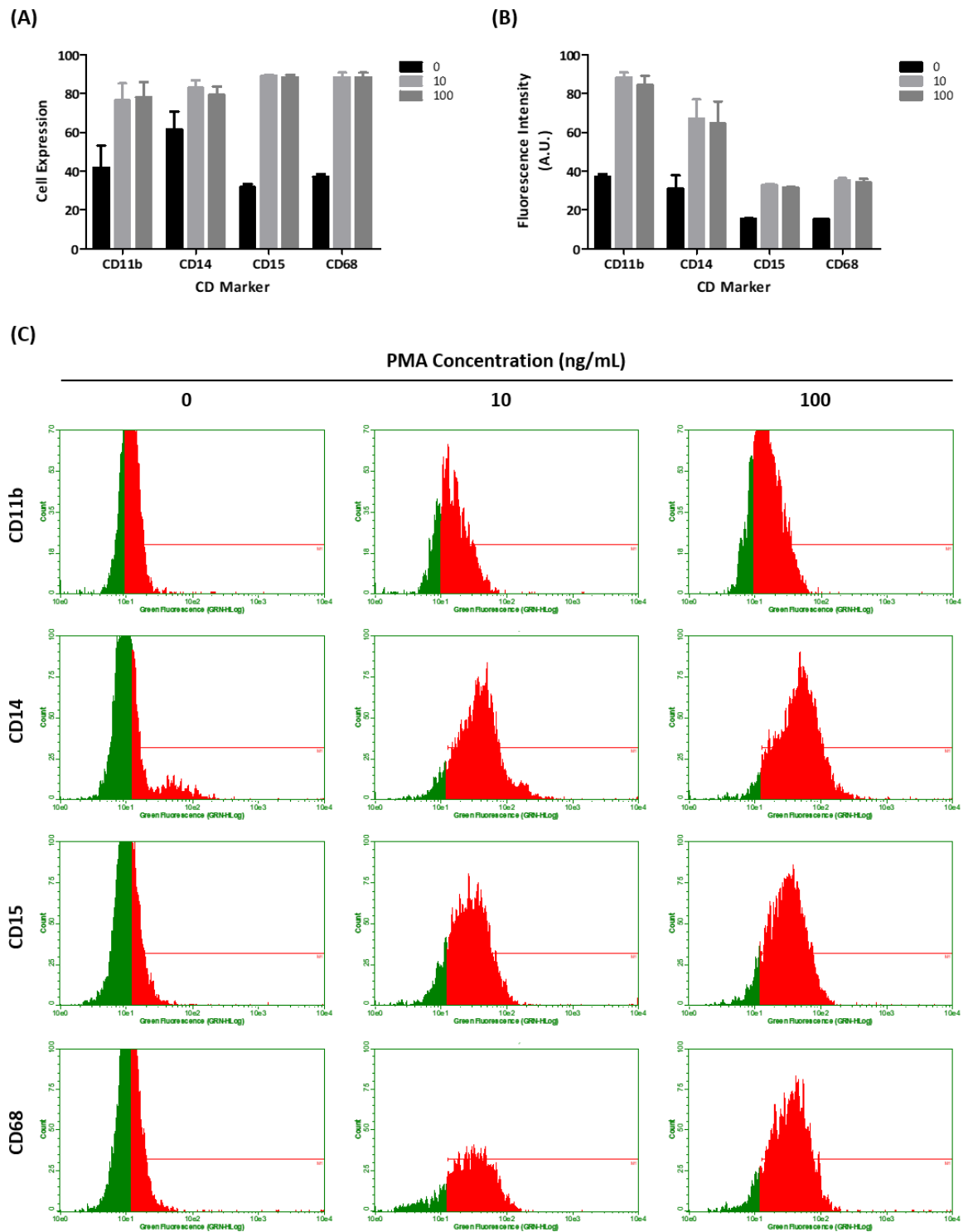


Figure 5.5. Characterisation of CD marker expression following differentiation of U937 cells with PMA. Flow cytometry analysis for expression of CD11b, CD14, CD15 and CD68 reveals an increased percentage of cells expressing these markers following treatment with PMA (A). The median fluorescence intensity of all tested markers also increased following differentiation with PMA (B). Representative histograms for all CD markers used in flow cytometry, gated as detailed in Chapter 2. Data represent mean $\pm$ SD, total $n=3-6$ from 2 independent test occasions.

5.4.1.2. Culture of U937 cells in DMEM

Inclusion of U937 cells within the intestinal mucosa *in vitro* models necessitates culture in DMEM, therefore the ability of cells to grow and differentiate in DMEM was examined to determine if all culture could proceed using one standardised culture media. Initial studies focused on the growth of undifferentiated cells in either RPMI or DMEM. As can be observed in Figure 5.6A, cells in both culture media display similar rounded morphology and typically grow as individual free-floating cells when maintained in suspension. Assessment of cell viability and cell yield via a trypan blue exclusion assay following subculture did not reveal any significant differences in the viability or growth of cells; cells remained over 99 % viable when the media was changed and also had comparable cell yield after 3 days of growth (Figure 5.6B, C). This indicated that DMEM was a suitable culture medium to use for the conventional suspension culture maintenance of U937 cells.

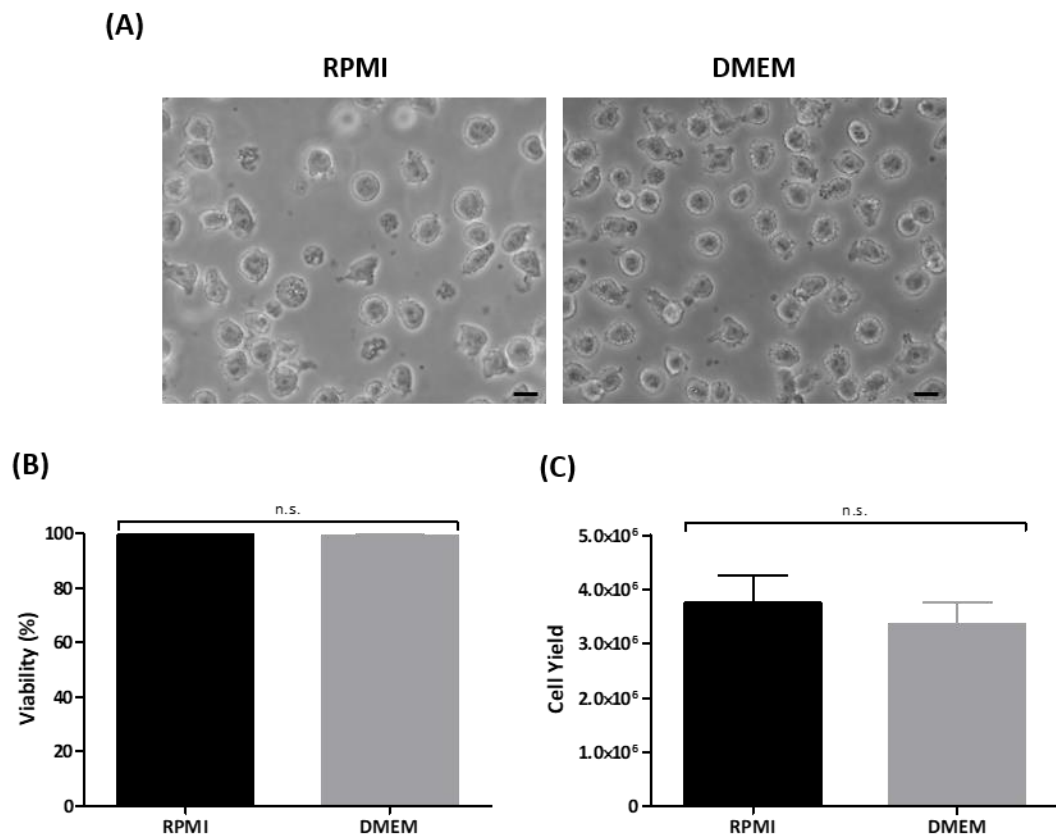


Figure 5.6. Assessment of undifferentiated U937 growth in RPMI and DMEM. Phase contrast images were taken of cells cultured in suspension and maintained in either RPMI or DMEM cell culture media (A). Images shown are representative of 9 cultures. The viability of cells was calculated following a trypan blue exclusion assay to quantify the number of live/dead cells (B). The total cell yield was assessed after the differentiation period through passaging of the cells and counting via a trypan blue exclusion assay (C). Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical replicates. Scale bars: 50 μ m. No significant differences found by Student's *t*-test.

As before, U937 cells were treated with 10 and 100 ng/mL PMA in the different culture media to determine if cells could be differentiated in DMEM. Figure 5.7A shows the gross morphology of the cells upon exposure to PMA. All cells gained an adherent phenotype and retained a rounded morphology, however increased clustering of cells could be observed in the cells grown in DMEM. U937 retrieval yield following differentiation and passaging was lower in cells maintained and differentiated in DMEM and was also lower than the number of initial cells that were seeded onto the tissue culture plastic (Figure 5.7B). This could be due to the cells not adhering as well in DMEM, or due to higher numbers of clustered cells. Furthermore, a slight decrease in the metabolic activity of cells differentiated in DMEM in comparison to those differentiated in RPMI was observed, though no overall differences in the total cellular viability were identified via a trypan blue exclusion assay (Figure 5.7C,D).

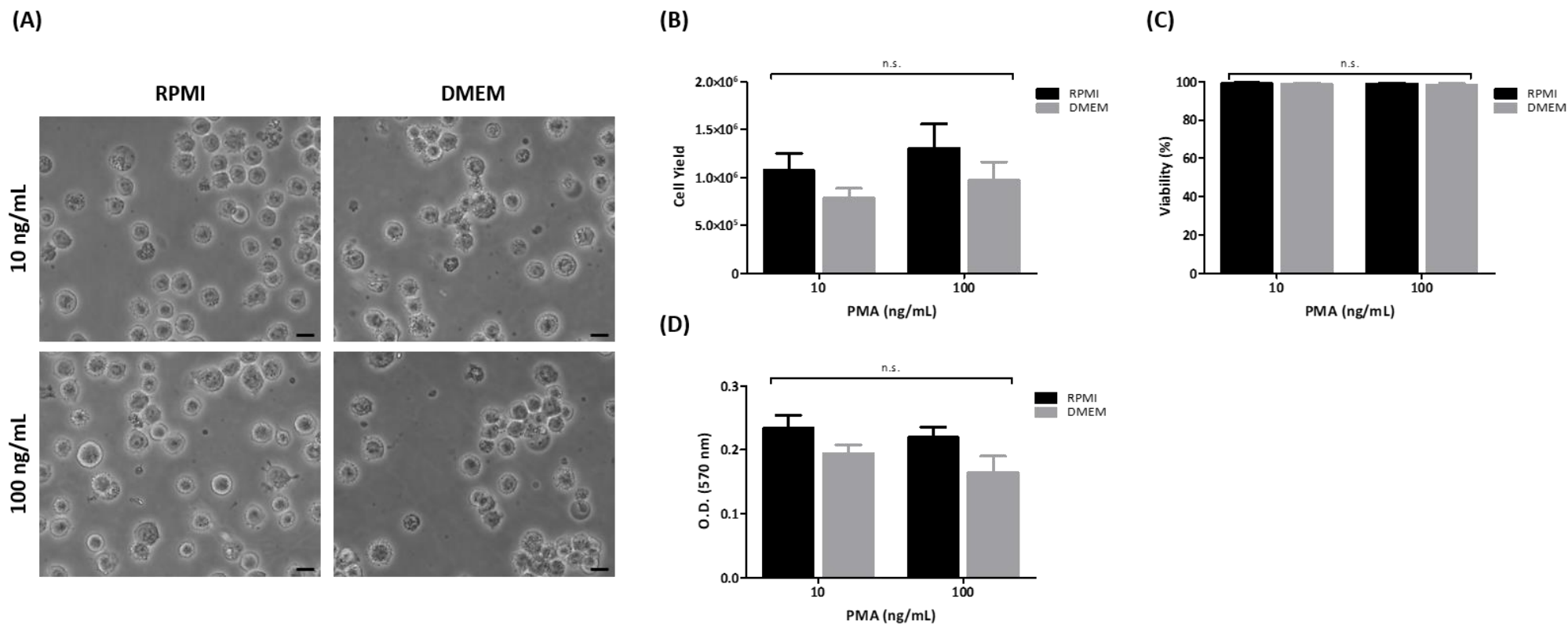


Figure 5.7. U937 differentiation in RPMI and DMEM. U937s cells maintained in complete RPMI or complete DMEM were treated with 10 or 100 ng/mL PMA to induce differentiation followed by a 24-hour recovery period. Phase contrast images were taken of cells maintained in either RPMI or DMEM cell culture media and differentiated with either 10 or 100 ng/mL PMA (A). Images shown are representative of 9 cultures. The total cell yield (B) was assessed after the differentiation period through passaging of the cells and counting via a trypan blue exclusion assay which was also used to quantify the number of live/dead cells (C). To further assess cellular viability, an MTT assay was performed after the recovery period on cells treated with PMA (D). Data represent mean $\pm$ SEM, total $n=9$ comprised of 3 independent test occasions, each with 3 technical repeats. Scale bars: 50 μ m. No significant differences identified by two-way ANOVA.

5.4.1.3. Finalised differentiation protocol for U937 cells

Overall DMEM resulted in a marginally diminished metabolic activity of the U937 cells and resulted in a lower cell yield than RPMI culture media. The number of cells retrieved after PMA exposure was also lower than the initial cell seeding density, thus RPMI was selected as the most appropriate culture medium for the maintenance and differentiation of U937 cells. Although little differences were detected between U937 cells exposed to 10 and 100 ng/mL PMA in regard to cell growth, viability and expression of differentiation markers, higher phagocytosis of *E. coli* particles was observed in those cells treated with 100 ng/mL PMA. Accordingly, the final optimised protocol for U937 differentiation is outlined in Figure 5.8: U937 cells were maintained in RPMI culture media, incubated with 100 ng/mL PMA for 48 hours followed by a 24-hour rest period to allow the cells to recover from treatment.

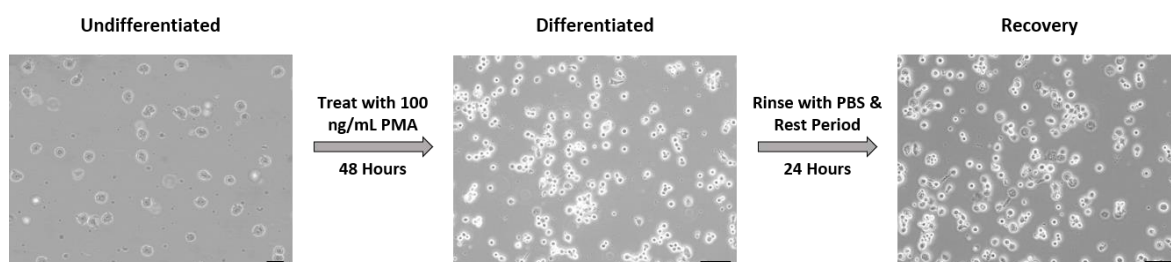


Figure 5.8. Optimised U937 differentiation protocol. U937 cells were maintained in RPMI and treated with 100 ng/mL PMA for 48 hours. Following a rest day, cells could then be used in future experiments. Phase contrast images taken using an EVOS FL microscope. Scale bars: 50 μ m.

U937 cells differentiated with this optimised protocol were subject to immunostaining to characterise cellular morphology in more detail (Figure 5.9). Phalloidin staining was used to visualise the actin cytoskeleton; positive staining was prominent particularly around the edges of the cells along the cell membranes. The rounded nature of the cells is clear with phalloidin staining, in addition to evidence of cell clustering, indicative of macrophage cells. Similarly, CD14 was predominantly localised to the edges of the cells as would be expected for a cell surface receptor. Unlike CD14, CD68 has a more diffuse expression pattern in differentiated U937 cells, though most positive staining could be detected around the cell periphery.

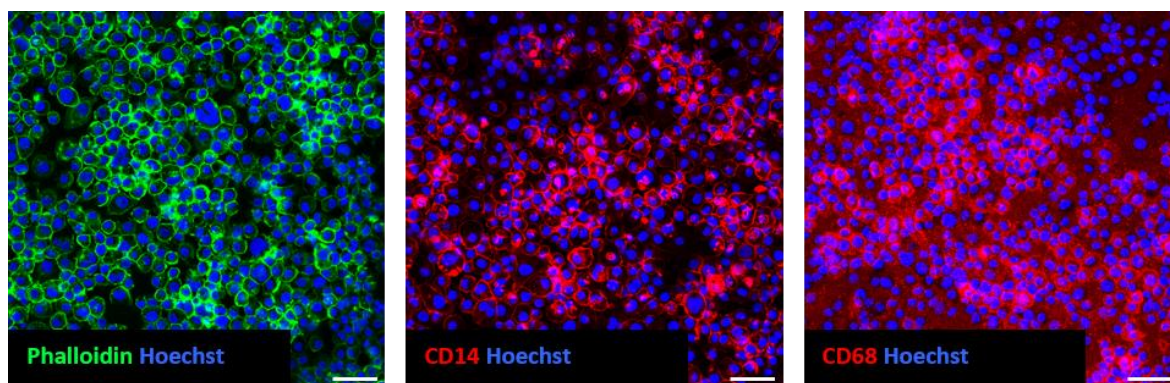


Figure 5.9. Characterisation of differentiated U937 cells. U937 cells differentiated according to the optimised protocol were fixed with 4 % PFA for 10 minutes before being immunolabelled for the actin cytoskeletal marker, phalloidin (green) and for the macrophage markers CD14 and CD68 (red). Nuclei were counterstained with Hoechst (blue). Images depicted are representative of 3 cultures. Scale bars: 50 μ m.

5.4.2. Development of an Epithelial-Immune Cell Co-Culture System

Numerous studies have focused on the development of a Caco-2-immune cell co-culture system using the Transwell® membrane system. THP-1, U937, RAW 264.7, and Raji B cells have all been utilised in such co-culture studies. Caco-2 cells are typically seeded onto Transwell® membranes and cultured as normal, while immune cells are seeded in a suitable well plate that the Transwell® insert can then be placed into following completion of immune cell differentiation. This would usually occur between days 14-16 of epithelial cell culture (277,279,302,495,512–514). Models then remain in co-culture for up to 7 days before introducing a proinflammatory stimulus, allowing the co-culture and TEER to stabilise. Thus, a co-culture model utilising Caco-2 Transwell® monolayers and differentiated U937 cells at the base of the well was developed.

5.4.2.1. U937 cells are viable following long-term culture after differentiation

In order to establish a co-culture model using U937 cells, it was necessary to confirm that the immune cells retained a high level of viability and remained metabolically active following long-term culture. Under normal 2D experimental conditions, U937 cells would be treated with PMA for 48 hours to induce differentiation into macrophage-like cells and left to recover for a further 24 hours. However, in co-culture systems, cells may be left for up to 7 days before exposure to an inflammatory stimulus, thus an MTT and trypan blue exclusion assay was performed at day 0 and 7 after cell differentiation had been completed. Cells were maintained in DMEM following differentiation as DMEM is the standard culture media for epithelial monolayers.

Differentiated U937 cells display a reduced metabolic activity after an additional 7 days of culture, however this was not a significant reduction compared to the metabolic activity of cells that had just completed differentiation (Figure 5.10A). There was also a reduction in the viability of cells following long-term culture after differentiation, however all cells retained a viability of over 97 % with cells at day 0 and day 7 having a viability of $98.9 (\pm 0.32) \%$ and $97.4 (\pm 0.64) \%$ respectively, suggesting that this was not a biologically significant effect (Figure 5.10B). These results demonstrate that although a reduction in both metabolic activity and viability were found after long-term culture of differentiated U937 cells, they retained a high level of viability and were healthy.

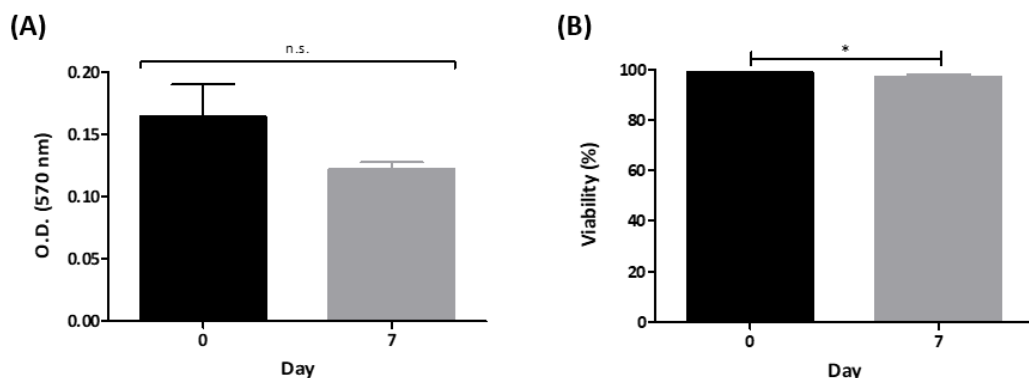


Figure 5.10. Differentiated U937 cells remain metabolically active and viable following long-term culture. U937 cells were differentiated as normal in 12-well plates, then switched to DMEM culture media. At days 0 and 7 and MTT assay (A) and trypan blue exclusion assay (B) were performed to assess the cellular metabolic activity and viability, respectively. Data represent mean $\pm$ SEM, total $n=9$, comprised of 3 independent experiments, each with 3 technical replicates. $*P \leq 0.05$ as determined by Student's t -test.

5.4.2.2. Co-culture with differentiated U937 cells does not disrupt epithelial barrier function

Following assurance that differentiated U937 cells remained both metabolically active and viable following long-term culture, work began on introducing the Caco-2 monolayer to the basally located immune cells. Caco-2 cells were seeded as normal on the apical surface of Transwell® membranes and left to differentiate for 14 days, at which point TEER started to plateau. Models were then transferred to another 12-well plate with fully differentiated U937 cells adhered to the wells and were left to stabilise for the remainder of epithelial cell differentiation. To determine if the immune cells negatively impacted on the epithelial structure or barrier function prior to use in an inflammatory stimulus experiment, assessment of the monolayer was performed through histology and apical junctional complex immunostaining, in combination with a series of transport assays.

H&E staining reveals that an epithelial monolayer successfully formed in the presence of U937 cells (Figure 5.11). In both culture systems, Caco-2 cells had a cuboidal phenotype yet when the monolayer is in co-culture with U937 cells, the nuclei have a more evident basolateral location than monolayers cultured without the presence of U937s. This suggests that co-culture of Caco-2 and U937 cells may induce a more polarised phenotype across the epithelium.

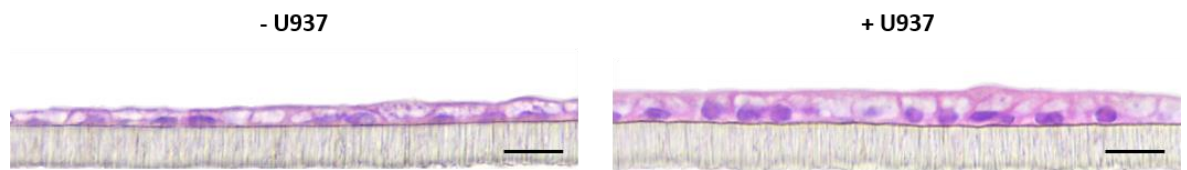


Figure 5.11. H&E staining of Caco-2 monolayers cultured in the absence or presence of differentiated U937 cells. Caco-2 cells were maintained on Transwell® inserts for 14 days before being moved to either fresh 12-well plates or 12-well plates with U937 cells differentiated on the base of the well. Monolayers display a more polarised morphology when co-cultured with differentiated U937 cells. Images shown are representative of 9 models. Scale bars: 20 μ m.

As can be seen in Figure 5.12, immunofluorescent staining of tight junction and adherens junction proteins displayed no obvious differences in the expression pattern or localisation of the apical junctional complex between control monolayers and those cultured in the presence of U937 cells. Occludin, claudin-1 and claudin-2 were selected to assess the tight junction components of the epithelial barrier. Occludin had a highly defined expression pattern surrounding the apical surface of the epithelium. Although not as distinct as occludin, claudin-1 and claudin-2 were also found at the apical pole of the monolayer and no differences were observed in either the “sealing” or “pore-forming” claudin protein expression between the monolayers. Similarly, E-cadherin was present across the entire epithelial monolayer cultured with and without differentiated U937 cells and demonstrates that adherens junctions are successfully forming in the co-culture system.

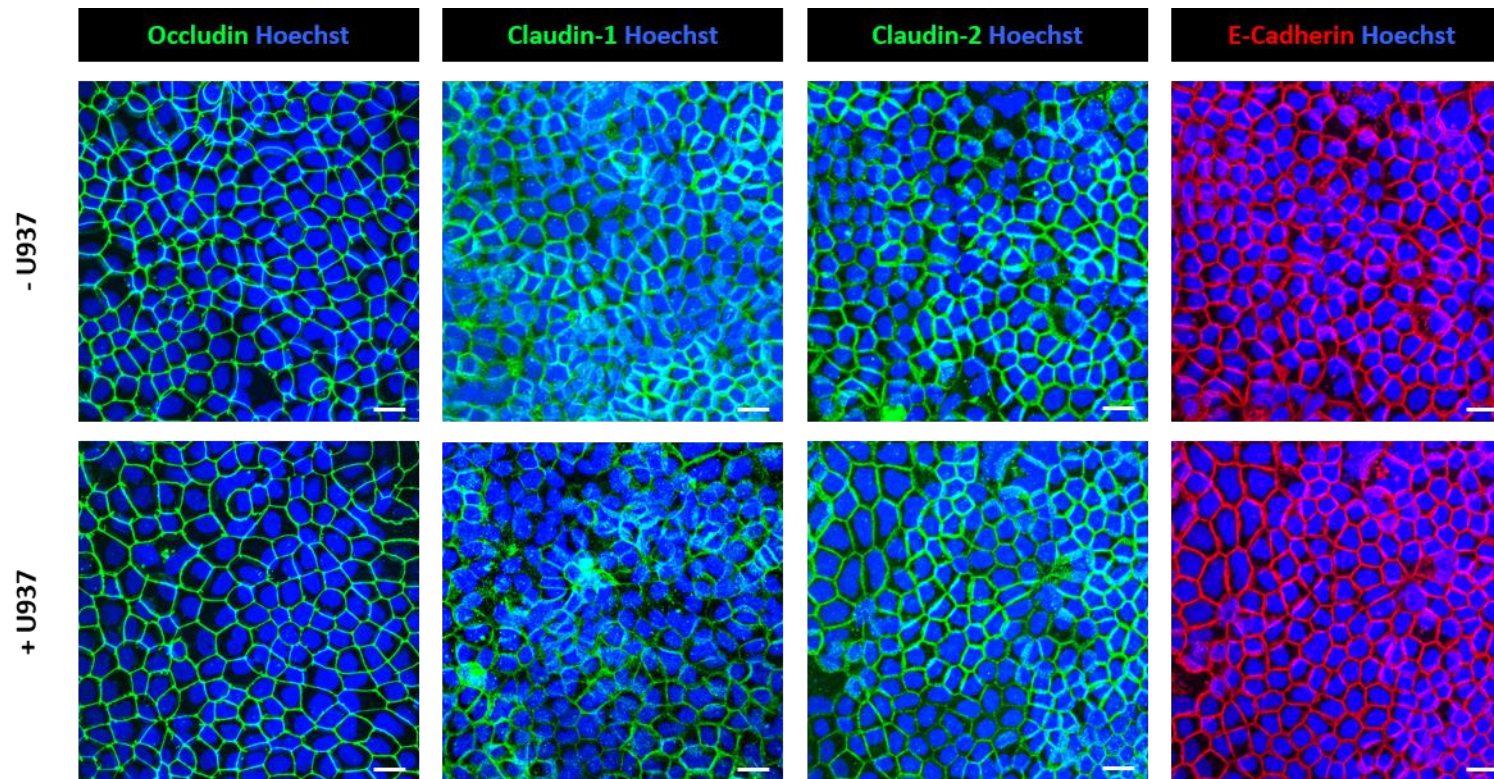


Figure 5.12. Immunofluorescent staining of the apical junctional complex in Caco-2 monolayers cultured in the absence or presence of differentiated U937 cells. Top-down compressed Z-stacks of Caco-2 monolayer. Models were immunolabeled for the tight junction protein: occludin, claudin-1 and claudin-2 (green) and for the adherens junction marker E-cadherin (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 3 models. Scale bars: 20 μ m.

Preceding co-culture, TEER was continuously assessed during the initial 14-day epithelial growth period, monolayers with an abnormally high or low electrical resistance were not used in subsequent co-cultures. TEER measurements were then taken following the establishment of co-culture once the epithelium had completed the full 21 days of differentiation. Figure 5.13A demonstrates that the immune cells did not affect the electrical resistance across the monolayer, suggesting that differentiated U937 macrophages did not negatively impact epithelial barrier function. This was confirmed by lucifer yellow rejection and transport experiments in addition to FD4 and FD20 permeability assays, where no differences in the permeability characteristics of the epithelial barriers was identified (Figure 5.13B-E). Assays were conducted in the apical-basolateral direction only as the absorptive capacity of the cells was of greater interest than the secretory capabilities.

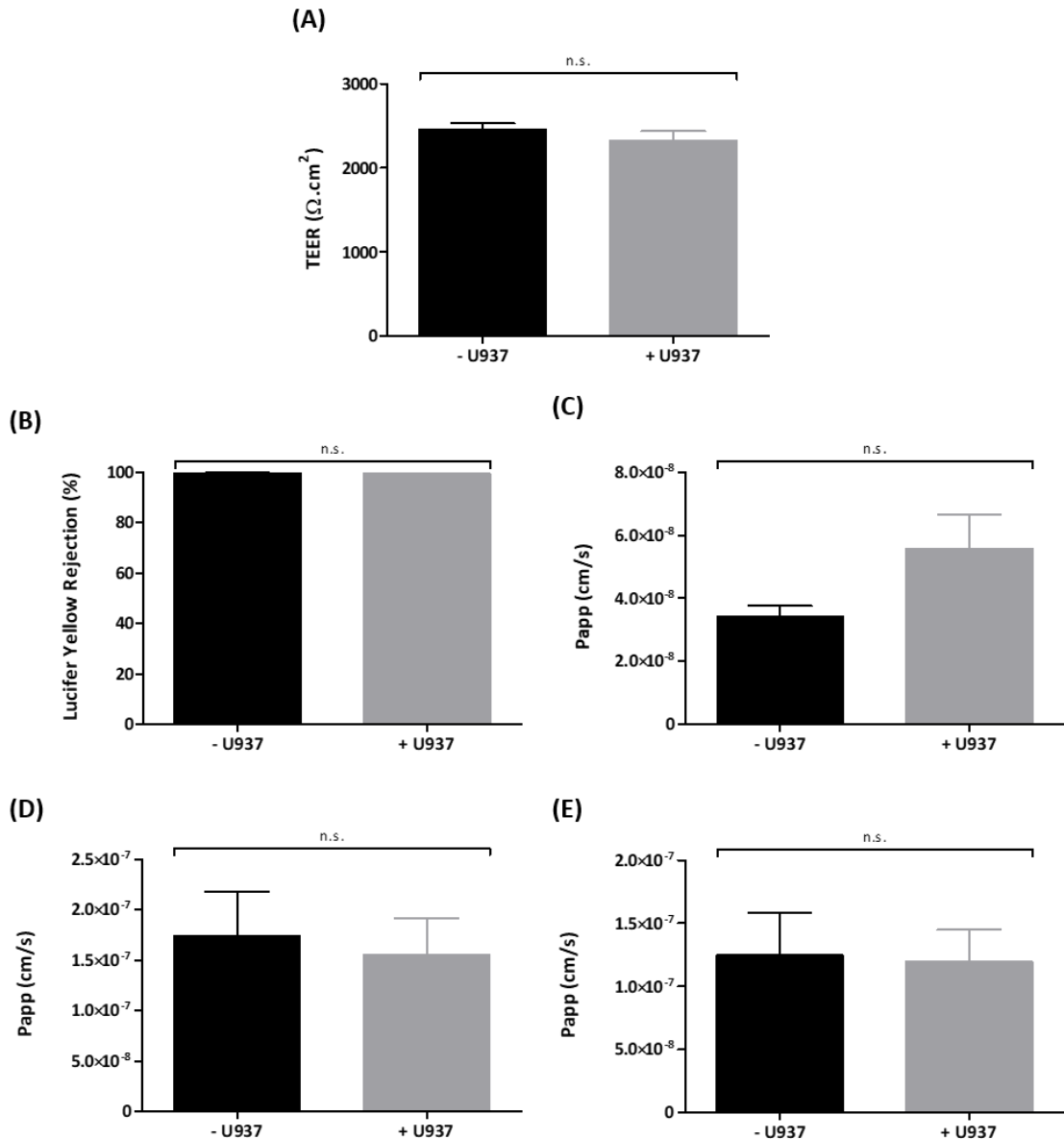


Figure 5.13. Epithelial barrier function is not diminished in co-culture with differentiated U937 cells. Caco-2 cells were maintained on Transwell® inserts for 14 days before being moved to either fresh 12-well plates or 12-well plates with U937 cells differentiated on the base of the well. TEER was assessed following completion of epithelial differentiation (A) and a series of transport assays were conducted including a lucifer yellow rejection assay (B), in addition to permeability assays in the apical-basolateral direction for the fluorescent compounds lucifer yellow (C), FD4 (D) and FD20 (E). No differences were found in any of the functional tests conducted. Data represent mean $\pm$ SEM, total $n=9-18$ from a minimum of 3 independent experiments, each with 3 technical replicates. No significant differences were found by Student's *t*-test.

5.4.3. Introduction of an Immune Component into the Lamina Propria Equivalent

Within the human intestinal mucosa, tissue-resident macrophages are usually found within gut-associated lymphoid tissues or within the lamina propria. Within diseased intestinal tissue such as Crohn's disease or ulcerative colitis, large immune cell infiltration into the lamina propria is a hallmark of the disease. Therefore, to create a structurally representative model of the mucosal tissues, U937 cells should be ideally located in the lamina propria compartment of the *in vitro* model with large numbers present.

5.4.3.1. Culture of differentiated U937 cells on alternative Alvetex® formats

Prior to use within the lamina propria equivalent, differentiated U937 cells were assessed for their ability to form attachments to Alvetex® substrates. Three Alvetex® substrates were utilised in this experiment: Polaris with voids of 3-4 µm and 0.4-0.6 µm interconnects; Strata with voids of 20 µm and 3 µm interconnects; and Scaffold with voids of 36-40 µm and 15 µm interconnects.

U937 cells were differentiated according to the optimised protocol and were then passaged with trypsin, seeded onto Alvetex® membranes and maintained for 2 days. Figure 5.14 shows the resulting H&E, very few cells remained on the surface of and in the Alvetex® membranes following differentiation and reseeding. U937 cells seeded on the apical membrane of Strata and Polaris were typically found on the surface of the cell culture support, however some cells can be found just below the surface in Strata inserts. Although these two Alvetex® formats are not used in the intestinal mucosa equivalent, they show that U937 cells can reform attachments to alternative tissue culture plastics following differentiation and trypsinisation. Unlike the cells on Polaris and Strata, sparse numbers of U937 cells can be seen within Alvetex® Scaffold, showing that the cells can infiltrate into the culture membrane and have the potential to be included in the HDFn lamina propria compartment of culture.

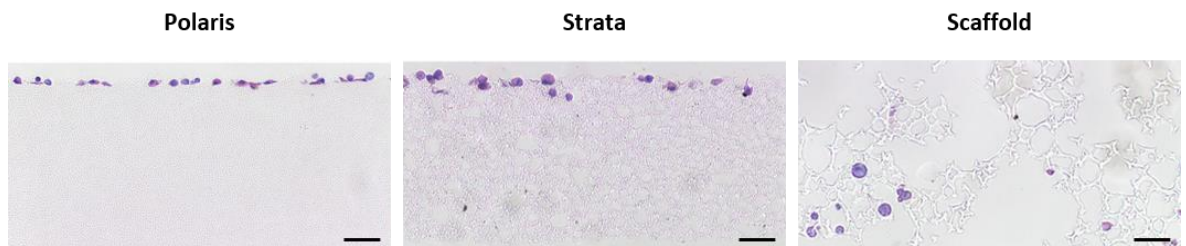


Figure 5.14. U937 culture on varying Alvetex® formats. H&E staining of 0.5×10^6 U937 cells seeded onto the surface of Alvetex® Polaris, Strata and Scaffold and maintained for 2 days prior to wax embedding. Images shown are representative of 3 cultures. Scale bars: 50 µm.

5.4.3.2. Optimisation of U937 and HDFn co-culture

Preliminary optimisation experiments for U937 and HDFn co-culture focused on the seeding density of U937 cells. As histological analysis of 0.5×10^6 U937 cells seeded into Alvetex® Scaffold showed few cells remaining within the cell culture membrane after 2 days of culture, HDFn cells were first seeded into the insert to provide a foundation in which to culture the of U937 cells. HDFn culture proceeded for 7 days in complete DMEM with TGF- β 1 and ascorbic acid supplements; 7 days of fibroblast pre-culture was initially selected as it was demonstrated to support the growth of Caco-2 cells in Chapter 3 with evidence of ECM production. U937 cells were then seeded onto the 7-day-old lamina propria equivalents at the following densities: 0, 1×10^4 , 0.5×10^5 , 1×10^5 , 0.5×10^6 , and 1×10^6 .

H&E staining (Figure 5.15) shows that U937 cells appear as densely stained rounded cells and are located within the fibroblast compartment; no cells matching these criteria were identified within the control model without U937 cells. Cells were largely identified in clusters of U937 cells and were rarely found spread evenly as individual cells throughout the model. At the three lower seeding densities of 1×10^4 and 5×10^4 and 1×10^5 , there were insufficient cells to truly mimic the structure of healthy and diseased intestinal tissue, thus these seeding densities were excluded from future experiments. At the two higher seeding densities of 0.5×10^6 and 1×10^6 U937 cells there appeared to be more even distribution of the cells along the length of the membrane, however they were still located within patches of U937 cells.

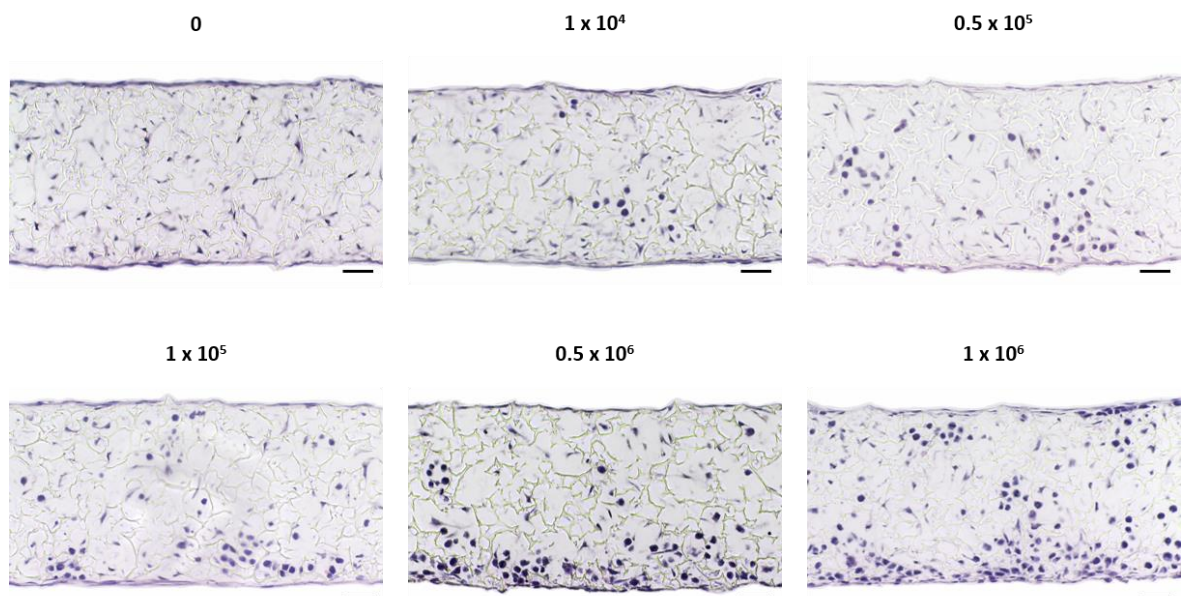


Figure 5.15. Initial optimisation of U937 seeding density for lamina propria culture. HDFn cells were maintained in Alvetex® Scaffold for 7 days in complete DMEM with TGF- β 1 and ascorbic acid supplements. U937 cells were seeded onto the 7-day lamina propria equivalents and culture proceeded for a further 7 days. The U937 seeding densities utilised included: 0, 10 000, 50 000, 100 000, 500 000 and 1 000 000 cells/insert. H&E staining of U937 and HDFn lamina propria equivalents. Images shown are representative of 6 models. Scale bars: 50 μ m.

Fibroblast culture time was then altered to determine if the level of fibroblast layering and ECM deposition affected the ability of the U937 cells to enter the lamina propria compartment. Days 7 and 10 of fibroblast growth in Alvetex® Scaffold were selected and U937 cells were seeded at 0.5×10^6 or 1.0×10^6 cells per membrane at the selected time points. H&E staining did not reveal any differences in the level of fibroblasts layering on the surface of Alvetex® Scaffold and the number of visible immune cells was comparable across all models, even when double the number of U937 cells were initially seeded (Figure 5.16). However, as the immune cells are required to remain viable and metabolically active for the remainder of lamina propria culture – up to day 14, and the full epithelial culture and differentiation period, a further 21 days - the later time point of U937 cell seeding was selected. This also allows for fibroblasts to be cultured in media containing TGF- β 1 and ascorbic acid for a further 4 days and encourages further deposition of ECM. Additionally, the lower seeding density of 0.5×10^6 cells/model was selected as there were no noticeable differences between the two seeding densities; the lower seeding density was thus less resource intensive to produce.

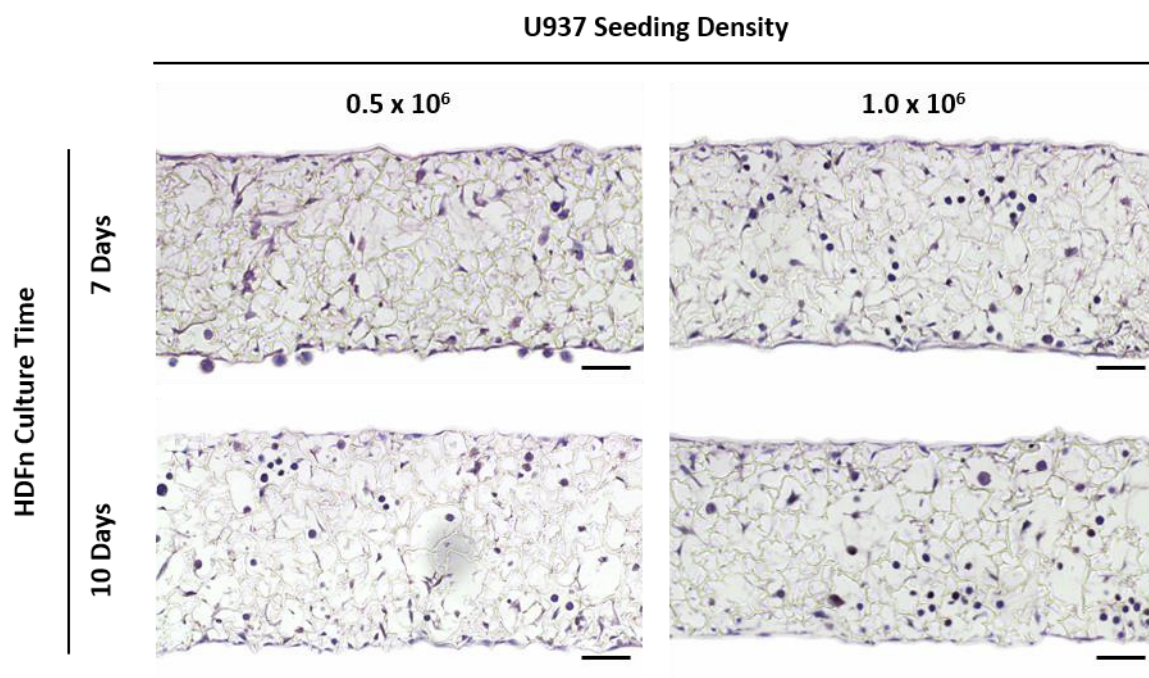


Figure 5.16. Optimisation of HDFn culture time and U937 seeding density. HDFn fibroblasts were maintained in Alvetex® Scaffold for 7 or 10 days in complete DMEM with TGF- β 1 and ascorbic acid supplements. U937 cells were seeded onto the lamina propria equivalents at either 0.5×10^6 or 1.0×10^6 cells/membrane and culture proceeded for a until day 14. Models were then harvested for wax embedding and H&E staining. Images shown are representative of 6 models. Scale bars: 50 μ m.

To ensure that the selected culture conditions were an appropriate choice for the lamina propria compartment. A brief characterisation of the immune cells and ECM was conducted through immunofluorescent analysis, depicted in Figure 5.17. Immune cells could be distinguished from the fibroblast component by CD14 immunostaining, though some background staining was detected throughout the membrane. As concluded from histological staining, the U937 cells typically form colonies within the lamina propria rather than appearing as individual macrophage-like cells.

Additionally, staining for the ECM components, collagens I and III was performed. Both collagens were found throughout the lamina propria indicating that the presence of immune cells did not impede the production and deposition of an ECM by the fibroblasts. However, less collagen deposition was evident along the edges of the Alvetex® membrane than described in previous chapters, potentially due to the reduced exposure of fibroblasts to TGF- β 1 and ascorbic acid.

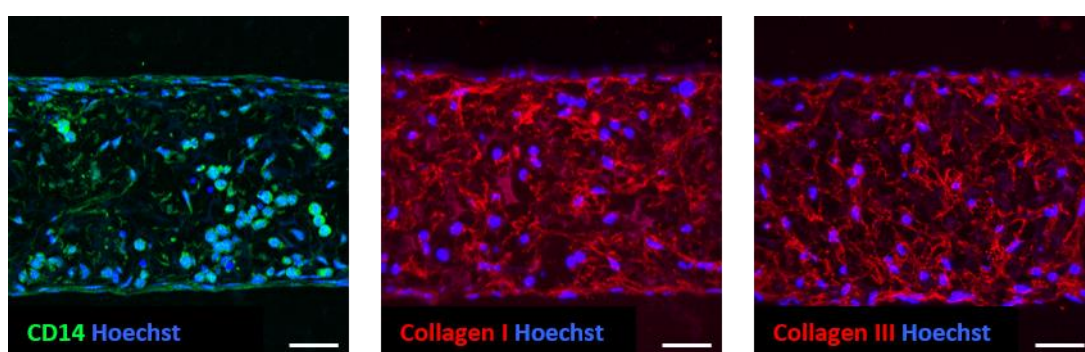


Figure 5.17. Immunofluorescent characterisation of the immune-competent lamina propria model. HDFn fibroblasts were maintained in Alvetex® Scaffold for 10 days in complete DMEM with TGF- β 1 and ascorbic acid supplements. U937 cells were seeded onto the lamina propria equivalents at 0.5×10^6 cells/membrane and culture proceeded for a until day 14. Models were then harvested for wax embedding and immunolabelled for the macrophage marker CD14 (green) and the ECM proteins collagen I and collagen III (red). Nuclei were counterstained with Hoechst (blue). Images shown are representative of 6 models. Scale bars: 50 μ m.

5.4.4. Development of an Immune-Competent Triple Co-Culture Model of the Intestinal Mucosa

The fibroblast-macrophage co-culture developed in Section 5.4.3 was utilised as a foundation on which to seed the Caco-2 epithelium. Caco-2 cells were seeded as normal on the surface of the lamina propria and remained in culture for 21 days ensuring complete enterocyte differentiation occurred. Resultant models were then referred to as the triple co-culture. For each experiment conducted, additional models were generated due to the lower reproducibility of the models; approximately 1 in 7/8 models would fail to form a successful epithelial monolayer as determined by TEER and histology. Failed models were excluded from all future analysis.

5.4.4.1. Characterisation of Caco-2 cells in co-culture with an immune-competent lamina propria equivalent

Figure 5.18 shows the overall morphology of the triple co-culture. A fully formed homogenous epithelium with polarised enterocytes can be observed resting on the surface of the Alvetex® membrane, within which HDFn and U937 cells reside. Layering of fibroblasts on the membrane surface was evident in the triple co-culture and immune cells were easily identified as the densely stained circular cells as described in Section 5.4.3. Diffuse, particulate staining was present surrounding some of the macrophage-like cells suggesting that there may be some apoptosis or necrosis of U937 cells occurring.

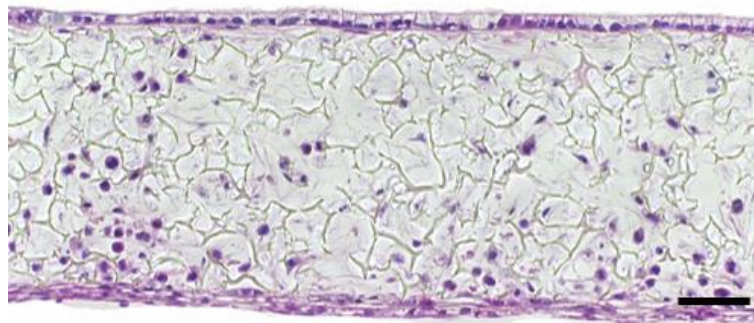


Figure 5.18. H&E staining of an immune-competent triple co-culture. HDFn and U937 cells were seeded into Alvetex® Scaffold membranes to create an immune-competent lamina propria foundation on which to build an epithelium. Caco-2 cells were seeded onto this foundation and maintained in complete DMEM for a further 21 days. Models were then harvested for wax embedding and representative images of H&E staining are depicted. Image depicted is representative of 9 models. Scale bar: 50 μ m.

Based on the histological staining, there was little evidence of ECM production in the triple co-culture, thus immunofluorescent staining techniques were employed to fully characterise the structure and constituents of the *in vitro* 3D culture (Figure 5.19). To confirm that the epithelium had fully differentiated into small intestinal enterocyte-like cells, immunostaining for the microvilli marker villin was utilised. Strong villin staining was evident along the entire epithelial surface indicating that the monolayer had an apically located microvilli brush-border characteristic of differentiated enterocytes. Further epithelial staining was conducted to identify components of the apical junctional complex, namely claudin tight junction proteins and E-cadherin. Like villin, all AJC markers were present throughout the entire epithelium, yet there were also high levels of claudin background staining localised to the U937 immunocytes. Close observation of the epithelial structure from these AJC immunofluorescent images revealed that the Caco-2 epithelium had a more cuboidal appearance rather than columnar, though the nuclei retained a basolateral location. Junctional staining was most obvious at the basolateral surface of the epithelium rather than along the lateral membranes as would be expected, this was particularly the case for claudin-1.

Immunofluorescent analysis of the lamina propria compartment was also conducted. Vimentin and α -SMA were used to define the HDFn component, while CD68 was utilised for the macrophages. CD68 positive cells displayed a circular morphology and were often found in clusters. The U937 cells were typically localised to the lower half of the Alvetex® Scaffold, rather than having an equal distribution throughout the lamina propria. On the other hand, fibroblasts could be identified throughout the entirety of the lamina propria compartment with but were most prominent at the basolateral surface of the Alvetex® membrane. Fewer α -SMA positive cells were detected than vimentin positive suggesting that there were fewer activated fibroblasts or myofibroblasts present within the lamina propria than normal fibroblasts. However, the macrophage-like cells were also identified by the vimentin antibody making it difficult to compare the levels of the two stromal cell types.

Evidence of ECM deposition was also identified through immunofluorescent staining techniques. Collagens I, III, and fibronectin were also shown to be deposited within the Alvetex® membrane demonstrating that the presence of immune cells within the 3D co-culture system did not disrupt the endogenous production of ECM proteins.

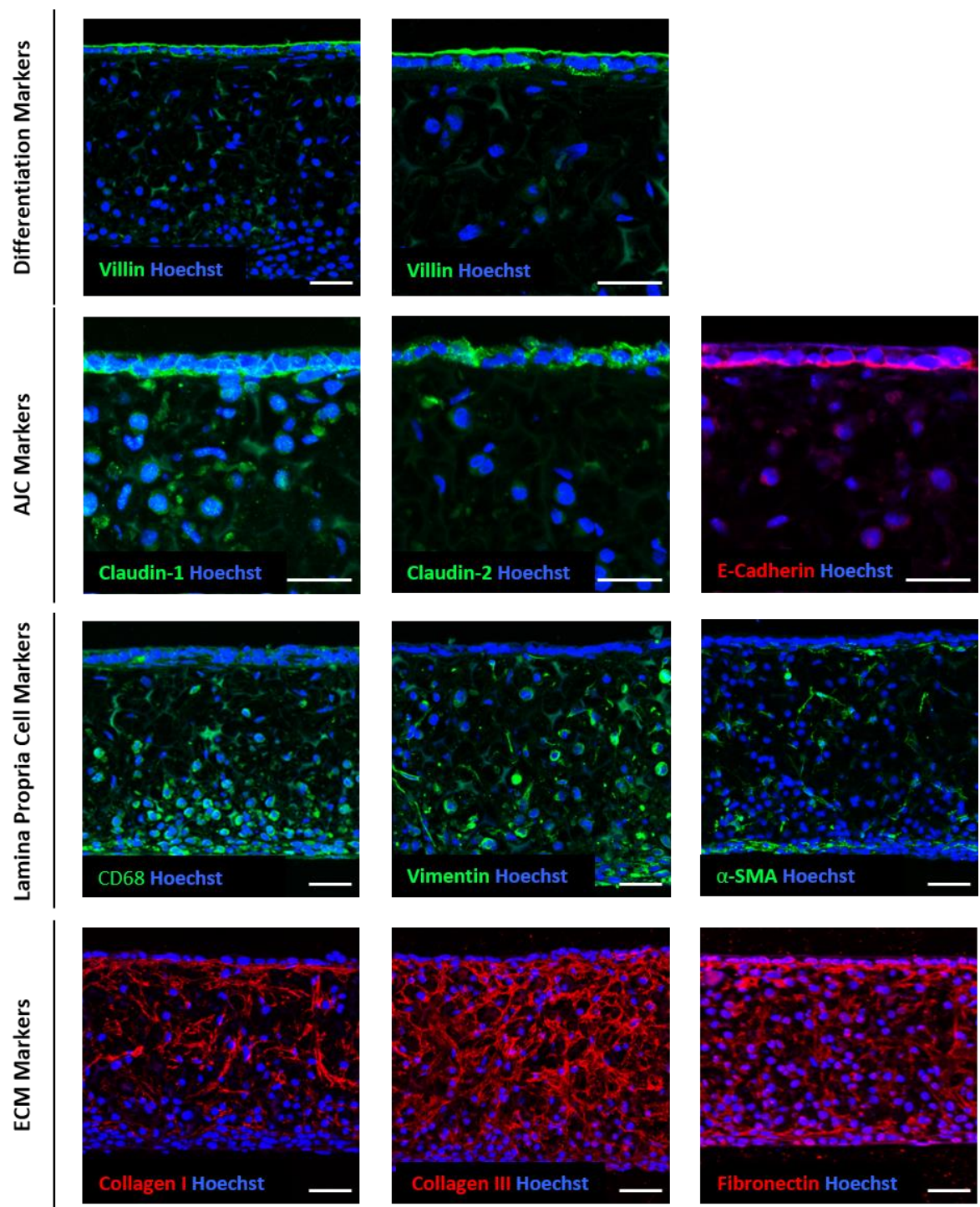


Figure 5.19. Immunofluorescent characterisation of the 3D triple co-culture. HDFn and U937 cells were seeded into Alvetex® Scaffold membranes to create an immune-competent lamina propria foundation on which to build an epithelium. Caco-2 cells were seeded onto this foundation and maintained in complete DMEM for a further 21 days. Models were then harvested for wax embedding and representative immunofluorescent images are depicted. Models were immunolabelled for the differentiation marker – vimentin; the AJC markers - claudin-1, claudin-2, and E-cadherin; lamina propria cell markers - CD68, vimentin and α-SMA; and the ECM markers - collagen I, III and fibronectin. Models were counterstained with Hoechst to visualise nuclei. Images shown are representative of 9 models. Scale bars: 50 μm.

A brief comparison of the triple co-culture and the Caco-2 HDFn co structure was also carried out through immunostaining. One marker representing the three main constituents common to both 3D co-cultures was selected: E-cadherin was chosen as a marker for the epithelium, vimentin was selected for fibroblasts, and collagen III was the final marker distinguishing the ECM. Representative immunofluorescent images are depicted in Figure 5.20. E-cadherin clearly defined the lateral membrane of adjacent epithelial cells in both cultures, yet the cells in the Caco-2 HDFn co-culture system demonstrated a stronger polarisation and had a highly elongated and columnar morphology. The epithelium in the triple co-culture also had a columnar morphology but this is not as obvious as in the previous model. The existence of E-cadherin across the monolayer in both models provides evidence that an intact epithelial barrier has formed.

Vimentin immunostaining highlighted the difference in fibroblast localisation between the two models. Regarding the Caco-2 HDFn co-culture, fibroblasts were evident throughout the membrane but displayed the strongest positive vimentin staining along the edges of the scaffold, indicating that the fibroblasts form layers at these locations. However, in the triple co-culture this layering effect was diminished, particularly at the apical surface of the membrane adjacent to the epithelium. This reduced layering could potentially impact the ability of the Caco-2 cells to polarise and may explain why the cells do not always have a distinctive columnar phenotype in the triple co-culture. On the other hand, no differences in the ability of HDFn cells to secrete ECM was detected, large deposits of collagen III could be seen through the whole model.

Additionally, model function was compared across the two different co-cultures. This was initially done through assessment of TEER following epithelial monolayer differentiation. A small reduction in TEER occurred when cells were cultured with differentiated U937 macrophages present in the lamina propria, though this was not significant (Figure 5.21A). A further functional assessment was performed through a lucifer yellow transport assay in the absorptive apical-basolateral direction, which also indicated that there were no differences in the function of the two culture systems and that the epithelial barrier retained a high level of integrity following the introduction of U937 macrophages to the *in vitro* model (Figure 5.21B). Combined, the structural and functional analyses conducted revealed no major difference to the structure and function of the epithelial monolayers of the Caco-2 HDFn and triple co-culture systems.

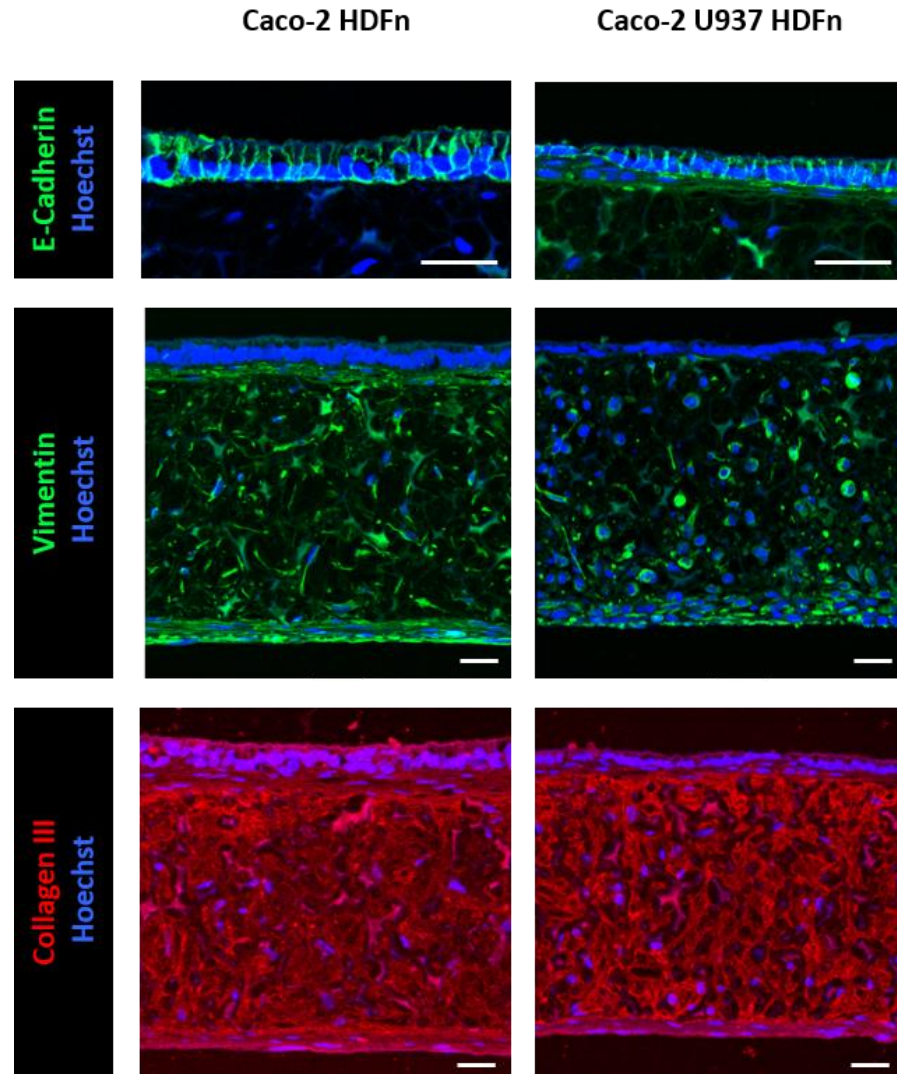


Figure 5.20. Comparison of the structural characteristics of the Caco-2 HDFn and the Caco-2 U937 HDFn 3D co-cultures. Caco-2 HDFn models were cultured as described in Chapter 3 and triple co-cultures were cultured as discussed above. To compare the structure of the epithelial and lamina propria compartments immunostaining for the junctional protein E-cadherin (green), the fibroblast marker vimentin (green) and the ECM protein collagen III (red) was performed. Nuclei were counterstained with Hoechst. Images shown are representative of 9 models. Scale bars: 50 μ m.

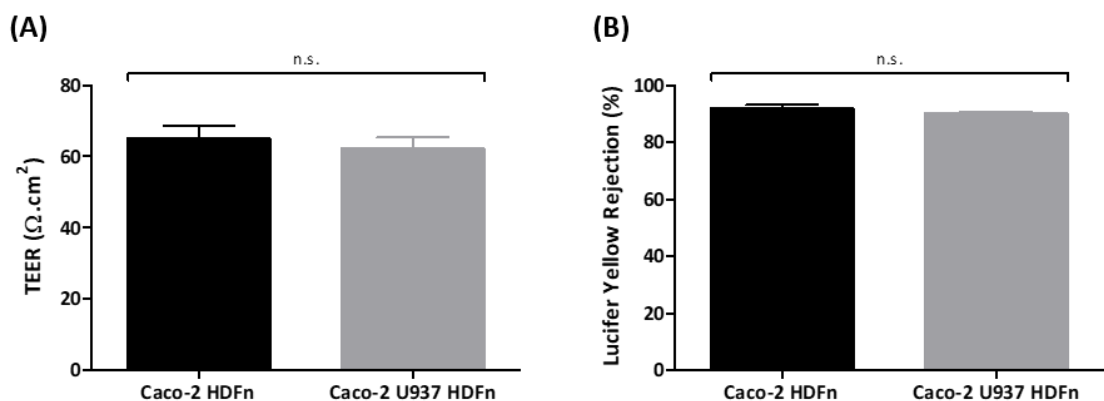
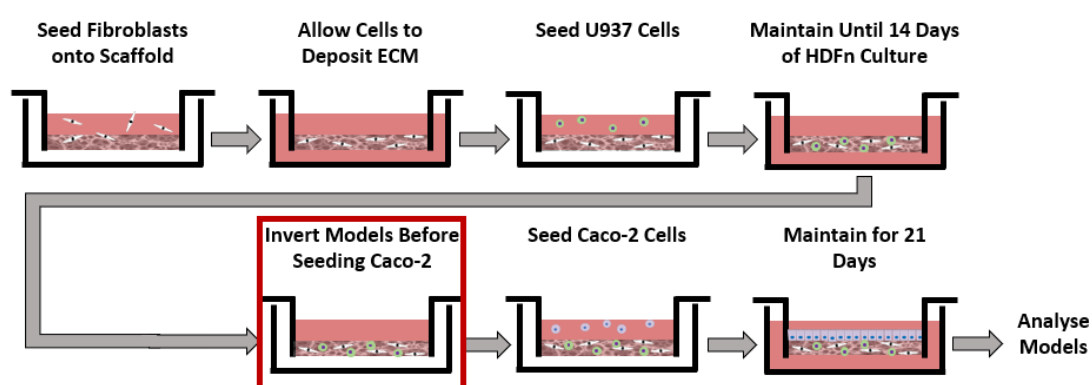


Figure 5.21. Comparison of the functional characteristics of the Caco-2 HDFn and the Caco-2 U937 HDFn 3D co-cultures. Caco-2 HDFn models were cultured as described in Chapter 3 and triple co-cultures were cultured as discussed above. Following 21-days of epithelial cell culture, TEER measurements were taken (A) and a lucifer yellow rejection assay was conducted (B). No significant functional differences were determined by these analyses. Data represent mean $\pm$ SEM, total $n=9$ from 3 independent experiments, each with 3 technical replicates. No statistically significant differences were identified by Student's t -test.

5.4.4.2. Inversion of *in vitro* model alters the distribution of U937 immune cells

One of the most discernible features of the triple co-culture was the location of the U937 macrophages. These immune cells were frequently located in the lower half of the Alvetex® membrane and were not immediately adjacent to the epithelium. Therefore, a pilot study was performed to determine whether the localisation of these U937 cells could be altered through physical inversion of the Alvetex® membrane. Alvetex® Scaffold has the unique ability to be unclipped from its surrounding plastic, therefore models were unclipped, inverted, and reinserted into the plastic supports at two time points during cell culture. The first time point selected was immediately prior to epithelial cell seeding, while the second time point was at day 14 of Caco-2 cell culture (Figure 5.22).

(A)



(B)

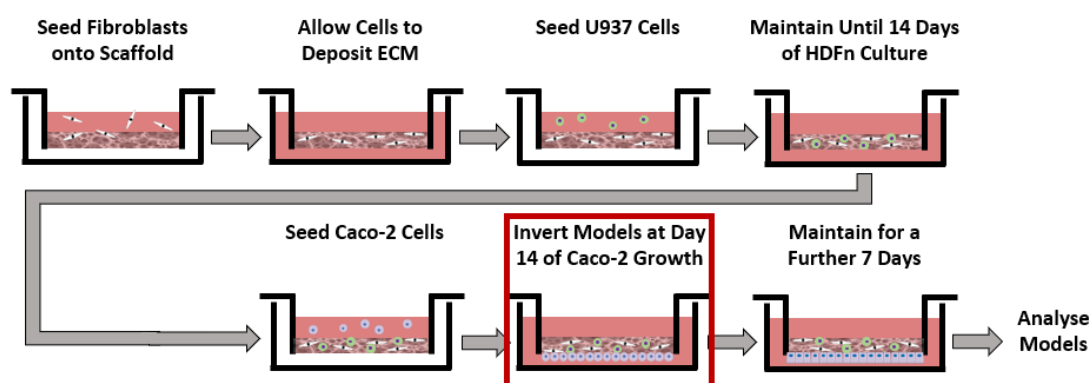


Figure 5.22. Schematic protocol detailing the model inversion processes of the triple co-culture. Fibroblast cells were seeded onto Alvetex® Scaffold and allowed to deposit ECM for a minimum of 10 days before differentiated U937 cells were trypsinised and seeded onto the model, culture then proceeded until day 14. Models could then be unclipped and inverted immediately prior to Caco-2 cell seeding (A) or inverted 14 days after Caco-2 cell seeding (B). Cultures were then harvested for wax embedding and histology 21 days after Caco-2 cells were initially seeded.

H&E staining of the resultant inverted triple co-cultures is shown in Figure 5.23. In all models, the epithelial monolayer was well polarised with columnar cells and basally located nuclei. In control models where no inversion event occurred, few U937 macrophages are visible, those that are present are primarily located in the basolateral section of the lamina propria, with some individual cells located in the upper segment. Contrastingly, in triple co-cultures where the model was inverted displayed greater numbers of immune cells present overall and more cells were present in the apical section of the lamina propria. This was especially evident when models had been inverted immediately before epithelial cell seeding where large clusters of cells are observed distributed throughout the lamina propria. Inverting the models during the culture period required manual handling of the Alvetex® membranes and increased the likelihood of physical damage to the *in vitro* cultures and also amplified the risk of infection. Thus, although the results showed a promising redistribution of the macrophage-like cells, the inversion of the models was not included in the finalised triple co-culture protocol.

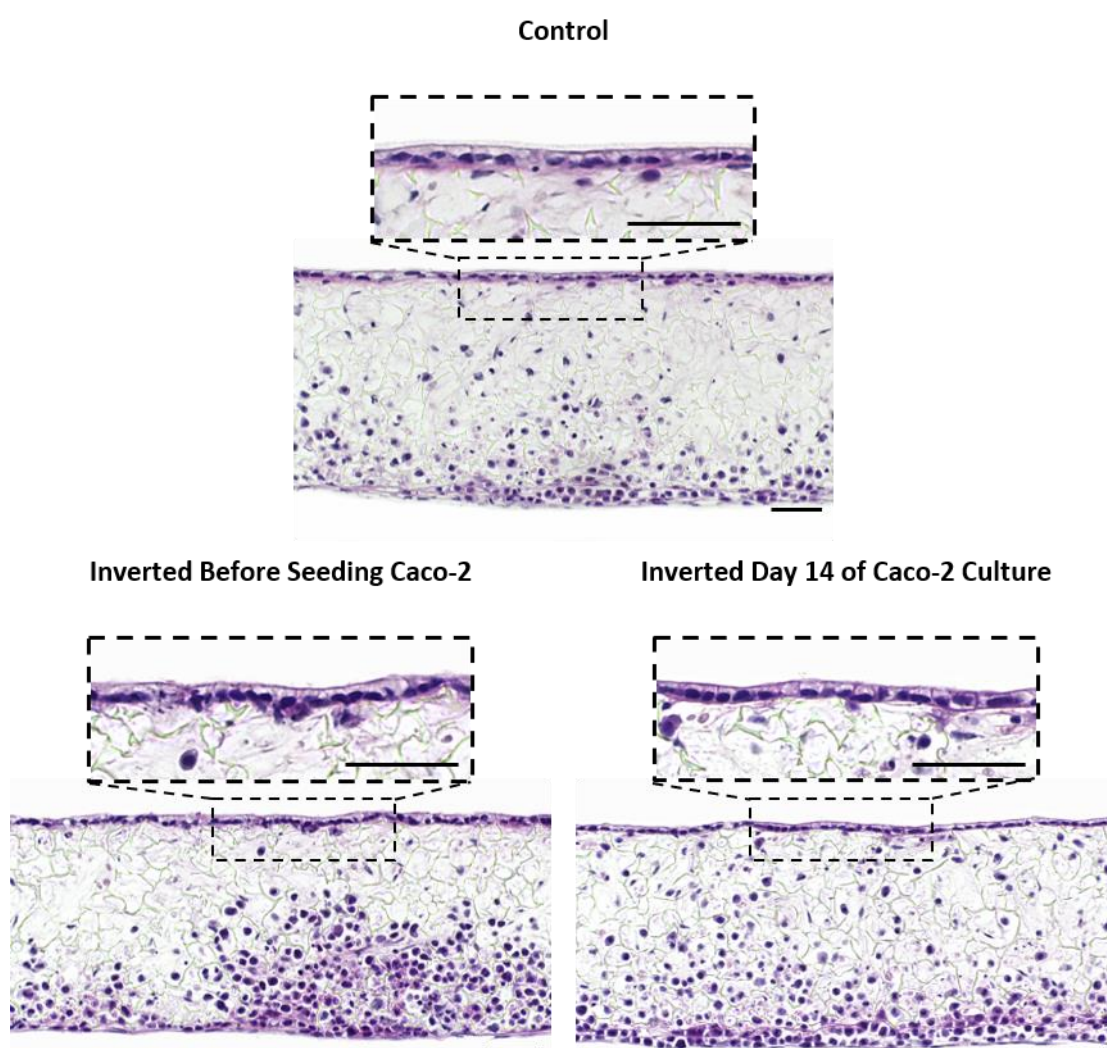


Figure 5.23. Inverting the 3D triple co-cultures alters the distribution of U937 macrophages. Triple co-cultures with HDFn, U937 and Caco-2 cells were cultured as normal or were inverted according to the protocol outlined in Figure 5.21 either before Caco-2 cell seeding or at day 14 of Caco-2 cell culture. Models were fixed and processed for H&E staining. Images shown are representative of 3 models. Scale bars: 50 μ m.

5.4.5. Summary of Experimental Findings

This chapter has focused on the development of two *in vitro* models of the intestine that contain an immunocyte cell lineage. U937 cells were selected for this purpose as they are known to differentiate into macrophages and respond to proinflammatory stimuli. The main findings are summarised below:

- U937 cells can be differentiated into adherent macrophage-like cells by incubation with 100 ng/mL PMA for 48 hours.
- Differentiated U937 macrophages can be indirectly co-cultured with Caco-2 Transwell® monolayers without negatively affecting the structure or function of the epithelium.
- Differentiated U937 macrophages can be incorporated into the lamina propria compartment and co-cultured with fibroblasts without inhibiting the production of ECM.
- A Caco-2 monolayer can be differentiated on the surface of a lamina propria compartment containing differentiated macrophages without damaging epithelial structure or function.

5.5. Discussion

Previous chapters have sought to establish co-culture models of the intestinal mucosa utilising intestinal epithelial cells and stromal fibroblasts, representing the epithelial barrier and lamina propria, respectively. However, in addition to fibroblasts, the lamina propria of the intestine is home to numerous other cell lineages including immune cells; 10-20 % of the mononuclear cells residing within the lamina propria are macrophages (515). Thus, this chapter set out to optimise the development of an *in vitro* intestinal mucosa tissue construct that incorporating macrophages, fibroblasts, and absorptive enterocytes.

5.5.1. U937 Cells are Differentiated into Macrophages upon Treatment with PMA

U937 cells are the most widely utilised *in vitro* model of macrophages. They were originally isolated from a 37 year old Caucasian male with diffuse histiocytic lymphoma, specifically from a pleural effusion where excess fluid had built-up between the layers of pleura membranes outside of the lungs (500). Numerous protocols have been developed to induce the differentiation of U937 cells into macrophages, including adding DMSO to the culture media for 4 days, 1,25-dihydroxyvitamin D₃ for 2 days and 200 IU/mL IFN- γ for 2 days (503,509,516). However the most common technique for inducing differentiating U937 cells is treatment with PMA, though the concentration and length of PMA treatment vary from 5-100 ng/mL PMA and from 24-96 hours (504,516–518). Treatment with PMA for 48 hours, however, did appear to be the most common incubation time and additional studies have identified that PMA concentration, rather than time, had a greater significant impact on the expression of macrophage markers (517). Therefore, 48 hours of PMA treatment was selected as the most appropriate incubation period and compound to induce U937 cells to differentiate into M0 macrophages, however the protocol required optimisation of the PMA concentration.

Two different concentrations of PMA were selected for use in this study 10 ng/mL and 100 ng/mL. Both concentrations were able to trigger U937 suspension cells to become adherent and form characteristic “clumps” on the tissue culture plastic. CD11b is a characteristic macrophage marker that has previously been demonstrated to increase in expression in differentiated macrophage cells (518). This was in agreement with the present study where flow cytometry analysis revealed that both 10 and 100 ng/mL PMA induced higher numbers of cells to express the marker; in addition, CD11b fluorescence intensity also increased when compared to untreated control cells that remained undifferentiated. U937 cells were also assessed for the differentiation marker CD14 which functions as a co-receptor in Toll-like receptor signalling pathways (510). CD14 has also been found to increase in U937 cells differentiated with both 1,25-dihydroxyvitamin D₃ and DMSO and is a crucial mediator of LPS signalling, thus was considered an important marker to assess. Similar to CD11b expression and fluorescence intensity, CD14 increased upon treatment with PMA irrespective of the concentration (519,520). Two additional markers were also found to increase with both PMA concentrations: CD68, a macrophage marker found to be induced in PMA-treated U937s through activation of the RhoA/ROCK signalling pathway, and CD15, a key protein involved in phagocytosis (521,522). No discernible differences were identified in the CD marker expression profile in U937 cells between 10 and 100 ng/mL PMA. However, when comparing the phagocytic ability of the cells through engulfment of

fluorescently labelled *E.coli* particles, U937 cells treated with 100 ng/mL PMA had marginally greater internalisation. U937 cells differentiated with PMA have also been shown to phagocytose *Mycobacterium tuberculosis* through TEM analysis, where the bacteria can be observed in membrane-bound vesicles (516). The PMA concentration that was therefore chosen for future studies was 100 ng/mL.

Researchers have demonstrated that the M0 macrophages differentiated from U937 monocytes can be further polarised into classical M1 proinflammatory macrophages or into alternative M2 anti-inflammatory macrophages. M1 subsets can be created through additional treatments of IFN- γ and LPS, while addition of the cytokines IL-4 and IL-13 can induce an M2 phenotype (523). The ability of U937 macrophages to further polarise was not assessed here as the co-cultures being developed were not intended to automatically generate an immune response and no reduction in epithelial barrier function was desired, though this will be the focus of Chapter 6.

Although differentiated U937 cells are widely used as *in vitro* models of macrophage function, it is important to note that the cells are of malignant origin, as such the functional properties of the cells may differ to those isolated from PBMCs. PBMCs are typically used as a whole range of cells though individual subsets such as dendritic cells and macrophages can be isolated from the suspension and have been used in *in vitro* co-cultures with Caco-2 cells to induce controlled (485). Cell subsets isolated from PBMCs are advantageous to use as they are not of cancerous origin and therefore should function as normal blood immune cells. Nevertheless, U937 macrophages are highly reproducible in comparison to PBMC isolates and are suitable substitutes for primary tissue resident macrophages that have yet to be successfully replicated due to their reproducibility (524).

5.5.2. U937 Macrophages do not affect Epithelial Barrier Structure or Function

The optimised U937 differentiation protocol described in this chapter induces a non-activated macrophage phenotype in the cells. The uncommitted state of the macrophages was preferred for use in model development as no harmful effects on epithelial barrier structure or function were desired; initial models were developed to be healthy, immune-competent cultures. Primary studies were focused on a Transwell®-based co-culture of Caco-2 monolayers and differentiated U937 cells. Comparing the structure of the epithelium in the presence or absence of U937 macrophages did not highlight any major differences in the composition of the apical junctional complex or the overall morphology of the monolayer. Caco-2 epithelial cultures presented as an intact monolayer under both culture conditions though a small degree of change to the enterocyte cell shape was noted whereby monolayers cultured in the presence of macrophages had a slightly more columnar phenotype. Similarly, no significant alterations in the barrier properties and function were identified between the culture set ups indicating that a “healthy” epithelial-immune cell co-culture system had been developed and could then be used in further experimental studies with an inflammatory stimulus. In addition, no adverse effects of the macrophages on the epithelial barrier were detected in the triple co-culture.

Although many studies have focused on the development of an immune-competent intestinal model, few have focused on co-culture of Caco-2 and U937 cells specifically. Those that have, employed a range of different methods to create *in vitro* intestinal models. For example, Yoshimoto et al., followed a protocol similar to the one described in this chapter wherein Transwell® Caco-2 monolayers were introduced to differentiated U937 macrophages at the base of the well, however unlike the results presented within, the research group found that TEER increased in co-cultures compared to monocultures (302). Other *in vitro* models developed using Transwell® membranes have instead differentiated U937 cells on the cell culture membranes and subsequently transferred these inserts into wells with Caco-2 or HT29 epithelial cells adhered to the base of the well (525). No differences in epithelial alkaline phosphatase activity were found under normal co-culture conditions, suggesting that the epithelium was intact and functioning normally as found in the experiments described above; however when U937s were directed to the M2 phenotype, a significant reduction in alkaline phosphatase activity was identified (525). Additionally, when undifferentiated monocytes were cultured with Caco-2 monolayers, epithelial cells retained high viability as determined by a trypan blue exclusion assay (310). Further co-culture models of Caco-2 and differentiated U937 M0 macrophages have been developed using microfluidic systems. Unlike the previously described study, Caco-2 cells exhibited a reduction in viability in co-culture when compared to monoculture though this effect could be diminished through the introduction of perfusion (300); highlighting the need to incorporate other aspects of intestinal biology into *in vitro* models

Though the Transwell® model established in this chapter revealed no adverse effects of U937 macrophages on the epithelium, contradictory evidence exists regarding the impact of THP-1 macrophages on the epithelium. Early Caco-2 THP-1 studies revealed that in the presence of IEC cells, THP-1 cells had enhanced phagocytic capabilities, though these cells were retained as a monocyte suspension rather than as differentiated macrophages (515). When Caco-2 monolayers were introduced to differentiated THP-1 macrophages, Satsu et al., recorded a reduction in TEER suggesting that epithelial barrier integrity had been depleted. Furthermore, an increase in lactate dehydrogenase (LDH) was present in co-cultures indicative of cell death; this was further corroborated by microscopic analysis of nuclei which showed signs of necrotic cell death (512,526). Disruption to the epithelium could, however, be reduced upon addition of anti-TNF α antibodies to the culture similar to IBD therapies suggesting that this could be an effective co-culture to mimic the TNF- α /IBD immune response (512,526). Conversely, other studies have demonstrated that co-culture of Caco-2 and THP-1 macrophages caused no reduction in TEER when compared to monocultures and co-cultures were deemed “stable” (301). Irrespective of the positive/negative effects of THP-1 macrophages on the intestine epithelial monolayers, these co-cultures are the most widely used to examine cellular communication between the intestine macrophages and epithelium in addition to investigating the inflammatory responses and protective effects of putative IBD therapies (301,513,527–529). However, the Caco-2 U937 co-culture provides an alternative platform to study inflammation and can be used to corroborate findings from Caco-2 THP-1 co-culture and hence reduce the numbers of animals used in the later stages of drug testing.

5.5.3. 3D Co-Culture Techniques have been used to Establish an Immune-Competent *In Vitro* Intestinal Model

The overarching aim of this chapter was to develop a 3D co-culture of the intestinal mucosa comprising of an epithelial monolayer and a lamina propria with fibroblasts, endogenous ECM, and immune cells. Following development of a Transwell®-based epithelial co-culture, experiments focused on incorporating U937 macrophages into the lamina propria previously developed in Chapter 3. Co-culture of HDFn and U937 macrophages did not affect the ability of fibroblasts to deposit endogenous ECM, nor was the presence of α -SMA positive cells affected. U937 macrophages have been proposed to promote a fibrotic phenotype in *in vitro* cultured fibroblasts due to increasing hyaluronic acid production and fibroblast contractility through the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signalling pathway (530). No evidence of fibrosis was found in the immune-competent lamina propria, and this was not a desirable phenotype as fibrosis can have consequences on the intestinal epithelium and the purpose of model development was to create a healthy, immune-competent of the intestinal mucosa.

In vitro models comprising of fibroblasts and macrophages are useful for assessing wound healing processes and monitoring migration, though this would prove a difficult task using the model developed within, due to the cells being contained in an inert polystyrene scaffold. Thus, the use of this fibroblast-U937 macrophage lamina propria model was to serve as a scaffold on which to build the intestinal epithelial barrier. Caco-2 cells successfully differentiated into an epithelial monolayer expressing the appropriate differentiation and junctional markers on the surface of the immune competent lamina propria. Additionally, no changes in TEER or lucifer yellow rejection were found, confirming that an intact barrier had been formed.

Various other techniques have been utilised to generate 3D models of the intestine that contain immune competent cells. Two different immune cell lineages, THP-1 and MUTZ3 have successfully been used to represent macrophages and dendritic cells, respectively (531,532). These cells were seeded into collagen gels with a Caco-2 monolayer lying on the apical surface to form a 3D intestinal mucosa. Ultrastructural analysis revealed an intact and differentiated epithelium with evidence of a microvilli brush border while TEER was comparable to Caco-2 monocultures suggesting that immune cells had no adverse consequences on the enterocytes. Treatment of the model with nanoparticle inflammatory stimuli resulted in the initiation of an immune response indicating the potential uses of this *in vitro* system for studying the toxic and inflammatory effects of nanoparticles (531). Another immune-competent model of the intestine has also been developed using macrophages and dendritic cells, though these were isolated from PBMCs (485). Immune cells were similarly embedded in a collagen gel coating the Transwell® membrane with Caco-2 enterocytes on the surface, which remained able to form a monolayer in the presence of immune cells, with evidence of tight junction formation. Cultures were then able to respond to inflammatory stimuli and have subsequently been used to investigate nanoparticle transport to identify optimal drug delivery systems under inflamed intestinal conditions (485,486).

However, the above models did not contain a fibroblast component and the only ECM component present within the models was from a rat-tail collagen gel. Thus, other studies have attempted to incorporate a fibroblast lineage into their cultures allowing for more cell-cell and cell-ECM interactions to be investigated under normal and inflamed conditions. A recent model has successfully embedded L929 murine fibroblasts isolated from subcutaneous areolar adipose tissue and U937 cells in a collagen gel on Transwell® membranes with a Caco-2 epithelium on the surface (311). However, the U937 cells utilised in this study were not differentiated into macrophages and instead remained as monocytes. This would allow for analysis of the effect of proinflammatory stimuli on the differentiation and polarisation of the monocytes. Furthermore, this *in vitro* model mimicking the gut wall has subsequently been used to investigate the inflammatory effect of dietary proteins, specifically wheat gliadins and glutenins (310,311). Another model has been developed that similarly incorporated fibroblasts and immunocytes in a collagen gel, though mouse embryonic fibroblasts and PMA treated THP-1 macrophages were the cell lineages selected in this study (313). In addition to Caco-2 enterocytes, this model also contained HT29-MTX goblet-like cells in the epithelium at a ratio of 90:10. A more *in vivo*-like phenotype was observed in the epithelium of the culture, with a reduced TEER and P-gp expression, however the impact of the macrophages was not discussed or investigated (313). A significant limitation of these 3D *in vitro* cultures containing fibroblasts is the origin of the fibroblasts - as they are both murine derived, they will therefore exhibit species differences to humans. Although the model presented within does not contain fibroblasts of intestinal origin, it is entirely humanised and does not contain exogenous collagen, thus could potentially be used to understand the signalling mechanisms and interactions occurring between human fibroblasts, macrophages, and enterocytes.

Chapter 4 discussed the importance of including a goblet-cell component to produce a mucus lining in *in vitro* intestinal models. A co-culture of Caco-2, HT29-MTX and THP-1 cells has been developed by Calatayud et al. Unusually, the THP-1 cells were embedded in a collagen gel as monocytes and were differentiated with PMA treatment during the epithelial cell culture on the surface of the collagen gel. The researchers discovered that PMA differentiation reduced both the TEER and ability of the epithelium to exclude lucifer yellow, however it is not certain whether this was due to the differentiated macrophages or as a direct consequence of PMA acting on the epithelium (533). Nevertheless, this model has also been used to establish an “ecosystem” model of the intestine through inclusion of a synthetic microbial consortium of 8 commensal bacterial strains, allowing for the host-microbe interactions to be studied in detail. HT29 spheroids have additionally been co-cultured with PMA-treated U937 macrophages in a bioreactor and utilised in colonisation studies of *Salmonella* wherein macrophages were demonstrated to display anti-microbial properties (534). Future experiments could therefore include HT29-MTX cells in the indirect Transwell® Caco-2 U937 co-culture and in the direct 3D co-culture models developed in this chapter.

Although an immune-competent triple co-culture has been established, the models suffered from a loss of reproducibility when compared to the co-cultures developed within the previous chapters, with 1 in 7 or 8 models failing to produce a successful epithelial monolayer on the surface of the lamina propria. This resulted in additional models having to be cultured to ensure that there were enough successful models to conduct

downstream transport and future model stimulation experiments. Additionally, high numbers of U937 cells would fall through the Alvetex® Scaffold membrane, thus required the cell culture plate holding the insert to be changed more frequently to prevent a build-up of cells on the base of the well.

5.6. Conclusions

Chapter 5 aimed to develop an immune-competent triple co-culture model of the intestinal mucosa. Existing models have primarily focused on THP-1 derived macrophages that have previously been demonstrated to have adverse effects on epithelial barrier structure and function. A similar immunocyte cell line, U937, can also be differentiated into macrophages and has been utilised in *in vitro* co-cultures. It was hypothesised that U937 macrophages could be incorporated into the Transwell® and Alvetex® models without disrupting the epithelial barrier, under normal “healthy” conditions.

Differentiation of U937 cells into M0 inactive macrophages has been accomplished through several different protocols. Therefore, initial experiments focused on optimising the concentration of PMA used to direct differentiation; 100 ng/mL was selected due to cells expressing high levels of macrophage markers and having phagocytic capabilities. Differentiated macrophages were then utilised in an epithelial co-culture with Caco-2 monolayers on Transwell® inserts. No alterations to the epithelial structure were identified, nor were any differences found between monocultures and co-cultures regarding barrier function, suggesting a healthy immune-competent co-culture had been developed.

Further experiments then demonstrated that differentiated U937 cells could also be incorporated into the lamina propria compartment and an epithelial monolayer could be established on the surface. Again, no differences were identified in epithelial structure or function when compared to a Caco-2 HDFn co-culture. Additionally, fibroblasts retained their ability to deposit endogenous ECM without showing evidence of becoming fibrotic in nature. Future studies could be conducted to determine the impact of mucus-producing goblet-like HT29-MTX cells on the indirect and direct, 3D co-culture systems.

Overall, this chapter has successfully developed an immune-competent triple co-culture that will be used to investigate the effects of proinflammatory stimuli on the structure and function of the epithelium in Chapter 6. The following chapter will additionally seek to characterise the immune response and identify similarities to inflammatory bowel diseases.

Chapter 6: Inducing an Inflammatory Response in the Immune-Competent 3D Intestinal Co-Culture Model

6.1. Introduction

The development of *in vitro* models that mimic the structure and function of the intestine mucosa is of vital importance to provide physiologically relevant tools to carry out investigations into the healthy and diseased states of the intestine. To best recapitulate the inflamed mucosa, incorporation of immune cells into *in vitro* models is extremely desirable as such cells are responsible for driving and exacerbating inflammatory responses. Therefore, this chapter will focus on introducing inflammatory stimuli to immune-competent *in vitro* models of the intestinal mucosa that were previously developed in Chapter 5 and characterise the resulting responses elicited within the models.

6.1.1. The Inflamed Intestinal Mucosa

Maintenance of homeostasis within the intestines is essential due to the high microorganism load present within the intestinal lumen that necessitates a highly regulated immune response and the establishment of tolerance. Disruptions to homeostasis can result in chronic inflammation that can contribute to inflammatory bowel diseases (IBD): ulcerative colitis (UC) and Crohn's disease (CD). IBD has an increasing worldwide prevalence and diseases present with complex, multifactorial aetiology and pathologies, requiring further investigations into the cellular interactions underpinning the diseases (93,535).

Both UC and CD exhibit many commonalities between their phenotypes yet also demonstrate numerous differences. A major characteristic of both IBD states is a reduction in the integrity of the epithelial barrier resulting in increased luminal antigen uptake that can further exacerbate inflammation via the stimulation of excessive immune cell migration and production of proinflammatory cytokines (536). Major differences in the disease pathologies, however, reside in the location of the affected tissues. CD can affect any part of the GI tract, yet it is most prevalent within the ileum of the small intestine, while UC is restricted to the colonic mucosa. Additionally, CD can be present in all four layers of the mucosal tissues, but UC is again more confined and only affects the innermost mucosal layer comprising of the epithelium, lamina propria and muscularis mucosae. However, the more restrictive nature does not necessarily correlate with disease severity as the entirety of the large intestine is affected during active states of UC whereas in CD, areas of active inflammation appear in patches and can be surrounded by non-involved tissue.

CD and UC both present with chronic inflammation that not only damages the epithelial barrier but induces an increased deposition of ECM leading to fibrosis of the mucosal tissues. Following repeated bouts of chronic inflammation, fibrosis can result in a stricturing effect of the intestinal lumen which becomes narrower, preventing the passage of luminal contents (95,537). This is particularly evident in CD. Furthermore, not only are epithelial and stromal cells implicated in the pathology of IBD, but numerous immune cells are also heavily involved and are the primary drivers of intestinal inflammation that aggravate the chronic effects of the disease. The involvement of immune cells in IBD and their secreted cytokines are discussed further in Chapter 1 (Section 1.4.5), only the role of macrophages will be further examined within this chapter as macrophages are the most dominant immune cell lineage within the intestinal mucosa and are associated with inflammatory diseases including IBD. Additionally, only macrophage-like cells have been incorporated into the intestinal mucosal models developed in the previous chapter.

6.1.2. Macrophages in the Intestine

Macrophages comprise the largest population of tissue-resident immune cells within the entire body. Under normal homeostatic conditions, macrophages are present in a state of anergy where they are termed M0. Anergic macrophages display a non-inflammatory profile when exposed to microbial stimuli to establish tolerance to the commensal microbiome. Additionally mucosal macrophages actively maintain the integrity of the epithelial barrier under homeostatic conditions by stimulating the proliferation of epithelial progenitor cells via the secretion of prostaglandin E₂ (538,539). In addition to maintaining healthy homeostatic conditions, macrophages are implicated in the development of IBD.

6.1.2.1. Classification of macrophages according to their activation status and function

Traditionally macrophages have been classified into two major subgroups once they are activated: classically M1 activated macrophages and alternatively M2 activated macrophages. M1 macrophages are differentiated in response to TNF- α , IFN- γ or LPS, either alone or in combination, while M2 macrophages are polarised following exposure to the anti-inflammatory cytokines IL-4 or IL-13 which are considered to have redundant functions (540–543). However, further classification systems have recently emerged focusing on grouping macrophages according to their function under homeostatic conditions: immune regulation, host defence and wound healing, yet there is also some overlap between the roles of macrophages (544,545). Within this paradigm, M1 macrophages largely fall under the host defence category due to their microbicidal functions, while M2 macrophages have roles related to wound healing. The final macrophage lineage are known as regulatory macrophages due to the production of high levels of the anti-inflammatory cytokine, IL-10, that has immunosuppressive roles and is crucial for the establishment of tolerance within the intestine. Although a new categorisation system of macrophage polarisation has been established, M1 and M2 macrophages are still widely referred to within the literature and there remains debate regarding the classification of intestinal lamina propria macrophages (165). Thus, for the purposes of this study and literature review regarding IBD, macrophages will be deemed classically activated M1 macrophages to indicate their role in the

proinflammatory response while anti-inflammatory macrophages will be referred to as alternatively activated M2 macrophages.

6.1.2.2. The balance of activated macrophages in inflammatory bowel diseases

Large scale macrophage infiltration into the lamina propria of patients with active IBD is one of the main causes of chronic inflammation. Interestingly CD was originally thought to be caused by a deficiency in macrophages, however patients with CD were considered to exhibit a Th1 immune response that is typically provoked by the secretion of an array of proinflammatory cytokines from M1 macrophages including TNF- α , IL-1 β , IL-6, IL-12, and IL-18 (546,547). Conversely, UC was traditionally associated with a Th2 immune response induced by M2 macrophages. However recent theories are moving away from this paradigm, in part due to the discovery of novel T lymphocytes such as Th17 and regulatory T cells (discussed further in Chapter 1).

In general, although both M1 and M2 macrophage numbers are elevated in IBD inflamed mucosa, proinflammatory M1 macrophages heavily outweigh the anti-inflammatory M2 macrophages (170). When present in the inflamed tissue, M1 macrophages secrete proinflammatory cytokines in addition to producing reactive oxygen species and proteases that degrade the ECM of the lamina propria allowing the infiltration of further immune cells (547). This imbalance was further supported by studies utilising DSS-induced colitis murine models where M1 macrophages were discovered to be amplified while M2 macrophages were reduced resulting in greater disease progression within the models (548). Additionally, YAP (yes-associated protein) of the Hippo signalling pathway promotes epithelial proliferation to alleviate some of the symptoms of IBD, yet alternative roles of YAP in IBD aggravation have also been demonstrated. By utilising murine models of IBD, YAP was shown to promote an M1 phenotype in macrophages while simultaneously inhibiting the polarisation of M2 macrophages (549).

The imbalance of macrophage subtypes has therefore been subject to investigations to determine whether macrophage cells are suitable therapeutic targets to treat IBD via correction of the disrupted ratio between M1:M2 cells. Studies to test such novel compounds have been conducted using both *in vivo* animal models and *in vitro* monocytes and macrophages. For example, when monocytes isolated from CD patients were treated with a variant of vitamin D they displayed enhanced polarisation of M2 vs M1 macrophages while simultaneously reducing the secretion of proinflammatory cytokines following stimulation with LPS (550). Similarly, the chemical compound, baicalin, alleviated DSS-induced colitis in a murine model by increasing the polarisation of M2 macrophages (551). These studies highlight the importance of maintaining a tight regulation of the balance of macrophage subtypes within the intestinal mucosa to prevent chronic diseases.

Therefore, to mimic aspects of the pathologies of IBD most accurately within the *in vitro* models used in this work presented within, differentiated U937 cells should be polarised towards M1 macrophages. This will be achieved by stimulating *in vitro* models with known M1 inducers including high concentrations of LPS alone, IFN- γ and LPS and TNF- α .

6.1.3. *In Vitro* Models of Intestinal Inflammation

Modelling IBD frequently requires the use of one of five murine models that are outlined in Chapter 1, Table 1.2. However, these models only recapitulate certain aspects of the disease pathology observed in humans and can only be used to model certain phenotypes. For example, the DSS colitis model is widely employed to mimic epithelial barrier dysfunction while the IL-10^{-/-} mouse model is utilised for investigations regarding immunoregulation and macrophage function. Due to these limitations of murine models, the most optimal method for investigating IBD is to use *ex vivo* tissue samples from patients with IBD, yet such tissue is short-lived, and availability is scarce. Therefore, *in vitro* models of the intestinal epithelium and mucosa can be used to study IBD in an effort to reduce animal usage and isolate the roles of signalling molecules on specific cell lineages.

6.1.3.1. Isolated cells from IBD patients are useful tools to study inflammatory bowel diseases

Immune cells isolated from patients with IBD provide useful models to study the cytokine profile and mechanisms underpinning disease pathology whilst distinguishing features unique to UC and CD. Studies using isolated T lymphocytes from the lamina propria of IBD patients revealed that T cells from the inflamed mucosa of CD patients exhibited increased IFN- γ secretion compared to control cells (552). Conversely, T cells from the inflamed UC lamina propria expressed higher levels of IL-5, supporting the original theory that CD manifested as a Th1-type immune response while UC displayed characteristics of a Th2 immune response (552).

Further investigations into immune cells isolated from IBD patients have sought to understand the mechanisms underpinning the action of specific cytokines and peptides. It was originally hypothesised that TNF- α was a cofactor for Th1 responses induced in mucosal cells of CD patients. TNF- α incubation of lamina propria mononuclear cells (LPMC) isolated from CD patients showed an increase in the production of IFN- γ , a Th1 cytokine, seeming to confirm this hypothesis (553). Conversely, data from peripheral blood mononuclear cells (PBMC) isolated from IBD patients demonstrated a reduction in IFN- γ compared to healthy donors following stimulation with a viral cytomegalovirus (CMV) peptide suggesting an impaired response to viral infection; this could explain why CMV induces colitis in some IBD patients (554). However, no differences were found in the cytokine production by mucosal T lymphocytes within the same study. Other studies utilising PBMC and LPMC have demonstrated that during active IBD tolerance to commensal bacteria is broken, yet it is re-established following disease remission (555).

Further studies have focused on investigating the effects of potential IBD treatments. For example, an analogue of vitamin D exerted an immunosuppressive effect on PBMCs isolated from CD patients via inhibition of proliferation and reduction of overall TNF- α and NF- κ B levels (556). Interestingly, in macrophage-depleted PBMC isolates from CD patients treated with vitamin D3, the proliferation of T cells was found to be elevated concurrent with increased IL-6 and IL-4 production (557). These results suggest that potential drug

candidates may elicit differential effects depending on the cells responding to the treatment and preclinical testing of such novel compounds should aim to investigate the responses of as many cell lineages as possible.

In addition to studies examining the role of various immune cells, patient-derived immune cells have recently provided useful information regarding the overall pathology of IBD. Immunological profiling studies of blood collected from UC patients has led to the discovery of two inflammatory conditions in the pathology of the disease. Immune cells including macrophages and monocytes were responsible for driving an acute inflammatory response followed by a later remodelling condition where NK cells, monocytes and TGF- β 1 secretion played larger roles (558). Furthermore, the study presented possible explanations for the increased stenosis and lumen narrowing observed in patients treated with the anti-TNF- α antibody, infliximab; as TNF- α production was identified in the acute stage of inflammation, suppression of TNF- α activity would therefore promote the remodelling phase of inflammation driving excessive ECM deposition. Additionally, the group postulated that anti-IL-13 antibody treatments, such as anrukizumab, may fail to reduce some symptoms in UC during the secondary remodelling inflammatory condition (558). It would therefore be interesting to conduct a similar study using cells derived from CD patients and determine whether there are multiple phases to the disease pathology.

6.1.3.2. The use of *in vitro* epithelial monocultures to understand mechanisms of inflammatory bowel disease

Though immune cells isolated from IBD patients have been demonstrated to be useful tools for investigating the inflammatory response, they lack cell lineages of epithelial origin, thus cannot be utilised to fully understand the impact of the epithelial barrier on disease initiation and progression. However, *in vitro* models of the intestinal epithelium have also been extensively used to investigate the impact of factors involved in IBD on epithelial structure and function.

6.1.3.2.1. Caco-2 monolayers and inflammatory bowel diseases

Caco-2 monolayers are the gold standard *in vitro* culture system to mimic healthy intestinal epithelial cell function. However, they can also be utilised to study aspects of inflammation via the introduction of noxious stimuli to their culture media and examining the effects on the structure and function of the epithelial barrier. Additionally, the production of proinflammatory cytokines in response to exposure to stimuli can be assessed.

Exposure of Caco-2 monolayers to cytokines and bacterial products thought to be involved in the acute inflammation phase of IBD including IFN- γ , TNF- α , IL-1 β and LPS, demonstrated that high concentrations of these molecules applied simultaneously were able to elicit an increase in epithelial permeability. In addition, the levels of downstream proinflammatory cytokines (IL-8 and IL-6) expressed by the Caco-2 monolayer were amplified (559). However the study highlighted the importance of IL-1 β in particular, indicating that IL-1 β should be the focus of future *in vivo* studies (559).

Furthermore, Caco-2 monolayers have been utilised to investigate the mechanisms underpinning the enhanced epithelial permeability observed in IBD. Application of TNF- α to Caco-2 monolayers revealed that TNF- α induces a reduction in epithelial barrier integrity due to disruption of the apical junctional complex; specifically, the protein occludin which was found to have reduced expression compared to control monolayers (560). Further studies then demonstrated that TNF- α induced the activation of myosin light chain kinase (MLCK) via the extracellular regulated protein kinase 1/2 (ERK1/2) signalling pathway resulting in a decrease in ZO-1 and increase in epithelial permeability, a finding that was also confirmed using mice (297,298). Anti-TNF- α therapy is a successful treatment to alleviate some of the symptoms of IBD and induce remission of the diseases, such studies utilising the Caco-2 monolayer could provide plausible explanations into the mechanisms of anti-TNF- α activity. Moreover, a novel anti-TNF- α antibody CT-P13 was demonstrated to have comparable roles to a widely used antibody, infliximab, on the Caco-2 monolayer indicating that the culture system has direct uses to test future IBD treatments (299).

In addition to being used to investigate the effects of proinflammatory stimuli that are present within the IBD mucosa, Caco-2 monolayers have been utilised to examine the mechanisms behind the DSS-induced colitis murine model of IBD as the reason behind the pathogenesis of DSS was not fully understood. The DSS model is primarily used to monitor epithelial barrier function, as such the Caco-2 monolayer was a suitable alternative *in vitro* model for further investigations. Initial studies determined that DSS induced cell cycle arrest within the Caco-2 monolayers and reduced the production of reactive oxygen species in addition to inducing ER stress as identified by markers including immunoglobulin-binding protein (BiP) and X-box binding protein (XBP1) (561,562). Interestingly, XBP1 loss has been found to induce basal activation of NF- κ B that propagates the proinflammatory response to various stimuli including TNF- α (12,123). Furthermore, TEER was reduced and trafficking of polarised enzymes, such as the apically expressed sucrase-isomaltase, was impaired. Together, these results demonstrated that DSS treatment perturbs ER homeostasis, cellular polarity, and membrane integrity resulting in an overall disruption to the barrier function (562).

6.1.3.2.2. Alternative intestinal epithelial monolayers to study aspects of inflammatory bowel diseases

Although Caco-2 epithelia are the primary monocultures used to model healthy and diseased intestinal functions, other epithelial cell lines have also been used to study the response of intestinal epithelial cells to proinflammatory stimuli. Like the Caco-2 monolayer, the action of TNF- α stimulation has been investigated using HT29 monolayers; TNF- α was found to impair epithelial barrier function, an effect which was further amplified by IFN- γ treatment (563). IFN- γ is known to enhance the expression of TNF- α receptors on cell membranes; following induction of the TNF- α receptors by IFN- γ , TNF- α was shown to reduce the number of tight junction strands and “kiss” sites while concomitantly increasing the permeability of the epithelium (307,564,565). Furthermore, the HT29 monolayer has been utilised to demonstrate the importance of the Th2 cytokine IL-13 in the pathology of UC; IL-13 was found to reduce TEER within the monolayers whereas an equivalent cytokine, IL-4 did not (566). Comparison of these monolayers to UC patient samples confirmed that the decrease in TEER was determined to be due to an increased level of apoptosis in combination with increased expression of claudin-2, a pore-forming tight junction protein (566). Interestingly, co-cultures of

Caco-2 and HT29-MTX cells maintained on hydrogel scaffolds have also been used to model IBD *in vitro*, the study identified that a combination of IL-1 β , TNF- α and hypoxia treatment were able to induce changes to the tight junctions, altered mucin secretion and reduced alkaline phosphatase activity across the epithelium – phenotypes that are often associated with IBD (320).

Alternatively, T84 cells have also been employed to understand inflammatory processes within the intestine. TNF- α application to T84 monolayers has been used as a comparison to *in vivo* studies investigating the uptake of endocytic antigens in CD. The study demonstrated that TNF- α resulted in enhanced endosomal uptake in the T84 monolayers supporting the theory that TNF- α has regulatory roles regarding antigen endosomal uptake in enterocytes (536). However, no alterations to TEER were evident within the T84 monolayer suggesting that the cells may not be the most useful to mimic barrier disruption. On the other hand, T84 monolayers have proven useful in investigating the role of bacteria in the pathogenesis of IBD, specifically the role of adherent-invasive *E. coli* (AIEC). Infection of T84 with AIEC resulted in a reduction in TEER and increased paracellular flux of FD10 accompanied by a redistribution of ZO-1 along with the formation of gaps in the monolayer (567). This study demonstrated that bacteria are linked to IBD via damage to the epithelial barrier to propagate disease pathogenesis.

6.1.3.3. Co-cultures of intestinal epithelial cells and immune cells to study inflammation

The models discussed above only utilise one cell lineage, to better understand the cellular and signalling interactions underpinning IBD, *in vitro* co-cultures that incorporate an epithelial lineage and one or more immune cell lineages offer a more complex and physiologically relevant model.

6.1.3.3.1. Co-cultures of epithelial and immunocyte to recapitulate inflammation *in vitro*

Indirect co-cultures of intestinal epithelial cells and immune cells of cancerous origin are widely used to study intestinal inflammation and crosstalk. Caco-2 cells are the most frequently used epithelial cell line in this regard, while macrophages differentiated from either U937, THP-1 or RAW 264.7 monocytes are also extensively used. TNF- α , a key proinflammatory cytokine in IBD that causes significant damage to the epithelium as discussed above; it is the subject of many antibody blocking therapies is primarily produced by macrophages. Therefore, co-culture with human macrophage lineages (not the murine RAW 264.7 cell line) will be primarily discussed within this section as they are also the lineage incorporated into the *in vitro* models developed in Chapter 5.

Initial studies of Caco-2 and THP-1 macrophages found that co-culture alone increased release of LDH - a marker of cell death, and decreased TEER within the monolayer (512). Furthermore, this epithelial damage could be suppressed by adding an anti-TNF- α antibody to the culture medium suggesting that the presence of THP-1 cells alone was sufficient to induce IBD-related phenotypes within the epithelium (512,526). Epithelial damage and IL-8 release induced by co-culture of Caco-2 and THP-1 macrophages could also be prevented by the addition of dietary taurine to the culture medium; similar results were found in DSS-induced

colitis mice indicating potential therapeutic effects of the compound (527). Though they have been proven to have adverse effects on the Caco-2 epithelium, THP-1 cells remain a popular choice for co-culture studies and have recently been demonstrated to mimic aspects of inflammatory bowel disease following IFN- γ and LPS stimulation, resulting in an upregulation of IL-8 and concurrent decrease in barrier integrity (301). This same model has subsequently been used to assess the vulnerability of the inflamed epithelium to silver nanoparticles that are prevalent in food products; studies found greater cell death in models that were representative of the inflamed mucosa compared to those of the healthy intestine (568). More complex co-cultures involving Caco-2 cells, THP-1 macrophages and MUTZ-3 dendritic cells have also been established and have showed that IL-1 β has significant effects on the production of IL-8 within the model and also altered the distribution of immune cells within the model to areas between adjacent immune cells (531). This is in agreement with earlier studies using only the Caco-2 monolayer that demonstrated a significant role of IL-1 β in inducing epithelial damage, and also confirmed that inflamed models were more susceptible to damage induced by silver nanoparticles than their non-inflamed controls (531,559). These results demonstrate that further efficacy and safety testing may be required on nanoparticles to ensure they do not elicit damaging responses in individuals with an inflamed intestine such as colitis or IBD patients.

As discussed, control co-cultures of Caco-2 and THP-1 cells incurred epithelial damage without the addition of further stimulatory molecules. However, no such disruption to the epithelium has been demonstrated in Caco-2 U937 co-cultures suggesting that they are a useful cell line to use as control models would be more representative of the healthy intestinal state. Co-cultures of Caco-2 and U937 cells have been utilised to investigate the effects of proinflammatory stimuli involved in IBD, such as LPS which has little effect on Caco-2 monolayers alone due to the hyporesponsive nature of these epithelial cells (484,569). In a microfluidic co-culture system, TNF- α was shown to be greatly induced when LPS was introduced to the model, however this was not the case in Caco-2 monocultures (300). Additionally, the protective effects of novel therapeutic compounds have been investigated using Caco-2 U937 co-cultures; phycocyanin was shown to attenuate damage to the tight junctions and suppressed the expression of IL-6 and IL-8 when models were stimulated with LPS (302). Alternative microbial metabolites that are present within the microbiota of IBD patients including butyrate and ammonium were not found to damage the epithelial barrier function, however TNF- α release from macrophages was stimulated while IL-10 secretion was inhibited (514). This implies that although these bacterial metabolites will not incur any further damage to the epithelium in IBD by themselves, they may exacerbate any pre-established proinflammatory response of macrophages.

The co-cultures discussed above only incorporate one epithelial cell line, yet to recapitulate the structure of the intestinal epithelium more holistically, Caco-2 and HT29-MTX cells have been utilised in co-cultures to represent enterocytes and goblet cells respectively. However, very few studies have then gone on to incorporate an immune lineage into the models. Of those models that have all three intestinal cell lineages represented, THP-1 macrophages appear to be the most widely used immunocyte and no triple co-cultures have, as of the time of writing, incorporated U937 cells. Stimulation of a Caco-2/ HT29-MTX/ THP-1 *in vitro* model with LPS demonstrated an increased production of the proinflammatory cytokines IL-8, IL-6 and TNF-

α , in addition to impairing the barrier function of the epithelial monolayer as assessed by TEER (533). Furthermore, this model was found to allow bacterial attachment to the epithelium which, when stimulated with LPS, resembled the inflamed mucosa of IBD patients (533). Moreover, the anti-inflammatory properties of dietary components have been assessed using a triple co-culture incorporating, Caco-2, HT29-MTX and THP-1 cells; plum and cabbage proteins were successfully able to reduce the expression of IL-6 and IL-8 elicited from the model following application of TNF- α , IL-1 β and LPS (570). Finally, a recent study investigating the impact of ingested microplastics was carried out using a triple co-culture of Caco-2, HT29-MTX, and THP-1 cells. In healthy models without application of proinflammatory stimuli, microplastics did not induce any effects, however, exposure to plastics following an induction of an immune response in the model to mimic the inflamed mucosa, further exacerbated the release of IL-1 β by the models and induced epithelial cell death (571). These results demonstrate the need for further testing of potentially hazardous compounds on inflamed models of the intestine to understand how patients with chronic inflammatory diseases may respond.

6.1.3.3.2. *In vitro* models of the intestinal mucosa using more physiologically relevant cells and patient-derived blood samples

Although not an *in vitro* co-culture model, Caco-2 cells maintained as 3D cyst-like structures have been utilised to assess the effects of plasma derived from IBD patients. Exposure of the Caco-2 cells to plasma from CD patients resulted in amplified permeability in a manner that was deemed to be activity-dependent based on the severity of the disease (572). Cysts exhibited decreased expression of ZO-1 and occludin when incubated with CD plasma, however introduction of an anti-TNF- α treatment attenuated the disruption observed in the tight junctions and overall barrier function suggesting that the primary defects were due to the presence of TNF- α (572).

PBMCs isolated from healthy individuals have been utilised in numerous co-culture models with Caco-2 cells. Stimulation of such co-cultures with pathogenic and non-pathogenic *E. coli* revealed a reduction in the level of proinflammatory cytokines produced in response to non-pathogenic bacteria in comparison to pathogenic bacteria (484). This indicated that the cells were able to distinguish between commensal bacteria that did not require initiation of immune response and those that did (484). Specific lineages from PBMCs have also been isolated and incorporated into co-cultures with Caco-2. Leonard and colleagues established a model of Caco-2 cells and PBMC-derived macrophages and dendritic cells; upon stimulation with IL-1 β the tight junctions were disrupted and the production of IL-8 was enhanced over Caco-2 monolayers (485). This model has subsequently been used to assess the efficacy of different drug formulations of budesonide during inflammation, an important factor not always fully taken into account when assessing the delivering of novel drug compounds (486).

Caco-2 cells have also been successfully used *in vitro* in combination with patient-derived PBMC cells, not just cells from healthy individuals, to more accurately represent IBD *in vitro*. Co-culture of Caco-2 cells and PBMC resulted in an increase in the number of CD8⁺ T lymphocytes that produced IFN- γ in the PBMC isolated from

patients with IBD, however no increase was identified in cells derived from healthy controls. Additionally, no such changes in the numbers of CD4⁺ lymphocytes were detected in either healthy or diseased states (573). Furthermore, no distinguishing features were found between patients with UC and CD and those with active or inactive diseased states. Moreover, this same study replicated these results garnered from Caco-2 co-cultures with freshly isolated intestinal epithelial cells (573). This study indicated that IFN- γ induction in CD8⁺ lymphocytes may play an important role during chronic inflammation in IBD as the increase in such cells in response to co-culture with epithelial cells suggests that the lymphocytes may target the epithelium and induce further damage that subsequently augments further inflammatory processes.

In addition to cell lines, primary epithelial cells have been co-cultured with immune cells derived from healthy individuals to further investigate the interactions between the epithelium and immune cells. Primary studies utilising murine cells were able to successfully combine primary epithelial enteroids with either CD8⁺ T lymphocytes or intraepithelial lymphocytes that were also isolated from mice (574,575). However, more recent advances have focused on human cells. Epithelial enteroids have been successfully co-cultured with macrophages derived from healthy PBMC samples; macrophages were found to improve the barrier function of the epithelium as indicated by augmented TEER in co-culture and additionally enhanced the columnar phenotype observed within the epithelial cells (576). Introduction of bacteria to the culture system, increased the number of macrophage projections within the model indicating that the normal function of the cells was retained in co-culture (576). This unique model has the potential to be altered to include primary cells isolated from patients with IBD to study disease pathology and thus may prove to be useful in personalised medicine investigations.

6.1.3.3.3. Co-cultures of the intestinal mucosa incorporating additional cell lineages to mimic aspects of IBD

Epithelial and immune cells are not the only cell lineages to consider when developing models to study IBD, however they should be considered the most important due to the primary pathologies of UC and CD affecting these two cell types. The role of endothelial cells is also an important factor when investigating inflammation due to the enhanced permeability of endothelial tissues to immune cells during active inflammation. A co-culture using Caco-2 cells on the apical surface and the ISO-HAS-1 endothelial cell line on the basolateral surface of a Transwell® membrane has been developed (577). Interestingly, when the co-culture was exposed to proinflammatory stimuli (IFN- γ , TNF- α , IL-1 β and LPS), the epithelial barrier integrity remained stable based on TEER, yet the permeability to NaFITC was unexpectedly decreased. Furthermore, the co-culture secreted proinflammatory cytokines in response to stimulation but these were only detected at the apical side adjacent to the epithelium and were not found at the basolateral side. However, in endothelial monoculture, a strong immune response was observed, indicating that the epithelial barrier may have a protective effect on the endothelium and prevent an exaggerated immune response within the cells. Hepatic cell lines have also been incorporated into triple co-culture systems mimicking the intestinal mucosa (Caco-2, undifferentiated THP-1 monocytes and HepaRG cells), yet these have not been utilised to investigate facets of IBD (578). Rather, the model was utilised to assess the cytotoxicity of mycotoxins found in fungi;

incorporation of hepatocytes into the model provided protective effects that blocked the cytotoxic side effects observed in Caco-2 THP-1 co-culture.

Recent investigations have highlighted the importance of fibroblasts and the ECM in the pathogenesis of IBD. Mesenchymal cells are, in part, responsible for maintaining a microenvironment enriched in anti-inflammatory molecules such as TGF- β and IL-10 (49,50). However, in IBD overexpression of TNF- α can induce the secretion of collagens, MMPs and TIMPs that can alter the composition of the ECM supporting the epithelium (147,148). Furthermore, high concentrations of TIMPs are present in fibrotic areas of the intestinal mucosa and increased levels of MMPs have been identified in the inflamed mucosa of active IBD patients (151). These findings have demonstrated that stromal cells such as fibroblasts are not bystanders in IBD and their incorporation into *in vitro* models of the intestine would prove invaluable for future studies investigating IBD. Recently, a model using Caco-2 cells, undifferentiated U937 monocytes and L292 mouse fibroblasts has been developed (310,311). Investigations into the effect of dietary wheat proteins on intestinal inflammation was assessed using this model, revealing that the proteins disrupted tight junctions, increased expression of the proinflammatory cytokine IL-8, and stimulated migration of the monocytes (310). This study demonstrated that dietary components could induce inflammatory responses and may augment inflammation already established within the IBD mucosa.

6.1.4. Conclusions

Many different *in vitro* models are utilised to assess the effects of inflammatory stimuli on the epithelial barrier and production of proinflammatory response. These models range from simplistic epithelial monolayers to more complex co-cultures using cell lines or primary cells derived from healthy individuals or IBD patients. However, very few model systems have been developed that incorporate a mesenchymal, fibroblast lineage into the co-culture and when fibroblasts are included, they are of animal origin limiting the use of the model in recapitulating a fully humanised response. Thus, the approach taken in this chapter was to utilise the *in vitro* models developed in Chapter 5 - epithelial-immune co-cultures and triple co-cultures that also incorporated a fibroblast lineage of human origin – and examine the effects of various stimuli on the epithelial barrier function and determine whether an immune response was elicited within the models. To best resemble the pathology of inflammatory bowel diseases, U937 macrophages required further polarisation to the M1 lineage, thus the known M1 inducers: LPS, IFN- γ and TNF- α will be utilised to induce this phenotype. Furthermore, as these cytokines and peptides are known to have roles in the pathogenesis of IBD, it is hypothesised that aspects of IBD pathology will be mimicked within the *in vitro* models.

6.2. Hypotheses and Aims

This chapter aims to utilise the *in vitro* co-culture models developed in Chapter 5 and apply stimulatory molecules to provoke an inflammatory response and attempt to recapitulate some aspects of inflammatory bowel disease. Three different compounds known to induce a proinflammatory M1 phenotype within macrophages will be examined: lipopolysaccharide (LPS), interferon-gamma (IFN- γ) and tumour necrosis factor α (TNF- α). It is hypothesised that a proinflammatory response will be induced in the *in vitro* models which will, in turn, alter the overall structure and function of the epithelial monolayer.

6.2.1. Objectives

- Characterise the structure of healthy, ulcerative colitis and Crohn's disease intestinal tissue samples utilising histological and immunofluorescent staining techniques.
- Optimise the concentration and assess the effect of acute LPS stimulation on 2D differentiated macrophages, Caco-2-U937 co-cultures and immune-competent 3D co-culture models.
- Determine whether repeated LPS doses induce an immune response more representative of chronic inflammation.
 - Apply sequential treatments of LPS to 2D cells and *in vitro* models and characterise the immune response and structural changes induced by repeated LPS.
- Assess the effect of IFN- γ on the structure and function of the epithelium in the two *in vitro* co-cultures.
- Characterise the effect of utilising IFN- γ as a priming agent prior to LPS exposure.
- Determine whether IFN- γ and TNF- α provoke a greater response when applied adjunctively or when models are pre-exposed to IFN- γ before TNF- α .

6.3. Experimental Design

Chapter 6 aims to employ the immune-competent *in vitro* co-cultures developed in the previous chapter to mimic aspects of IBD through the generation of a proinflammatory response. LPS, IFN- γ and TNF- α were selected for use as proinflammatory stimuli as they are all physiologically relevant factors involved in the pathogenesis of IBD. LPS has been demonstrated to be elevated in the mucosal tissues and blood of IBD patients and under normal circumstances should not invoke an immune response (579–581). IFN- γ is a known M1 macrophage inducer and has also been used to prime *in vitro* intestinal models to respond to LPS, thus was utilised to determine if the immune response generated by LPS could be enhanced (301,523). Models stimulated with LPS alone or in combination with IFN- γ had a total $n=9$ conducted over 3 independent experimental occasions, each with 3 technical replicates (Section 2.7.1). Finally, a series of pilot studies investigating the effect of co-stimulation of models with both IFN- γ and TNF- α were conducted. IFN- γ has been shown to enhance the expression of TNF- α receptors and enhance its effect within cells; additionally, TNF- α is a key factor in IBD that can be directly targeted during treatment regimens (189,190,582). Models subject to TNF- α treatment had a total $n=6$ from 2 independent experiments, each with 3 technical repeats (Section 2.7.1).

Following optimisation of the concentration of the inflammatory inducer molecules on 2D cells, Transwell® immune competent immune competent co-cultures and 3D Alvetex® co-cultures, models were assessed structurally and functionally for evidence of epithelial barrier dysfunction and the establishment of an immune response (Sections 2.6 and 2.7). Characterisation of the immune response was conducted primarily via qPCR analysis to determine whether the gene expression levels of immune cell markers had been altered. Additionally, when triple co-cultures were repeatedly stimulated by LPS, the cell culture supernatant from 9 models set up over 3 separate days was collected, and samples were analysed in duplicate by an ALBIA assay to determine the overall concentration of secreted cytokines (Section 2.7.3).

6.4. Results

6.4.1. Characterisation of IBD Tissue Samples in Comparison to Healthy Intestinal Tissue

To understand the structural changes that occur in the IBD intestinal mucosa, histological and immunofluorescent staining techniques were used to characterise the overall intestine morphology along with characterising the components epithelial barrier and lamina propria. Human intestinal mucosa tissues were kindly provided by Reprocell; healthy human tissue was shipped living and processed for wax embedding within the laboratory at Durham University, whilst diseased tissue samples of UC and CD were provided embedded in wax and were sectioned in-house prior to analysis. Such tissue samples were specifically biopsies of the large intestine, due to UC only affecting the colonic tissue. Caco-2 cells, though they differentiate into intestinal epithelial cells that are structurally and functionally like small intestinal enterocytes, are colonic in origin and thus can be used in further comparisons with the human tissue samples following inflammation.

Histological staining techniques, H&E and PAS/Alcian blue, were employed to visualise the overall anatomy and morphology of healthy and diseased tissue (Figure 6.1). Mucosal samples from healthy individuals have long crypts that lie parallel to each other and extend from the luminal surface to the muscularis mucosa. These crypts are strongly stained by PAS/Alcian blue indicating the presence of goblet cells throughout the crypts. Moreover, a thick layer of secreted mucus covering the luminal surface of the healthy tissue samples was identified. Both IBD tissue samples present with a large, diffuse, transmucosal inflammatory infiltrate where large numbers of plasma cells accumulated in the lamina propria. The crypt architecture of the healthy tissue is not preserved in IBD, where there is evidence of mild crypt disruption and atrophy. Crypts lost their parallel structure and had a distinctly shortened appearance; this is particularly evident in the PAS/Alcian blue stained images. Furthermore, there is evidence of mucin depletion within the crypts and no mucus layer was identified lining the epithelial surface, however, as discussed in Chapter 4 this may be due to the use of solvents in the dehydration and wax embedding processes. Also of note, is evidence of muscularis mucosa thickening in both UC and CD samples compared to the H&E-stained images of healthy intestinal tissue.

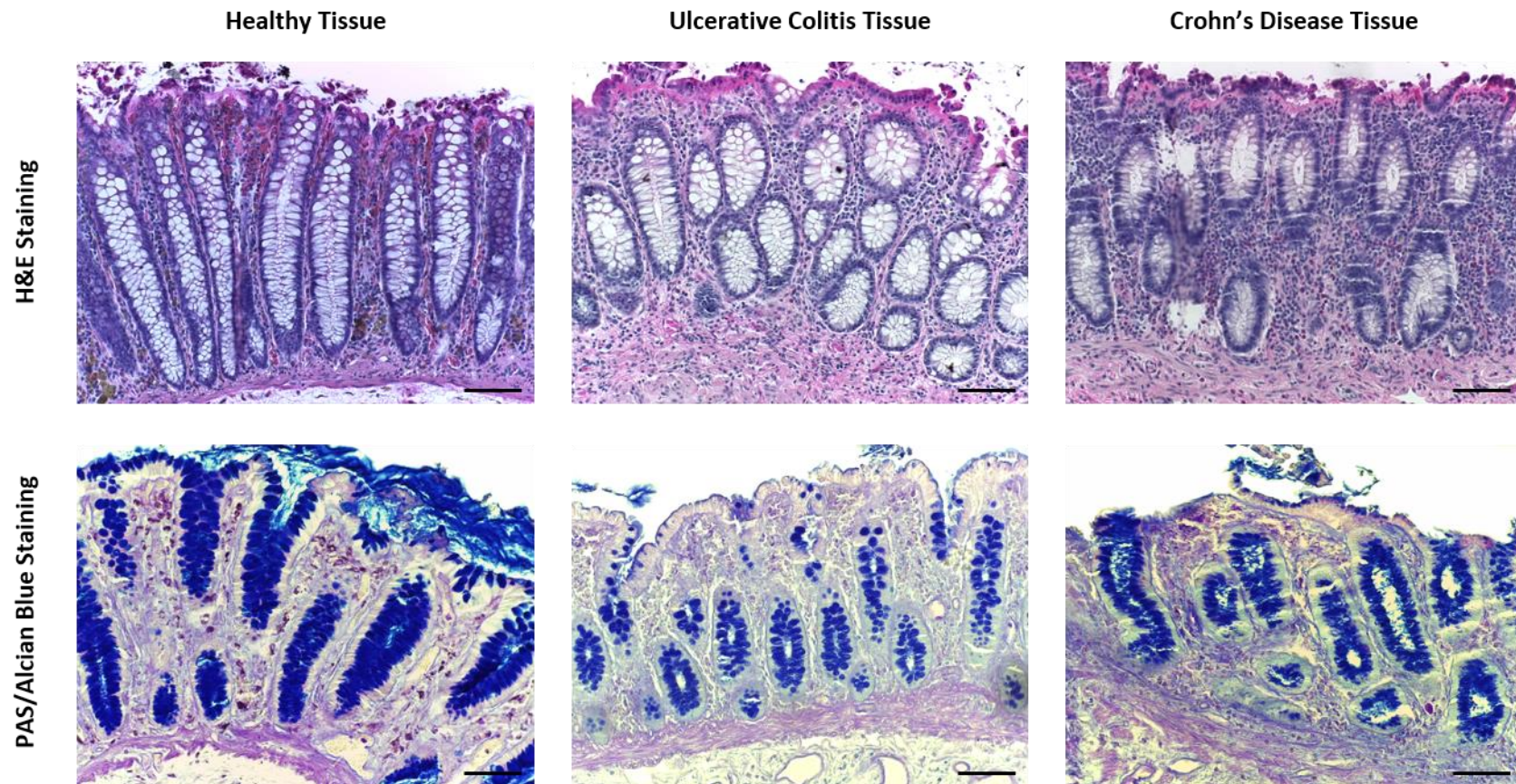


Figure 6.1. Histological staining reveals characteristic crypt architecture disruption in IBD intestinal tissue samples. Wax embedded samples of healthy, UC and CD intestine mucosa were sectioned and subject to H&E staining (top), showing gross mucosal anatomy and PAS/Alcian blue staining (B) to indicate the presence of mucus produced by goblet cells. Long parallel crypts are visible in healthy tissue and are shortened and disrupted in diseased states. Scale bars: 100 μ m.

A known aspect of IBD is increased intestinal permeability due to a disrupted epithelial barrier. Therefore, the structure of the epithelial monolayer was assessed through immunofluorescent staining for components of the apical junctional complex; namely claudin-1, claudin-2, and E-cadherin (Figure 6.2). Claudin-1 had very weak expression in native intestinal tissue and was identified at the epithelial monolayer in both UC and CD tissue samples. Unusually, almost no positive claudin-1 was detected in healthy tissues. Claudin-2 on the other hand, had a much more obvious expression profile, being primarily located to the epithelial monolayer lining the luminal surface of the mucosal tissue, rather than extending down into the crypts. Claudin-2 staining highlighted the distinctive columnar phenotype of the cells due to its expression along the lateral membranes of adjacent epithelial cells. No discernible differences were found between either the healthy or IBD intestine samples regarding the expression of claudin-2. Finally, E-cadherin was used as a marker for adherens junctions. Like Claudin-2, strong immunostaining was detected for E-cadherin in all three tissue types but had the strongest staining in healthy intestine samples. Positive E-cadherin staining was also detected in the lamina propria adjacent to the luminal surface, this was most obvious in the healthy intestine, though it is not known what specific cells were expressing the marker. Strong staining was present in both IBD samples, though greater epithelial cell definition was evident in UC compared to CD, wherein E-cadherin was apparent surrounding entirety of the intestinal epithelial cells, including the apical surface of the cells, rather than being restricted to the lateral membranes.

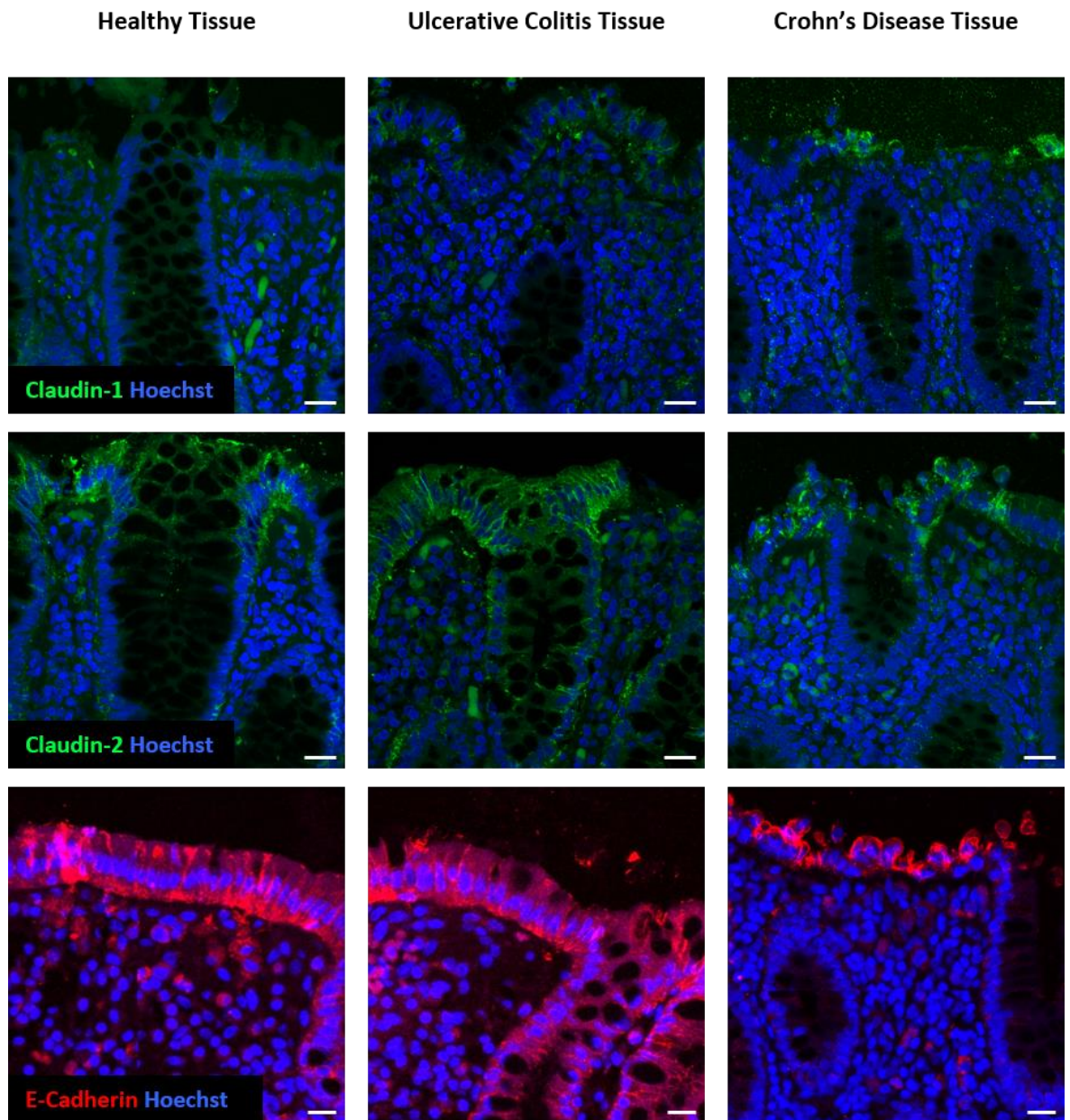


Figure 6.2. Immunofluorescent analysis of junctional markers in healthy, UC and CD reveals the AJC is intact in healthy and diseased tissue. Wax embedded blocks of healthy, UC and CD intestinal mucosa tissues were sectioned and immunolabelled for claudin-1 (green, top), claudin-2 (green, middle) and E-cadherin (red, bottom). Nuclei were counterstained with Hoechst. Representative images from compressed Z-stacks are shown. Scale bars: 20 μ m.

To further understand the composition of the immune cell infiltrate observed in both UC and CD tissues, staining for the marker, CD68 was carried out. CD68 is highly expressed in cells of the monocyte lineage including both circulating and tissue macrophages and is considered to be a pan-macrophage marker. CD68 is often used to identify inflammation associated with macrophages. Its precise function is poorly understood but is hypothesised to have roles in antigen processing and scavenging, i.e., clearing cellular debris and promoting phagocytosis. Figure 6.3. shows that in the healthy intestine mucosa, CD68 positive cells are predominantly located at the base of the lamina propria adjacent to the muscularis mucosa, where the mesenteric lymph nodes are likely to be located. Conversely, no large clusters of CD68 positive cells were identified adjacent to the presumed lymph node location in IBD tissues and, instead, CD68 expressing cells can be identified throughout the lamina propria. In UC particularly, the strongest staining was found directly below the luminal surface. This indicates that macrophages likely constitute a large component of the transmural immune cell infiltrate.

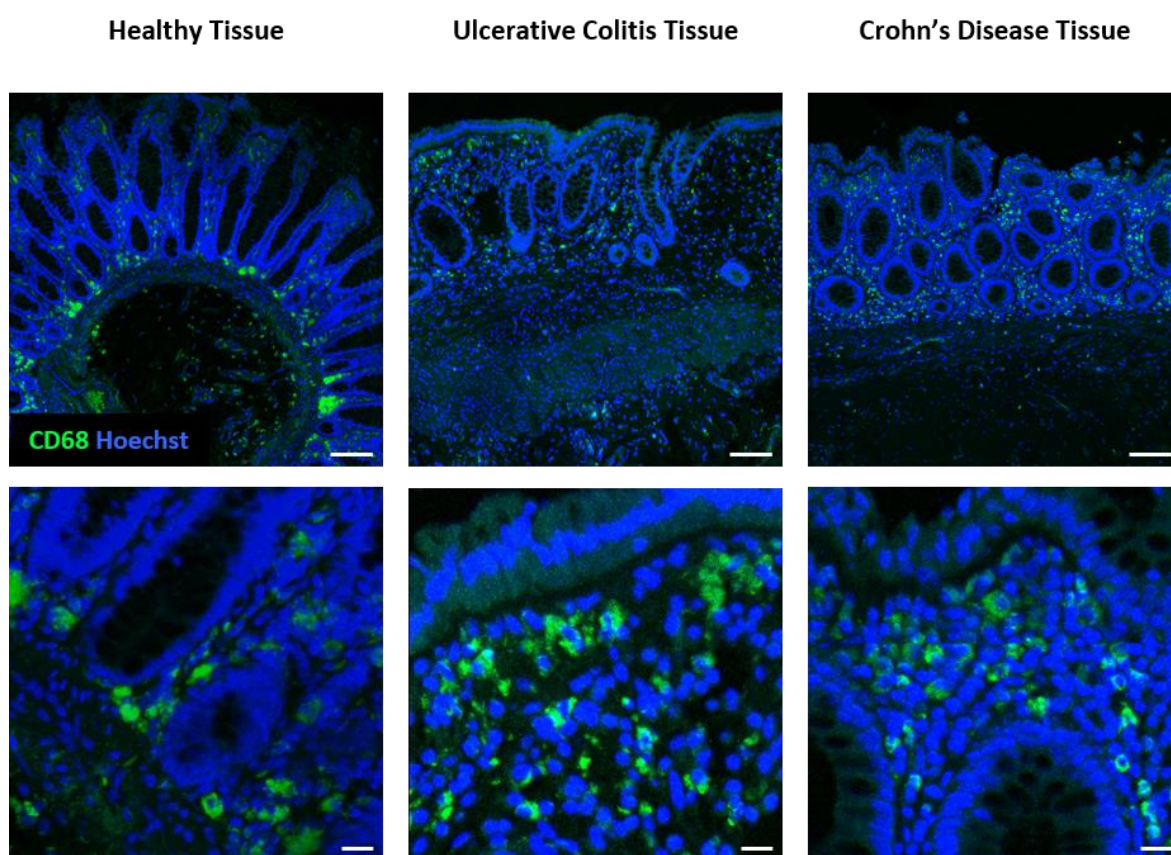


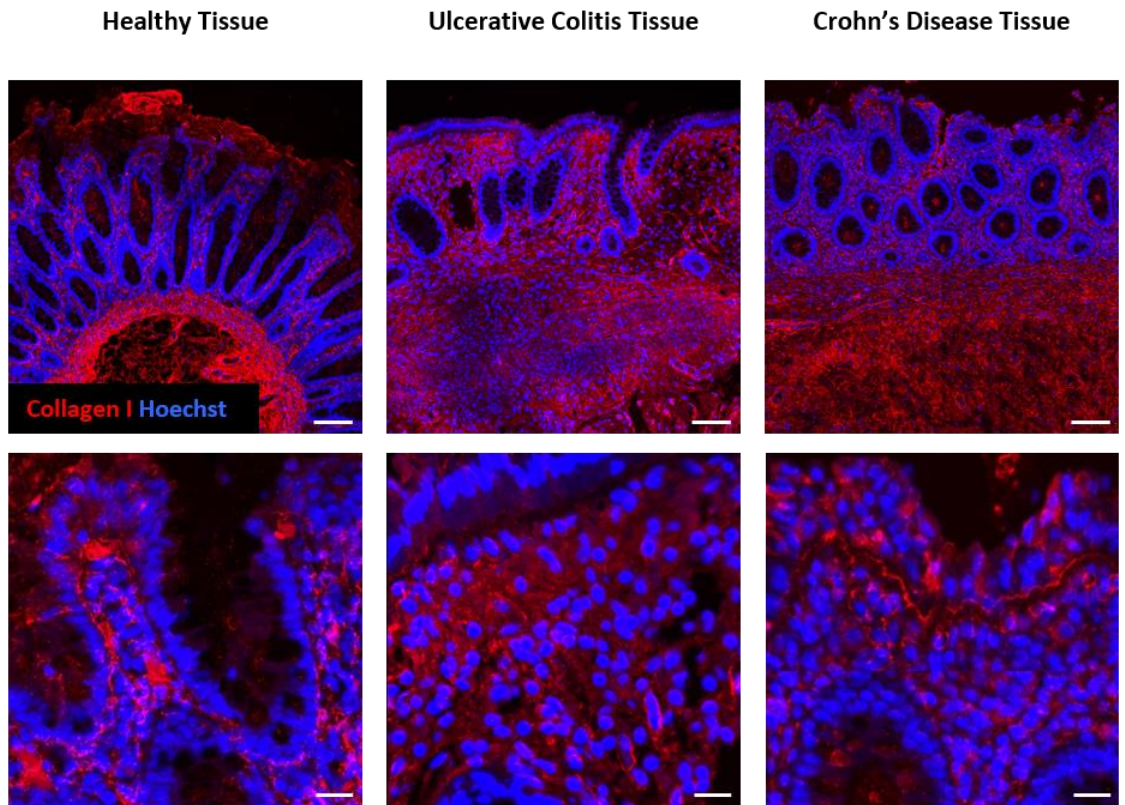
Figure 6.3. Cells expressing the macrophage marker, CD68, are present throughout the lamina propria of IBD tissues. Healthy, UC and CD intestine mucosa samples were immunolabelled for the pan-macrophage marker CD68 (green) and nuclei were counterstained with Hoechst. Representative images of compressed Z-stacks are depicted. CD68 positive cells are primarily present adjacent to the muscularis mucosa in the healthy intestine but have a more diffuse localisation in both IBD tissues. Scale bars: 100 μm (top), 20 μm (bottom).

Both UC and CD are chronic inflammatory diseases which can affect ECM composition, thus immunofluorescent analysis of ECM components were conducted. Collagen I can be seen to be expressed throughout the lamina propria and submucosa and was particularly strong in the submucosa of the healthy tissue (Figure 6.4A). Strong background staining within the mucus layer of the healthy intestine was also present. In regard to the lamina propria, collagen I expression was most noticeable in the diseased tissues, though this may be due to the crypt disruption phenotype that amplifies the area of lamina propria available for ECM deposition. Collagen I could be clearly identified immediately subjacent to the intestinal epithelial cell monolayer where it functions to provide structural support to the epithelium.

Collagen III had a similar expression profile to collagen I, where positive staining was observed in both the lamina propria and submucosa (Figure 6.4B). However, the staining of collagen III in the submucosa did not appear to be as intense as collagen I. Very strong positive signal was present in the lamina propria and was most obvious in areas subjacent to the epithelia at the luminal surface in both the healthy and UC intestine. CD collagen III expression was extremely apparent throughout the entire lamina propria though the distinctive fibre-like nature of the protein was not evident in this location. Similarly, expression of fibronectin appeared to be more punctate in the lamina propria of all tissue samples compared to its presence in the submucosa (Figure 6.5A). Fibronectin deposition was extremely obvious in the submucosa but was more diffuse in the lamina propria, especially in the UC intestine. Overall, the strongest staining for fibronectin was in the healthy intestine.

Finally, immunofluorescent analysis of α -smooth muscle actin was performed to assess the level of the cells considered to be responsible for fibrosis (Figure 6.5B). α -SMA could be clearly localised to the muscularis mucosa in the healthy and UC samples, though the muscularis mucosa was not visible in the CD tissue. Furthermore, the numbers of α -SMA positive cells overall were generally low in all three samples. Regarding the lamina propria, cells expressing α -SMA tended to line the intestinal crypts and no positive staining was detected adjacent to the epithelial monolayer.

(A)



(B)

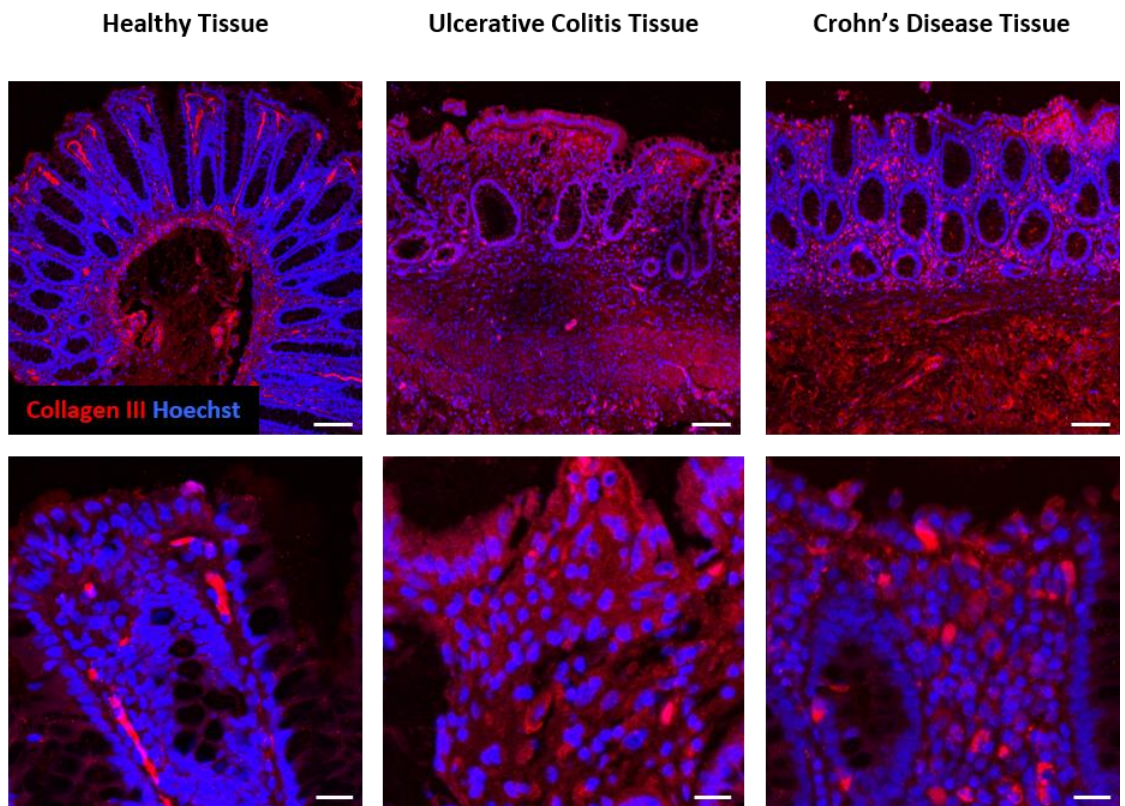
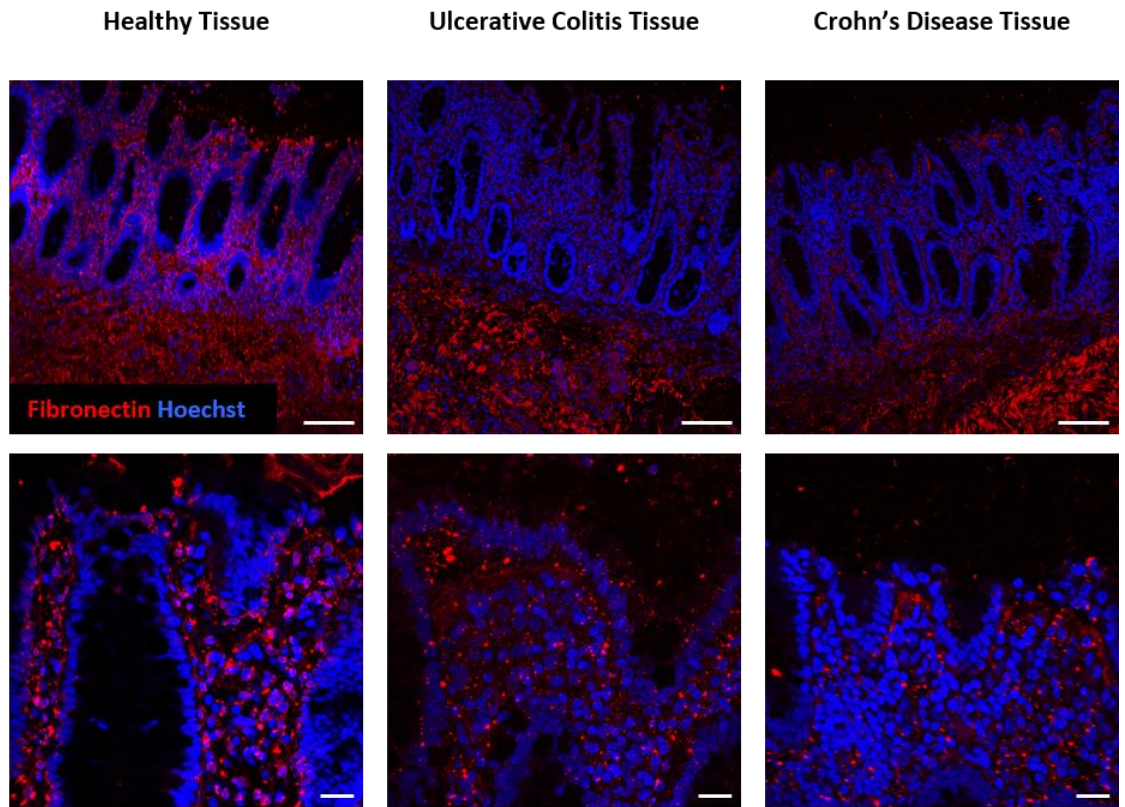


Figure 6.4. Collagens are expressed in the healthy and IBD intestinal mucosa and submucosa. Collagen I (A) and collagen III (B) antibodies were used to stain healthy, UC and CD intestine tissues. Nuclei were counterstained with Hoechst and representative, compressed Z-stacks are shown. Scale bars: 100 μ m (A,B top rows), 20 μ m (A,B bottom rows).

(A)



(B)

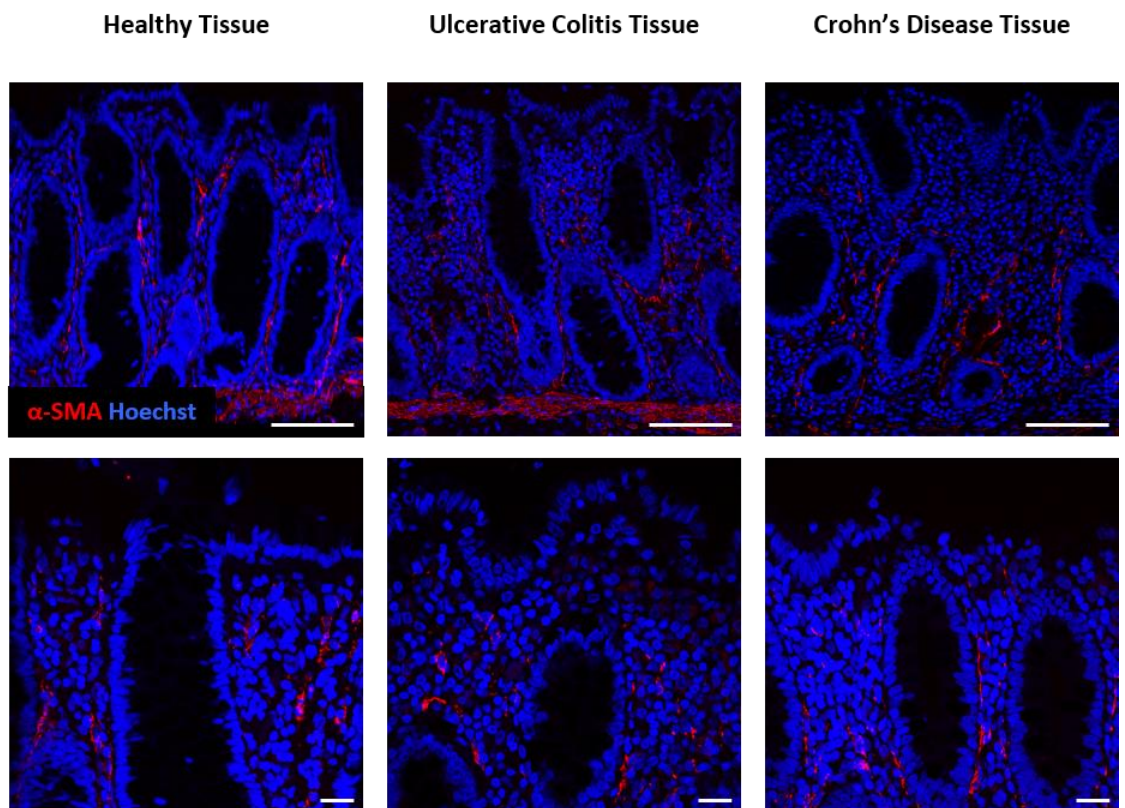


Figure 6.5. Immunofluorescent staining of fibronectin and α -SMA. Fibronectin (A) and α -SMA (B) antibodies were used to stain healthy, UC and CD intestine tissues. Nuclei were counterstained with Hoechst and representative, compressed Z-stacks are shown. Scale bars: 100 μ m (A,B top rows), 20 μ m (A,B bottom rows).

6.4.2. Characterisation of the Responses Induced by Acute LPS Stimulation

LPS is a major component of the outer membrane of Gram-negative bacteria and is a potent activator of both monocytes and macrophages, often being used to induce an M1 pro-inflammatory phenotype in U937 and THP-1 derived macrophages (523,524,583,584). Additionally, LPS has been postulated to have pathogenic roles in the intestinal inflammation of Crohn's disease and ulcerative colitis due to elevated levels in the intestinal mucosal tissues and blood serum of these patients (579–581). Therefore, LPS was selected as a physiologically relevant effector molecule for use in the primary studies to stimulate an inflammatory response within the cells and *in vitro* models developed in Chapter 5.

6.4.2.1. Stimulation of 2D-cultured U937 macrophages with LPS

Prior to stimulating *in vitro* models with LPS, the ability of 2D cultured U937 macrophages to respond to LPS treatment was assessed. U937 cells were differentiated following the protocol established in Chapter 5. Following their rest day, macrophages were incubated with 0–10 µg/mL LPS for 24 hours before downstream analysis. Cells were first analysed by immunofluorescent staining to visualise the distribution of actin via Phalloidin staining, and to detect the expression pattern of two CD molecules: CD14 and CD68. Phalloidin is a bicyclic peptide that binds to actin filaments (F-actin), and Figure 6.6A shows that actin clearly surrounds the rounded nucleus of unstimulated U937 cells indicating that the cells have a circular morphology overall. Similar staining can be observed when U937 macrophages were incubated with 1 and 10 µg/mL LPS, however phalloidin is not always immediately adjacent to the cells, indicating that the cell cytoplasm is more spread out on the surface of the glass coverslips than in unstimulated controls. Additionally, cells were often found as clumps when treated with LPS and the actin staining is not as defined.

Figure 6.6A also shows representative immunofluorescent images of CD14 and CD68. Both CD14 and CD68 display a similar localisation to F-actin in the macrophages and are found surrounding the cell membranes, though the expression is more diffuse. CD14 is a co-receptor for TLR4 and is responsible for facilitating the downstream signalling processes induced by LPS. Greater numbers of U937 macrophages were positively stained for CD14 when treated with LPS, additionally the fluorescence intensity appeared to increase. Similarly, the pan-macrophage marker CD68 had higher numbers of cells expressing the marker when exposed to LPS. Quantification of LPS-induced changes to the expression of CD14 and CD68 was performed via qPCR analysis; mRNA was extracted from U937 macrophages treated with 0, 0.1, 1 and 10 µg/mL LPS and the expression of CD14 and CD68 was normalised first to the mRNA expression of actin and then to the control cells without LPS treatment (Figure 6.6B,C). No large fold changes occurred in the expression of CD68 yet CD14 was found to increase upon incubation with LPS, though the highest levels were detected in the cells treated with 1 µg/mL LPS, rather than 10 µg/mL. Assessment of the gene expression changes at an earlier time point could be performed to determine whether the lower CD14 expression observed with 10 µg/mL LPS is due to the time taken to elicit the greatest being prior to the time lysates were harvested.

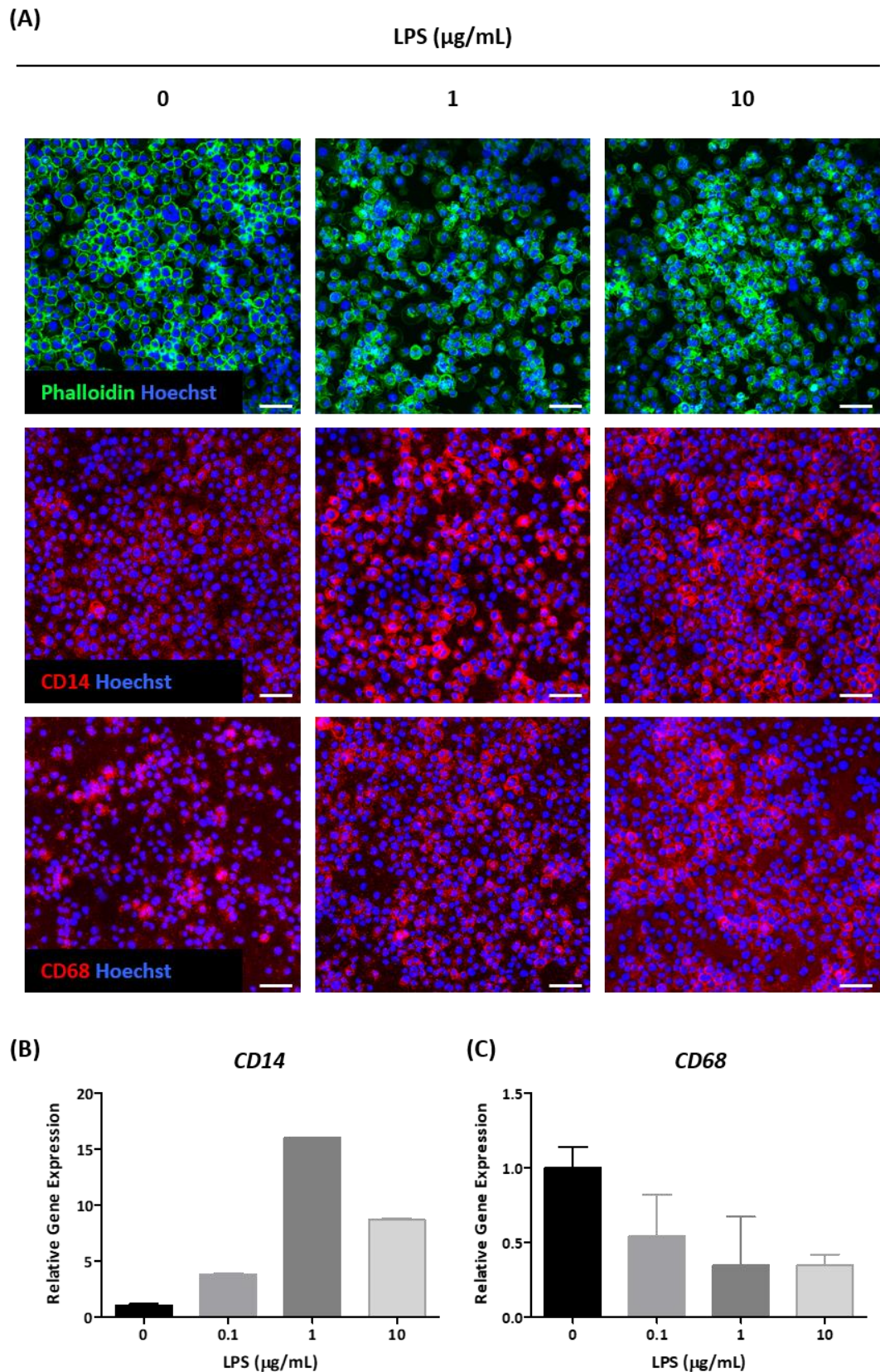


Figure 6.6. Assessment of CD marker expression following LPS stimulation of differentiated U937 macrophages. PMA-differentiated macrophages were incubated with 0-10 $\mu\text{g/mL}$ LPS for 24 hours. Cells were then either fixed in 4 % PFA and immunolabelled for Phalloidin (green), CD14 or CD68 (red) and counterstained with Hoechst (A) or were subject to qPCR analysis. For qPCR, lysates were processed to cDNA and were analysed for the expression of CD14 (B) or CD68 (C); expression levels were first normalised to the level of actin then to the unstimulated control cells. Images shown are representative of 3 cultures. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction. Scale bars: 50 μm .

The maximum LPS concentration, 10 $\mu\text{g/mL}$, resulted in a lower mRNA expression of CD14 than 1 $\mu\text{g/mL}$ LPS. Therefore, to ensure that the highest concentration of LPS was not cytotoxic to the cells an MTT assay was performed at 4 and 24 hours following the initial treatment (Figure 6.7). No differences in the metabolic activity of the macrophages were detected when cells were incubated with different concentrations of LPS at either 4 hours or 24 hours indicating that all concentrations of LPS used in this study were non-cytotoxic and suggesting that the cells remained viable following stimulation. Although it appears that after 24 hours of LPS exposure the cells had a higher metabolic activity, the two time points could not be directly compared as the MTT assays were not conducted at the same time.

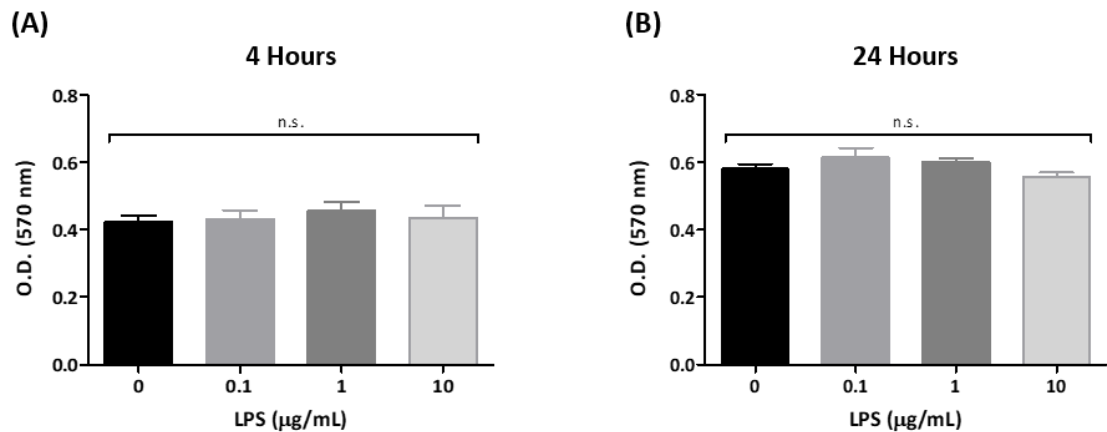


Figure 6.7. Cells remain metabolically active following LPS treatment. Differentiated U937 macrophages were incubated with 0, 0.1, 1 and 10 $\mu\text{g/mL}$ LPS for either 4 or 24 hours. An MTT assay was then performed to assess the metabolic activity of the cells and no significant differences were found. Data represent mean $\pm$ SEM, total $n=6-9$ from 3 independent experiments, each with 2-3 technical replicates. No significant differences were found by one-way ANOVA with Tukey's post-test.

Finally, the inflammatory response of 2D-cultured U937 macrophages in response to incubation with different LPS concentrations for 24 hours was assessed. This was done through qPCR analysis to assess the mRNA expression levels of *IFNG*, *TNF*, *IL6* and *CXCL8* that are the genes encoding the proinflammatory cytokines IFN- γ , TNF α , IL-6 and IL-8 respectively, in addition the anti-inflammatory cytokine IL-10, which is encoded for by the *IL10* gene, was also assessed. IFN- γ is a proinflammatory cytokine, considered to be a primary activator of macrophages, yet the ability of macrophages to produce their own source of IFN- γ remains controversial (585). Examination of the mRNA levels of IFN- γ in the LPS-treated macrophages revealed a small increase upon incubation with 1 μ g/mL LPS, however no such increases were observed with 0.1 or 10 μ g/mL LPS and the overall levels remained low (Figure 6.8A). It is important to note, however, that these U937 cells are of malignant origin, thus may not respond with the same signalling pathways as found *in vivo*.

TNF- α , on the other hand, is a proinflammatory cytokine known to be produced by macrophages during acute inflammation, displayed an increase in expression levels in all concentrations of LPS (586). 0.1 μ g/mL LPS had a small increase while 1 and 10 μ g/mL LPS had further increases in TNF- α expression levels, suggesting that a dose-dependent pro-inflammatory response has been generated by LPS (Figure 6.8B). Interestingly, TNF- α signalling can induce apoptosis and necrosis in cells, and although no differences in metabolic activity were observed across LPS concentrations, it would be prudent to conduct further assays such as TUNEL staining (terminal deoxynucleotidyl transferase dUTP nick end labelling) or quantifying the activity of Caspase 3, to determine if this was the case.

IL-6 can be produced by macrophages in their non-activated state and primarily functions as a proinflammatory cytokine, yet also has roles as an anti-inflammatory myokine (587). Its' mRNA expression levels exhibited a stepwise increase with higher levels of LPS stimulation, in a manner similar to the expression of TNF- α (Figure 6.8C). While IL-8, another proinflammatory cytokine strongly produced in the intestinal mucosa, had a similar expression profile to CD14 (Figure 6.6B) and IFN- γ (Figure 6.8A,D), whereby U937 macrophages incubated with 1 μ g/mL LPS had the highest levels of expression.

Finally, mRNA expression of the anti-inflammatory cytokine IL-10, was also assessed through qPCR (Figure 6.9). IL-10 expression has been shown to be induced by LPS-treated macrophages concurrently with the expression of proinflammatory cytokines though the signalling pathways leading to its induction differs from other proinflammatory mediators (588–591). IL-10 functions to dampen down the immune response and maintain epithelial barrier integrity, thus was considered an important cytokine to assess the expression of. Similar to CD14, IFN- γ and IL-8, the expression levels of IL-10 mRNA were highest in macrophages stimulated with 1 μ g/mL LPS, though it was still found to be produced in cells incubated with 0.1 and 10 μ g/mL LPS.

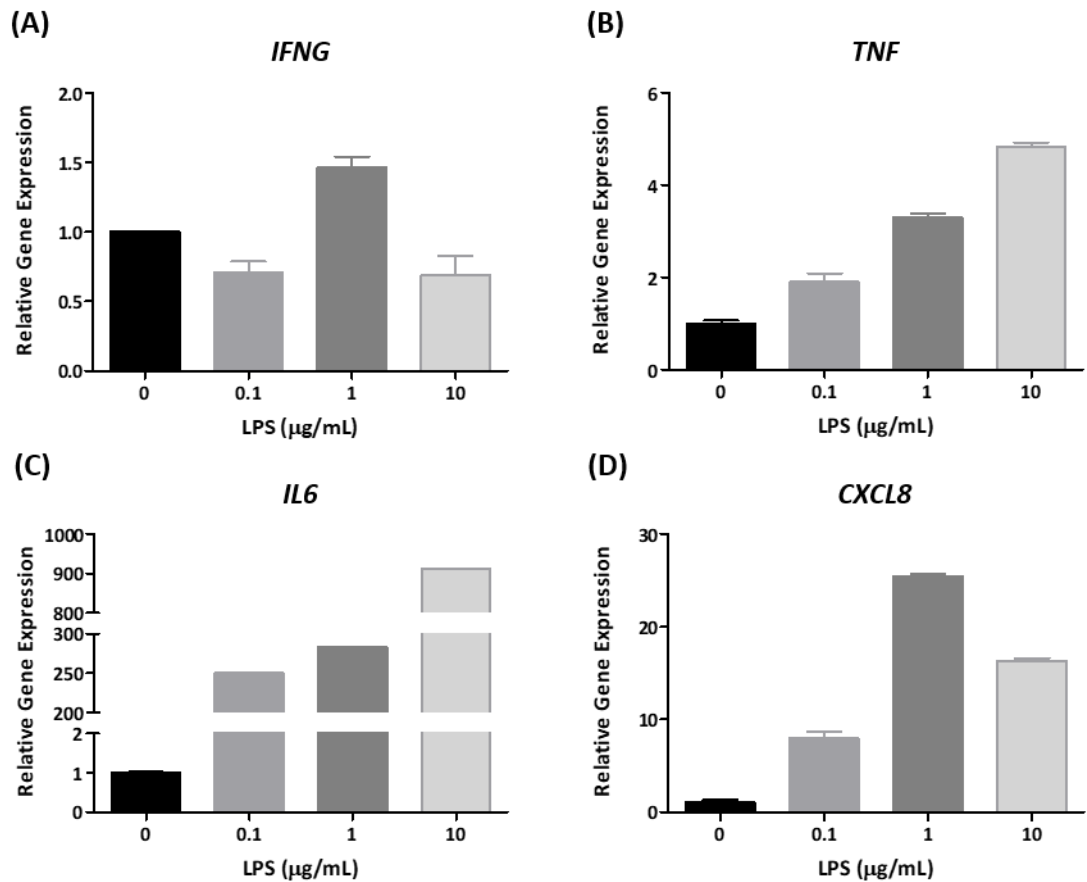


Figure 6.8. Pro-inflammatory cytokines are expressed by U937 macrophages after treatment with LPS. Differentiated macrophages were treated with 0-10 µg/mL LPS for 24 hours before qPCR analysis of the mRNA expression levels of IFNG gene encoding the IFN-γ protein (A), TNF encoding TNF-α (B), IL6 encoding IL-6 (C) and CXCL8 encoding IL-8 (D). Results were first normalised to the mRNA expression levels of actin then to 0 µg/mL LPS control samples. Data represent mean ± SEM, total n=6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

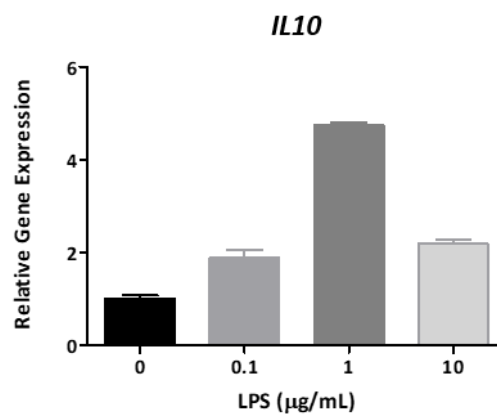


Figure 6.9. The anti-inflammatory cytokine, IL-10, is expressed in LPS-treated macrophages. Differentiated macrophages were treated with 0-10 µg/mL LPS for 24 hours before qPCR analysis of the mRNA expression levels of the gene IL10 encoding the IL-10 protein. Data was first normalised to actin expression levels then to 0 µg/mL LPS control samples. Data represent mean ± SEM, total n=6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

The level of phagocytosis induced by LPS on 2D macrophages was also assessed. Values of phagocytosis were normalised against unstimulated controls to give an overall percentage effect i.e., the phagocytic response induced by the effector molecule (Figure 6.10). All concentrations of LPS examined successfully increased phagocytosis compared to control cells with the lowest concentration of LPS, 0.1 $\mu\text{g/mL}$, driving an increase by almost 100 %. Phagocytosis then increased incrementally, in a dose-dependent manner, with higher concentrations of LPS confirming that U937 macrophages were able to respond appropriately to stimulatory compounds.

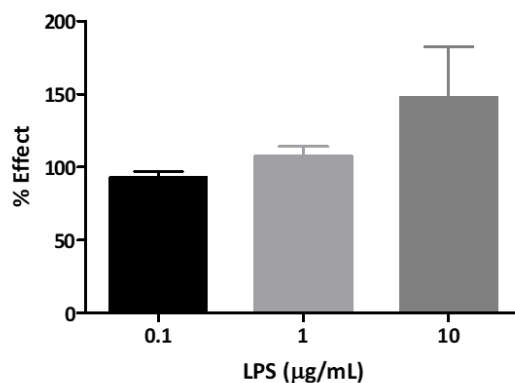


Figure 6.10. LPS increases the level of phagocytosis by differentiated macrophages in a dose-dependent manner. Differentiated U937 macrophages were treated with 0, 0.1, 1 and 10 $\mu\text{g/mL}$ LPS and were subject to a Vybrant™ phagocytosis assay as described in Chapter 5, Section 5.3.4. The percentage effect was calculated by normalising the fluorescence intensity of stimulated cells to the fluorescence intensity of unstimulated control macrophages. Data represent mean $\pm$ SD, total $n=6$ from 2 independent stimulation experimental, each with 2 technical repeats.

6.4.2.2. LPS incubation of Caco-2 U937 macrophage co-cultures reduces epithelial barrier integrity

Following preliminary testing of different LPS concentrations on U937 macrophages maintained in traditional 2D culture, LPS was then applied to the Caco-2 U937 co-culture developed in Chapter 5. Once the epithelial monolayer had fully differentiated and was in the presence of U937 macrophages, LPS at 0.1, 1 and 10 $\mu\text{g/mL}$ was applied to models and incubated for 24 hours as before. Intestinal epithelial cells are normally in contact with bacterial components including LPS that reside in the intestinal lumen and under normal conditions, no inflammatory response is generated. However, if LPS passes through the epithelial barrier, it can encounter various immune cells including macrophages and instigate an immune response. Thus, to simulate an immune response more representative of IBD tissues, LPS was applied to the basolateral side of the indirect *in vitro* co-culture where the macrophages are located.

Figure 6.11 shows the resulting H&E staining of the Caco-2 monolayers following co-culture and LPS treatment. Epithelial monolayers remained intact under all concentrations of LPS treatment, and the Caco-2 enterocytes appear well polarised with distinctly basal nuclei. Though a monolayer is present upon incubation with 10 $\mu\text{g/mL}$ LPS, the structure differs from the other monolayers. Nuclei, although still basally located, appear to be absent in some individual cells, though this may be due to the plane of sectioning. Additionally, the apical pole of a minority of the cells appears concave, as denoted by the asterisks.

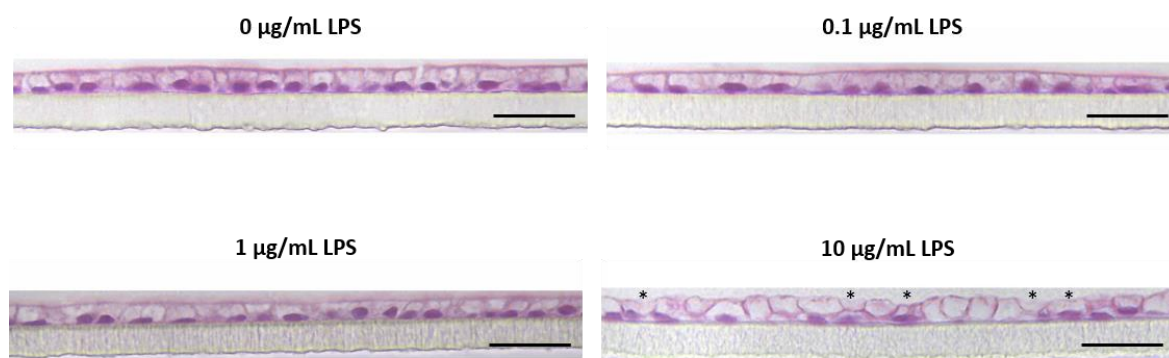


Figure 6.11. Histological staining of Caco-2 U937 co-cultures stimulated with LPS for 24 hours. Caco-2 U937 co-cultures were established as described in Chapter 5. Following complete differentiation of the epithelium, LPS was introduced to the basolateral side of the well for 24 hours. Models were then processed for wax embedding, sectioned, and stained with H&E. * indicate individual cells with a concave apical membrane. Images shown are representative of 9 models. Scale bars: 20 μm .

Further assessment of the structure of the epithelial barrier was then carried out through wholemount immunofluorescent staining for claudin-1, claudin-2, and E-cadherin (Figure 6.12). Claudin-1 exhibited a highly defined expression pattern with an apical localisation surrounding each individual cell in untreated monolayers. Upon stimulation with LPS, the expression appeared more diffuse rather than as continuous filaments, yet claudin-1 was still identifiable surrounding every cell. Similarly, claudin-2 and E-cadherin had an apical localisation and were present under all conditions. E-cadherin was of particular interest, as although claudins are responsible for forming the tight junction seal and maintaining the epithelial barrier, the adherens junctions are responsible for recruiting tight junction components and maintaining the overall stability of the overall AJC complex. Thus, if the expression of E-cadherin was altered, so too would the expression of tight junction proteins. No difference in E-cadherin was visible between the different treatments, however, as the incubation period of LPS was only 24 hours, changes of expression at the protein level may not yet be detected.

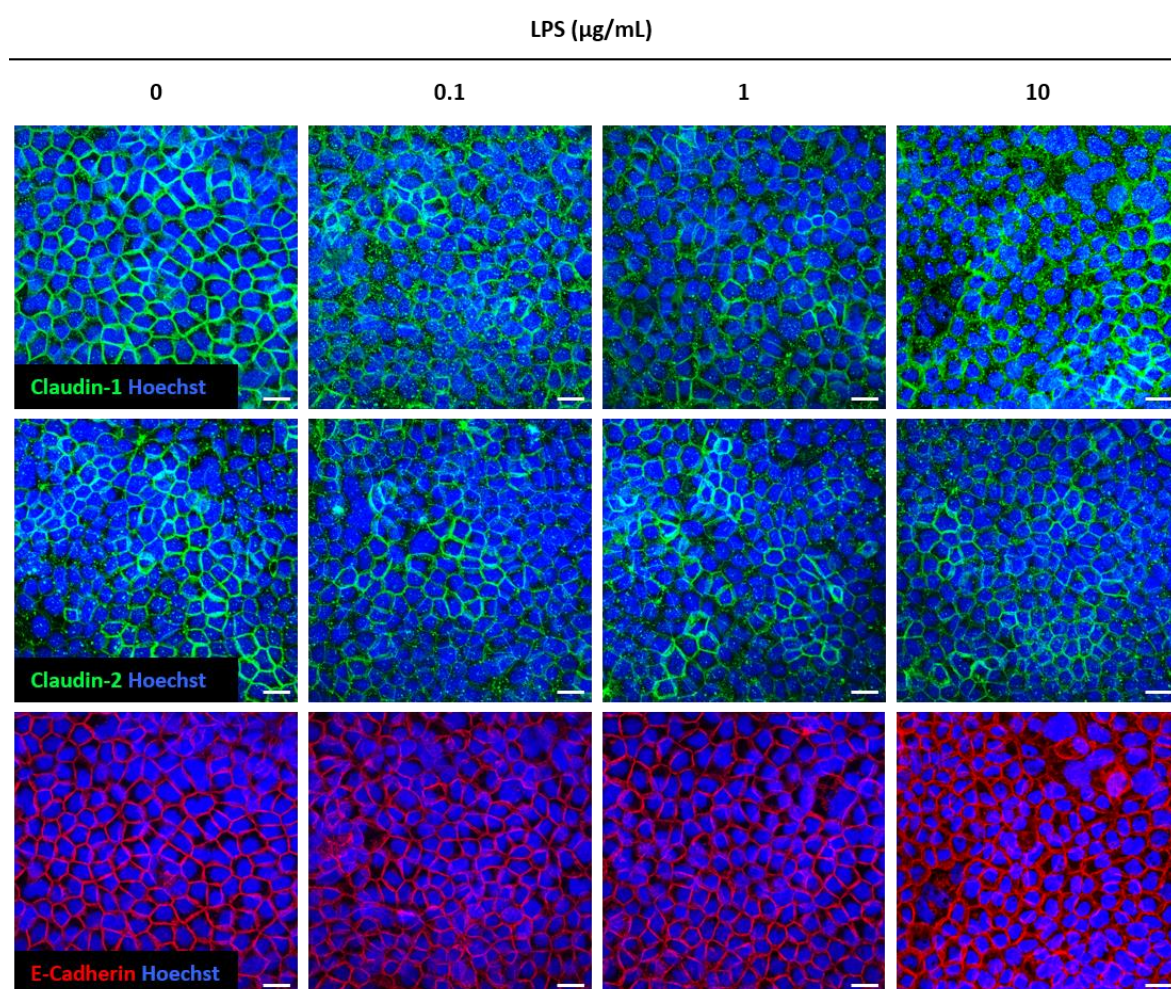


Figure 6.12. The structure of the apical junctional complex is not affected after an acute 24-hour incubation period with LPS. Caco-2 U937 co-cultures were established as described in Chapter 5. Following complete differentiation of the epithelium, LPS was introduced to the basolateral side of the well for 24 hours. Models were then fixed in 50:50 methanol: acetone and immunolabelled for the tight junction proteins: claudin-1 and claudin-2 (green) in addition to the adherens junction marker E-cadherin (red). Nuclei were counterstained blue with Hoechst. Representative compressed Z-stacks of the top-down view of 3 epithelial monolayers are shown. Scale bars: 20 μm .

Although few structural changes in the epithelial barrier were detected, alterations to the barrier function were identified. The effect of LPS concentration on TEER was assessed after 24 hours of incubation by calculating the percentage change from untreated control co-cultures. An ~8 % reduction in TEER was induced by 0.1 $\mu\text{g/mL}$ LPS and was reduced by a further 6 and 20 % when incubated with 1 and 10 $\mu\text{g/mL}$ LPS respectively (Figure 6.13A). This reduction in TEER is suggestive of a weakened barrier and an increased permeability, thus a lucifer yellow rejection assay, in addition to permeability assays, was performed.

Dye exclusion and transport assays were only performed in the apical-basolateral direction as absorptive permeability is of more interest in cases of intestinal inflammation and IBD. Lucifer yellow rejection remained high in all co-cultures irrespective of LPS concentration, with less than 1 % lucifer yellow leaking through the monolayer to the basolateral side (Figure 6.13B). Upon closer examination of the transport of lucifer yellow, a small increase in permeability was detected with increasing LPS concentration, though this was not found to be significant (Figure 6.13C). This trend was more obvious regarding the transport of FD20 which also displayed a higher permeability correlating with higher LPS concentrations (Figure 6.13D).

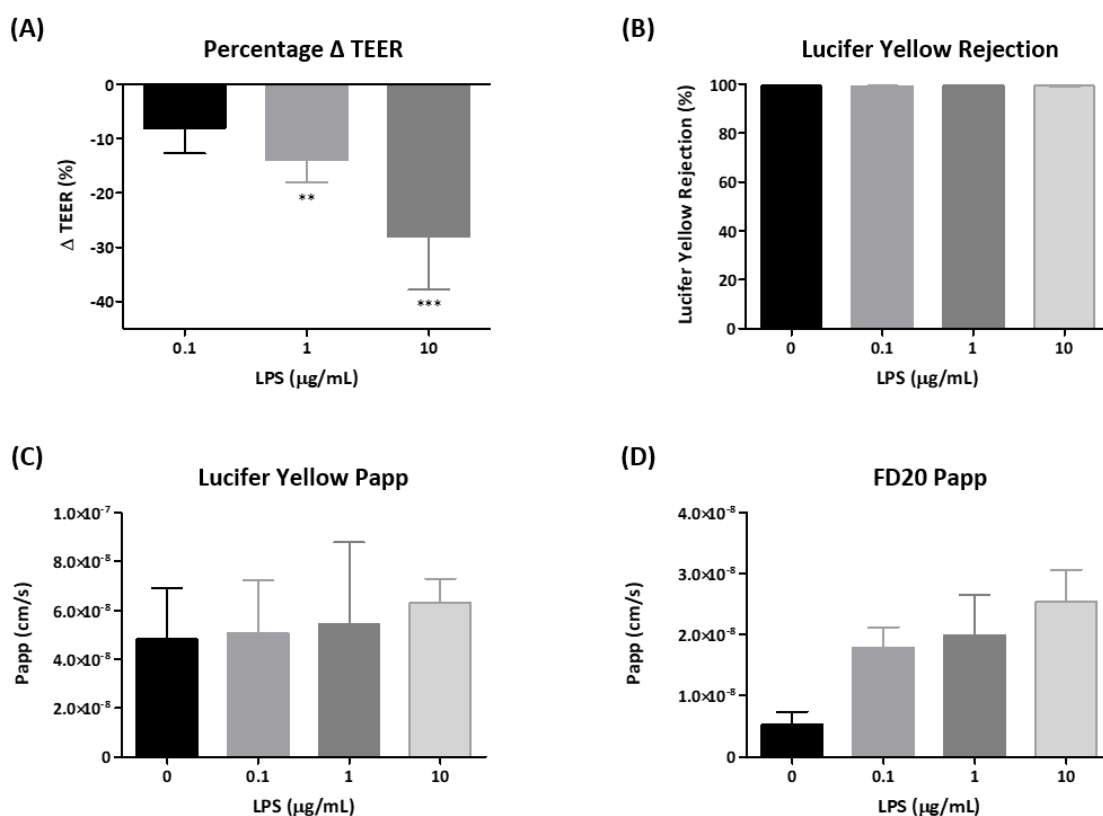


Figure 6.13. LPS causes a reduction in TEER and an increase in permeability in Caco-2 U937 co-cultures. Caco-2 U937 co-cultures were established as described in Chapter 5. Following complete differentiation of the epithelium, LPS was introduced to the basolateral side of the well for 24 hours. Following incubation, TEER was assessed across the different models and the percentage delta TEER was calculated against the untreated control model (A). A lucifer yellow rejection assay (B) and transport assay (C) were also performed, in addition to assessment of FD20 transport. Data represent mean $\pm$ SEM, total $n=9$ from 3 independent experiments (A) and mean $\pm$ SD, total $n=6$ from 2 independent experiments (B-D). Within each independent experiment, 3 models were cultured as technical repeats. ** $P \leq 0.001$ and *** $P \leq 0.0001$ as determined by one-way ANOVA with Dunnett's post-test.

6.4.2.3. Acute LPS treatment induces a proinflammatory response in the immune-competent triple co-culture

The effect of acute LPS treatment was also assessed on the triple co-culture. As before, LPS was added basolaterally to the cell culture media at 0, 0.1, 1 and 10 µg/mL and incubated for 24 hours. Initial characterisation of the effect of LPS on the models focused on histological staining to show the overall structure and morphology of the *in vitro* cultures. Figure 6.14 shows H&E staining of the 3D co-cultures incubated with LPS. Untreated co-cultures present with a well differentiated monolayer and evidence of epithelial polarisation into columnar shaped cells with basally located nuclei. U937 macrophages can be observed in the lamina propria, typically clustered together in the lower half of the membrane and are abundant in the layers of fibroblasts lining the lower edge of the membrane, though some cells are present in the upper half. Incubation with 0.1 and 1 µg/mL LPS does not appear to affect epithelial structure, yet the immune cells seem more evenly distributed throughout the Alvetex® Scaffold. Additionally, there appears to be greater numbers of macrophages visible within the bulk of the lamina propria overall, suggesting that a migratory phenotype may have been induced within the macrophages. However, this was not the case when models were treated with 10 µg/mL LPS for 24 hours. The resulting H&E images from these models show an intact epithelium but fewer noticeable immune cells in the lamina propria; this was evident in all biological and technical repeats. Furthermore, the fibroblast layering on the basolateral surface of the Alvetex® membrane appears diminished, possibly due to cell death or due to sample processing. Diffuse pink staining could be observed in all models, suggesting that there may be some cell death in the lamina propria compartment, however whether the debris is from fibroblast or macrophage cell death is not possible to distinguish, though the staining is more prominent adjacent to macrophage cells.

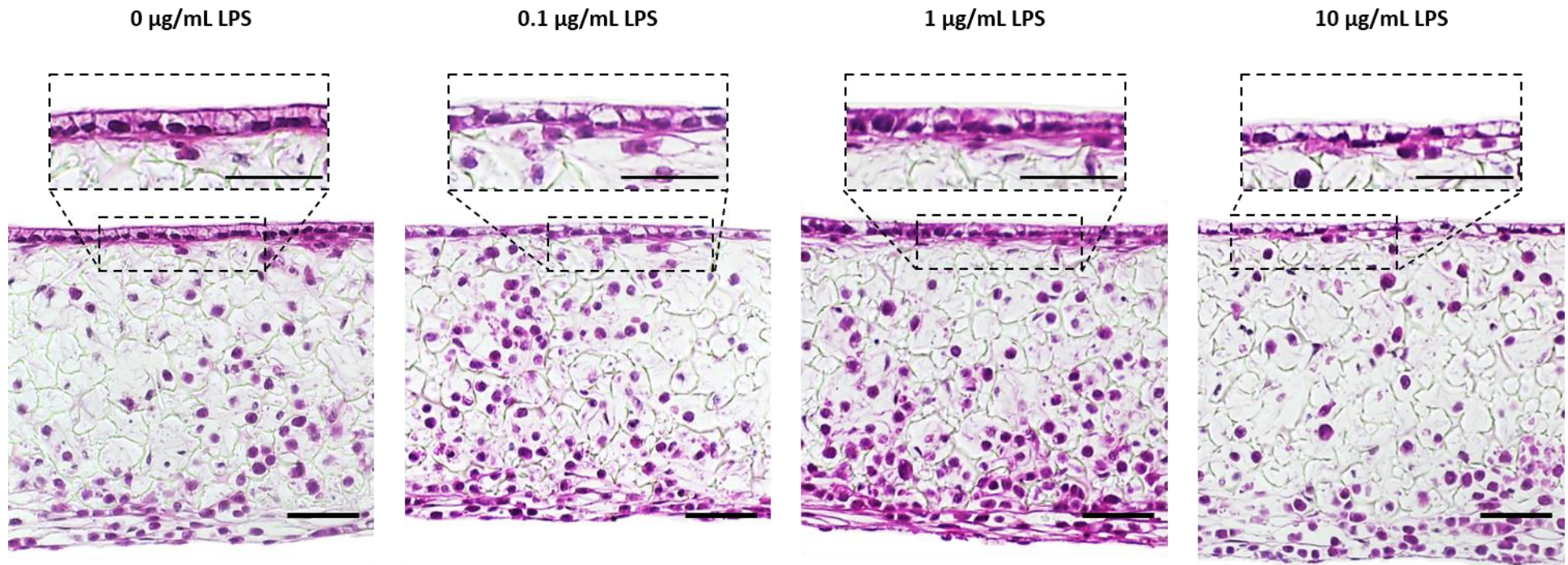


Figure 6.14. Acute LPS stimulation of the immune-competent triple co-cultures does not appear to affect epithelial morphology. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with LPS in the basolateral compartment for 24 hours before harvesting of the models. Representative H&E staining of 9 3D co-cultures treated with 0, 0.1, 1 and 10 µg/mL LPS is shown. Scale bars: 50 µm.

The lamina propria of the immune-competent triple co-cultures that had been treated with acute LPS stimulation was then characterised. This was primarily done via immunofluorescent staining for the ECM proteins collagen I, collagen III, and fibronectin (Figure 6.15A). Collagen I was abundantly expressed throughout the lamina propria and is most prominently expressed immediately subjacent to the epithelium and along the basolateral surface of the Alvetex® Scaffold membrane where fibroblast layering occurred. In untreated control models, collagen I appeared to be associated with the rounded nuclei of the U937 macrophages, yet in treated models, there are some individual U937 nuclei that are not in contact with collagen suggesting that the cells may have become migratory. Overall collagen I expression remained unchanged across the co-cultures incubated with different concentrations of LPS for 24 hours. Collagen III was also expressed throughout the lamina propria, though to a lesser extent than collagen I. It was also not as predominantly expressed subjacent to the epithelium and appeared to have a slightly reduced expression upon 0.1 µg/mL LPS incubation, though this was not evident at higher concentrations. Finally, immunostaining for fibronectin revealed an abundant expression in the immune-competent triple co-cultures and, like collagen I, was strongly localised below the epithelium in all treatment conditions. The only discernible difference between the untreated and treated models was the nature of fibronectin staining; in untreated controls fibronectin can be observed as fibres while in stimulated cultures it is visible as densely stained compact areas, rather than its usual fibrous nature.

Western blot analysis of two lamina propria components, TIMP-1 and α -SMA, was also performed though no densitometry was carried out as the representative images shown in Figures 6.15B and 6.15C are only from two biological repeats. Tissue inhibitor matrix metalloproteinase 1 (TIMP-1) is a natural inhibitor of matrix metalloproteinases that are responsible for degrading the ECM and has additional roles in promoting cell proliferation and inhibiting apoptosis (592). The expression levels of the TIMP-1 protein increased upon incubation with LPS and were further amplified with higher concentrations of LPS (Figure 6.15B). The expression of α -SMA, the myofibroblast marker that is associated with tissues fibrosis, did not have any noticeable changes between the unstimulated and stimulated models (Figure 6.15C). The overall lack of major changes within the lamina propria composition may be due to the short LPS exposure period and more prolonged incubations may be required to observe greater changes.

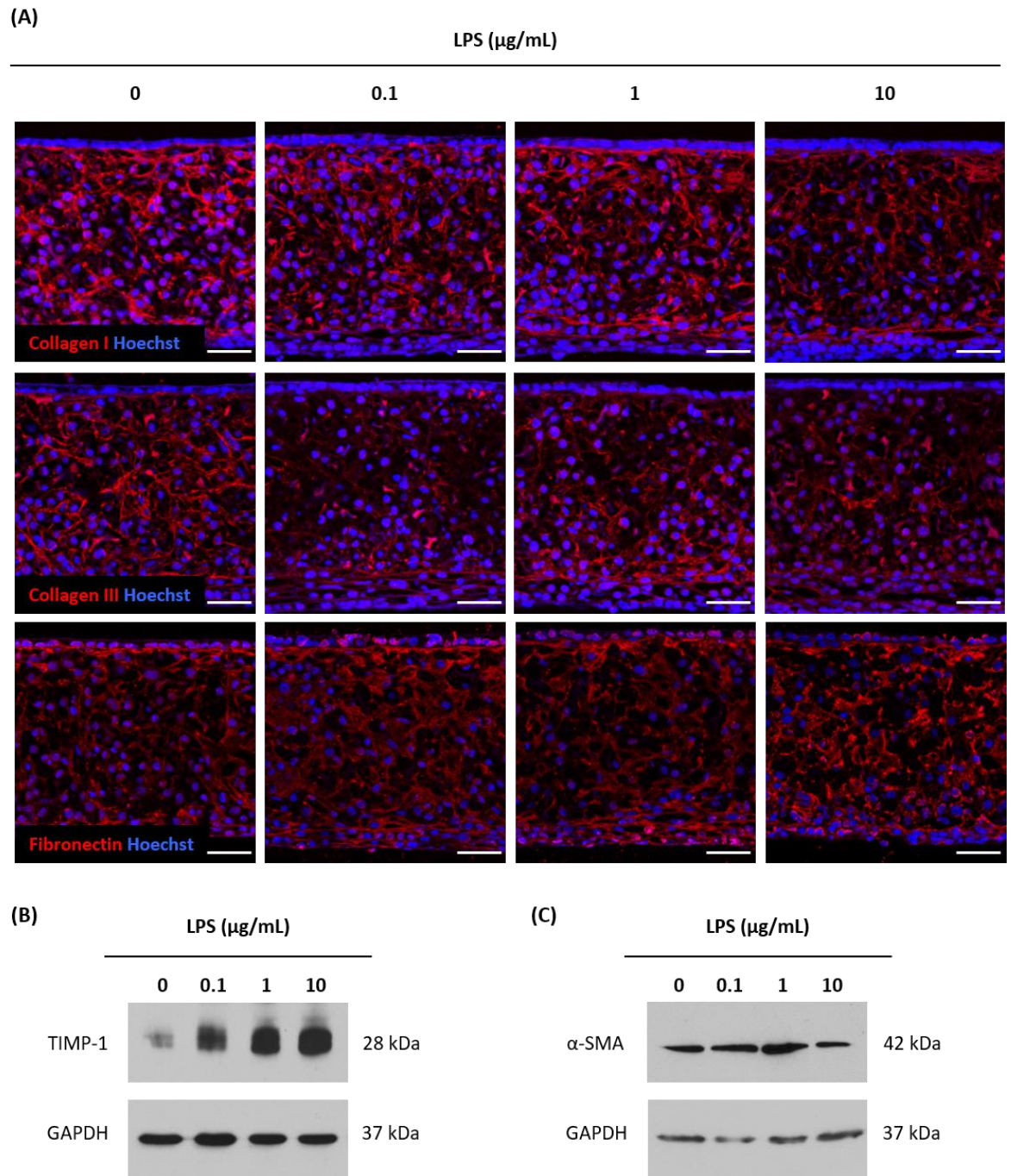


Figure 6.15. Acute LPS treatment does not have immediate effects on the lamina propria ECM. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with LPS in the basolateral compartment for 24 hours before harvesting of the models. ECM characterisation was then performed through immunofluorescent staining for collagen I, collagen III, and fibronectin (red), nuclei were counterstained blue with Hoechst, representative images of 9 models are shown (A). Western blot analysis for TIMP-1 (B) and α -SMA (C) were additionally performed with GAPDH loading controls. Representative western blot images of 2 SDS-PAGE gels are depicted. Scale bars: 50 μm .

To assess the effect of acute LPS stimulation on the epithelial barrier integrity, initial characterisation focused on the components of tight junctions. Immunofluorescent images of claudin-1 and claudin-2 staining are shown in Figure 6.16A. No alterations in either the distribution or expression levels of either protein were detectable through immunofluorescent analysis; both junctional markers were expressed along the lateral membranes of every individual Caco-2 enterocyte within the epithelium. As the incubation period for LPS treatment was limited to 24 hours, changes at the protein level may not yet be evident, therefore qPCR analysis of occludin, claudin-1 and ZO-1 which are encoded by the genes *OCN*, *CLDN1*, and *TJP1* respectively, was carried out. Occludin mRNA levels were amplified upon stimulation with LPS and were further enhanced with higher concentrations of LPS (Figure 6.16B). This unexpected result may be due to signalling processes that aim to prevent any epithelial damage induced by the LPS. On the other hand, both claudin-1 and ZO-1 (Figures 6.16C and D, respectively) mRNA expression levels decreased with increased levels of LPS suggesting a breakdown in barrier integrity, yet the overall fold change is low and as such the biological impact may be minimal.

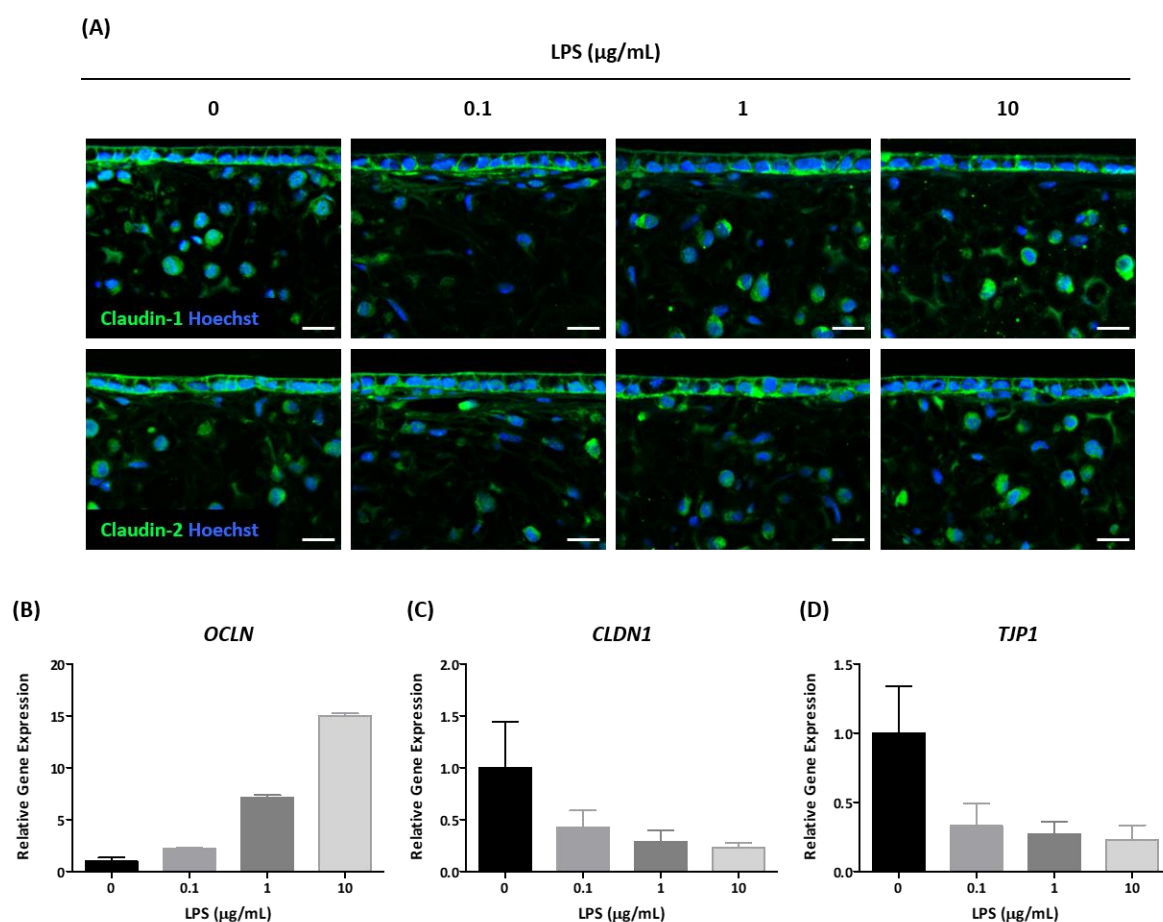


Figure 6.16. Acute LPS treatment of immune-competent triple co-cultures induces differential effects on tight junction components. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with 0-10 $\mu\text{g/mL}$ LPS in the basolateral compartment for 24 hours before harvesting of the models. Representative immunofluorescent images of 9 models; claudin-1 and claudin-2 (green) and nuclei counterstained with Hoechst (blue) revealed no differences in tight junction expression or localisation upon treatment with LPS (A). Quantitative PCR analysis for occludin (*OCN*) (B), demonstrated an increased mRNA expression upon LPS incubation while *CLDN1* (claudin-1) (C) and *ZO-1* (*TJP1*) (D) decreased with LPS treatment. qPCR data was first normalised to *GAPDH*, then to the gene expression levels of untreated control models. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction. Scale bars: 50 μm .

Further characterisation of the adherens junctions was also performed, as such junctions have important roles in stabilising the apical junctional complex and therefore have crucial roles in maintaining epithelial barrier integrity. E-cadherin and β -catenin are two key constituents of adherens junctions; immunofluorescent staining of the two proteins reveals that treatment of models with LPS for 24 hours does not induce structural changes in the expression of either protein (Figure 6.17A). Both E-cadherin and β -catenin were strongly expressed in all models and were primarily located at the lateral membranes of enterocytes. Quantification of the mRNA expression levels of E-cadherin, however, revealed that LPS causes a reduction in *CDH1*, the gene encoding for E-cadherin (Figure 6.17B). This reduction however was not enhanced by higher concentrations of LPS; levels of *CDH1* were similar between all stimulated co-cultures.

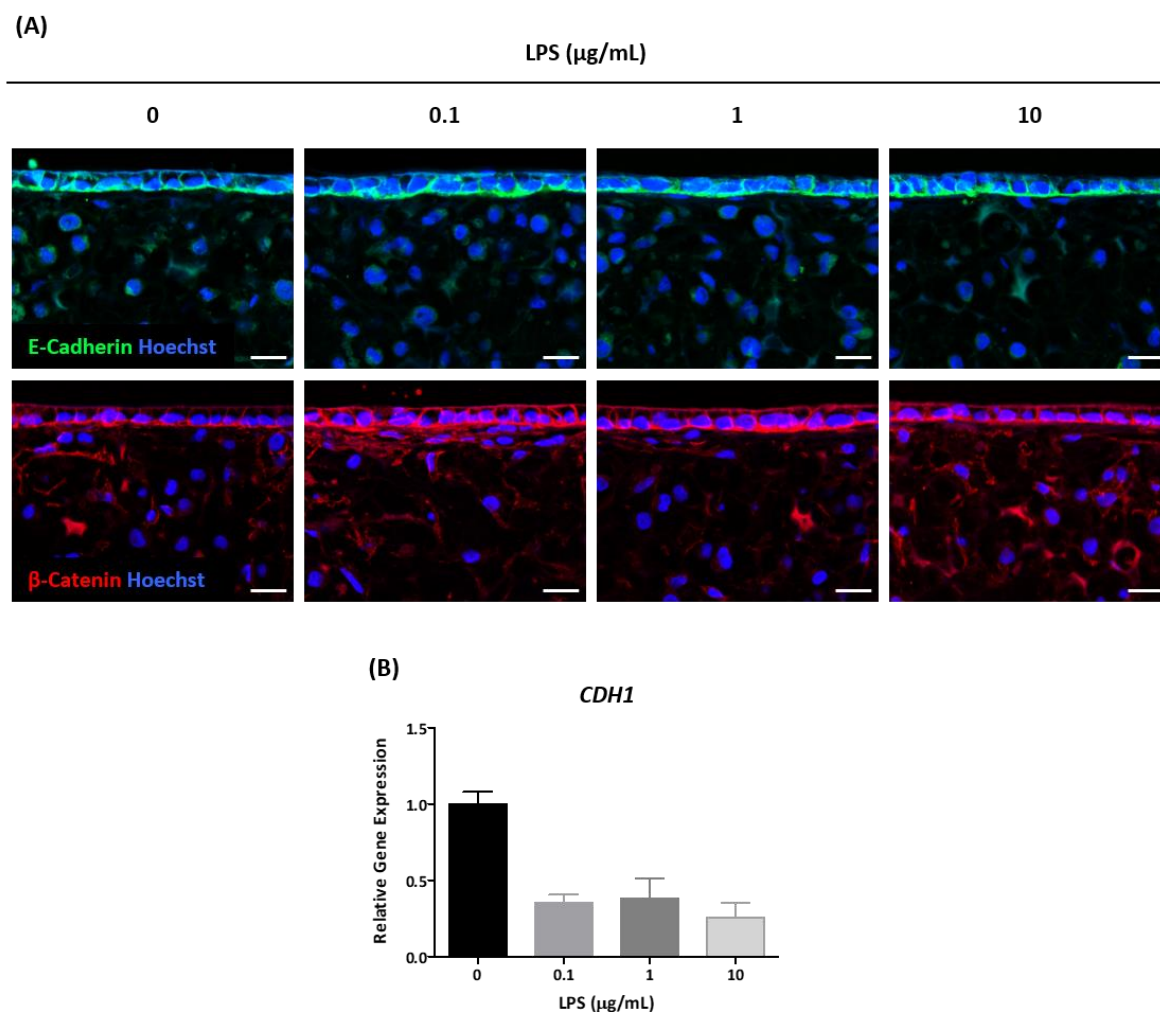


Figure 6.17. Acute LPS treatment does not alter the adherens junctions at the protein level but reduces the expression of mRNA. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with 0-10 $\mu\text{g/mL}$ LPS in the basolateral compartment for 24 hours before harvesting of the models. Representative immunofluorescent images of 9 models; E-cadherin (green) and β -catenin (red) and nuclei counterstained with Hoechst (blue) revealed no differences in tight junction expression or localisation upon treatment with LPS (A). Quantitative PCR analysis for E-cadherin (*CDH1*) (B) demonstrated that LPS treatment reduces the mRNA expression levels. Data was first normalised to GAPDH, then to the gene expression levels of untreated control models. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction. Scale bars: 20 μm .

Structural assessment of the epithelial barrier revealed no discernible changes at the protein level regarding the apical junctional complex, however expression levels of mRNA of both tight and adherens junction components were demonstrated to alter upon acute LPS incubation. To better characterise the full impact of LPS treatment on the integrity of the barrier, functional characterisation was carried out. This was initially done via analysis of the electrical resistance across the epithelium through TEER measurements. TEER was expressed as the percentage change from untreated control models with 0 µg/mL LPS (Figure 6.18A). The lowest concentration of LPS, 0.1 µg/mL caused a significant reduction in TEER by almost 22 %, and this was further augmented by 1 µg/mL LPS which induced a 40 % decrease in resistance compared to control cultures. However, only a minimal additional decrease in electrical resistance was caused by 10 µg/mL LPS where the TEER was diminished to 46 % of the control levels.

A reduced TEER is suggestive of a diminished epithelial barrier function which could have important consequences on the transport characteristics. Therefore, further assessment of the functional changes induced by short-term LPS treatment was performed through a lucifer yellow rejection assay (Figure 6.18B), in addition to quantification of the permeability of lucifer yellow, FD4 and FD20 (Figures 6.18C-E). No significant alterations to the absorptive passive paracellular transport were identified through these transport assays though a minor increase in the transport of FD4 was found upon LPS treatment. This suggests that LPS has an immediate effect on the electrical resistance properties of the epithelial barrier but does not cause an instant increase in permeability, possibly due to unaltered protein expression in the apical junctional complex.

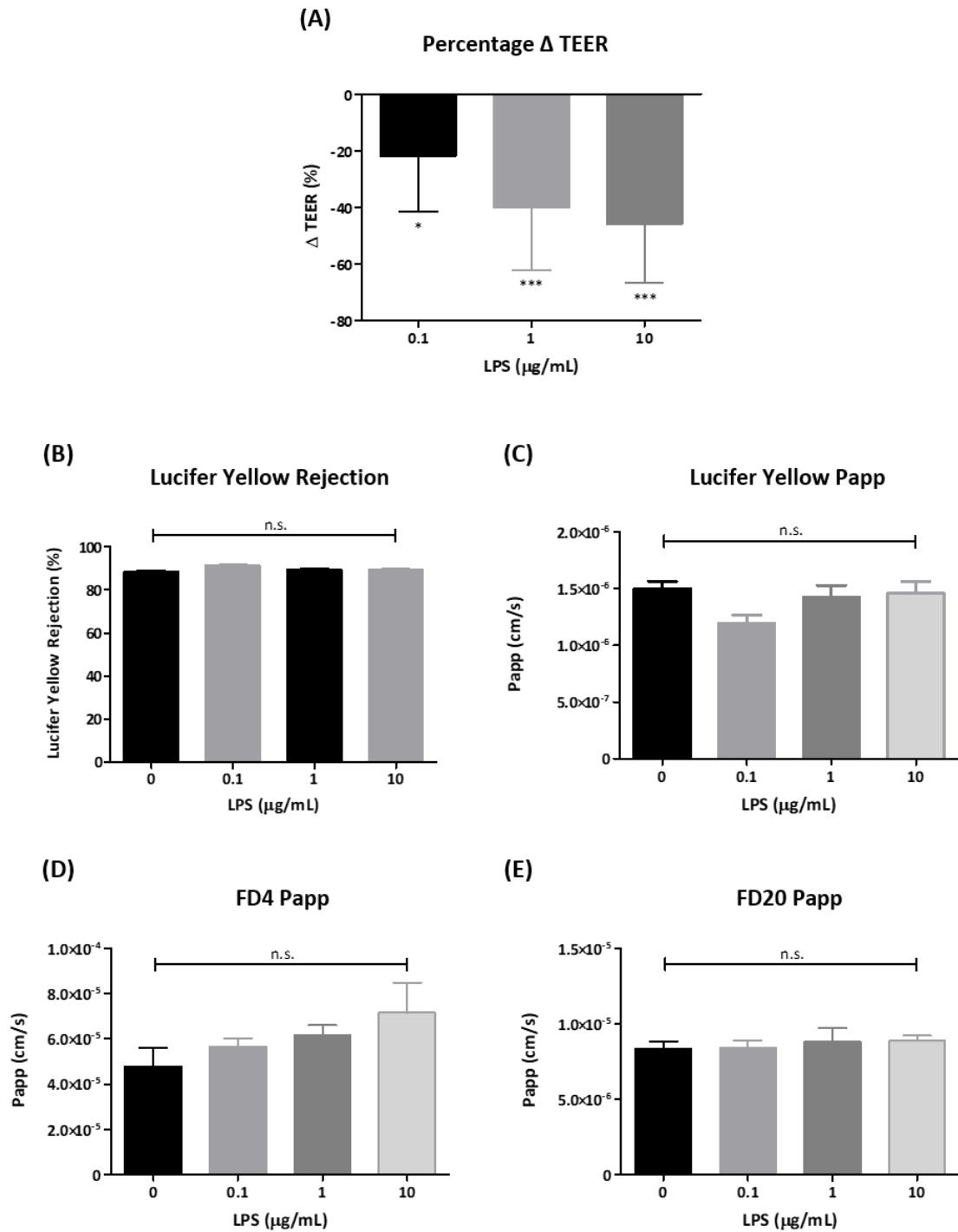


Figure 6.18. Acute LPS treatment significantly reduces TEER but does not alter the permeability of immune-competent triple co-cultures. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with 0-10 $\mu\text{g/mL}$ LPS in the basolateral compartment for 24 hours before characterising the function of the epithelial barrier. TEER was assessed across the different models following LPS incubation and the percentage delta TEER was calculated against the untreated control model (A). A lucifer yellow rejection assay (B) and transport assay (C) were also performed in addition to assessment of FD4 (D) and FD20 (E) permeability; no significant differences were found in the permeability of any of the assessed compounds. Data represent mean $\pm$ SEM, total $n=9$ from 3 independent experiments, each with 3 technical repeated. * $P\leq 0.05$ and *** $P\leq 0.0001$ as determined by one-way ANOVA with Dunnett's comparison against 0 $\mu\text{g/mL}$ LPS control co-cultures.

To understand the inflammatory response induced by LPS, qPCR analysis of pro- and anti-inflammatory cytokines was conducted. However, it was not possible to determine the contribution of individual cell lineages precisely, though it can be hypothesised that the U937 macrophages are the most prominent source of the cytokine mRNA due to their inherent nature to respond to inflammatory stimuli. TNF- α (*TNF*) expression levels increased in models treated with LPS compared to controls, with the greatest increase identified in models treated with 10 $\mu\text{g/mL}$ LPS which almost doubled the expression (Figure 6.19A).

Interleukin 1 β is a proinflammatory cytokine that functions as a key mediator of the inflammatory response to pathogenic stimuli such as LPS and additionally exacerbates damage caused by chronic inflammation (593). A concentration-dependent increase in the mRNA expression levels of IL-1 β (*IL1B*) was found across the models treated with 0-10 $\mu\text{g/mL}$ LPS suggesting that IL-1 β may play a key role in conducting the inflammatory response generated by LPS stimuli in the tissue constructs (Figure 6.19B). Interleukin 6 mRNA was also found to be increased in the models treated with LPS, though the highest levels were present in co-cultures incubated with 1 $\mu\text{g/mL}$ LPS (Figure 6.19C). Conversely, the proinflammatory cytokine interleukin 8 did not display a stepwise increase upon LPS treatment (Figure 6.19D).

In addition to quantification of the gene expression of proinflammatory cytokines, the levels of the anti-inflammatory cytokines interleukin 10 (*IL10*) and TGF- β 1 (*TGFB1*) were also assessed by qPCR. Interestingly, the concentration of LPS utilised for treatment induced differential responses in the expression of IL-10 (Figure 6.20A). Lower LPS concentrations (0.1 $\mu\text{g/mL}$ LPS) induced an increase in IL-10 mRNA expression following a 24-hour incubation period of the triple co-cultures, while the two higher concentrations (1 and 10 $\mu\text{g/mL}$ LPS) resulted in a decreased level of expression. No large alterations were identified in the expression of TGF- β 1 (Figure 6.20B).

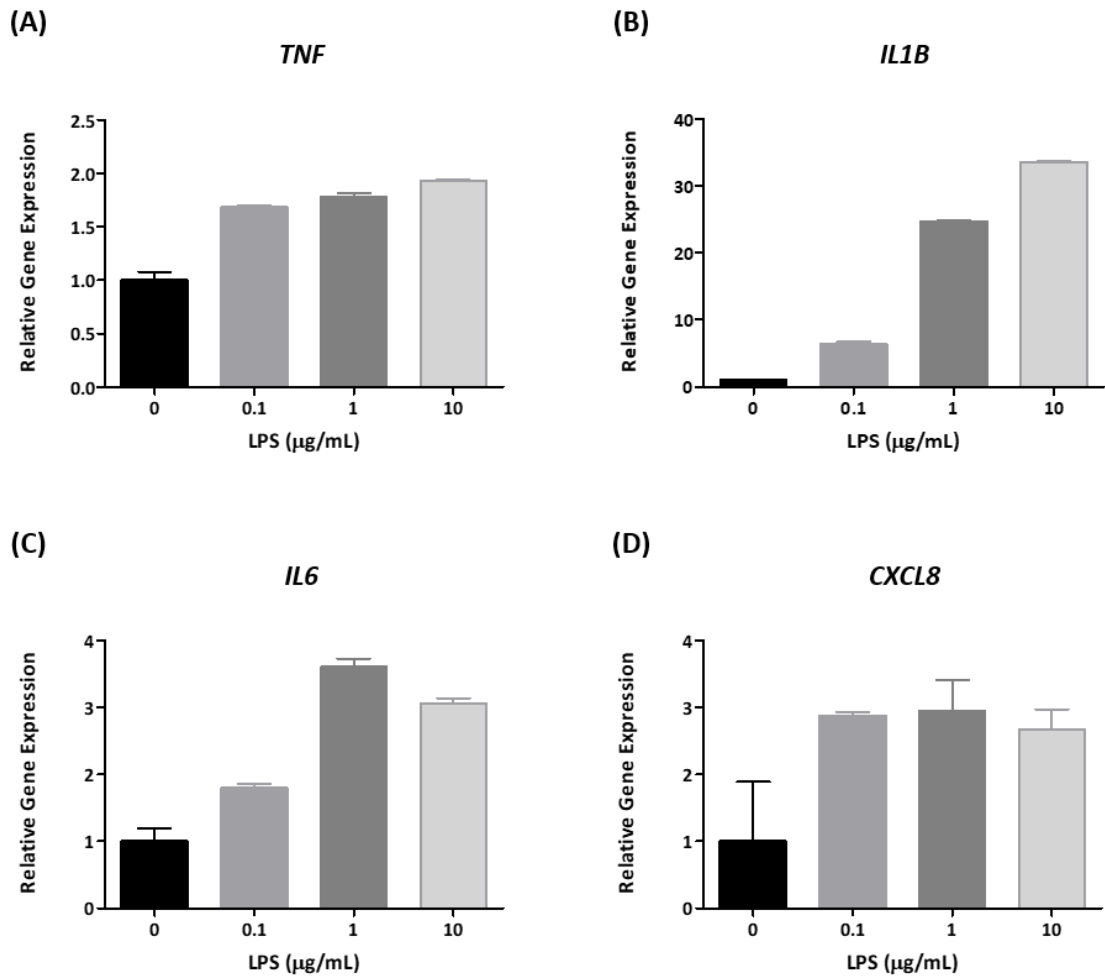


Figure 6.19. Acute LPS treatment induces an increase in the expression of proinflammatory cytokines as determined by qPCR. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with 0-10 µg/mL LPS in the basolateral compartment for 24 hours before lysing the models for qPCR analysis. Gene expression levels of tumour necrosis factor- α (A), interleukin 1 β (B), interleukin 6 (C) and interleukin 8 (D) were first normalised to the mRNA expression levels of GAPDH then to untreated control samples. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

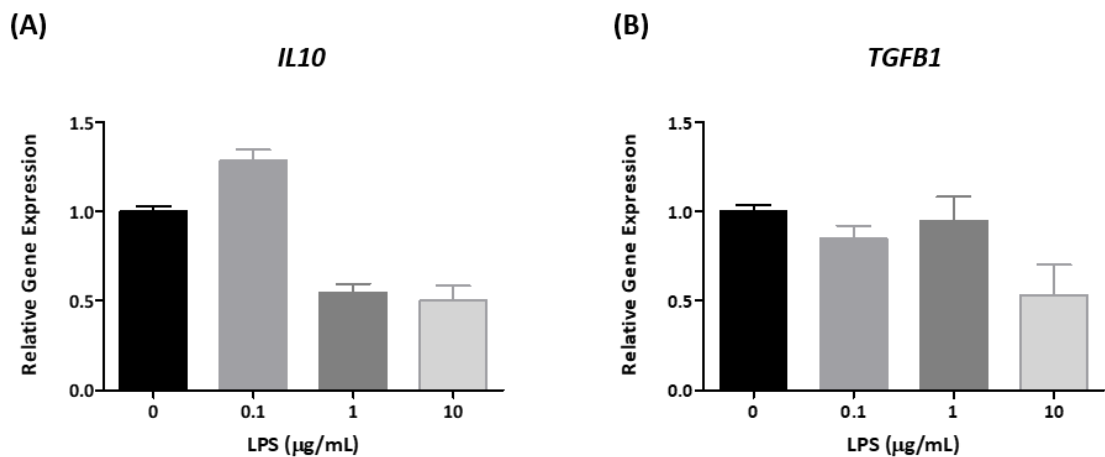


Figure 6.20. Acute LPS treatment causes changes in the mRNA expression levels of anti-inflammatory cytokines. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with 0-10 µg/mL LPS in the basolateral compartment for 24 hours before lysing the models for qPCR analysis. Gene expression levels of interleukin 10 (A) and transforming growth factor β 1 (B) were first normalised to the mRNA expression levels of GAPDH then to untreated control samples. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

Previous experiments focused on characterising the effects of acute LPS stimulation after 24 hours of incubation. Minimal changes in the structure were identified but some reduction in the integrity of the epithelial barrier was observed. It is possible that changes induced by LPS take longer than 24 hours to become apparent, thus one concentration of LPS (1 µg/mL) was selected to assess the functional changes induced in the immune-competent after 48 and 72 hours of LPS incubation. Time “0 h” hereby refers to untreated models with no LPS stimulus, while 24 h, 48 h and 72 h refer to the length of time models were incubated with LPS.

TEER was initially found to decrease upon treatment with LPS and this reduction was amplified after 48 hours (Figure 6.21A). However, by 72 hours of incubation with LPS, the electrical resistance began to recover and started to return to normal levels observed before LPS stimulation. Furthermore, the ability of the epithelium to exclude the fluorescent dye lucifer yellow was attenuated after 24 hours of LPS treatment, yet by 72 hours the barrier had fully recovered and was able to exclude more dye than untreated co-cultures (Figure 6.21B). No significant differences were found when the Papp of lucifer yellow was calculated, indicating that although the barrier properties of the model were weakened, the biological impact of this was negligible (Figure 6.22). Additionally, no significant differences were found in the apical-basolateral permeability of FD4 or FD20, however a similar trend is observed in the transport of the two compounds (Figure 6.21D, E): incubation with LPS for 24 hours induced the greatest increase in permeability and this was gradually recovered to baseline levels over the 48-hour and 72-hour long treatments. This demonstrated that longer incubation times following acute stimulation did not induce further changes in the epithelial barrier integrity.

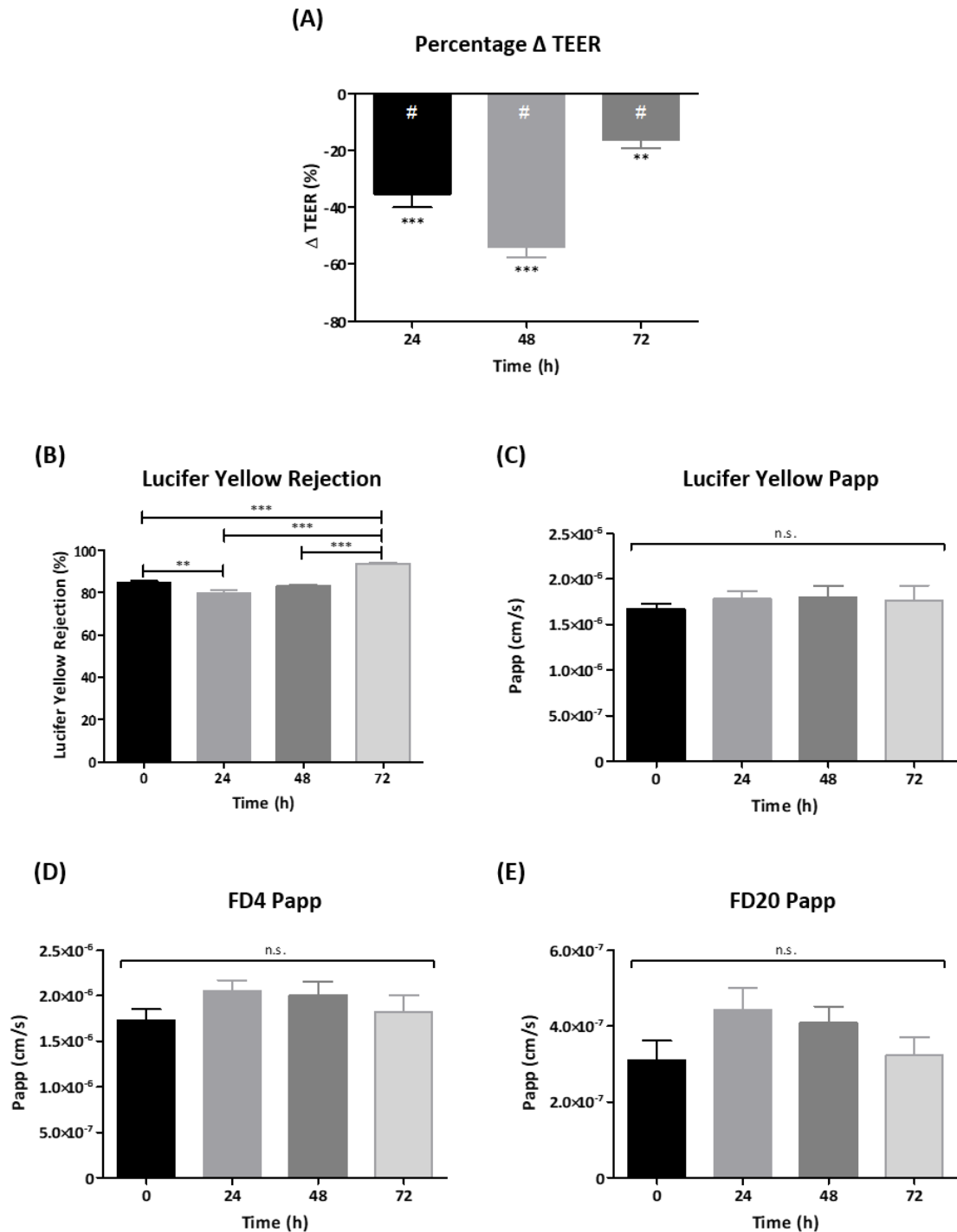


Figure 6.21. Longer LPS incubation times do not potentiate the reduction in epithelial barrier integrity. Immune-competent triple co-cultures were established as outlined in Chapter 5. Following enterocyte differentiation, the arms of the plastic insert were snapped off and models were transferred to 12-well plates where they were incubated with 0-10 $\mu\text{g/mL}$ LPS in the basolateral compartment for 24 hours before characterising the function of the epithelial barrier. TEER was assessed across the different models following LPS incubation and the percentage delta TEER was calculated against the untreated control model (A). A lucifer yellow rejection assay (B) and transport assay (C) were also performed in addition to assessment of FD4 (D) and FD20 (E) permeability; no significant differences were found in the permeability of any of the assessed compounds. Data represent mean $\pm$ SEM, total $n=9$ from 3 independent experiments. Within each independent experiment, 3 models were cultured per experimental condition. ** $P \leq 0.001$ and *** $P \leq 0.0001$ compared to untreated controls, # signifies $P \leq 0.0001$ between treatment incubation times as determined by one-way ANOVA with Tukey's post-hoc test.

6.4.3. Repeated LPS Applications to Mimic Chronic Inflammation

Acute LPS treatment demonstrated that an immune response was generated in 2D macrophages and the *in vitro* culture systems. However, in pathological states such as IBD, the intestinal mucosa is chronically affected by inflammatory stimuli, thus the effect of repeated “chronic” LPS applications to individual cell lineages and to the 3D immune-competent co-culture was investigated. This was done through repeated doses of LPS treatment every 24 hours up to 3 days. Although 10 µg/mL induced the greatest changes in the epithelial barrier in both *in vitro* models (demonstrated in Section 6.4.2), it was hypothesised that this high concentration may reduce cellular viability if repeated applications were used. Hence, the lower concentration of 1 µg/mL LPS was selected as this also reduced epithelial barrier integrity and successfully induced an inflammatory response in both differentiated macrophages cultured in 2D and in immune-competent co-cultures. Cells and models therefore remained either untreated controls or had 1-3 doses of 1 µg/mL LPS applied to their culture media sequentially every 24 hours and were then harvested for downstream analysis 24 hours after receiving their last dose of LPS (Figure 6.22).

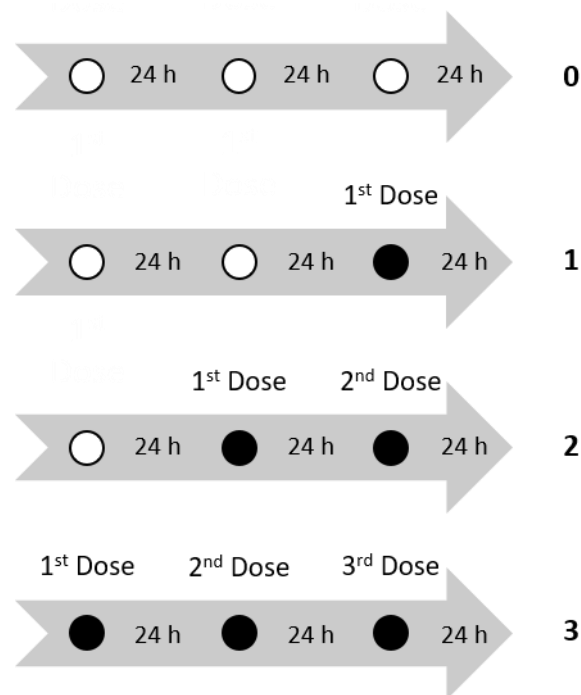


Figure 6.22. Schematic representation of “chronic” LPS stimulation. Cell lines and immune-competent *in vitro* models were subject to serial doses of LPS every 24 hours before sample processing for further downstream analysis. Untreated control samples had 0 doses of LPS, and the remaining samples were treated with LPS either 1, 2 or 3 times at 24-hour intervals as indicated by the shaded circles. 24 hours after the last dose of LPS, samples were harvested.

6.4.3.1. Chronic LPS stimulation induces a proinflammatory response in 2D cultured cells

Prior to investigating the impact of chronic LPS stimulation on the *in vitro* models, repeated LPS doses were applied to differentiated macrophages maintained in 2D, in addition to 2D cultured HDFn and Caco-2 cells as outlined in Figure 6.22. The inflammatory response following chronic stimulation was then quantified by qPCR to examine differences in cytokine gene expression and to determine how each individual cell lineage that constitutes the immune-competent triple co-culture responds to repeated stimulation with LPS.

Following differentiation of U937 cells, chronic LPS treatments were applied to the macrophages. Cells were then lysed for RNA and gene expression analysis was conducted. The gene expression two proinflammatory cytokines, IFN- γ and TNF- α , increased with repeated LPS treatments (Figure 6.23A,B). One application of LPS more than doubled the levels of both IFN- γ and TNF- α from baseline levels where cells were not exposed to the inflammatory stimulus. Furthermore, multiple LPS doses amplified the production of *IFNG* and *TNF* mRNA in a stepwise manner with 3 LPS treatments inducing the greatest increase in expression, with almost a 30-fold increase in the expression of *IFNG* and a 10-fold increase in *TNF*. Conversely, the expression levels of IL-1 β were highest following a one-off treatment with LPS and mRNA levels then exhibited an incremental decrease with additional doses, though expression levels remained over 10X greater than untreated cells (Figure 6.23C).

Gene expression levels of interleukins 6 and 8 are shown in Figures 6.23D and 6.23E; both cytokines are part of the proinflammatory response of macrophages to noxious stimuli. *IL6* mRNA induction reached maximal levels following two repeated LPS treatments, then experienced a slight reduction in expression, while IL-8 continued to cumulatively increase with further LPS incubations. Contrastingly, the anti-inflammatory cytokine, IL-10, demonstrated no large fold changes in expression levels, although marginally higher levels of *IL10* mRNA were present in all treated macrophages (Figure 6.23F).

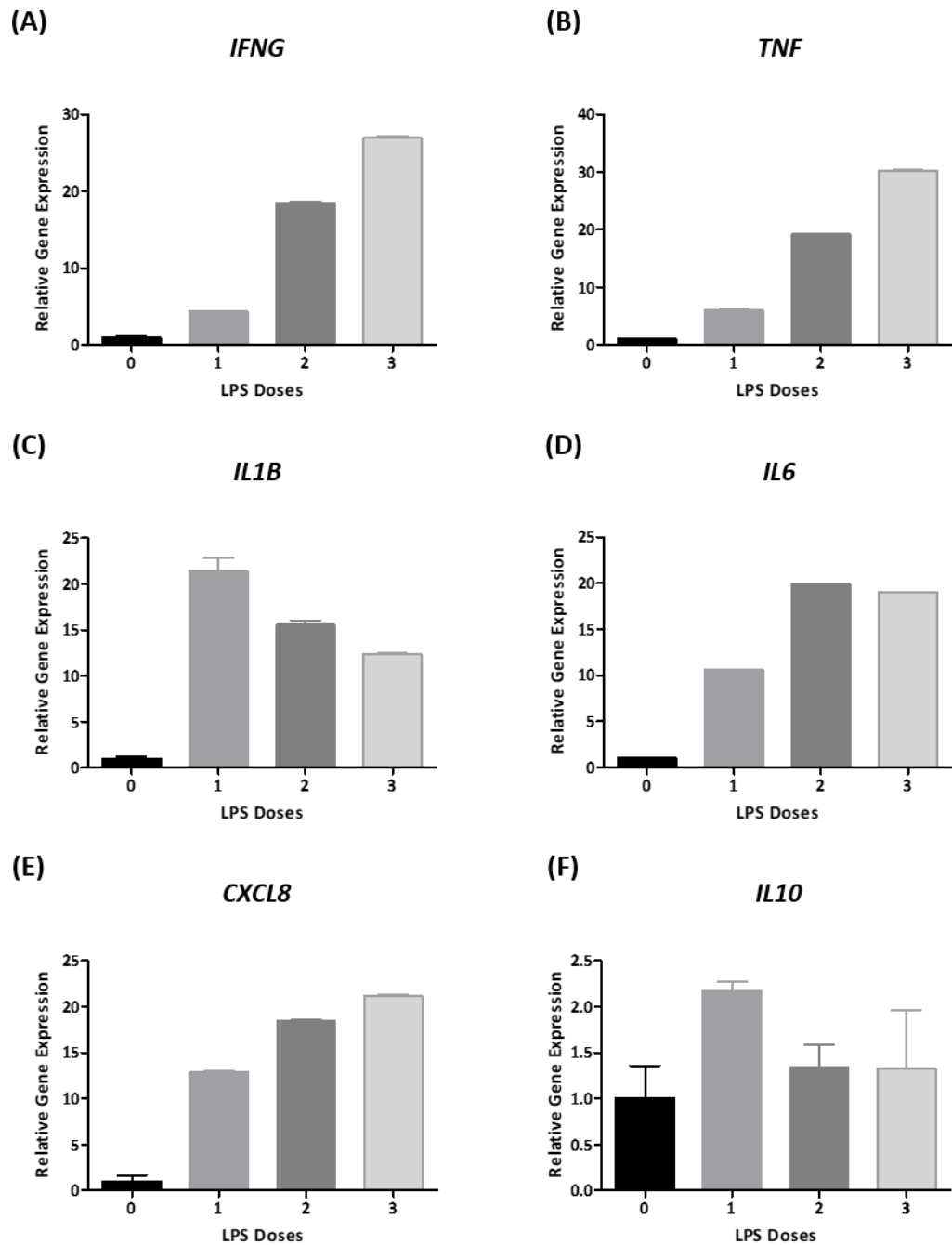


Figure 6.23. A strong proinflammatory immune response is induced in differentiated U937 macrophages treated with chronic, repeated LPS doses. Differentiated U937 macrophages were treated with repeated applications of 1 μ g/mL LPS as outlined in Figure 6.22. Cells were then harvested for qPCR analysis to examine the gene expression levels of IFNG (A), TNF (B), IL1B (C), IL6 (D), CXCL8 (E) and IL10 (F). Expression levels were first normalised to the mRNA expression levels of Actin then to untreated control samples. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

Fibroblasts are considered to have significant roles in maintaining immune cell and epithelial cell homeostasis and are thought to play a role in the pathogenesis of inflammatory bowel diseases, therefore the cytokine profile of 2D cultured fibroblasts subjected to repeated LPS stimulations was assessed via qPCR analysis. Fibroblasts are not thought to secrete high levels of IFN- γ ; thus, this cytokine was not investigated. TNF- α exhibited a small increase in expression following 1 and 2 doses of LPS treatment, however 3 repeated LPS applications resulted in the highest level of *TNF* mRNA induction (Figure 6.24A). A one-time dose of LPS also did not cause an increase in the expression of IL-1 β within the dermal fibroblasts, however repeated doses did induce increased expression with the highest levels being present following two applications of LPS (Figure 6.24B). Interleukins 6 and 8 both had a similar expression profiles whereby incremental increases in the mRNA levels were present in HDFn cells following multiple LPS treatments (Figures 6.24C,D). Finally, fibroblasts were assessed for their ability to produce the cytokine IL-10 as its production can have significant consequences on the fibrosis of the intestinal mucosa in chronic IBD cases. *IL10* mRNA increased over 150-fold with LPS treatment and maximal mRNA levels were detected following two repeated incubation periods followed by a slight decrease in gene expression after a third dose (Figure 6.24E). It is important to note that these fibroblasts were not maintained in a 3D microenvironment and did not deposit significant levels of ECM which would have further signalling and feedback roles within the 3D immune-competent co-culture and in the *in vivo* intestinal mucosa.

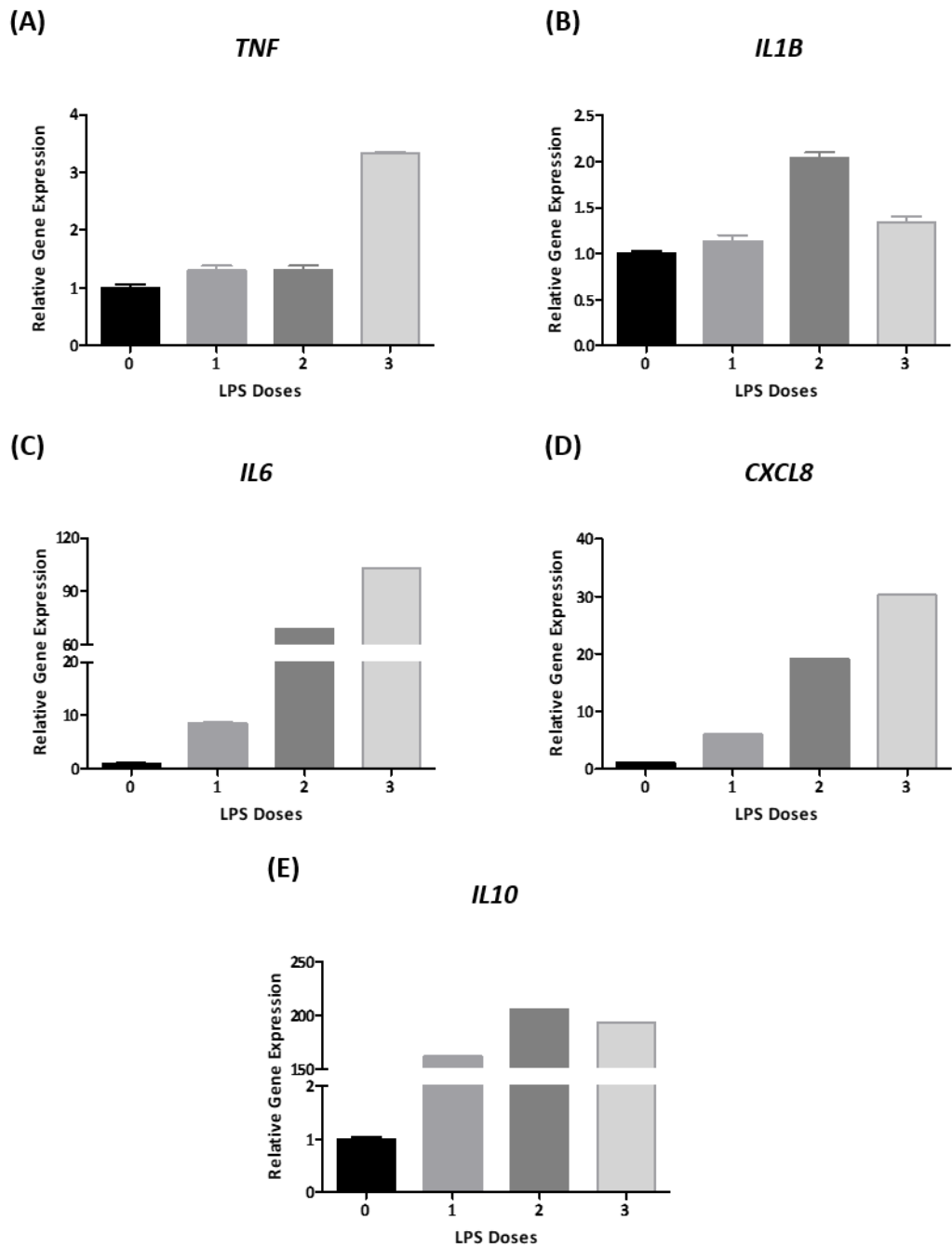


Figure 6.24. LPS treatment induces a proinflammatory cytokine response in fibroblasts that is further amplified by repeated doses. HDFn cells were seeded into 6-well plates and maintained for four days before cells were treated with repeated applications of 1 μ g/mL LPS as outlined in Figure 6.22. Cells were then harvested for qPCR analysis to examine the gene expression levels of TNF (A), IL1B (B), IL6 (C), CXCL8 (D) and IL10 (E). Gene expression levels were first normalised to the mRNA expression levels of Actin then to untreated control samples. Data represent mean $\pm$ SEM, total n=6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

Finally, assessment of the inflammatory response of Caco-2 cells, in addition to their ability to produce components of the apical junctional complex, was also conducted through qPCR analysis. TNF- α and IL-6 shared a similar expression profile in response to LPS stimulation within 2D cultured Caco-2 cells (Figures 6.25A,B). The gene expression of both cytokines, increased in an accumulative manner from one to two LPS doses, however 24 hours after the third application of LPS, the expression levels then dropped off. In contrast, interleukins 8 and 10 reached the highest gene expression following three LPS treatments (Figures 6.25C,D). Interestingly the increased gene expression of all cytokines never exceeded 4X baseline levels, much lower than in fibroblasts and macrophages indicating that Caco-2 epithelial cells may not be major contributors of the inflammatory response induced by LPS stimulation directly, but indirect inflammatory activation is also likely to occur. It would also be of interest to determine if there are any differences in the cytokine response generated between Caco-2 cells cultured in 2D on tissue culture plastic to those that have formed a differentiated epithelial monolayer maintained on Transwell® inserts, however initial pilot studies resulted in poor RNA yields from Transwell® monolayers.

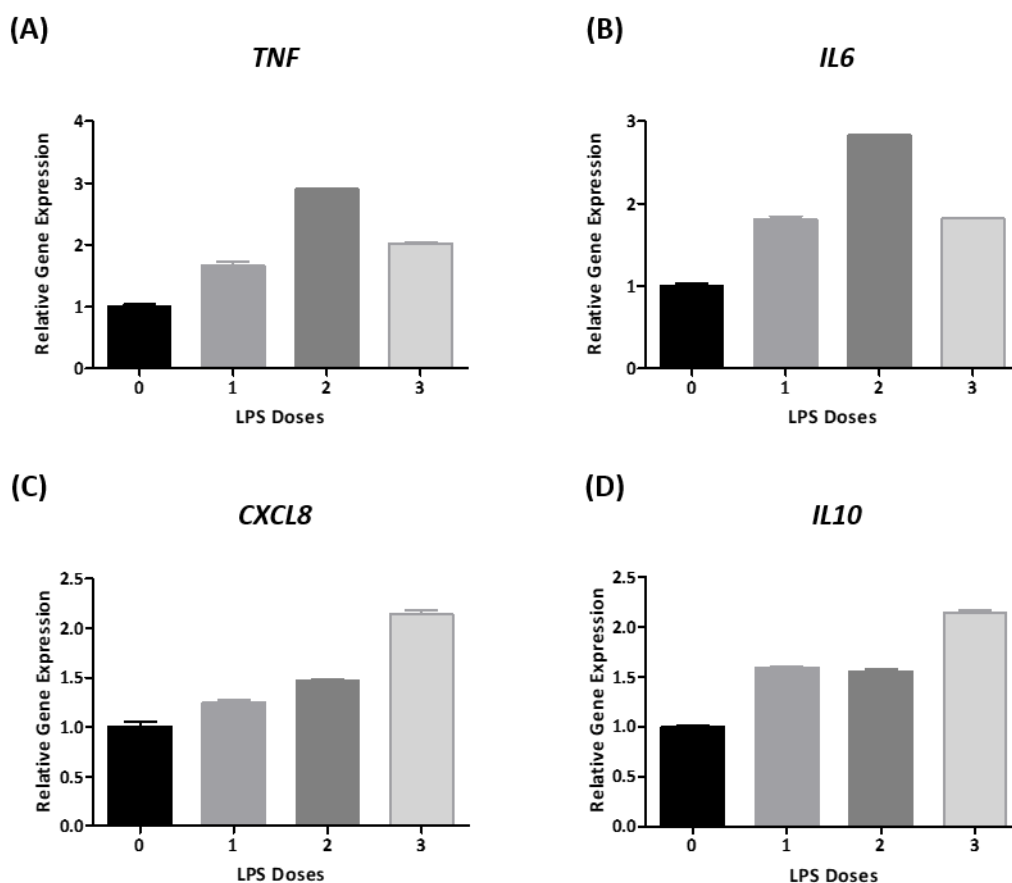


Figure 6.25. Caco-2 cells maintained in 2D produce an inflammatory cytokine response when subjected to repeated LPS treatments. Caco-2 cells were seeded into 6-well plates and maintained for four days before cells were treated with repeated applications of 1 μ g/mL LPS as outlined in Figure 6.22. Cells were then harvested for qPCR analysis to examine the gene expression levels of TNF (A), IL6 (B), CXCL8 (C) and IL10 (D). Gene expression levels were first normalised to the mRNA expression levels of Actin then to untreated control samples. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

Figure 6.26 shows the gene expression analysis of the apical junctional complex constituents. The tight junction components occludin, claudin-1 and ZO-1 were initially found to have an increase in their mRNA levels yet following the third dose the levels were reduced; this was particularly evident regarding occludin and ZO-1. This follows the same trend as observed in the expression profiles of TNF and IL-6 and could suggest that the cells are experiencing some cell death due to the chronic stimulation. Furthermore, E-cadherin was initially increased following one LPS incubation, yet subsequent doses reduced the levels closer to those of untreated samples. Although these results were generated from 2D cultured cells, they demonstrate that chronic stimulation could have significant effects on the ability of the Caco-2 cells to form an intact epithelial barrier when maintained in a 3D co-culture system.

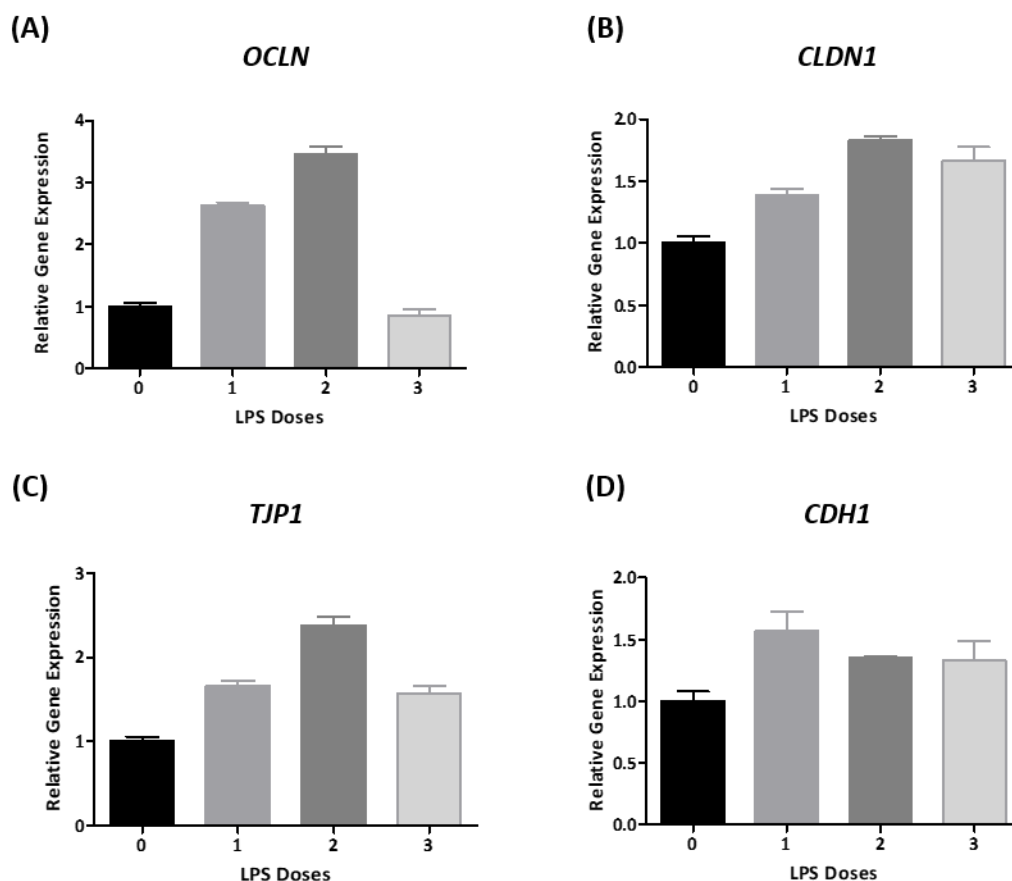


Figure 6.26. Caco-2 cells initially increase the mRNA levels of AJC proteins following LPS treatment followed by a subsequent drop off after repeated stimulations. Caco-2 cells were seeded into 6-well plates and maintained for four days before cells were treated with repeated applications of 1 $\mu\text{g}/\text{mL}$ LPS as outlined in Figure 6.22. Cells were then harvested for qPCR analysis to examine the gene expression levels of OCLN (A), CLDN1 (B), TJP1 (C) and CDH1 (D). Gene expression levels were first normalised to the mRNA expression levels of Actin then to untreated control samples. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

6.4.3.2. Chronic LPS treatment of the 3D immune-competent triple co-culture reduces epithelial barrier integrity

Conducting qPCR analysis of 2D cultured cells revealed that all three lineages present in the immune-competent triple co-culture responded to chronic LPS stimulation by inducing an inflammatory response via the production of pro- and anti-inflammatory cytokine mRNA. Subsequently the effect of repeated LPS applications were then evaluated on the 3D *in vitro* model. Preliminary assessment of the morphology of the *in vitro* models was performed through H&E staining (Figure 6.27). Untreated co-cultures present with a highly polarised, columnar epithelial monolayer overlying a lamina propria compartment comprising of fibroblasts and U937 macrophages that are typically found in the lower half of the Alvetex® Scaffold. A one-time application of LPS for 24 hours induces a change in the localisation of these immune cells which can then be identified subjacent to the epithelium, suggesting that a migratory phenotype had been induced in the macrophages. Furthermore, one 24-hour treatment of LPS appeared to reduce the degree of polarisation in the epithelium; the cells did not appear as elongated and less cellular cytoplasm was visible above the nuclei of the cells (highlighted by the arrows). Similarly, repeated doses of LPS caused the epithelium to gain a more flattened and cuboidal appearance which could have important consequences on epithelial barrier function, this phenotype was most evident following the maximal number of LPS doses where almost every individual cell had a flattened structure compared to control epithelial monolayers, as indicated by the arrows. Fewer immune cells were evident throughout the lamina propria of models stimulated multiple times, possibly due to migration out of the Alvetex® Scaffold or due to cell death, evidence of which can be observed in models treated three times with LPS where large levels of debris are present in the lamina propria.

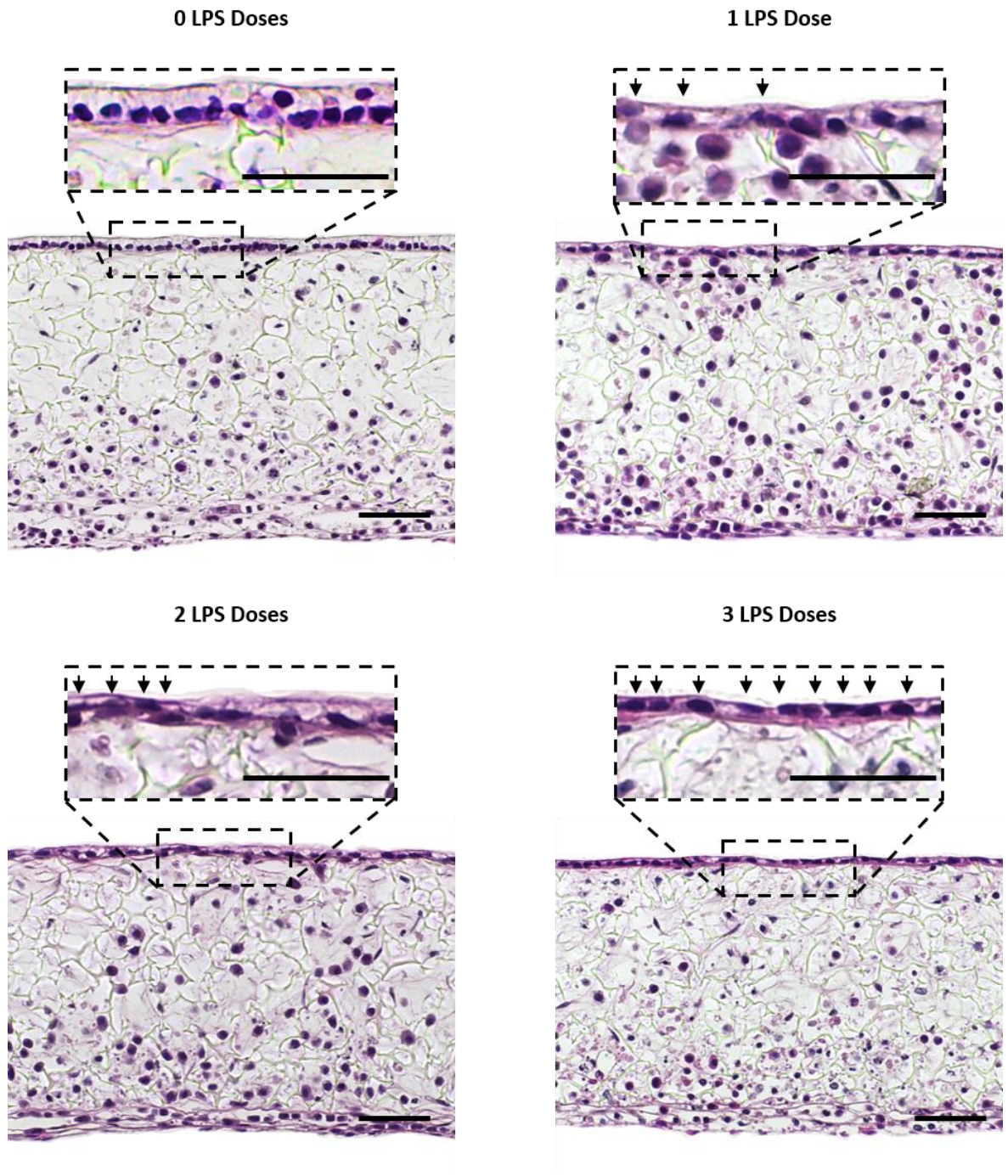


Figure 6.27. Repeated LPS stimulation reduces the degree of epithelial polarisation in the immune-competent triple co-cultures. Triple co-cultures were established as described in Chapter 5. Following enterocyte differentiation, the arms Alvetex® Scaffold were snapped off and the insert was transferred to a fresh 12-well plate where upon models were stimulated with repeated doses of LPS as outlined in Figure 6.22, before being harvested for histological staining. Representative H&E images of the untreated and treated models are shown. Arrows denote epithelial cells that possess a flattened morphology, with limited cytoplasmic staining evident above the nucleus. Images shown are representative of 9 models. Scale bars: 50 μ m.

Primary inflammatory responses typically induce a degradation of ECM to allow for immune cell infiltration, while chronic inflammation can lead to an overproduction of collagens resulting in fibrosis of the tissue. Therefore, the ECM of the lamina propria, specifically the collagen content, was investigated via immunofluorescent staining (Figure 6.28). Abundant collagen I and III were present in the Alvetex® Scaffold membrane of immune-competent triple co-cultures that were not subjected to LPS incubations. A similar level of both collagens I and III were visible in cultures exposed to one dose of LPS, yet following multiple doses there appeared to be a reduction in the levels of deposited collagen and large unstained areas were present. This apparent reduction was particularly evident following three treatments of LPS and was most prominent for collagen I. However, the overall levels of collagen in the different models were not quantified either by western blot analysis or a hydroxyproline assay wherein the levels of the amino acid responsible for stabilising the helical structure of collagen (hydroxyproline) is quantified. If confirmed to be reduced, this would suggest that the immune response generated in the co-culture model is not yet at the chronic stage and may require further proinflammatory stimuli to be applied.

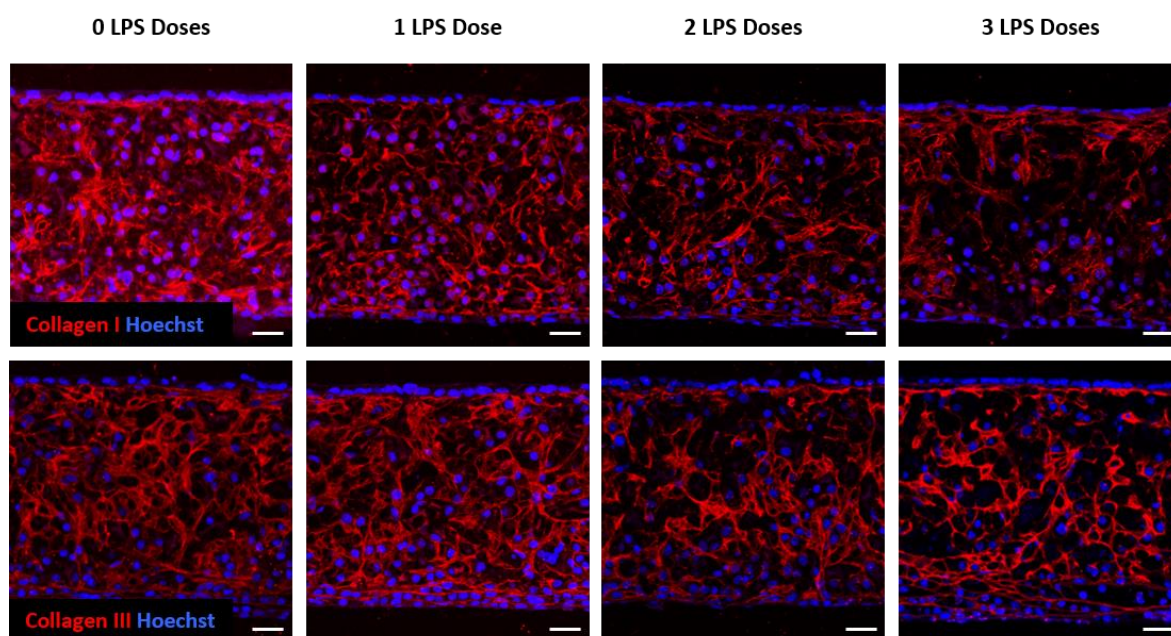


Figure 6.28. Collagen appears to be degraded upon repeated doses of LPS in the lamina propria of the immune-competent triple co-culture. Triple co-cultures were established as described in Chapter 5. Following enterocyte differentiation, the arms Alvetex® Scaffold were snapped off and the insert was transferred to a fresh 12-well plate where upon models were stimulated with repeated doses of LPS as outlined in Figure 6.22, before being harvested for wax embedding. Models were then sectioned, and immunofluorescent staining was performed for collagen I and collagen III (red), Hoechst staining was used to visualise nuclei (blue). Images shown are representative of 9 models. Scale bars: 50 μ m.

Preliminary assessment of the impact of repeated LPS treatments on the epithelial barrier was conducted through qPCR to quantify the gene expression levels of apical junctional complex proteins. Occludin, claudin-1 and ZO-1 were selected to represent the tight junctions, while E-cadherin was chosen to characterise the adherens junctions. Following a single dose of LPS, the gene expression of *OCLN* increased almost 4-fold and then decreased following repeated doses (Figure 6.29A). Occludin was the only gene found to initially increase upon LPS treatment, all other genes either remained the same (Figure 6.29B) or decreased (Figures 6.29C,D). After two or three incubations with LPS, the levels of claudin-1, ZO-1 and E-cadherin were drastically reduced in comparison to untreated controls, suggesting that the epithelial barrier may be weakened due to a lack of protein turnover and may display an increased permeability.

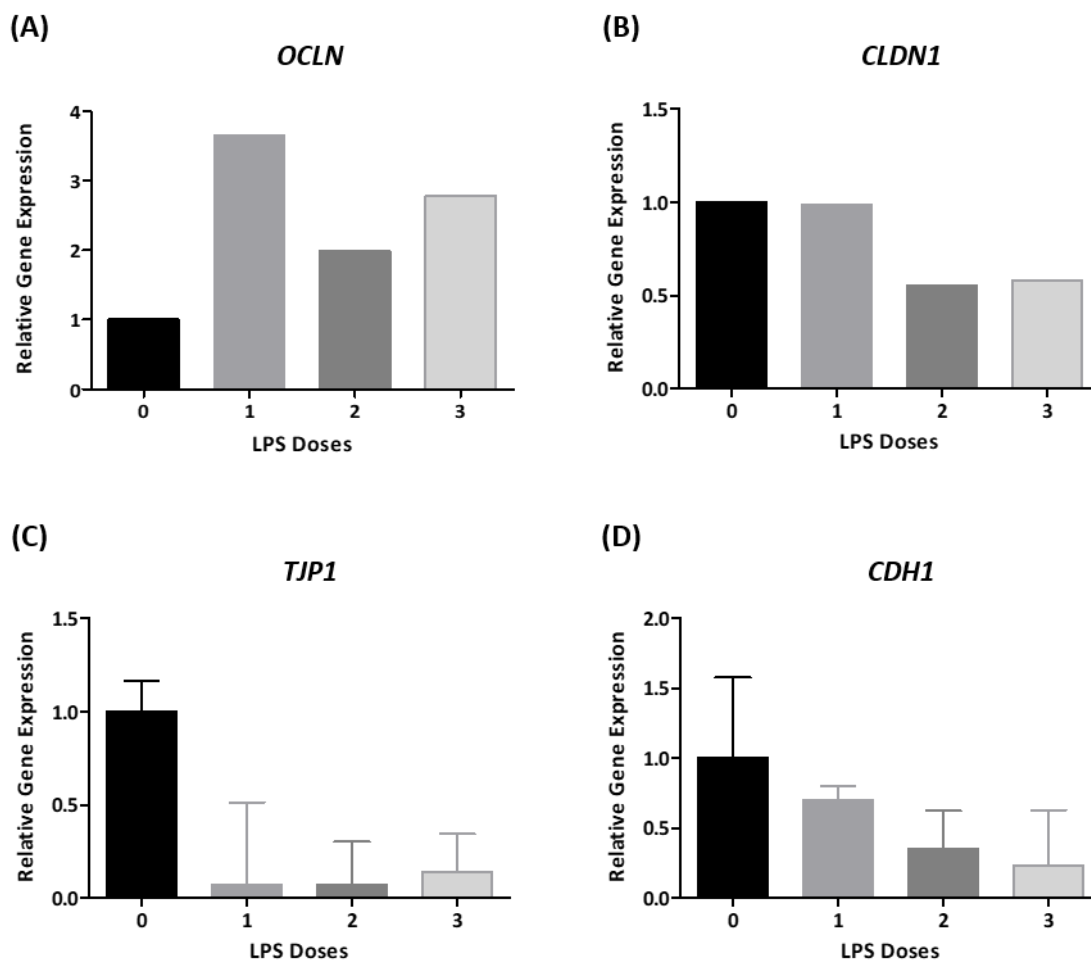


Figure 6.29. Repeated LPS treatments reduce the gene expression levels of apical junctional complex components. Triple co-cultures were established as described in Chapter 5. Following enterocyte differentiation, the arms Alvetex® Scaffold were snapped off and the insert was transferred to a fresh 12-well plate where upon models were stimulated with repeated doses of LPS as outlined in Figure 6.22, before being lysed for qPCR analysis. The gene expression levels of *OCLN* (A), *CLDN1* (B), *TJP1* (C) and *CDH1* (D) were first normalised to *GAPDH* then to untreated models. Data represent mean $\pm$ SD, total $n=3-6$ from 2-3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

In addition to qPCR analysis, the function of the epithelial barrier was assessed via TEER and a series of transport assays conducted in the absorptive direction. A one-time incubation with LPS resulted in a decreased TEER. This reduction was further exacerbated with repeated treatments of LPS, with 51 % and 67 % decrease from untreated controls when 3D *in vitro* cultures were treated with two or three doses of LPS respectively (Figure 6.30A). Although not significant, minor increases in the permeability of lucifer yellow with additional LPS incubations were observed (Figure 6.30B), yet this was much more apparent and significant regarding the transport of both FD4 and FD20 where the epithelial permeability demonstrated an incremental increase with additional treatments of LPS (Figures 6.30C,D). Lucifer yellow CH dipotassium salt has a molecular weight of 521.57 g/mol, while FD4 and FD20 have molecular weights of approximately 4000 g/mol and 20 000 g/mol respectively. This difference in molecular weight could account for the differences in permeability detected; lucifer yellow is almost 8X smaller than the smallest FITC-dextran compound and should pass through the epithelial barrier more easily than the dextran compounds, irrespective of barrier integrity. However, the dextrans may require a less restrictive epithelial barrier in order to pass through adjacent enterocytes due to their larger size; thus, a damaged epithelium caused by repeated doses of LPS may permit greater passage of the dextran compounds.

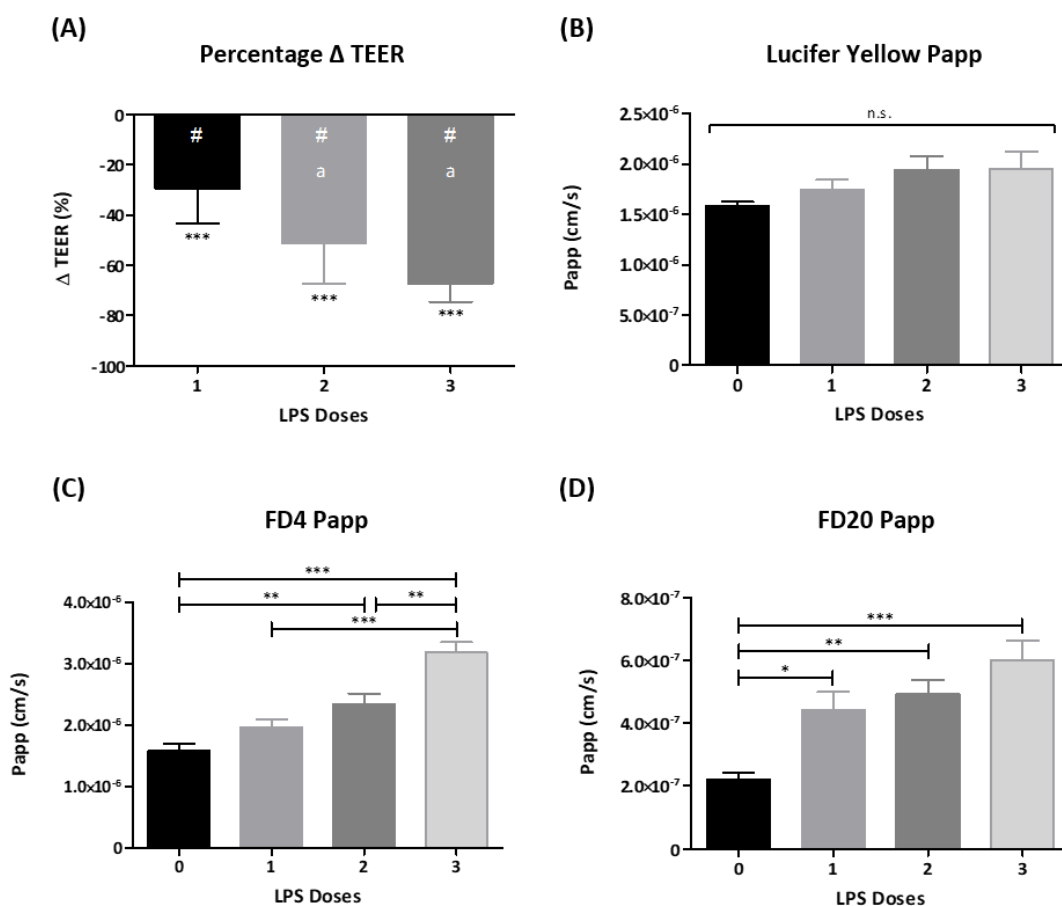


Figure 6.30. Repeated doses of LPS reduce the epithelial barrier integrity of immune-competent triple co-cultures. Triple co-cultures were established as described in Chapter 5. Following enterocyte differentiation, the arms Alvetex® Scaffold were snapped off and the insert was transferred to a fresh 12-well plate where upon models were stimulated with repeated doses of LPS as outlined in Figure 6.22, before conducting TEER analysis (A) and transport assays in the apical-basolateral direction and calculating the apparent permeability for lucifer yellow (B), FD4 (C) and FD20 (D). Data represent mean $\pm$ SEM, total n=12 from 4 independent experiments, each with 3 models set up per condition. * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$, # $P \leq 0.0001$ and ^a $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post-hoc test. * denotes were TEER differs from untreated control models, while # and ^a indicates significant differences between the number of LPS doses (A).

6.4.3.3. Repeated LPS treatment induces a strong proinflammatory response in the immune-competent triple co-culture

To characterise the immune response induced in triple co-cultures by repeated doses of LPS, gene expression analysis of both pro- and anti-inflammatory cytokines was carried out. A single 24-hour incubation period of LPS resulted in an induction of proinflammatory genes including *TNF*, *IL1B*, *IL6* and *CXCL8* which can be seen in Figure 6.31. However, repeated LPS doses did not induce subsequent increase in gene expression, and instead the mRNA levels of all assessed genes were found to decrease after two and three treatments. On the other hand, the expression of *IL10* did not differ much from untreated control models, however a minor increase was found after three repeated doses (Figure 6.32A). TGF- β 1 is an anti-inflammatory cytokine, that when constitutively expressed, can cause fibrosis; a frequent phenotype observed in the intestinal mucosa of IBD patients. Interestingly, the gene expression levels of *TGFB1* increased in a stepwise manner following multiple applications of LPS (Figure 6.32B), which if converted to protein, could eventually induce a fibrotic nature in the *in vitro* model with increased ECM deposition.

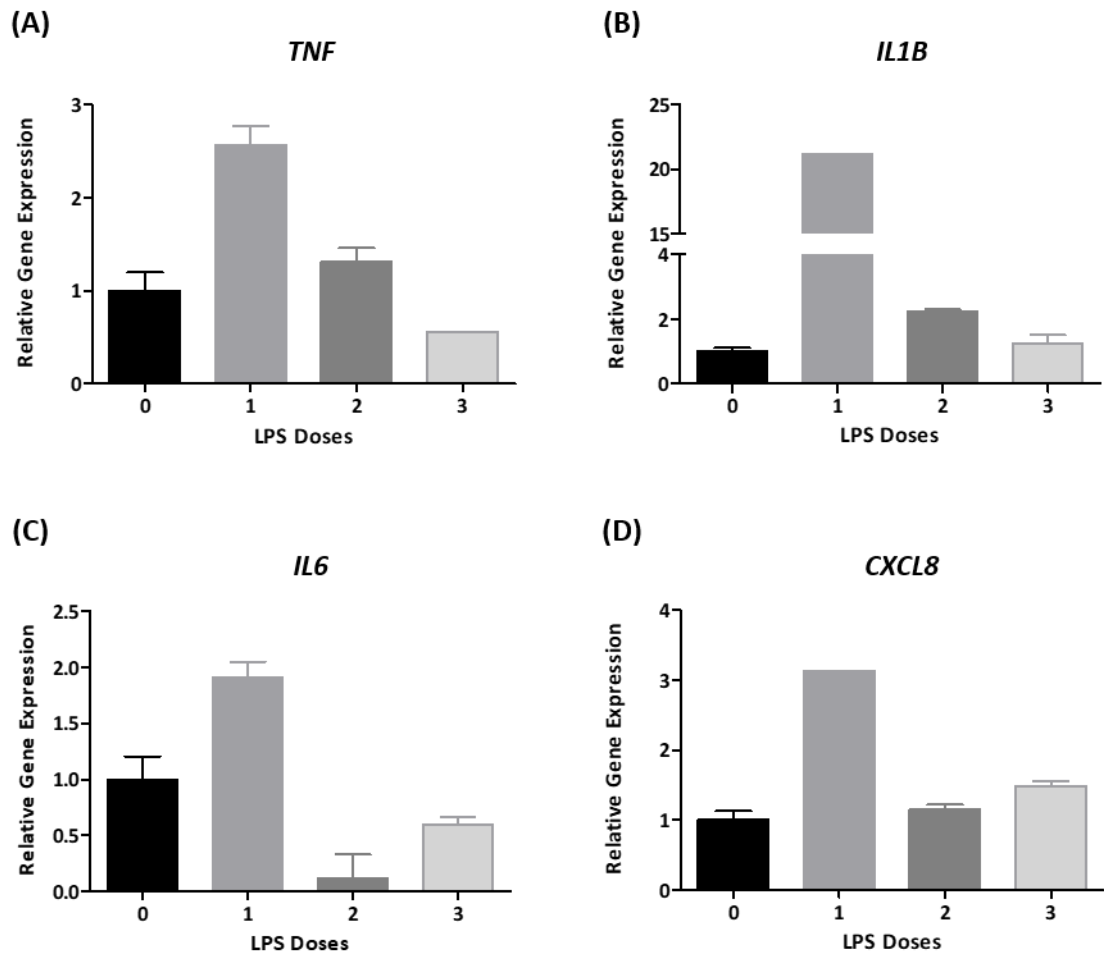


Figure 6.31. Repeated LPS treatments reduce the gene expression levels of proinflammatory cytokines. Triple co-cultures were established as described in Chapter 5. Following enterocyte differentiation, the arms Alvetex® Scaffold were snapped off and the insert was transferred to a fresh 12-well plate where upon models were stimulated with repeated doses of LPS as outlined in Figure 6.22, before being lysed for qPCR analysis. The gene expression levels of TNF (A), IL1B (B), IL6 (C) and CXCL8 (D) were first normalised to GAPDH then to untreated controls. An initial increase in the expression of all genes was observed following one incubation with LPS, however multiple treatments reduced the gene expression levels of the proinflammatory cytokines assessed here. Data represent mean $\pm$ SD, total $n=3-6$ from 2-3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

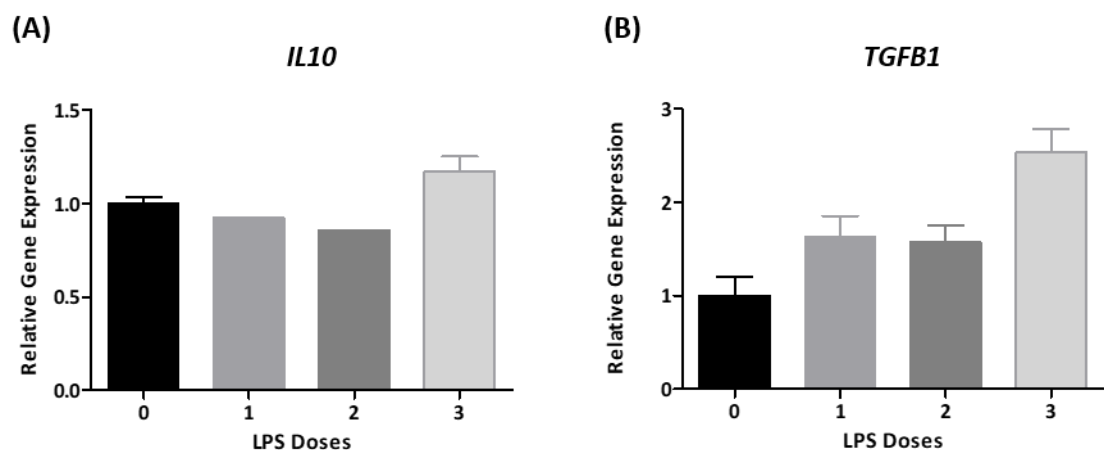


Figure 6.32. Anti-inflammatory genes are expressed following repeated LPS incubations. Triple co-cultures were established as described in Chapter 5. Following enterocyte differentiation, the arms Alvetex® Scaffold were snapped off and the insert was transferred to a fresh 12-well plate where upon models were stimulated with repeated doses of LPS as outlined in Figure 6.22, before being lysed for qPCR analysis. The gene expression levels of IL10 (A) and TGFB1 (B) were normalised to GAPDH then to untreated control levels. IL10 displayed minimal changes upon stimulation with LPS while TGFB1 displayed an incremental increase in expression. Data represent mean $\pm$ SD, total $n=3-6$ from 2-3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

Although qPCR provides a good indication of the degree of gene expression in the 3D models, it does not directly translate to the levels of protein present in the culture system. To fully characterise and understand the induction of proinflammatory cytokines following repeated LPS incubations, a cytokine array was conducted on cell culture media harvested from untreated control models and models treated with 3 doses of LPS, herein termed “repeated stimulation”. The human cytokine/chemokine array 48-Plex (HD48) service provided by Eve Technologies was utilised to assess for a variety of proinflammatory cytokines and chemokines present in the cell culture media following stimulation with LPS. Prior to conducting analysis on conditioned media collected from models, a preliminary analysis on fresh complete DMEM cell culture media that had not been in contact with cells or models was assessed to determine if there were any significant background levels of cytokines present within the media. No cytokines were detected within the control cell culture media samples; thus, any cytokines present in culture media harvested from models are produced by the cells within the immune-competent 3D co-cultures.

Colony stimulating factors (CSF) are part of the innate immune response and are considered to be master regulators of granulocytes - such as neutrophils, eosinophils, and basophils - and macrophage cell populations. They are hematopoietic growth factors that are produced by macrophages, T lymphocytes, stromal and endothelial cells, as such their presence within the 3D co-cultures could be due to either the fibroblasts or U937 macrophages or a combination of both cell lineages. CSFs can act on responding granulocytes and macrophages in a number of different ways; they can prevent apoptosis, induce proliferation, influence lineage commitment, and regulate the maturation of such lineages and finally, they can stimulate the functions of the mature macrophages and granulocytes by increasing phagocytic ability or stimulating the production of cytokines (594).

Three of the four colony stimulating factors were identified within the media collected from *in vitro* models as shown in Figure 6.33: G-CSF (granulocyte colony stimulating factor), GM-CSF (granulocyte-macrophage colony stimulating factor) and M-CSF (macrophage colony stimulating factor), however the presence of Multi-CSF, also known as IL-3 was not detected in the media. High levels of all three CSFs were present in the culture media harvested from untreated models, yet an increase was observed upon repeated stimulation with LPS. G-CSF was found to have the highest concentration within the media, over 2800 pg/mL, while GM-CSF was present at over 520 pg/mL: both G-CSF and GM-CSF were significantly increased in treated models and although the concentration of M-CSF increased upon LPS incubations, the effect was not determined to be significant. In addition to the three CSFs, the expression of the related molecule fms-related tyrosine kinase 3 ligand (Flt3-L), a key regulator of haematopoiesis, was also found to increase in concentration, but the overall levels were much lower than for the CSFs; models with repeated LPS stimulations only exhibited 2.4 pg/mL Fmt3-L in the media (Figure 6.33D).

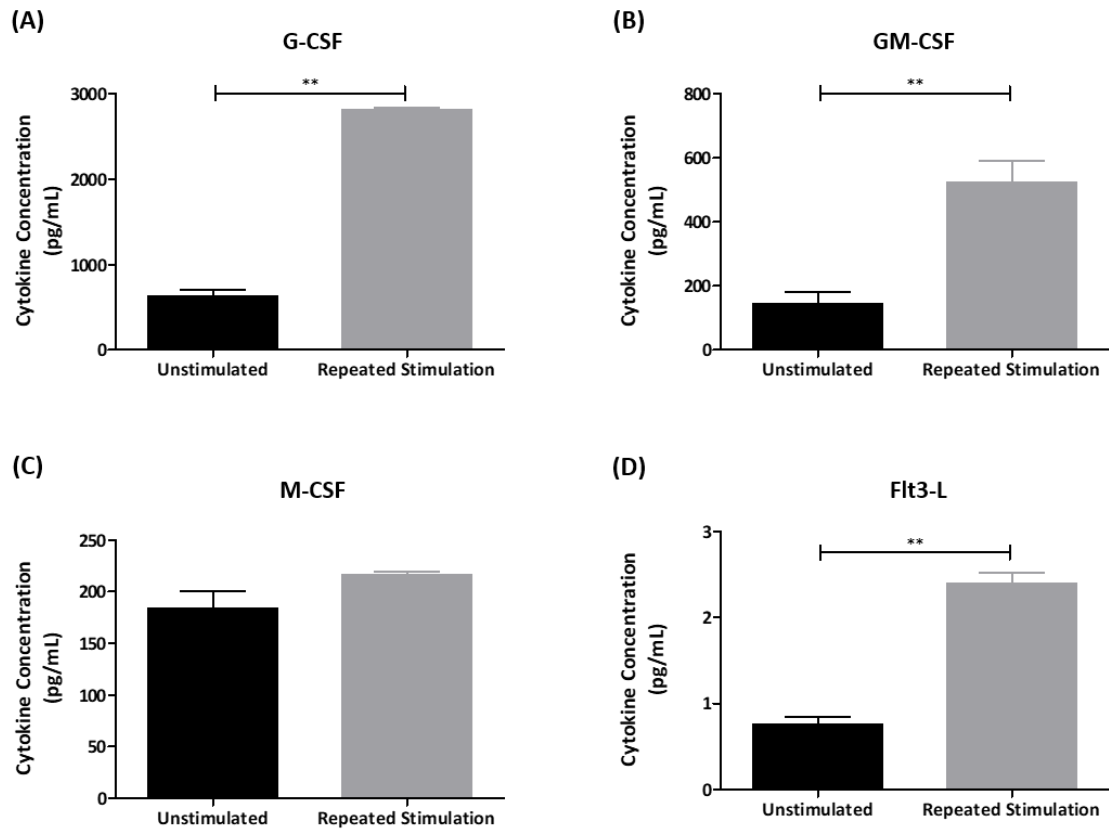


Figure 6.33. Repeated LPS incubations increase the concentration of colony stimulating factors in the cell culture media. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 μ g/mL LPS. Cell culture media was then harvested for cytokine analysis for G-CSF (A), GM-CSF (B), M-CSF (C) and the related CSF protein: Flt3-L (D). Data represent mean $\pm$ SEM, total n=6 from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. ** $P \leq 0.001$ as determined by Mann-Whitney test.

Next, the levels of two interferons were assessed. IFN- α 2, a type I interferon produced following exposure to viral and microbial products, including LPS, can promote antigen presentation in addition to activating the adaptive immune response (595). The concentration of IFN- α 2 in the culture media significantly increased from 5.43 pg/mL in unstimulated models to 12.29 pg/mL in models repeatedly stimulated with LPS (Figure 6.34A). Similarly, the expression of the type III interferon, IFN- γ , increased upon repeated stimulations, however this increase was very minor from 0.60 pg/mL to 0.69 pg/mL indicating that IFN- γ , although a primary activator of macrophages, does not play a major role in the immune response established in the immune-competent triple co-cultures in response to LPS stimulation as it is not secreted in significant quantities by either intestinal epithelial cells, macrophages or fibroblasts (Figure 6.34B).

In addition to the interferon family, two members of the TNF superfamily were also assayed for. These were TNF- α and TNF- β which are responsible for controlling apoptosis and regulation of both the innate and adaptive immune systems through the activation of various immune cells including natural killer cells and B and T lymphocytes. TNF- α almost doubled in concentration within the media yet no difference in the concentration of TNF- β was detected when models were exposed to multiple LPS doses (Figure 6.35).

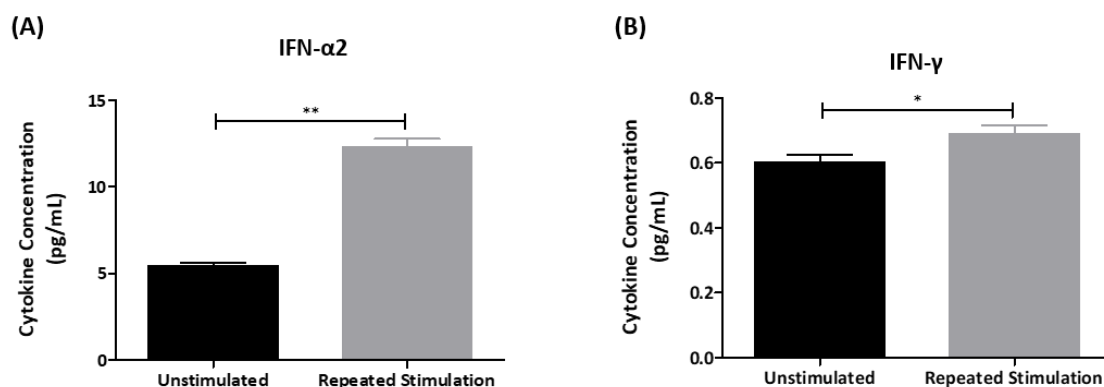


Figure 6.34. Repeated LPS incubations increase the concentration of type I and type III interferons in the cell culture media. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 μ g/mL LPS. Cell culture media was then harvested for cytokine analysis for IFN- α 2 (A) and IFN- γ (B). Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. * $P \leq 0.05$ and ** $P \leq 0.001$ as determined by Mann-Whitney test.

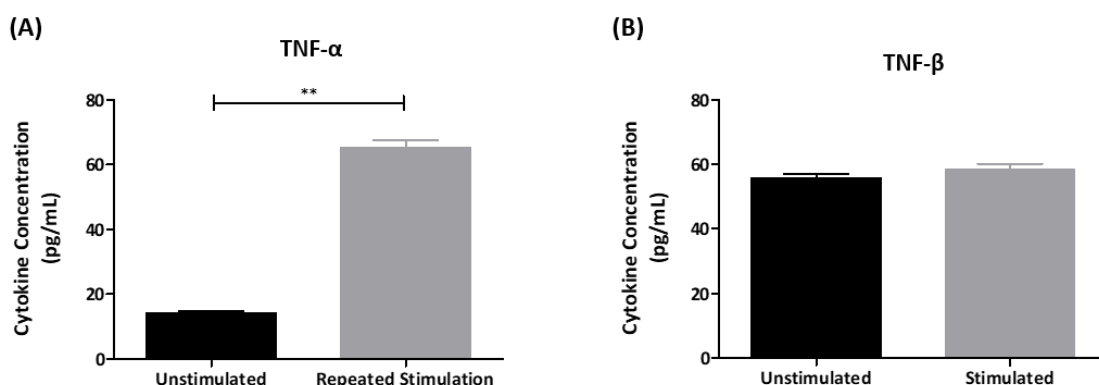


Figure 6.35. Repeated LPS incubations increase the secreted concentration of TNF- α but not TNF- β . Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 μ g/mL LPS. Cell culture media was then harvested for cytokine analysis for TNF- α (A) and TNF- β (B). Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. ** $P \leq 0.001$ as determined by Mann-Whitney test.

Interleukins are a group of immunomodulatory proteins that are responsible for mediating the inflammatory response between different cells. The concentrations of various interleukins were assessed via the cytokine array including those belonging to the IL-1 family. IL-1 is a proinflammatory cytokine that elicits a wide range of biological effects as part of the immune response including regulating the acute immune response, which may be why the highest levels of IL-1 β mRNA were found after 24 hours rather than later time points (Figure 6.30). IL-1 is composed of two distinct proteins IL-1 α and IL-1 β , both of which significantly increased to similar levels following repeated LPS stimulations (Figure 6.36A,B). Interestingly, IL-1RA also exhibited an increase after LPS treatment (Figure 6.36C). IL-1RA is a receptor antagonist that prevents the binding of IL-1 α and IL-1 β to their receptor (IL-1R1) thereby inhibiting downstream proinflammatory signalling processes induced by IL-1. The higher concentration of IL-1RA suggests that the cells within the model are attempting to attenuate the proinflammatory response.

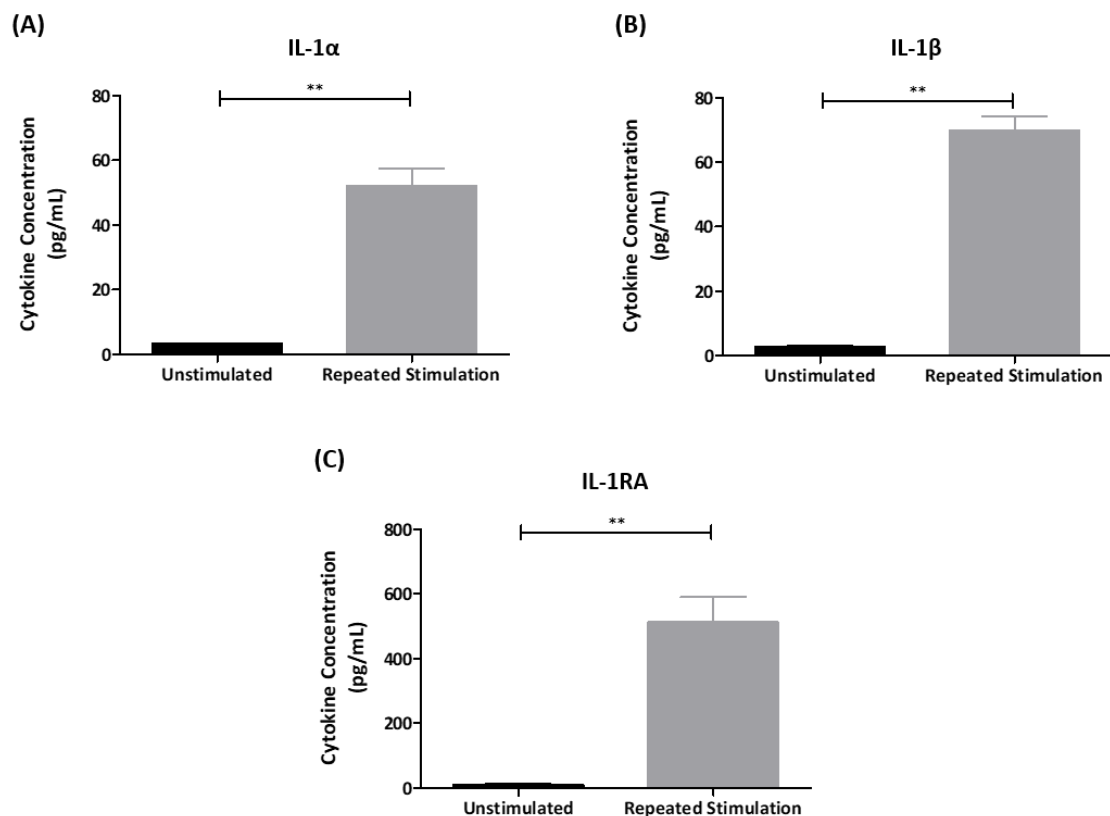


Figure 6.36. Repeated stimulation with LPS increases the secreted concentration of the IL-1 cytokine family of cytokines. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 μ g/mL LPS. Cell culture media was then harvested for cytokine analysis for IL-1 α (A), IL-1 β (B) and the receptor antagonist IL-1RA (C). Data represent mean $\pm$ SEM, total n=6 from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. ** $P \leq 0.001$ as determined by Mann-Whitney test.

IL-6 is the primary member of the IL-6 cytokine family which are a group of proinflammatory cytokines. IL-6 specifically, has critical roles in regulating the acute response to exogenous stimuli and potentiating downstream inflammation. With regards to intestinal macrophages, IL-6 prevents apoptosis and stimulates colony expansion to perpetuate inflammation. Additionally, IL-6 has been demonstrated to have roles in chronic inflammation and autoimmunity if it becomes dysregulated (596). IL-6 was found to be present in the cell culture media at very high levels; this was evident in both unstimulated control models and in models subject to repetitive LPS stimulation (Figure 6.37). The values generated for the IL-6 concentration in stimulated models exceeded the range of the standard curve but were close enough to allow extrapolated values to be estimated. IL-6 did display a minor increase in concentration compared to controls. However, such high levels of IL-6 present in untreated models suggests that there are some underlying proinflammatory signalling events occurring prior to stimulation that may have negative consequences on the overall health of the model.

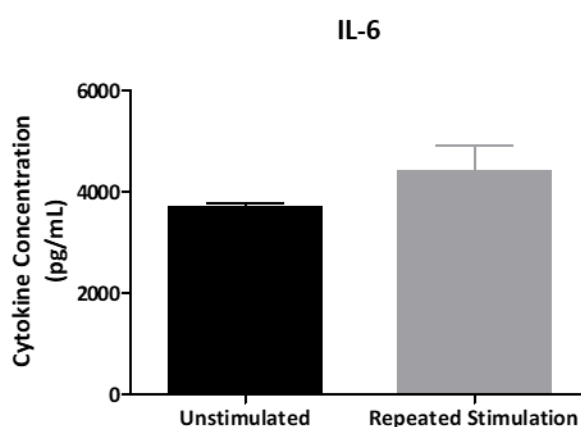


Figure 6.37. IL-6 is present at high concentrations in untreated co-cultures. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 $\mu\text{g/mL}$ LPS. Cell culture media was then harvested for cytokine analysis for IL-6. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. No significant differences were identified by Mann-Whitney test.

Members of the IL-12 family of cytokines were also investigated including IL-12p40, IL-12p70 and IL-27, all of which are produced by cells of the mononuclear phagocyte system comprised of monocytes, macrophages, and dendritic cells. IL-12 signals to the adaptive immune system and increases the differentiation of Th1 cells from naïve CD4⁺ T lymphocytes and also signals to the innate immune system by stimulating the production of IFN- γ in natural killer cells which consequently activate macrophage lineages. IL-12p70 was not detected within range of the standard curve, the concentration present in the media samples was very low, however, IL-12p40 levels were significantly enhanced upon repeated LPS treatment (Figure 6.38A). Similarly, IL-27 was also increased upon repeated LPS stimulations (Figure 6.38B). IL-27 is a unique cytokine that is also a member of the IL-6 family and has both pro- and anti-inflammatory effects. It exerts its proinflammatory effects in a similar manner to IL-12 through committing CD4⁺ T lymphocytes to the Th1 lineage. Conversely, IL-27 also drives the anti-inflammatory immune response by converting effector T cells into T regulatory cells that produce IL-10 and additionally inhibits Th17 cell differentiation (597,598). It would therefore be interesting to include naïve CD4⁺ T cells into the culture system to determine which lineage they would become committed to.

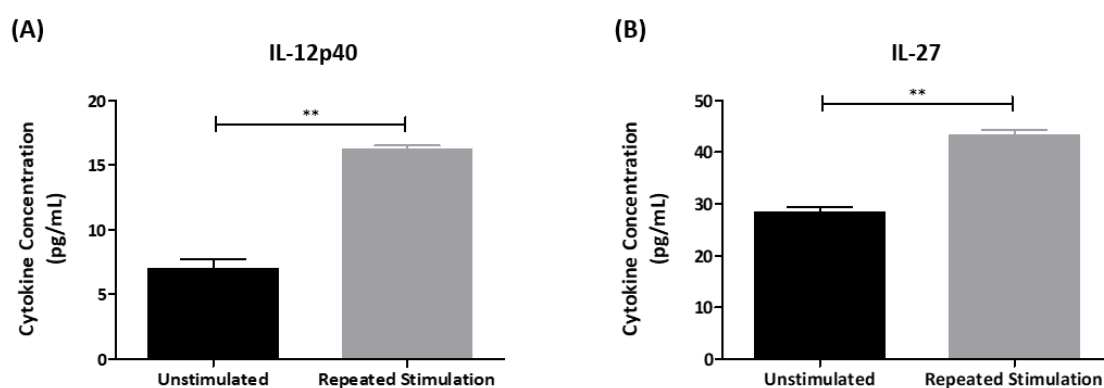


Figure 6.38. The IL-12 family of cytokines increase in the immune-competent triple co-cultures following repeated LPS stimulations. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 μ g/mL LPS. Cell culture media was then harvested for cytokine analysis for IL-12p40 (A), IL-27 (B) and IL-12p70 though no detectable levels of IL-12p70 were present. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. ** $P \leq 0.001$ as determined by Mann-Whitney test.

IL-15 and IL-2 are two structurally similar cytokines that were also assayed for; IL-2 was not detected within the cultures, but IL-15 was present in both untreated and treated models with higher levels identified in models that were stimulated repeatedly with LPS (Figure 6.39). IL-15 is produced by monocytes; it signals to both the innate and adaptive immune systems to activate and ensure the maintenance of a wide range of cells including natural killer cells and CD8⁺ T lymphocytes and thus helps maintain homeostasis under normal conditions and drive inflammation upon exposure to a proinflammatory stimuli.

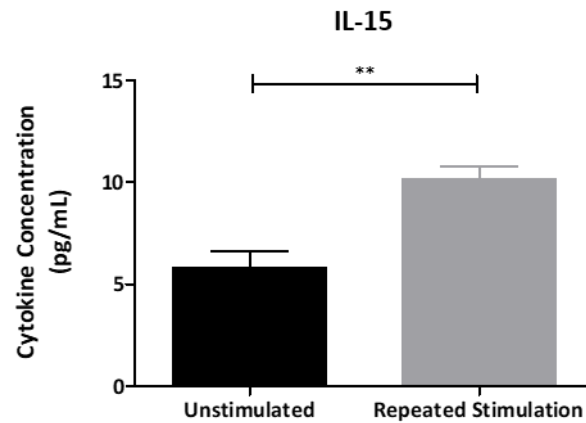


Figure 6.39. IL-15 increases upon multiple applications of LPS in the triple co-culture. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 µg/mL LPS. Cell culture media was then harvested for cytokine analysis for IL-15. Data represent mean ± SEM, total n=6 from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. **P≤0.001 as determined by Mann-Whitney test.

The final family of proinflammatory interleukins assessed was the IL-17 family which are produced by many different cell types including epithelial cells and macrophages. When overexpressed the IL-17 family of cytokines can have deleterious effects including contributing to the pathogenicity of IBD wherein IL-17 drives the production of further proinflammatory cytokines by myeloid, epithelial, and stromal cells resulting in macrophage recruitment (210). All three members of the IL-17 family, IL-17A, IL-17E/IL-25, and IL-17F significantly increased in concentration following repeated LPS stimulation compared to controls (Figure 6.40A-C). IL-17A is more potent than IL-17F though both have very similar functions; however, IL-17E has a more distinct role in inflammatory signalling processes and was present at much higher levels than the other two cytokines. IL-17E can act as an anti-inflammatory cytokine in addition to its role as a proinflammatory cytokine promoting the induction of Th2 and Th9 T lymphocytes via stimulation of the production of IL-4, IL-5, and IL-13. Of these three cytokines, only IL-13 was secreted by the models at detected levels yet just a marginal increase in its expression was present upon repeated LPS exposure (Figure 6.40D). Therefore, the precise role of the IL-17 signalling pathway within the culture system cannot be fully determined.

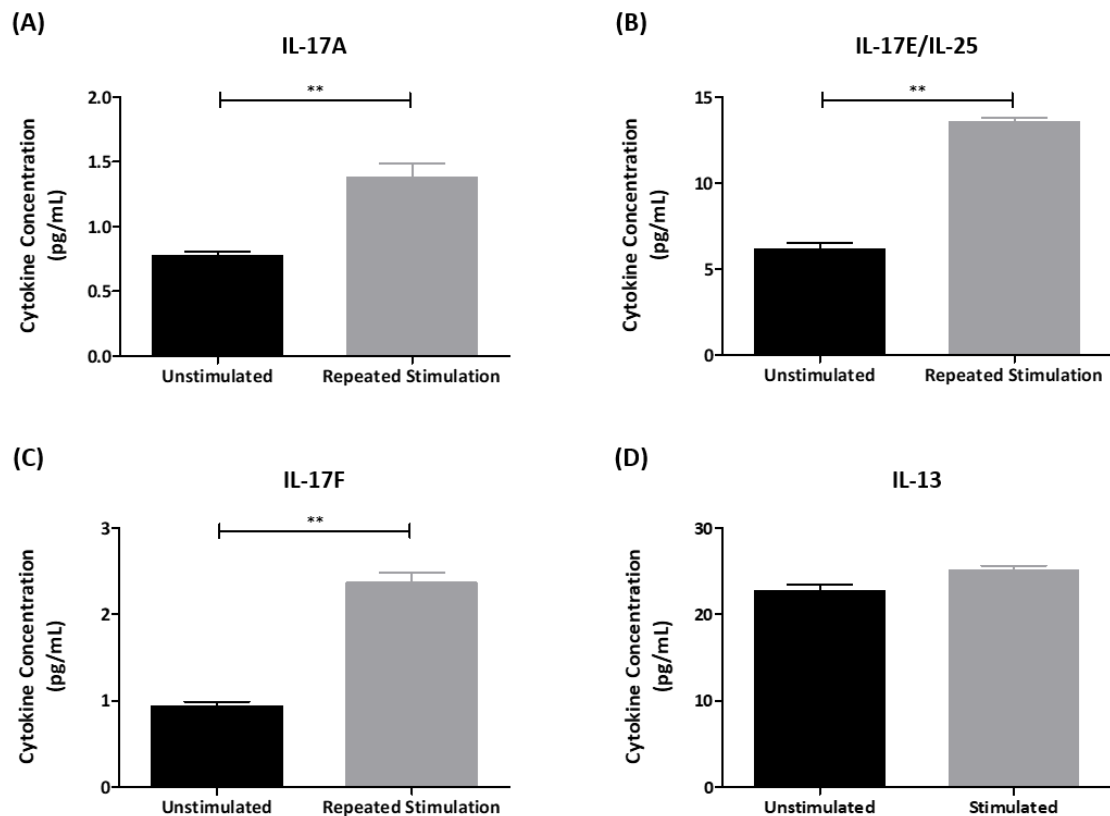


Figure 6.40. Repeated LPS stimulation of the immune-competent triple co-cultures induces the IL-17 family of cytokines to be secreted. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 $\mu\text{g/mL}$ LPS. Cell culture media was then harvested for cytokine analysis for levels of IL-17A (A), IL-17E/IL-25 (B), IL-17 (F). In addition, IL-13, a downstream protein of the IL-17E pathways was assessed (D), no significant differences in its secretion were determined in response to the increased IL-17E levels. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. **, *** $P \leq 0.001$ as determined by Mann-Whitney test.

Along with proinflammatory interleukins, two members of the anti-inflammatory IL-10 family of interleukins were assessed: IL-10 and IL-22. Due to differential receptor expression, the IL-10 family of cytokines exert their effects in a variety of ways. For example, IL-10 receptors are primarily located on immune cells including macrophages, thus IL-10 functions to dampen down the proinflammatory response driven by these cells. Conversely, receptors for IL-22 are present on non-immune cells including the epithelial and stromal cells of the intestine. In the intestine, IL-22 is produced in response to proinflammatory cytokines induced by microbial stimuli such as LPS, where it then stimulates proliferation and inhibits apoptosis and has important consequences on epithelial barrier function and the establishment of tolerance (599). Both IL-10 and IL-22 were found to increase in concentration upon repeated LPS doses, however only IL-22 exhibited a significant increase (Figure 6.41).

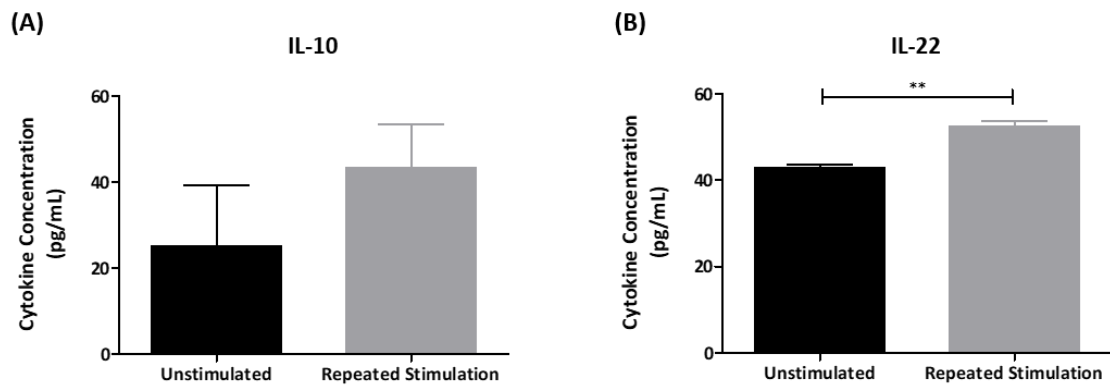


Figure 6.41. Anti-inflammatory cytokines, IL-10 and IL-22 have increased expression following LPS stimulation. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 $\mu\text{g}/\text{mL}$ LPS. Cell culture media was then harvested for cytokine analysis for levels of the IL-10 cytokine family: IL-10 (A) and IL-22 (B). Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. **, $P \leq 0.001$ as determined by Mann-Whitney test.

Following examination of the interleukins, the production of chemokines was then characterised. Chemokines are a large family of chemotactic cytokines that are further categorised into four sub-families according to their structure: the CC chemokines, the CXC chemokines, CX3C chemokines and finally the C chemokines, the latter of which were not investigated. The following discussions will be grouped according to cytokine families.

CC chemokines are also known as β chemokines. Macrophage inflammatory proteins 1 α (MIP-1 α or CCL3) and 1 β (MIP-1 β or CCL4) are both β chemokines that are produced by macrophages in response to bacterial endotoxins including LPS, and in response to other proinflammatory cytokines. Both chemokines were present at very low levels in unstimulated models, however following exposure to LPS secretion of the proteins was drastically enhanced to over 1000 pg/mL and 500 pg/mL for MIP-1 α and MIP-1 β respectively (Figure 6.42A,B). MIP-1 α and MIP-1 β are responsible for driving further migration of macrophages - within the immune-competent triple co-culture – along with monocytes and natural killer cells in tissues. Furthermore, MIP proteins function to enhance interactions between dendritic cell and T lymphocytes *in vivo* and are increased in IBD (600,601).

Monocyte chemotactic protein-1 (MCP-1 or CCL2), that has important roles in monocyte trafficking, was found to be produced at very high levels in both control and stimulated models. However, the exact concentration could not be determined due to the levels greatly exceeding the limits of the assay, no extrapolated values could be determined. It was also not possible to determine whether the production of MCP-1 was increased following stimulation. On the other hand, the related MCP-3 (CCL7) protein that is responsible for mobilising and recruiting monocytes could be detected by the assay. High levels of MCP-3 were present within the control models at over 150 pg/mL, yet this increased significantly when treated with multiple doses of LPS to almost 600 pg/mL (Figure 6.42C).

RANTES (regulated on activation, normal T cell expressed and secreted) also known as CCL5 is a trafficking protein normally expressed by T cells and activated platelets, however it was detected at high levels within the unstimulated model and even higher levels in the stimulated model suggesting an alternative source of the protein (Figure 6.42D). RANTES mediates macrophage chemotaxis, aggregation and phagocytosis through interactions with P-selectin and also exerts its effects on other innate immune cells including monocytes and natural killer cells (602).

Finally, macrophage-derived chemokine (MDC or CCL-22) and eotaxin-1 (CCL-11) were examined (Figure 6.42D,E). MDC is produced by macrophages in response to proinflammatory stimuli and recruits naïve T lymphocytes to the site of inflammation while eotaxin is responsible for recruiting eosinophils and basophils. Both were found to be significantly elevated upon LPS treatment though levels of eotaxin were much lower than the other CC chemokines assayed for.

Together, these results regarding the production and secretion of CC chemokines demonstrate that the macrophages present within the *in vitro* 3D co-cultures were receiving signals to mobilise and migrate to the site of inflammation.

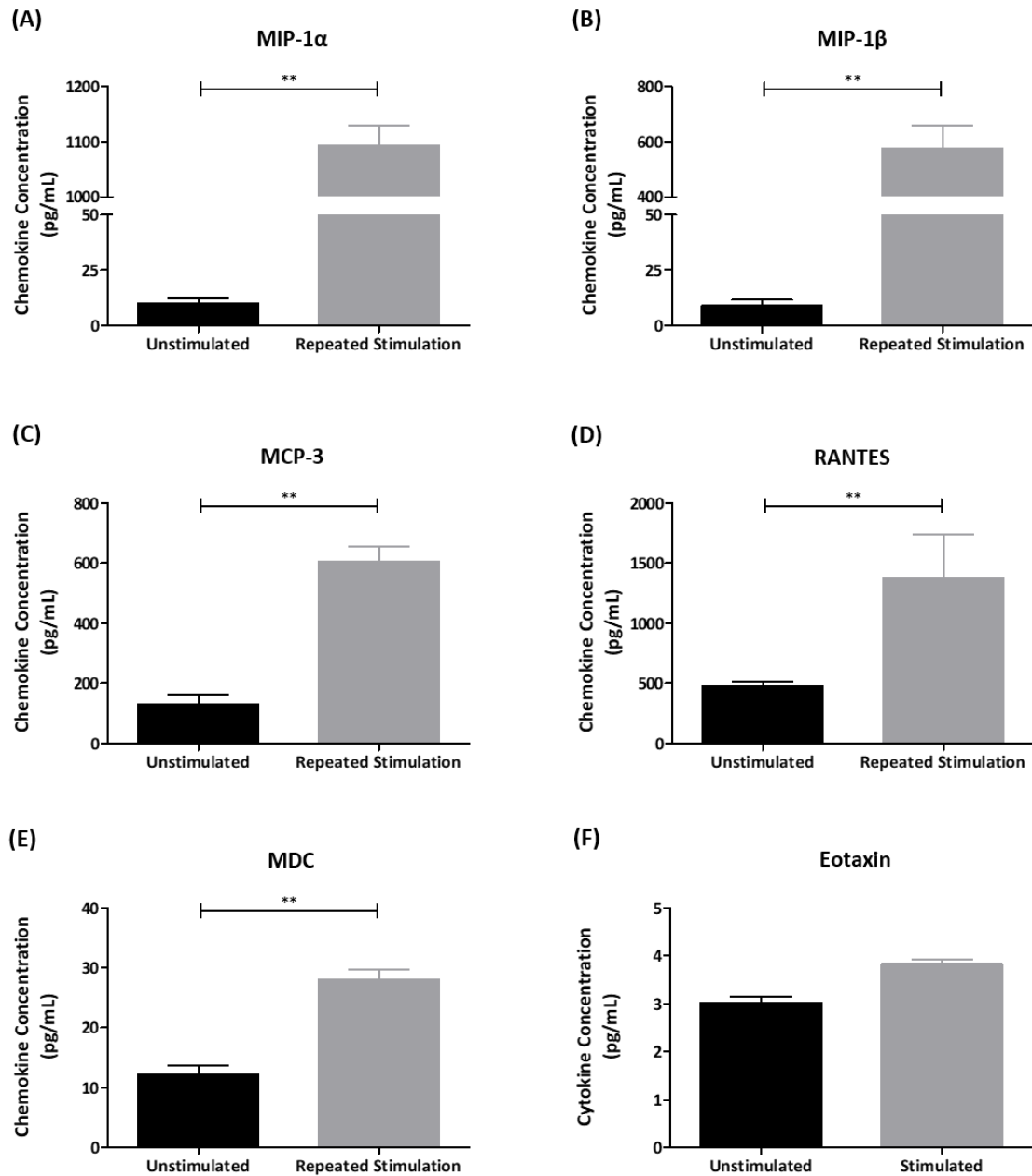


Figure 6.42. Members of the CC chemokine family are increased upon exposure to multiple doses of LPS in the immune-competent triple co-culture. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 μ g/mL LPS. Cell culture media was then harvested for cytokine analysis for levels of the CC chemokine family: MIP-1 α (A), MIP-1 β (B), MCP-3 (C), RANTES (D), MDC (E), and eotaxin (F). MCP-1 was also assayed for, but the levels of expression were beyond that of the standard curve thus no extrapolated data could be gathered. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. ** $P \leq 0.001$ as determined by Mann-Whitney test.

In addition to the CC chemokines, assessment of the secreted levels of the CXC chemokines was also conducted. GRO α (growth regulated oncogene- α), also known as CXCL1, is a chemotactic cytokine that primarily functions to enhance neutrophil trafficking to sites of inflammation. Additionally, GRO α has been demonstrated to increase the invasiveness of colorectal cancer cells such as Caco-2, thus if overexpressed within the models, Caco-2 cells could garner a migratory phenotype and disrupt the epithelial barrier in that regard (603). Production of GRO α did increase upon repeated LPS treatments, but already exhibited high levels within the culture media of control models (Figure 6.43A); this may be due to GRO α being constitutively expressed by some tumour-derived cell lines, particularly those of the colon, in addition to its induction by the presence of serum which is present within the culture media normally (604).

Interleukin 8 (CXCL8) is another CXCL chemokine that can be produced by both macrophages and intestinal epithelial cells to recruit neutrophils to the lamina propria of the intestinal mucosa. Similar to IL-6 production, IL-8 was present at high levels in the cell culture supernatant of untreated models, therefore repeated incubations with LPS did not induce a significant increase in IL-8 secretion (Figure 6.43B). This again suggests that there are proinflammatory signalling events already occurring within immune-competent triple co-cultures, thus they may not be reflecting a truly “healthy” intestinal mucosa.

The final two CXC chemokines investigated were monokine induced by interferon-gamma (MIG) and interferon-gamma induced protein 10 (IP-10) that are also named CXCL9 and CXCL10 respectively. As their names suggest, they are predominantly induced by the presence of IFN- γ ; both chemokines demonstrated an increase in concentration following multiple LPS treatments though the overall levels of IFN- γ remained very low (Figure 6.43C,D). MIG and IP-10 have roles in trafficking innate immune cells including macrophages as well as driving the Th1 proinflammatory response.

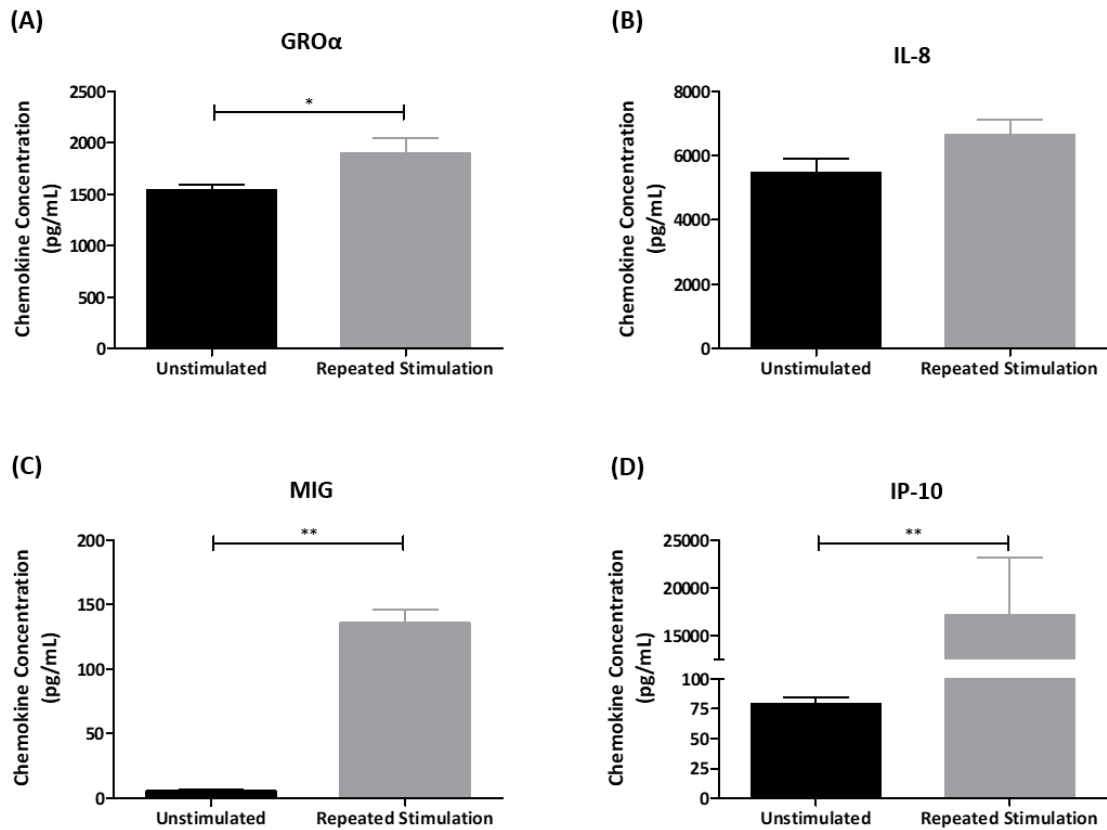


Figure 6.43. CXC chemokine secretion is increased in the immune-competent triple co-culture following repeated doses of LPS. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 μ g/mL LPS. Cell culture media was then harvested for cytokine analysis for levels of the CXC chemokine family: GRO α (A), IL-8 (B), MIG (C), and IP-10 (D). Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. * $P \leq 0.05$ and ** $P \leq 0.001$ as determined by Mann-Whitney test.

Only one member of the CX3C chemokine family has thus far been identified and is known as fractalkine. Fractalkine is either present as a soluble protein or as a transmembrane chemokine that is present on both macrophages and fibroblasts, therefore its presence within the cell culture supernatant could either be derived from HDFn or differentiated U937 cells. Furthermore, it can be released from cells undergoing apoptosis in order to recruit phagocytic cells such as macrophages and also has numerous roles in mediating trafficking of other leukocytes. Fractalkine expression was elevated upon LPS stimulation, though its overall levels were low in comparison to most other chemokines assessed (Figure 6.44). This may be due to the assay only detecting the secreted fractalkine component rather than the membrane bound protein which is responsible for conducting intracellular signalling.

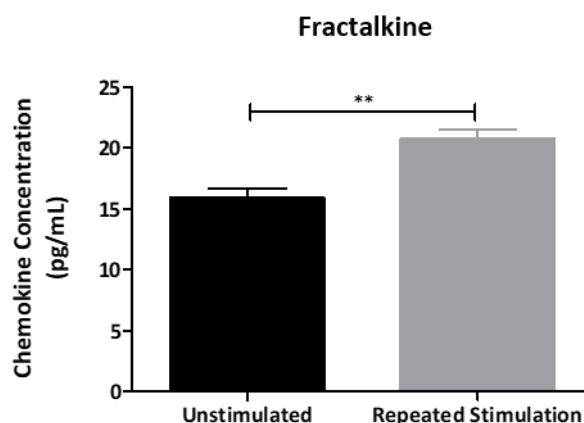


Figure 6.44. The CX3C chemokine, fractalkine, is increased following LPS stimulation in the triple co-culture but is present at lower levels than other chemokine families. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 $\mu\text{g/mL}$ LPS. Cell culture media was then harvested for cytokine analysis for levels of the only CX3C chemokine family member; fractalkine. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. $**P \leq 0.001$ as determined by Mann-Whitney test.

Supplementary to interleukins and chemokines three further growth factor families were investigated due to their roles in conducting the proinflammatory response. The first family was the fibroblast growth factor (FGF) superfamily wherein the secreted FGF2, also known as basic FGF, was identified as the only protein to decrease in concentration following LPS stimulation, though this reduction in expression was not significant (Figure 6.45). FGF-2 is normally induced during wound healing and its overexpression has been implicated in fibrosis, a common aspect of the IBD intestinal mucosa. This reduction in FGF-2 levels would suggest that the model is not yet mimicking chronic inflammation but may be more representative of an acute inflammatory response even with repetitive LPS applications.

The epidermal growth factor (EGF) family members EGF and TGF- α both increased following the inflammatory stimulus but were not present at high levels within the culture media (Figure 6.46). Both proteins are responsible for driving proliferation and differentiation within the epithelium and can also reduce inflammation. The observed increase in concentration would suggest that the *in vitro* model is undergoing processes to repair any damage induced in the epithelial barrier and maintain its integrity.

Finally, the concentration of vascular endothelial growth factor (VEGF) was also investigated. VEGF has crucial roles in promoting angiogenesis of endothelial cells and vascular permeability to allow immune cell infiltration into sites of inflammation and has also been shown to induce epithelial cell migration (605). VEGF is produced by many different cell types including stromal cells and immune cells such as macrophages, while its receptors are present on endothelial cells as well as intestinal epithelial cells. Figure 6.46 shows that VEGF-A, the specific family member evaluated in the assay, was present at high levels in untreated control models with over 3000 pg/mL in the cell culture media. VEGF-A levels were significantly elevated following repeated LPS doses to over 5000 pg/mL (Figure 6.47). It would therefore be of interest to assess Caco-2 migration or to include an endothelial cell line within directly the co-culture or the indirectly at base of the well to determine the angiogenic properties of the model prior to and following LPS treatment.

Overall, the cytokine assay has demonstrated that a strong proinflammatory response has been induced in the 3D co-culture with a wide range of cytokines being produced. This would drive a diverse range of inflammatory responses in a number of different immune cells from both the innate and adaptive immune system which could potentially be included either directly or indirectly within the model. Alternatively incorporating other immune cells within the model rather than macrophages would allow for comparisons of the direct signalling roles of individual cells in an inflammatory response.

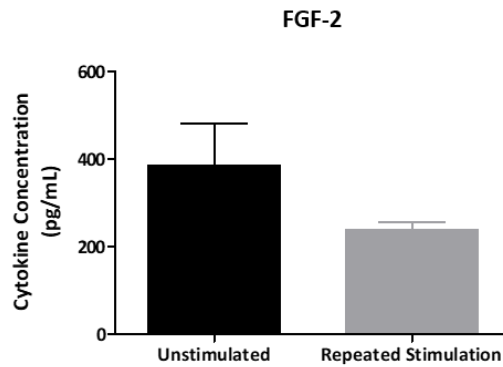


Figure 6.45. FGF-2 was reduced following LPS stimulation. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 $\mu\text{g/mL}$ LPS. Cell culture media was then harvested for cytokine analysis for levels of FGF-2, a member of the fibroblast growth factor superfamily. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. No significant differences determined by Mann-Whitney test.

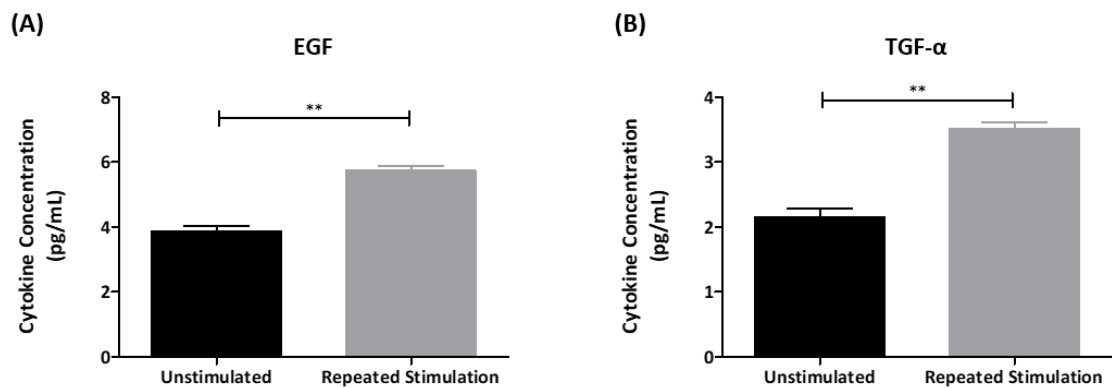


Figure 6.46. Expression of the EGF superfamily is induced following LPS stimulation in the triple co-culture. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 $\mu\text{g/mL}$ LPS. Cell culture media was then harvested for cytokine analysis for levels of the epidermal growth factor family: EGF (A) and TGF- α (B). Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. $**P \leq 0.0001$ as determined by Mann-Whitney test.

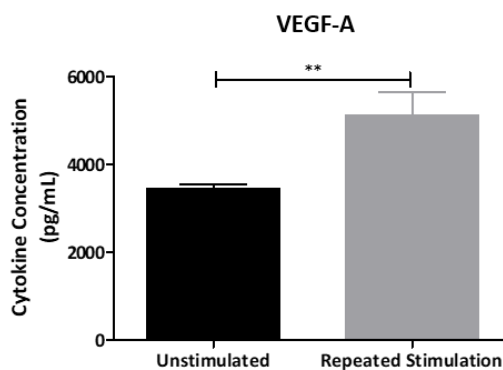


Figure 6.47. VEGF-A expression is high in untreated triple co-cultures and is further elevated upon repeated LPS stimulation. Immune-competent 3D co-cultures were either unstimulated or subject to three repeated 24-hour stimulations with 1 $\mu\text{g/mL}$ LPS. Cell culture media was then harvested for cytokine analysis for levels of VEGF-A, a member of the vascular endothelial growth factor family. Data represent mean $\pm$ SEM, total $n=6$ from 3 independent experimental repeats. Media from 3 technical replicate models were pooled together for each experiment and samples were analysed in duplicate. $**P \leq 0.0001$ as determined by Mann-Whitney test.

6.4.4. Application of the Proinflammatory Cytokine Interferon-Gamma to *in vitro* Models

LPS is an exogenous bacterial endotoxin that successfully induced an immune response in *in vitro* models. Although LPS is present within the mucosa of the IBD intestine, regarding the healthy intestine, LPS is only present within the intestinal lumen and does not stimulate an inflammatory response due to the establishment of tolerance. Therefore, another stimulus was selected to assess how the structure and function of the models respond to proinflammatory stimuli. IFN- γ is a proinflammatory cytokine that has been postulated to drive the pathogenesis of IBD and also has been shown to induce an M1 phenotype within macrophages; therefore IFN- γ was selected as a proinflammatory stimulus.

6.4.4.1. Optimisation of interferon-gamma concentration

Typically, IFN- γ is applied to *in vitro* cultures for 48 hours, this incubation period was chosen for the purposes of these sets of experiments, however the concentration of IFN- γ required to elicit effects on the models remained to be determined. Therefore, a brief pilot study to determine the optimal IFN- γ concentration to use to stimulate *in vitro* models was carried out. Two concentrations of IFN- γ were frequently used within the literature: 10 IU/mL and 100 IU/mL corresponding to approximately 50 and 500 ng/mL respectively. The pilot study was conducted only on the immune-competent 3D co-culture. Figure 6.48A shows the resulting histology after models were incubated with 0, 10 or 100 IU/mL IFN- γ for 48 hours; wherein the epithelium remains well structured. The lamina propria compartments appear similar between unstimulated models and in those treated with 10 IU/mL IFN- γ , U937 macrophages primarily localised to the lower segment. However, in models incubated with 100 IU/mL IFN- γ , greater numbers of macrophages are visible below the epithelium suggesting that they have been exposed to inflammatory cytokines and chemokines that induced their migration.

Characterisation of epithelial barrier function was carried out by calculating the percentage delta TEER from unstimulated control cultures. 10 IU/mL IFN- γ induced a very minor reduction in TEER which was further amplified upon stimulation with a higher concentration of IFN- γ (100 IU/mL); 10 and 100 IU/mL decreased the electrical resistance across the epithelium to approximately -6 % and -25 % respectively (Figure 6.48B). The ability of the models to exclude the lucifer yellow dye was assessed in the absorptive transport direction; although no major differences were detected, passage of lucifer yellow increased incrementally with 10 and 100 IU/mL IFN- γ (Figure 6.48C). As 10 IU/mL IFN- γ did not induce significant changes in either the morphology or function of the model, 100 IU/mL IFN- γ was selected for further experimentation.

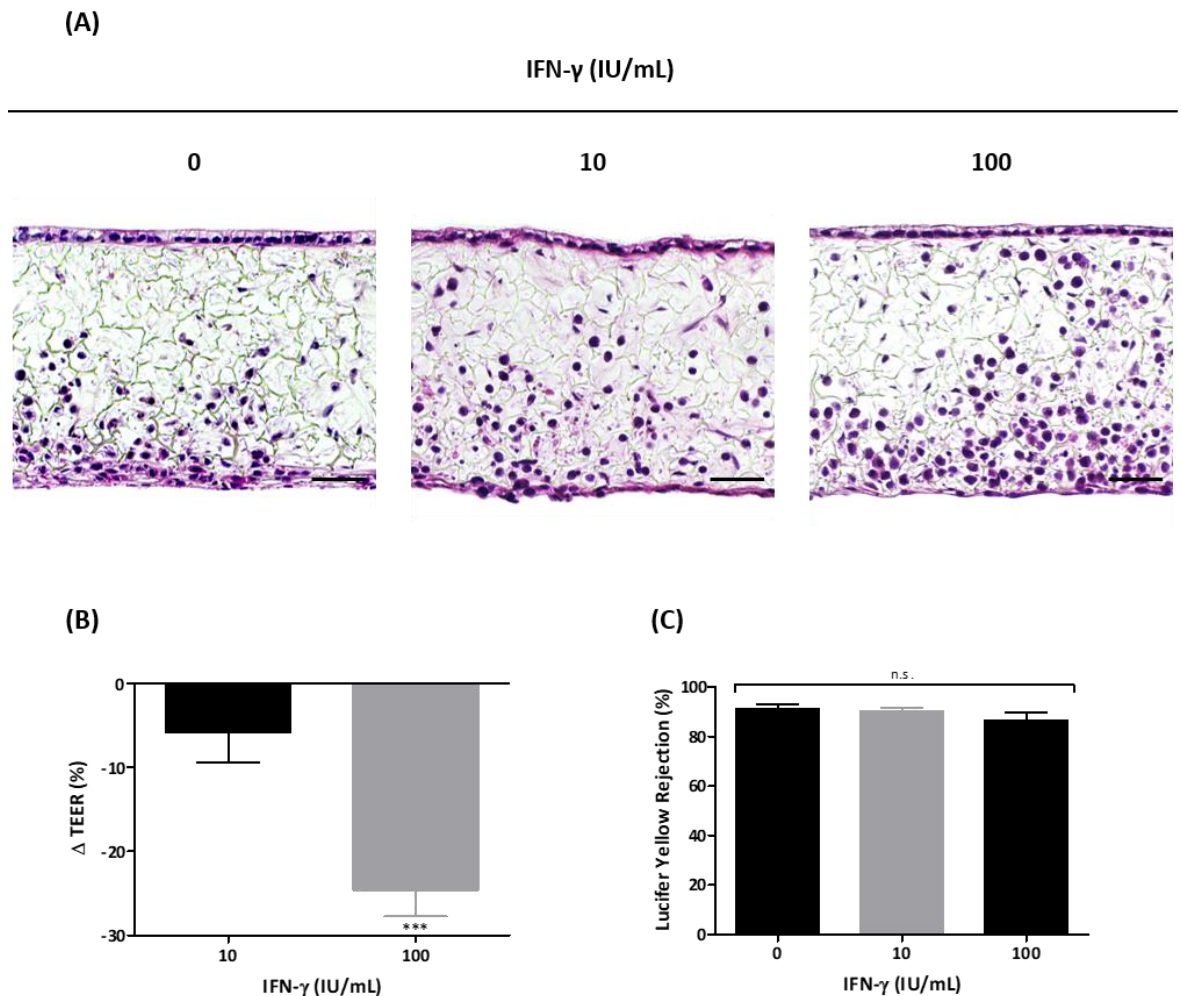


Figure 6.48. 100 IU/mL IFN- γ induces greater structural and functional changes in the immune-competent 3D triple co-culture models than 10 IU/mL IFN- γ . Following differentiation of the *in vitro* triple co-culture, models were transferred to a 12-well plate and incubated with either 0, 10 or 100 IU/mL IFN- γ for 48 hours in the basolateral compartment. The resulting model histology is shown through H&E staining (A). 100 IU/mL IFN- γ induced greater changes than 10 IU/mL IFN- γ in both the percentage decrease in TEER from control models (B) and in the rejection of lucifer yellow in the apical-basolateral direction. Images shown representative of 9 models. Data represent mean $\pm$ SEM, total $n=3$ from 3 independent experiments, each with 3 technical replicates of models. *** $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post-hoc test. Scale bars: 50 μ m.

6.4.4.2. Stimulating intestinal mucosa models with IFN- γ alone does not significantly impact the epithelial barrier

The effect of the optimised IFN- γ concentration was initially assessed on the Caco-2-U937 *in vitro* model that utilises the Transwell® cell culture platform. This was done through introduction of 100 IU/mL IFN- γ into the cell culture media in the basolateral compartment and incubating the models for 48 hours prior to downstream evaluation of model structure and function.

Wholemout immunostaining of the Caco-2 monolayer was utilised to visualise the distribution of claudin-1 and E-cadherin, components of tight and adherens junctions respectively (Figure 6.49A). Both proteins can be observed outlining the nuclei of individual cells in control and stimulated models; nuclei also have a very regular appearance to give the overall cobblestone morphology of the epithelium in both models. The lack of disruption in the arrangement of the apical junctional complex suggests that the effect of IFN- γ stimulation on the epithelial-macrophage co-culture has little effect on the integrity of the barrier. This was confirmed through functional characterisation of the model which was initially done through TEER analysis which was determined to be decreased by 13 % upon IFN- γ treatment. Furthermore, no significant differences were detected in the ability of the unstimulated and stimulated models to exclude the fluorescent dye, lucifer yellow (Figure 6.49B). Additionally, no major changes were induced regarding the permeability of lucifer yellow, FD4 or FD20; however, a minor increase in the permeability of lucifer yellow and FD4 was noted (Figure 6.49C-E).

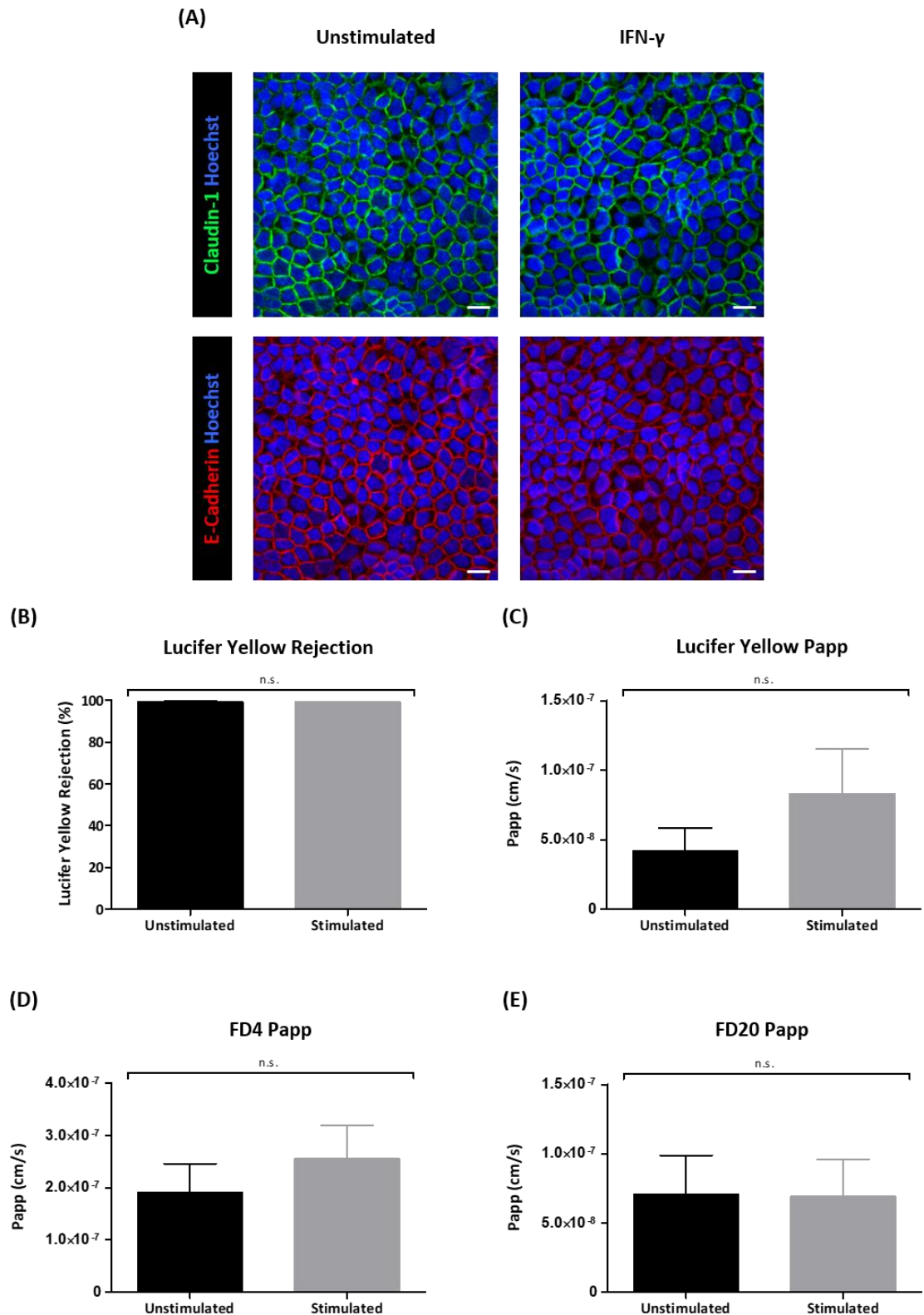


Figure 6.49. IFN- γ does not reduce the epithelial barrier integrity within *Caco-2-U937* co-cultures. *Caco-2-U937* co-cultures were established as described in Chapter 5. Following epithelial differentiation, 100 IU/mL IFN- γ was introduced into the basolateral compartment of the Transwell® culture system and incubation proceeded for 48 hours before harvesting of models for downstream analysis. Wholemount immunostaining of claudin-1 (green) and E-cadherin (red) are depicted in compressed Z-stacks where nuclei are visualised via Hoechst (blue) Images shown are representative of 3 models (A). A lucifer yellow rejection assay revealed no differences in the ability of the models to exclude the dye (B). The permeability of lucifer yellow (C), FD4 (D), and FD20 (E) were not found to be statistically significant via a Student's t-test. Data represent mean $\pm$ SEM, total $n=3$ from 3 independent experiments, each with 3 technical replicates of models. Scale bar: 20 μ m.

The impact of IFN- γ on 3D co-cultures was also assessed, the histology of which can be seen in Figure 6.47 where no alterations to the morphology of the epithelial monolayer were observed. To better characterise the composition of the epithelium following IFN- γ treatment, the gene expression of components of the apical junctional complex was assessed through qPCR (Figure 6.50). Expression of the genes encoding occludin, ZO-1 and E-cadherin all decreased following exposure to IFN- γ , contrastingly, the expression of the gene for claudin-1 increased slightly. This suggests that slight changes are induced within the epithelium after IFN- γ treatment, though the full impact was assessed through functional characterisation.

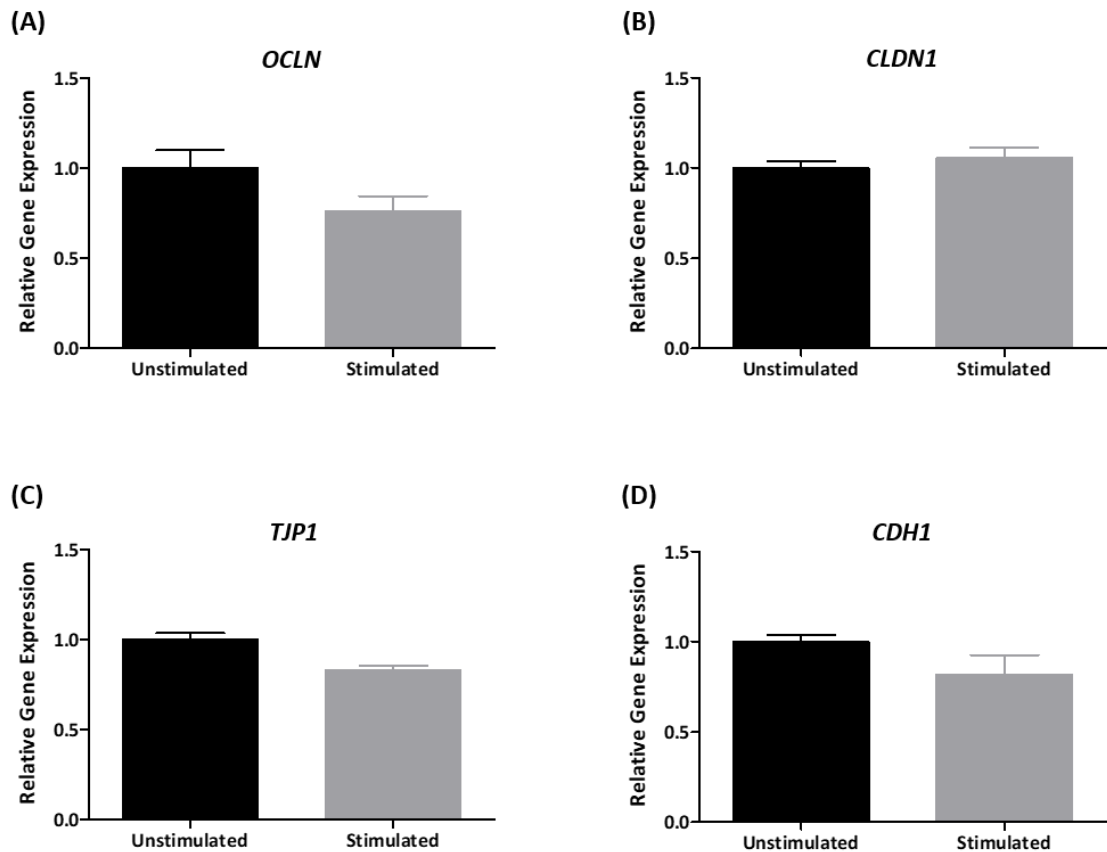


Figure 6.50. IFN- γ does not have a great effect on the gene expression of the apical junctional complex components. Immune-competent triple co-cultures, established as outlined in Chapter 5, were incubated with 100 IU/mL IFN- γ for 48 hours before harvesting for qPCR analysis. Expression of the following genes was assessed: OCLN (A), CLDN1 (B), TJP1 (C), and CDH1 (D). Gene expression levels were first normalised to GAPDH then to the expression levels unstimulated control models. Data represent mean $\pm$ SEM, total 6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

TEER had previously been found to decrease by approximately 25 % following triple co-culture incubation with 100 IU/mL IFN- γ for 48 hours (Figure 6.48). However, this decrease in TEER was not reflected in further functional testing. Unstimulated and stimulated co-cultures were both able to exclude over 90 % of lucifer yellow applied to the apical compartment (Figure 6.51A). Although minor increases could be detected in the absorptive permeability of lucifer yellow, FD4 and FD20, these were not statistically significant. This indicates that IFN- γ treatment alone is not sufficient to disrupt the integrity of the epithelial barrier.

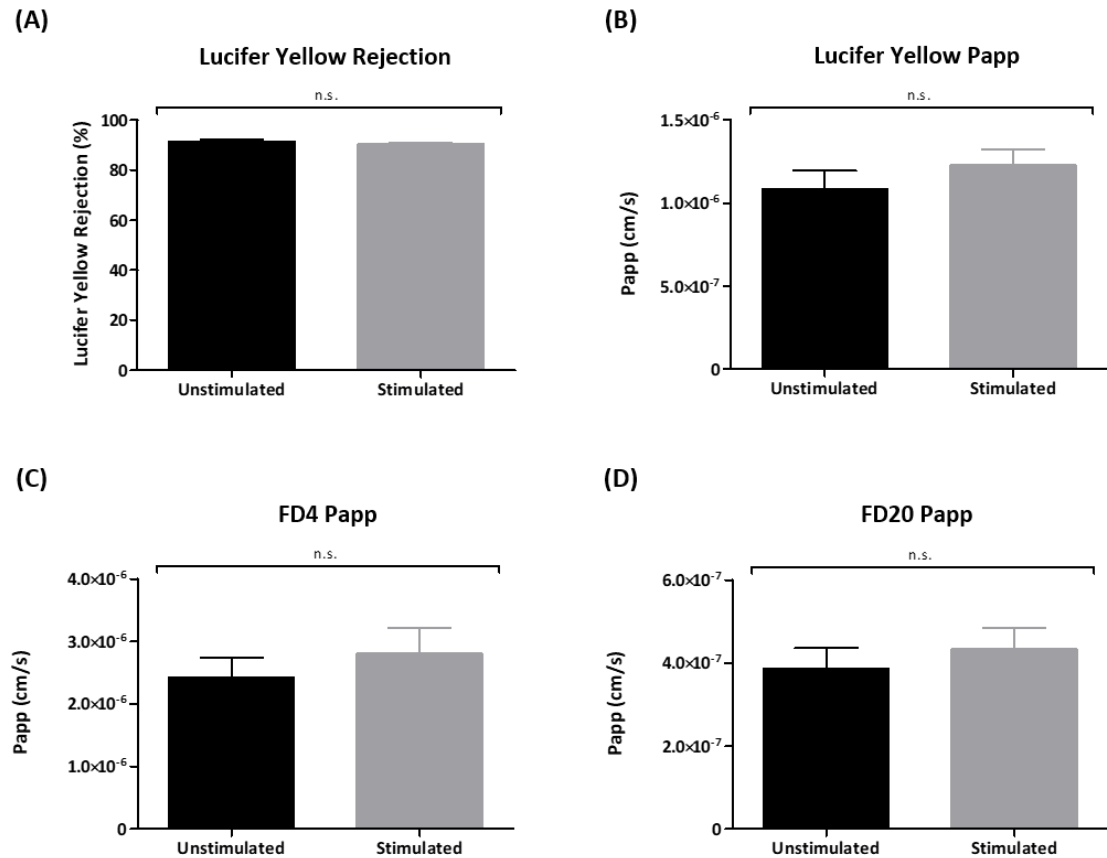


Figure 6.51. Stimulation with IFN- γ alone does not induce significant alterations in the permeability of the immune-competent triple co-culture. Immune-competent triple co-cultures, established as outlined in Chapter 5, were incubated with 100 IU/mL IFN- γ for 48 hours. Models were then subjected to a lucifer yellow rejection assay (A), in addition to calculating the permeability of lucifer yellow (B), FD4 (C), and FD20 (D) in the apical-basolateral direction. Data represent mean $\pm$ SEM, total $n=9$ from 3 independent experimental set ups, each with 3 models cultured as technical replicates. Data were found not significant (n.s.) by Student's t test.

Previous studies revealed that IFN- γ exerted minimal effects on the epithelial barrier structure and function, therefore the expression of genes involved in inflammatory signalling pathways were assessed by qPCR to determine if an immune response was induced within the triple co-culture upon IFN- γ treatment.

IFN- γ has previously been shown to induce the production of TNF- α in a time-dependent manner (606). Contrary to previous studies, expression of the gene encoding TNF- α did not noticeably change upon IFN- γ stimulation within the co-culture model assessed here (Figure 6.52A). On the other hand, *IL1B* did increase within the model following IFN- γ incubation, though it was the only proinflammatory cytokine to do so (Figure 6.51B). Expression of *IL6* did not change following stimulation (Figure 6.52C), however previous experiments within this chapter have demonstrated that IL-6 levels are constitutively high in unstimulated models, thus the presence of an additional stimulus may not be sufficient to further amplify the production of IL-6. The final proinflammatory cytokine assessed was IL-8, encoded by the *CXCL8* gene and was the only proinflammatory cytokine to decrease after exposure to IFN- γ . Similar to IL-6, IL-8 levels have also shown to be high in untreated models and IFN- γ may, again, not be enough to further enhance the gene expression of *CXCL8*. Supplementary to the proinflammatory cytokines, the gene expression of the anti-inflammatory cytokines IL-10 and TGF- β 1 was also assessed (Figure 6.53). Both genes decreased after stimulation suggesting that they may have been suppressed. Overall, IFN- γ induces a weak inflammatory response in the triple co-culture.

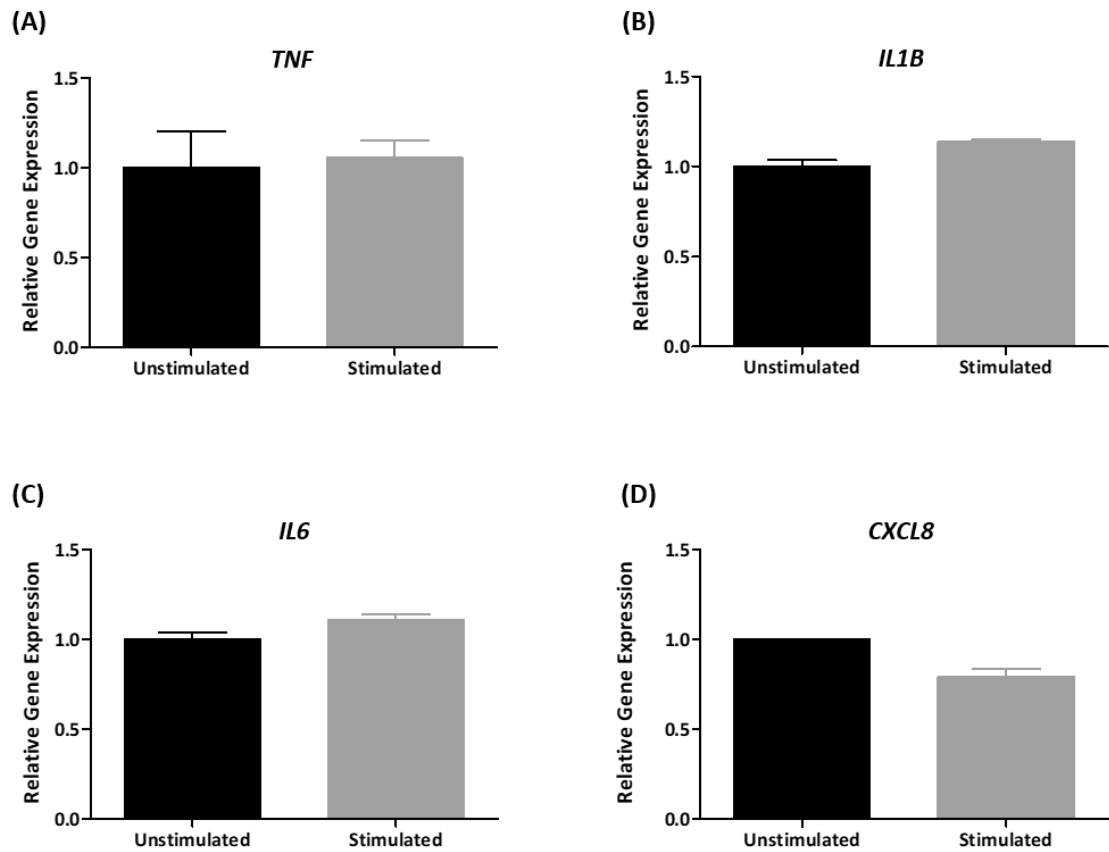


Figure 6.52. IFN- γ does not induce a strong proinflammatory response in the immune competent triple co-culture. Immune-competent triple co-cultures, established as outlined in Chapter 5, were incubated with 100 IU/mL IFN- γ for 48 hours before harvesting for qPCR analysis. Expression of the following genes was assessed: TNF (A), IL1B (B), IL6 (C), and CXCL8 (D). Gene expression was first normalised to GAPDH then to the expression levels of unstimulated control models. Data represent mean $\pm$ SEM, total 6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

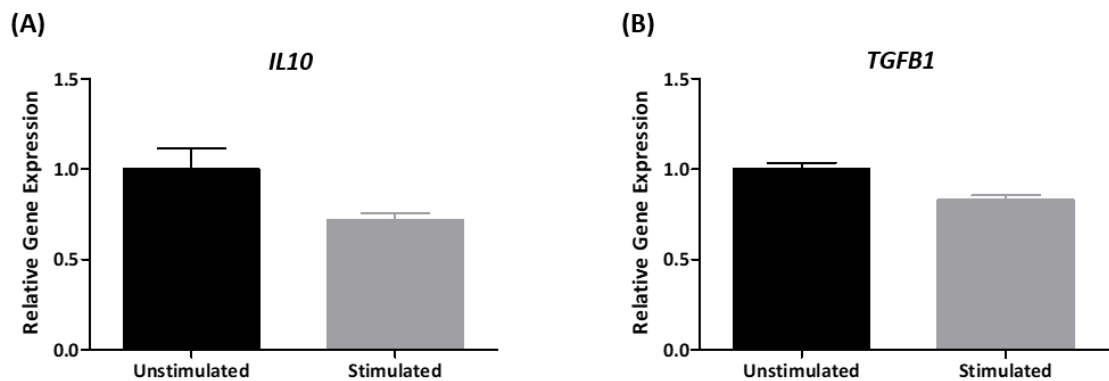


Figure 6.53. IFN- γ induces a slight reduction in the expression of anti-inflammatory genes. Immune-competent triple co-cultures, established as outlined in Chapter 5, were incubated with 100 IU/mL IFN- γ for 48 hours before harvesting for qPCR analysis. Expression of the following genes was assessed: IL10 (A) and TGFB1 (B). Gene expression was first normalised to GAPDH then to the expression levels of unstimulated control models. Data represent mean $\pm$ SEM, total 6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

6.4.5. Utilising Additional Proinflammatory Stimuli to Enhance the Cellular Response to Interferon-Gamma

Application of IFN- γ to the *in vitro* intestinal models alone did not provoke a significant immune response or reduce the integrity of the epithelial barrier. However, numerous studies have utilised IFN- γ treatment in combination with LPS or TNF- α to induce a proinflammatory phenotype in both 2D cells and *in vitro* models. Therefore, the effect of combining both LPS and TNF- α with IFN- γ was examined.

6.4.5.1. Characterising the effect of IFN- γ priming prior to LPS treatment of intestinal mucosa models

IFN- γ is known to differentiate macrophages to a proinflammatory phenotype, additionally IFN- γ is frequently utilised as a priming agent to augment cellular responses to LPS. Priming with IFN- γ prior to LPS treatment has been demonstrated to enhance the production of proinflammatory cytokines including TNF- α and IL-8 in both monocytes and macrophages (607–610). Furthermore, pre-exposure of Caco-2 monolayers co-cultured with THP-1 macrophages before application of LPS, potentiated the damage incurred on the epithelial barrier (301,611). Thus, the aim of this next section was to determine whether IFN- γ could be used as a priming agent in the two immune-competent intestinal co-cultures developed in Chapter 5. This was achieved through incubating models with 100 IU/mL IFN- γ for 48 hours in the basolateral compartment followed by incubation with 1 μ g/mL LPS in either the basolateral or apical compartments to represent increased serosal LPS during IBD and luminal LPS from commensal bacteria respectively.

Investigations initially focussed on the Caco-2 enterocyte–U937 macrophage co-culture model. Wholemout immunostaining of the resultant Caco-2 monolayers was conducted following stimulation to assess the structure of the apical junctional complex (Figure 6.54). Occludin was present in all epithelial monolayers and exhibited a regular polygonal architecture in both unstimulated control models and those treated only with IFN- γ . However, subsequent LPS stimulation did result in some structural abnormalities. For instance, when LPS was applied basolaterally, there were areas where occludin was not found surrounding the apical pole of each nucleus within the epithelium and there appeared to be some nuclear deformities indicative of cell death. Regarding the apical application of LPS, there were many areas of very compact polygonal occludin staining compared to the unstimulated control epithelia which could have an impact on the overall function of the barrier. Claudin-1, on the other hand, had a very similar expression profile across the different models irrespective of stimulation. Claudin-2 exhibited strong staining in some areas of the epithelial models yet weak staining in other areas; its expression was most consistent within the control monolayers. Large areas of discontinuous staining where no nuclei were present in the overall distribution of cells could be observed in models primed with IFN- γ and then stimulated with LPS basally. This phenomenon could be due to cell death of the Caco-2 enterocytes or could potentially be a processing artefact, though this was not seen in other models. Finally, the adherens junction element, E-cadherin, was assessed across the different culture conditions. E-cadherin was consistently expressed in control models but had areas of strong and weak staining in other models, which in turn could potentially impact the epithelial barrier properties.

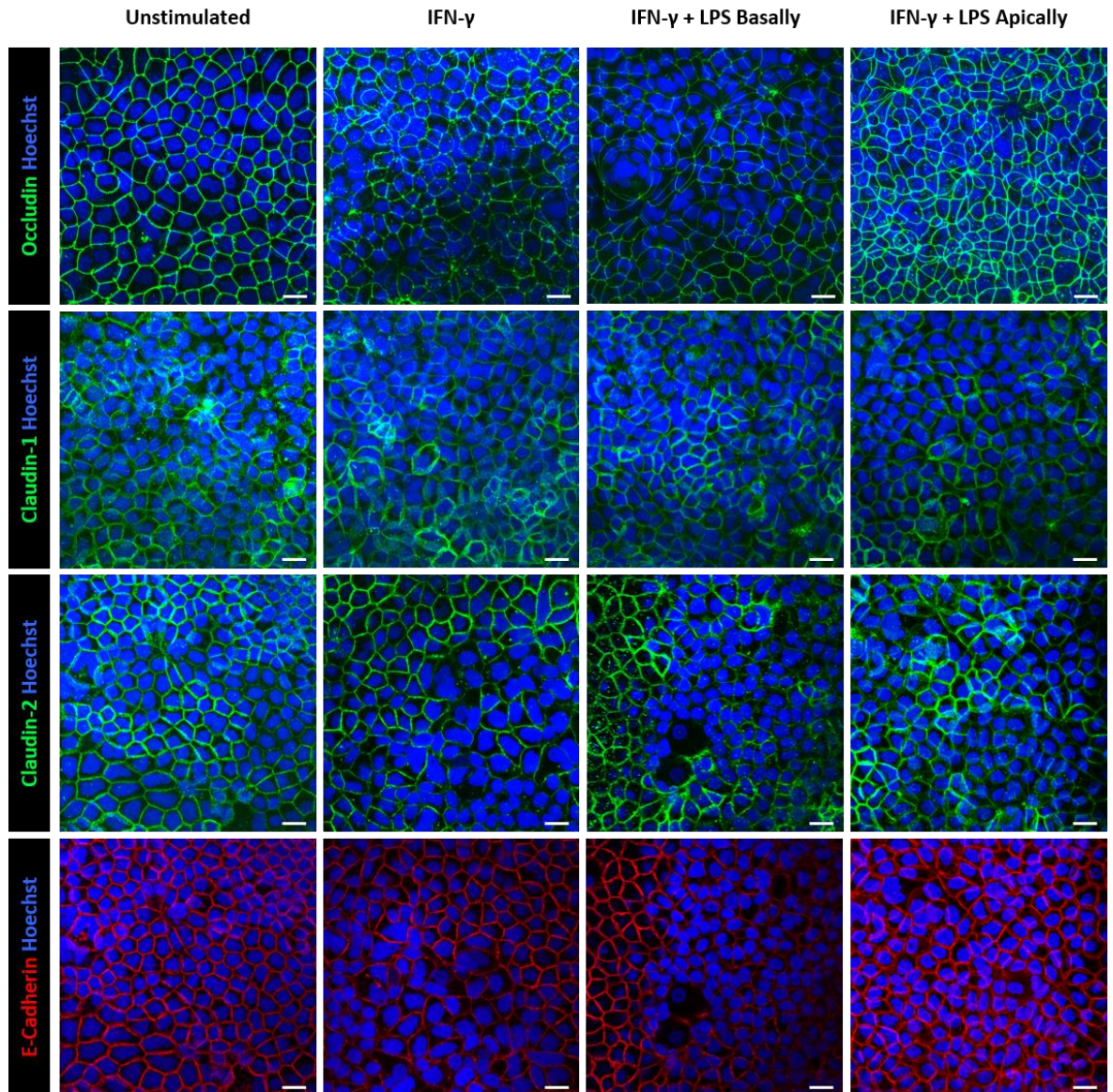


Figure 6.54. IFN- γ priming prior to LPS stimulation induces structural changes in apical junctional complex of the Caco-2 monolayer. Caco-2-U937 co-cultures were established as described in Chapter 5. Following epithelial differentiation, 100 IU/mL IFN- γ was introduced into the basolateral compartment of the Transwell® culture system and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. Models were then harvested for wholemount immunostaining. Compressed Z-stacks for the expression of occludin, claudin-1 and claudin-2 (green) and E-cadherin (red) are depicted. Nuclei were counterstained with Hoechst (blue). Images depicted are representative of 3 models. Scale bars: 20 μ m.

Characterisation of the functional properties of the monolayers following IFN- γ and LPS stimulation revealed that priming the cultures with IFN- γ prior to LPS treatment significantly reduced the TEER of the epithelium compared to control monolayers, an effect that was most pronounced when LPS was introduced into the basolateral side of the Transwell® membrane. (Figure 6.55A). LPS at 1 $\mu\text{g/mL}$ had previously been demonstrated to reduce TEER by 13 % from control monolayers (Figure 6.13), this was further reduced to 16 % following IFN- γ priming, suggesting that IFN- γ priming may augment the response of the epithelium to LPS. However, upon characterisation of the monolayer permeability, no significant differences were detected, similar to LPS treatment alone. All monolayers were successfully able to exclude almost 100 % lucifer yellow dye from penetrating to the basolateral compartment when applied apically (Figure 6.55B). Furthermore, the absorptive transport of lucifer yellow, FD4 and FD20 remained low, though a minor trend of increased permeability could be observed following stimulation (Figure 6.55C-D).

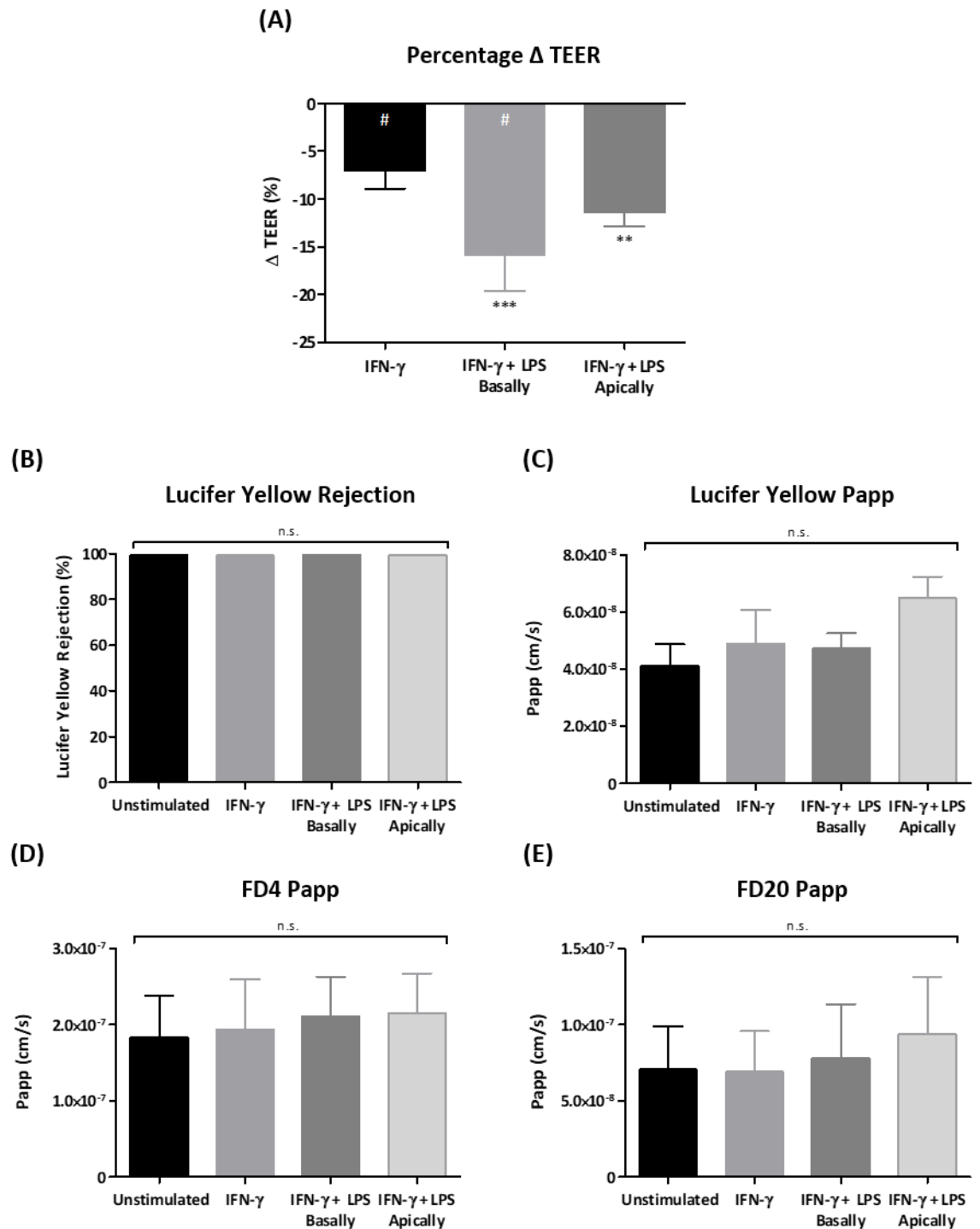


Figure 6.55. Priming of Caco-2-U937 co-cultures with IFN- γ does not significantly affect the permeability of epithelial monolayers. Caco-2-U937 co-cultures were established as described in Chapter 5. Following epithelial differentiation, 100 IU/mL IFN- γ was introduced into the basolateral compartment of the Transwell® culture system and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. Models were then subject to TEER analysis (A) and a lucifer yellow rejection assay (B). Additionally, Papp was calculated for the absorptive transport of lucifer yellow (C), FD4 (D) and FD20 (E). Data represent mean $\pm$ SEM, total n=9 from 3 independent experiments, within each experiment, a total of 3 models were cultured as technical repeats. ** $P \leq 0.001$, *** $P \leq 0.0001$, and # $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post-hoc test. * denotes that TEER was significantly different from unstimulated controls, while # indicates significant differences between treatment conditions.

The effect of IFN- γ and LPS treatment was then examined using the immune-competent triple co-culture. As with the Transwell®-based model, 100 IU/mL IFN- γ was applied to the basolateral compartment for 48 hours before treating the models with 1 μ g/mL LPS for a further 24 hours, LPS was either applied apically or basolaterally. Figure 6.56 shows representative H&E staining from the resultant models. IFN- γ treatment alone had little effect on the structural arrangement of the epithelial monolayer which presented with a distinctive columnar morphology. Greater numbers of U937 macrophages were present in the upper segment of the lamina propria suggesting they have received chemotactic signals to migrate towards the epithelium. Successive applications of IFN- γ and LPS induced greater changes within the overall histology of the model when compared to controls and those treated with IFN- γ . Examination of the lamina propria revealed high levels of debris (diffuse pink staining) within the model which could be attributed to either components of degraded ECM or, more likely, cellular debris from apoptotic and necrotic cells. This could indicate that treatment of models with both IFN- γ and LPS may have detrimental effects to the viability of the model, however as macrophages are known for their ability to clear apoptotic cells through phagocytic processes, this debris could further enhance the immune response.

Alterations to the epithelial monolayer were prominent; when LPS was introduced basolaterally following IFN- γ priming there was evidence of epithelial cell shedding (indicated by #) wherein Caco-2 cells were identified in the luminal space above the monolayer resulting in areas where the epithelial barrier was not fully intact. Some evidence of sparse epithelial shedding was also identified when LPS was applied apically, however the most dominant phenotype was the change to the elongated columnar shape of the epithelium; the epithelium displayed a cuboidal or flattened morphology, as highlighted by the arrows, along the majority of the monolayer, though some columnar cells remained. This altered, heterogenous architecture could be due to cellular damage that would, again, impact the structural integrity of the epithelial barrier. Such phenotypes were not evident in within models treated with IFN- γ alone, nor were these effects observed in models treated with 1 μ g/mL LPS for 24 hours as demonstrated previously within this chapter in Sections 6.4.2 and 6.4.3.

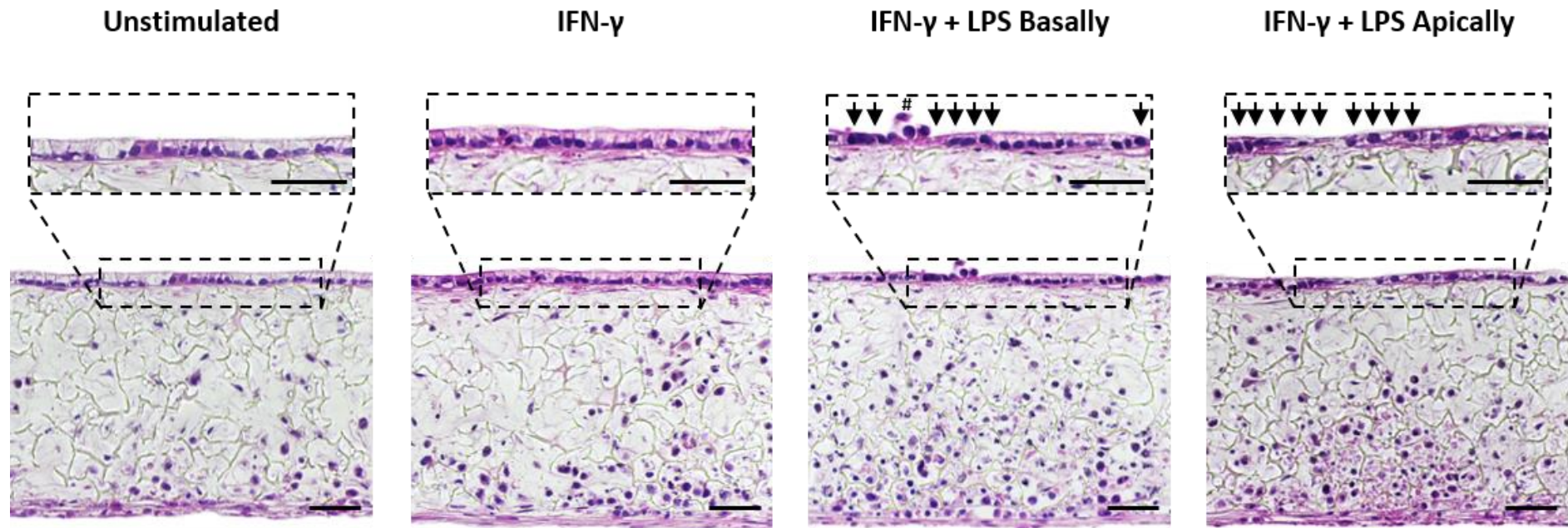


Figure 6.56. Successive treatments of IFN- γ and LPS induce structural changes in the epithelial monolayer of the *in vitro* triple co-culture and produce high levels of debris within the lamina propria. Immune-competent triple co-cultures were established as described in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where 100 IU/mL IFN- γ was introduced into the basolateral compartment and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. Models were then harvested for wax embedding and histology. Representative H&E images of 9 control, IFN- γ only and IFN- γ + LPS treated models are shown. Debris within the lamina propria is visible in models treated with both IFN- γ and LPS as diffuse pink staining within the Alvetex® Scaffold membrane. Additionally, these two models display alterations to the structure of the epithelium: evidence of epithelial cell shedding is denoted by # while flattened epithelial cells that appear to have lost their polarisation are indicated by arrows. Scale bars: 50 μ m.

To better visualise the morphological changes of the epithelium associated with IFN- γ and LPS, immunofluorescent analysis of two apical junctional complex proteins was conducted – representative images of which are depicted in Figure 6.57. Claudin-1, a sealing junctional protein, was utilised to visualise aspects of the tight junction. In both control models and those subject to IFN- γ , claudin-1 is discernible along the lateral membranes of adjacent epithelial cells, though staining is also present along the basolateral surface of the epithelium. Examination of models treated with both IFN- γ and LPS highlighted that some areas of the epithelial monolayer have compressed nuclei and an overall flattened cellular shape with limited claudin-1 staining between adjacent cells. On the other hand, claudin-1 was expressed very strongly along the basolateral membrane of the flattened epithelial monolayers and some positive staining was also found on the apical surface of the cells, confirming that the normal epithelial architecture was disrupted. Additionally, claudin-1 was found to stain the U937 macrophages in all models; though this is likely to be background staining, there is some evidence that both macrophages and dendritic cells express tight and adherens junctions (612).

Supplementary to claudin-1, the adherens junction marker, β -catenin was also visualised by immunofluorescent staining. β -catenin was selected for staining rather than E-cadherin as it is also a transcription factor that functions as part of the Wnt/ β -catenin signalling pathway and has crucial roles in macrophage differentiation and in promoting fibrosis. Expression of β -catenin was strongest within the epithelium; control models and those treated with IFN- γ demonstrated a similar expression pattern and localisation to claudin-1, with strong lateral membrane staining but some evidence of basolateral membrane staining that was most noticeable in models treated with IFN- γ for 48 hours. β -catenin was also evident in the epithelium of models treated successively with IFN- γ and LPS, though models treated with LPS apically do not have a clear expression profile between adjacent cells and positive staining overall was less obvious compared to other models. Regarding the role of β -catenin as a transcription factor, positive cytoplasmic staining of the protein can be observed. Within control models, β -catenin is present within both the rounded and more spindle shaped cells of the lamina propria, suggesting it is active as a transcription factor in both fibroblasts and macrophages. Similar positive staining is observed in models treated with IFN- γ alone and models incubated with IFN- γ then LPS basally, yet limited positive staining was present within the cells of the lamina propria in models treated with IFN- γ then LPS apically.

Though most cells within the lamina propria contain nuclei with a rounded architecture, in the model treated with IFN- γ only, evidence of lobed nuclei indicative of monocytes rather than macrophages was visible (denoted by #). This implies that there was either incomplete differentiation of the U937 cells prior to seeding within the model or that the macrophages have dedifferentiated, a phenotype previously demonstrated to occur in U937 macrophages cultured with frequent media renewals as happens within the triple co-culture (613). Interestingly there is evidence of nuclear blebbing (highlighted by *) within the lamina propria, indicative of apoptosis, confirming that some cellular death occurs within the lamina propria and may be responsible for the debris identified via histological staining.

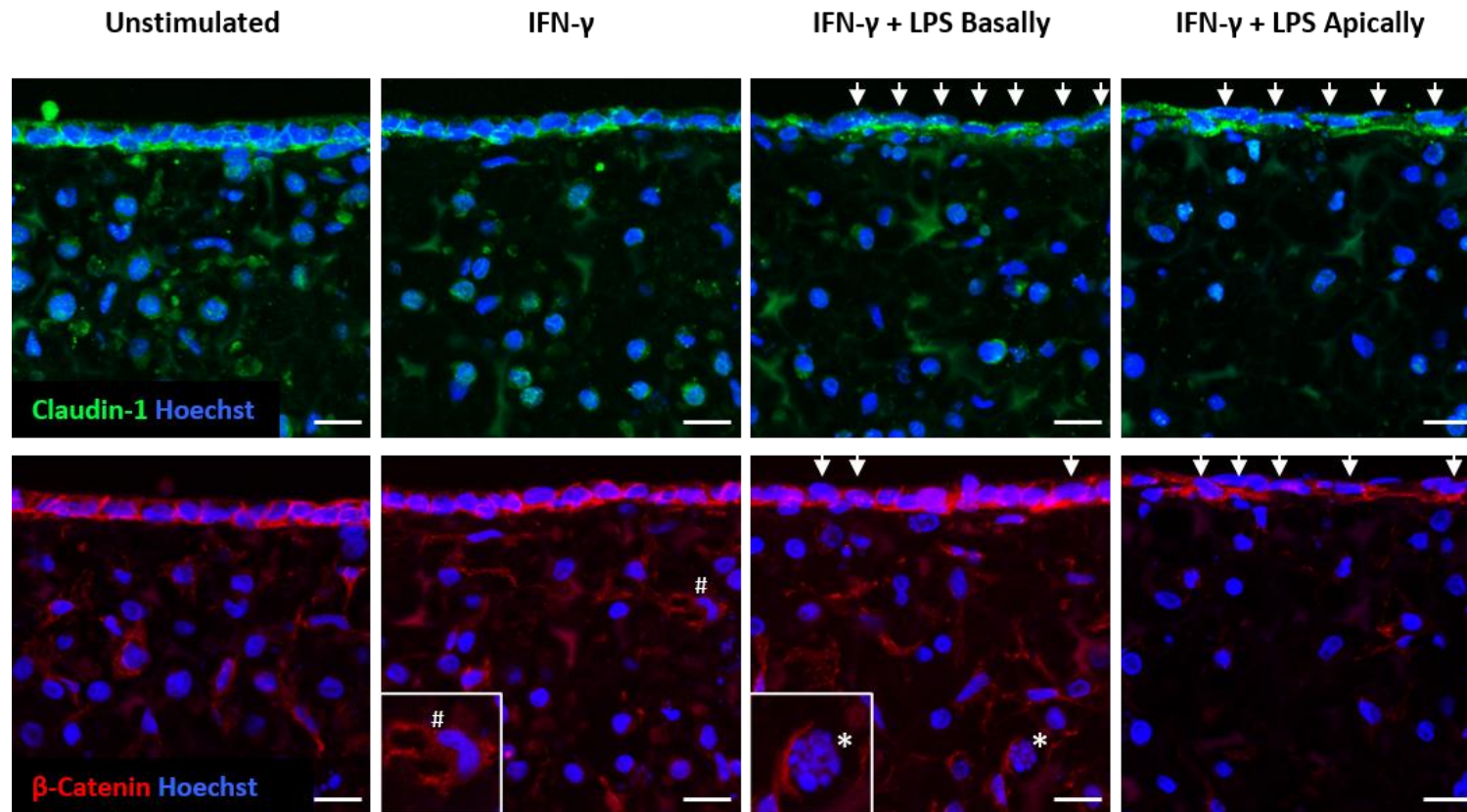


Figure 6.57. Successive treatments of IFN- γ and LPS alter the localisation of tight and adherens junctions' components. Immune-competent triple co-cultures were established as described in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where 100 IU/mL IFN- γ was introduced into the basolateral compartment and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. Models were then harvested for wax embedding and immunofluorescent analysis. Representative compressed Z-stacks of 9 control, IFN- γ only and IFN- γ + LPS treated models stained for claudin-1 (green) and β -catenin (red) are shown. Nuclei were counterstained by Hoechst (blue). Arrows highlight epithelial cells with a flattened nuclear morphology indicative of a flattened cellular morphology overall, # indicates a lobed nucleus, while * shows evidence of apoptosis through nuclear blebbing. Scale bars: 20 μ m.

Clear evidence to the disruption of the localisation of apical junctional complex was provided by histological and immunofluorescent analysis. However, the ability of the epithelium to continue to produce new AJC proteins remained unclear, thus analysis of the expression of genes involved in the AJC was conducted via qPCR. IFN- γ incubation alone for 48 hours induced minimal changes in the gene expression levels of both occludin and claudin-1 (Figure 6.58A,B). When successively treated with both IFN- γ and LPS, a reduction in the levels of both *OCN* and *CLDN1* was clear, suggesting that the epithelial barrier would be further compromised if continued to be maintained in culture as any protein damage sustained will likely not be replaced with new proteins. Of note was that LPS application apically did not reduce the levels of *OCN* and *CLDN1* like that of basolateral addition of LPS, this was unexpected as, structurally, the epithelium incurred more visible alterations and damage when LPS was given apically

The *TJP1* (ZO-1) gene was found to decrease with IFN- γ treatment alone (Figure 6.58C). However, following IFN- γ treatment with LPS restored levels of TJP-1 slightly beyond that of controls suggesting some compensatory signalling mechanisms are present within the culture system to prevent the collapse of the epithelial barrier function. Regarding the gene expression of E-cadherin (*CHD1*), a trend of reduced gene expression with IFN- γ treatment that was further decreased with the subsequent application of LPS was visible (Figure 6.58D).

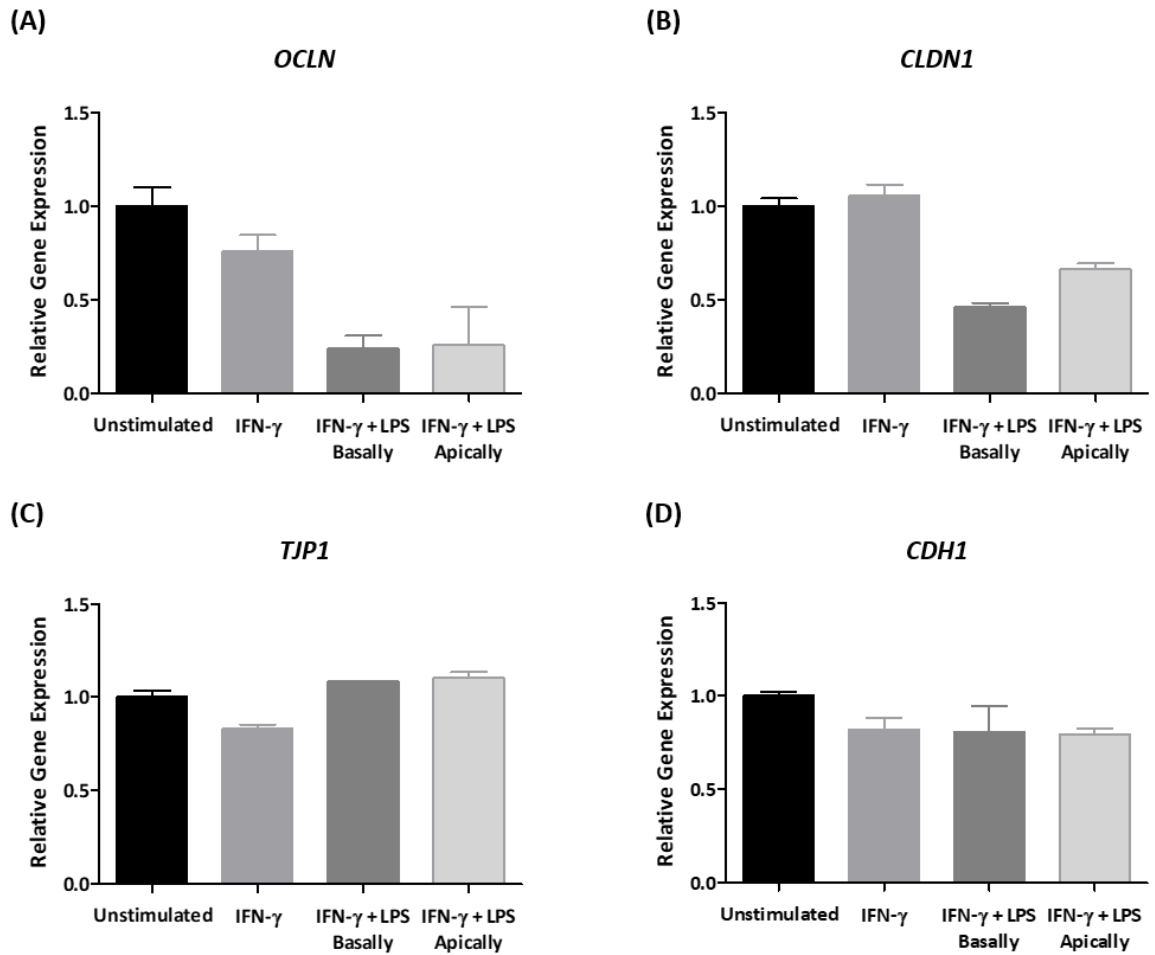


Figure 6.58. IFN- γ treatment followed by LPS generally induces a decrease in the expression of apical junctional complex genes. Immune-competent triple co-cultures were established as described in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where 100 IU/mL IFN- γ was introduced into the basolateral compartment and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. Models were then lysed for qPCR analysis. Gene expression levels of OCLN (A), CLDN1 (B), TJP1 (C) and CDH1 (D) were first normalised to levels of GAPDH then to unstimulated control models. Data represent mean $\pm$ SEM, total 6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

Final characterisation of the epithelium was performed through analysis of the functional barrier properties. This was initially done through TEER assessment where the percentage delta was calculated from control models (Figure 6.59A). IFN- γ alone was sufficient to cause a reduction in TEER by approximately 30 %, this was surprising as the overall structure of the monolayer remained intact and well differentiated. Treatment with IFN- γ followed by LPS caused a reduction in TEER by up to 70 % irrespective of where LPS was applied. This demonstrated that LPS significantly amplified the reduction in TEER caused by IFN- γ alone. Furthermore, this reduction in electrical resistance was also stronger than that of LPS treatment alone for 24 hours which had previously been demonstrated to induce a reduction in TEER by less than 40 %.

A lucifer yellow rejection assay was conducted to observe the ability of the monolayers to form an intact barrier and exclude the passage of the dye (Figure 6.59B). Control models and those stimulated with IFN- γ alone were able to exclude over 90 % of the apically applied lucifer yellow. However, treatment of models with both IFN- γ and LPS reduced the level of rejection to 89 % and 87 % respectively when LPS was applied basolaterally and apically, suggesting that the barrier formed is less tight and has become leakier. To confirm this, a series of transport assays were conducted in the apical-basolateral absorptive transport direction to calculate the apparent permeability coefficient of lucifer yellow, FD4 and FD20 (Figure 6.59C-E). Although no significant differences were identified, clear trends were apparent. Control models always exhibited the lowest level of transport while IFN- γ induced a minor increase. Further increases in the paracellular permeability were found when LPS was subsequently applied basolaterally with the greatest permeability present in models treated with LPS apically consistent with the degree of structural changes observed in the epithelium of the stimulated *in vitro* models.

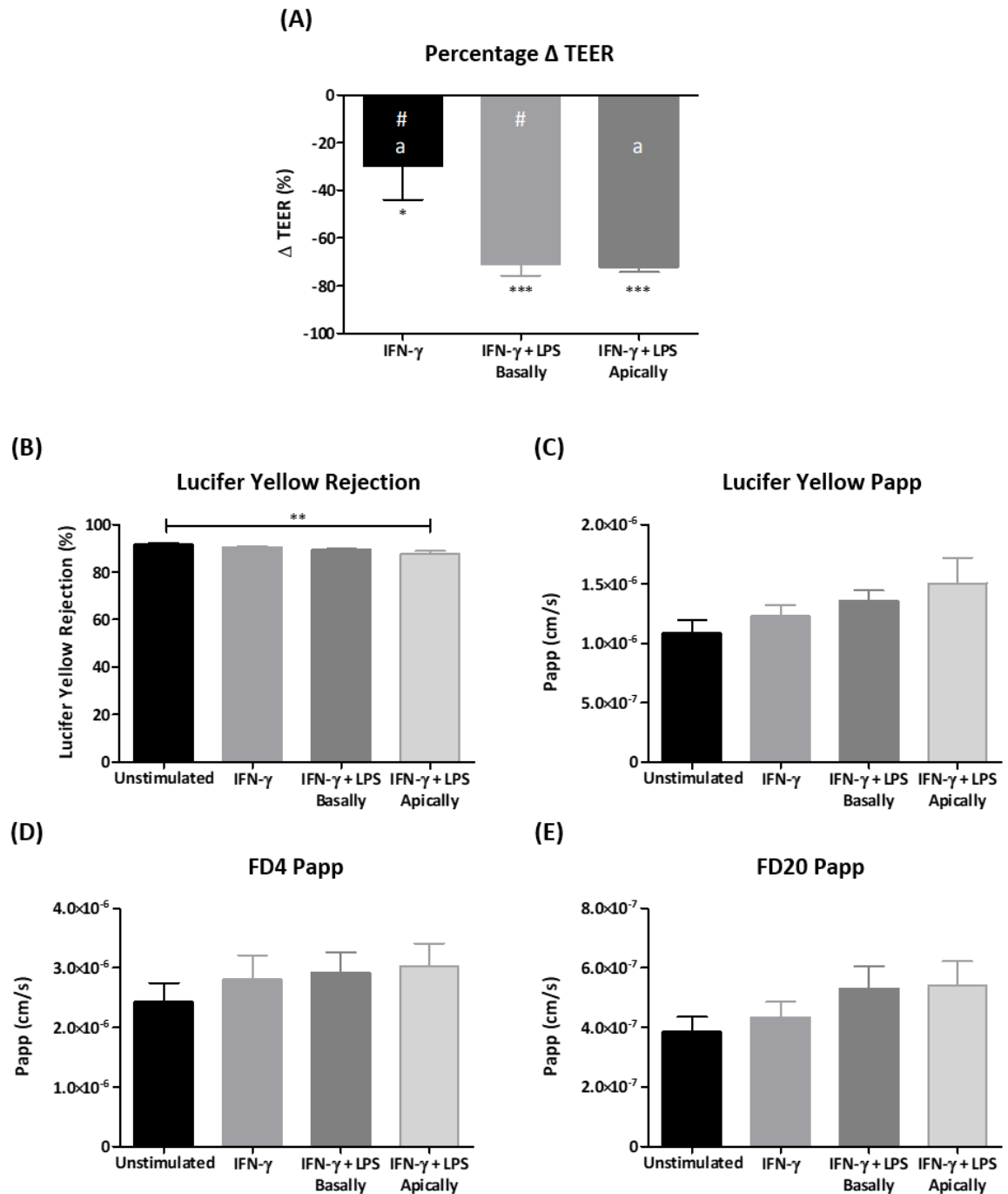


Figure 6.59. IFN- γ and subsequent LPS treatment induces greater functional changes in the epithelium than IFN- γ alone. Immune-competent triple co-cultures were established as described in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where 100 IU/mL IFN- γ was introduced into the basolateral compartment and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. TEER measurements were then taken of the models and the percentage delta TEER compared to unstimulated control models was calculated (A). A lucifer yellow rejection assay was performed in the apical-basolateral direction to determine the ability of the stimulated models to exclude the fluorescent dye (B). Additional transport assays of lucifer yellow (C), FD4 (D) and FD20 (E) were also performed in the apical-basolateral transport direction. Data represent mean $\pm$ SEM, total $n=9$ from 3 independent experimental repeats. * $P\leq 0.05$, ** $P\leq 0.001$, *** $P\leq 0.0001$, # $P\leq 0.0001$ and ^a $P\leq 0.0001$ as determined by one-way ANOVA with Tukey's post-hoc test. Regarding graph A, * denotes significant differences to unstimulated control models while # and ^a denote differences between different stimulation treatments.

It was hypothesised that LPS treatment following IFN- γ priming would induce a greater inflammatory response than IFN- γ alone. Thus, assessment of the inflammatory response induced by IFN- γ and LPS stimulations was performed through gene expression analysis of the levels of mRNA present within the models. Four proinflammatory cytokine genes (*TNF*, *IL1B*, *IL6* and *IL8*) were assessed in addition to two anti-inflammatory cytokines (*IL10* and *TGFB1*).

TNF- α is known to have synergistic effects with IFN- γ and has also been shown to induce its expression, yet within the triple co-culture presented here, incubation with IFN- γ alone did not increase mRNA levels of TNF- α (Figure 6.60A). However, providing a dose of LPS after IFN- γ , successfully induced TNF- α gene expression; overall expression levels of *TNF* were almost doubled with application of the two stimuli. A similar gene expression pattern was perceived with *IL1B*, where IFN- γ alone did not induce any changes in its expression but IFN- γ priming followed by LPS enhanced the induction of the *IL1B* gene (Figure 6.60B). Interestingly, IFN- γ and LPS stimuli appeared to reduce the overall levels of IL-6 being produced (Figure 6.60C), however, it is important to note that IL-6 was produced at extremely high levels by control models without stimulation (Figure 6.37), thus it may not be necessary for further induction of IL-6 when models are subjected to a proinflammatory stimulus. Finally, expression of the proinflammatory chemokine *CXCL8* was assessed (Figure 6.60D). Like the expression of *TNF* and *IL1B*, *CXCL8* did not alter much with only IFN- γ stimulation but was elevated following an additional stimulation with LPS. This was particularly evident when LPS was applied basolaterally to the lamina propria compartment where the macrophages are located.

Gene expression levels of *IL10* and *TGFB1* were also investigated. *IL10* was found to decrease following stimulation, irrespective of the secondary stimulation with LPS, however none of the observed decreases were greater than a 1-fold change and as such were not determined to be biologically significant (Figure 6.61A). On the other hand, *TGFB1* displayed an increase in expression following stimulation with both IFN- γ and LPS, with the greatest induction observed when LPS was applied apically (Figure 6.61B). IFN- γ incubation alone was not sufficient to drive an increase in the expression of *TGFB1*. Induction of *TGFB1* suggests that some anti-inflammatory signalling pathways have been induced within the model and could potentially result in fibrosis if models were maintained for longer.

Clear evidence of the induction of an inflammatory response to IFN- γ and LPS treatment has been shown, however, analysis focused on the induction of mRNA rather than protein levels. As there can be disparities between the level of gene expression and proteins, it would be interesting to determine the concentration of pro- and anti-inflammatory cytokines secreted into the media through either ELISA analysis or a further cytokine array.

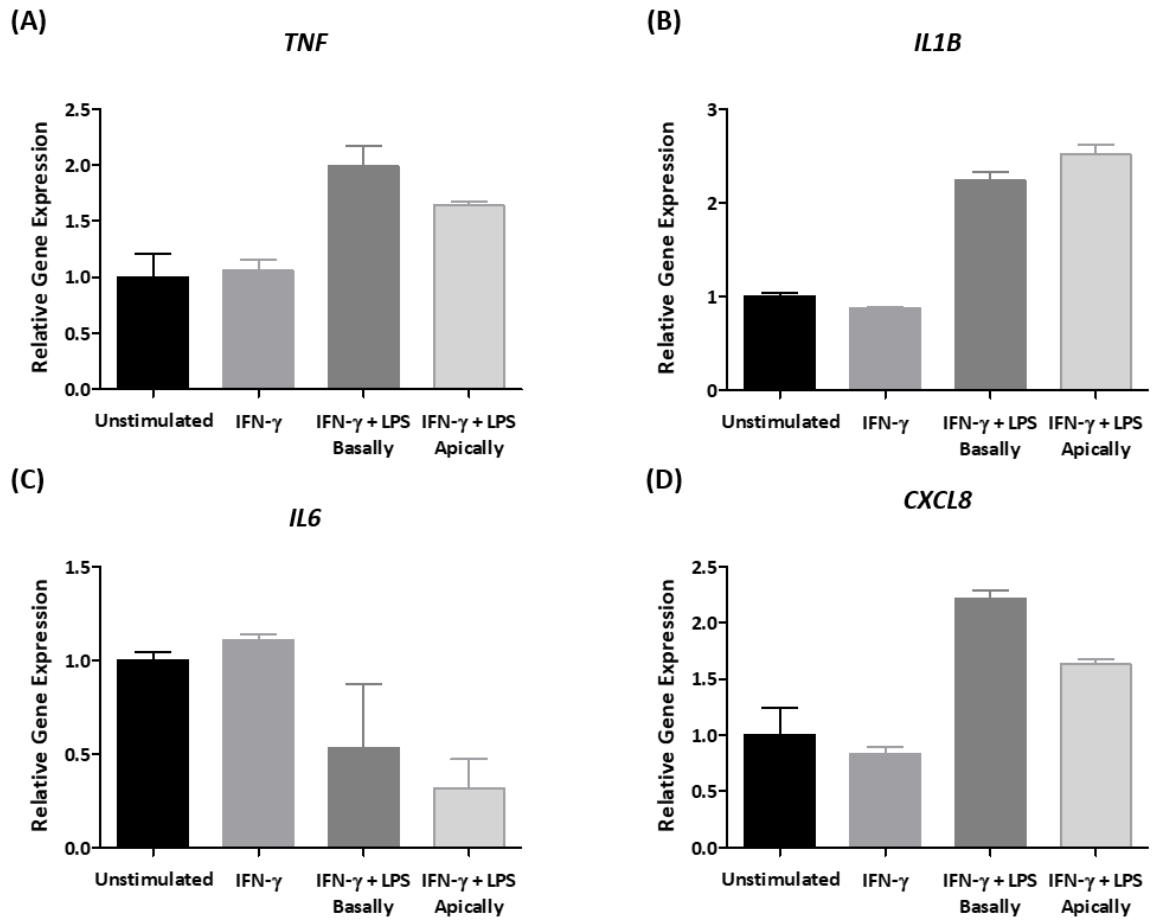


Figure 6.60. IFN- γ followed by a secondary treatment of LPS induces the gene expression of proinflammatory cytokines. Immune-competent triple co-cultures were established as described in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where 100 IU/mL IFN- γ was introduced into the basolateral compartment and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. Models were then lysed for qPCR analysis to quantify the gene expression of the proinflammatory cytokines TNF (A), IL1B (B), IL6 (C) and CXCL8 (D). Gene expression levels were first normalised to GAPDH then to unstimulated control models. Data represent mean $\pm$ SEM, total 6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

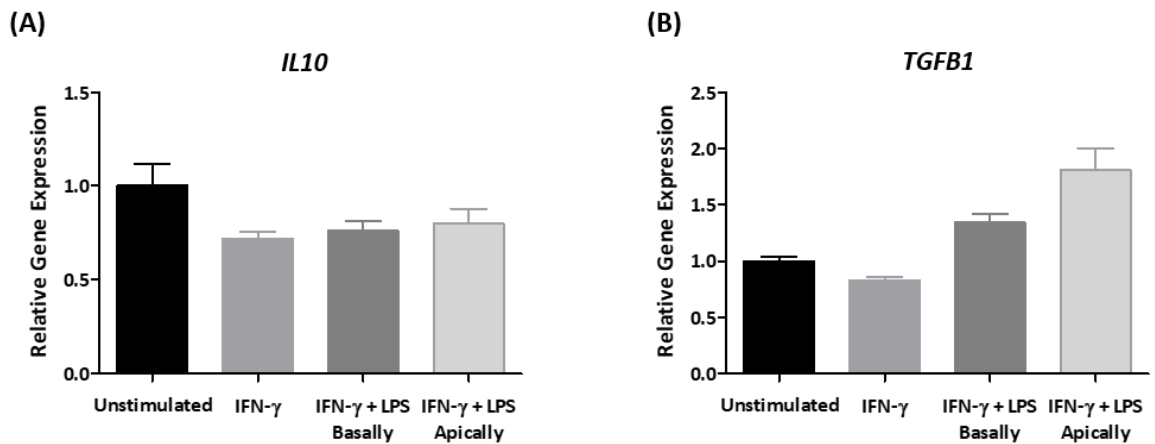


Figure 6.61. IFN- γ and subsequent LPS stimulation affects the gene expression levels of anti-inflammatory cytokines. Immune-competent triple co-cultures were established as described in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where 100 IU/mL IFN- γ was introduced into the basolateral compartment and incubation proceeded for 48 hours before further stimulation with 1 μ g/mL LPS either basally or apically for a further 24 hours. Models were then lysed for qPCR analysis to quantify the gene expression of the anti-inflammatory cytokines IL10 (A) and TGFB1 (B). Gene expression levels were first normalised to GAPDH then to unstimulated control models. Data represent mean $\pm$ SEM, total 6 from 3 independent stimulation studies, each with 2 technical repeats performed in the qPCR reaction.

6.4.5.2. Assessing the effect of IFN- γ and TNF- α stimulation on *in vitro* intestinal models

IFN- γ has been proposed to act in synergy with TNF- α to perpetuate the proinflammatory response by inducing the expression of TNF- α receptors in target cells (564,565). However, the influence of IFN- γ co-stimulation of cells adjunctively with TNF- α is not widely reported. Therefore, this section aimed to conduct a series of pilot studies on the immune-competent 3D co-culture to determine whether co-stimulation of IFN- γ and TNF- α or pre-exposure of *in vitro* models to IFN- γ and subsequent TNF- α stimulation induced a greater change in the properties of the epithelial barrier.

Preliminary experiments focused on determining the appropriate concentration of TNF- α to use in further studies with IFN- γ . TNF- α is utilised extensively at 10 ng/mL but some studies have used TNF- α at up to 100 ng/mL (559,560). Therefore, the effect of 0, 10, 50 and 100 ng/mL TNF- α treatment for 24 hours on the structure and function of epithelial monolayer was assessed. Similar to other stimulants, TNF- α was applied basolaterally while the apical media remained unaltered. No substantial differences could be identified regarding the structure of the epithelium when models were treated with various concentrations of TNF- α were analysed via histological staining; all enterocytes appear well polarised with a homogenous columnar phenotype suggesting that no damage to the barrier has been incurred as a result of the application of TNF- α (Figure 6.62). However, the lamina propria of the different models did demonstrate noticeable differences in morphology when TNF- α had been present at both 50 and 100 ng/mL; high numbers of macrophages were identified in the fibroblast layer surrounding the basolateral surface of the Alvetex® Scaffold membrane, resulting in a large distention of the model. Although some U937 macrophages were present adjacent to the epithelium, the predominant location of the immunocytes remained basal suggesting that the chemotactic signals were driving a basolateral migration of the cells. This phenotype was not found when models were treated with the lowest concentration of TNF- α or in unstimulated controls.

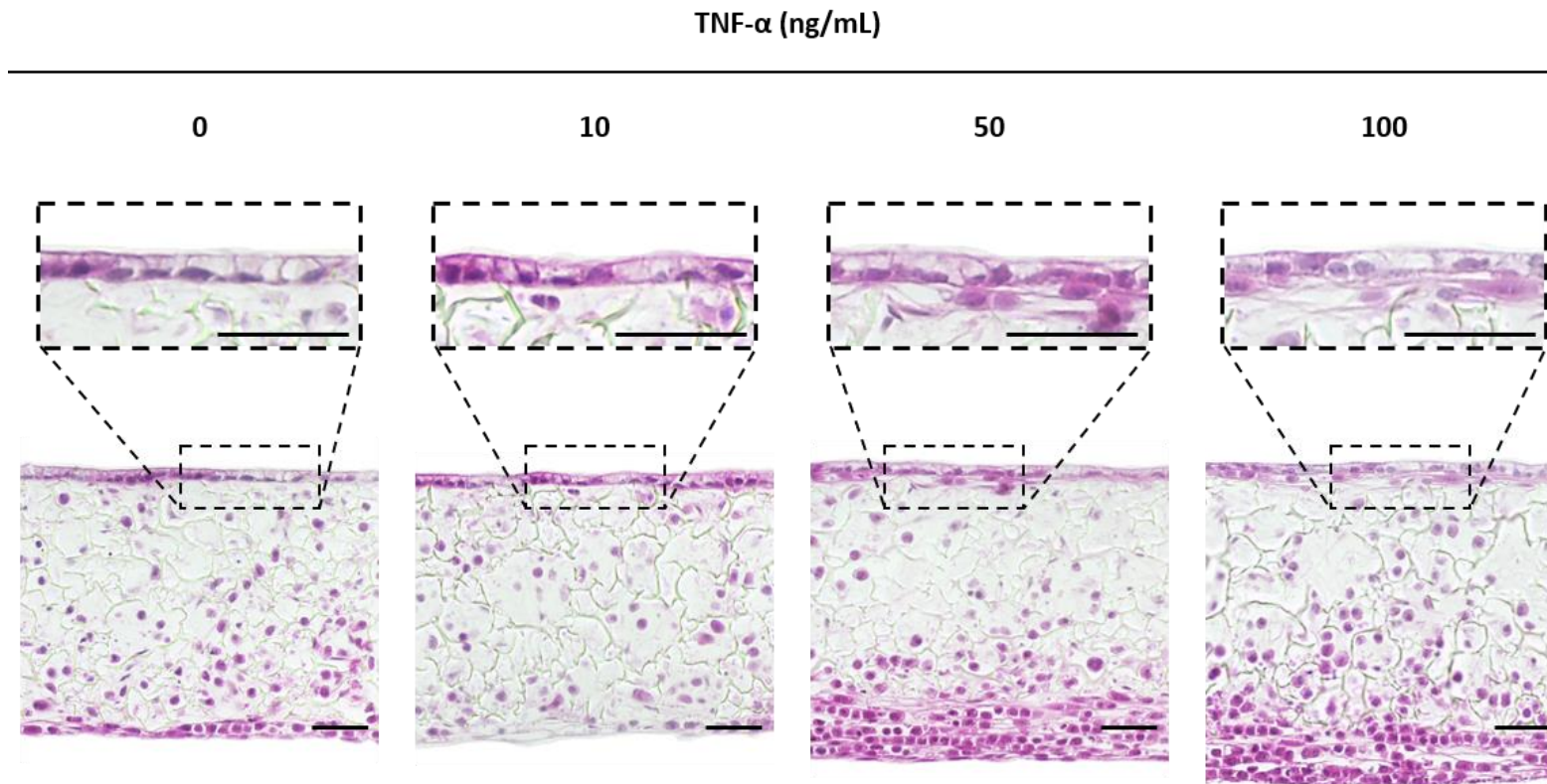


Figure 6.62. High concentrations of TNF- α do not affect the structure of the epithelium but induce a basolateral accumulation of macrophages. Immune-competent 3D co-cultures were established as defined in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where they were incubated with 0-100 ng/mL TNF- α for 24 hours before harvesting of models for wax embedding and histological analysis. Representative H&E-stained images of 6 models treated with 0, 10, 50 and 100 ng/mL TNF- α are shown. Scale bars: 50 μ m.

Further investigations of the effect of different concentrations of TNF- α on the *in vitro* models focused on characterising the functional properties of the epithelial barrier. Initial analysis concentrated on assessment of the electrical resistance across the epithelium. When compared to unstimulated control models, TNF- α induced a decrease in resistance by approximately 11-13 % irrespective of concentration (Figure 6.63A). Moreover, this reduction in TEER was not deemed to be significant suggesting that TNF- α alone does not elicit a strong response in the epithelium. To corroborate this hypothesis, various transport assays were conducted to assess the ability of the monolayer to reject lucifer yellow and determine the apparent permeability of models in the apical to basolateral absorptive transport direction (Figure 6.63B-E). No significant differences or discernible trends were observed with increasing TNF- α concentrations; the permeability remained similar to control models incubated without TNF- α .

The data presented here suggests that varying the concentration of TNF- α does not elicit a significant response on epithelial structure or function, however future studies would focus on determining whether the incubation period with TNF- α would have a greater impact on the barrier of the models. Although minimal alterations were induced in the epithelium, structural differences were observed within the lamina propria when higher concentrations of TNF- α were utilised. However, the observed localisation of macrophages in these models was not the overall desired location, thus taking together the overall model morphology and the lack of differences regarding epithelial function, 10 ng/mL TNF- α was selected for future experiments with IFN- γ .

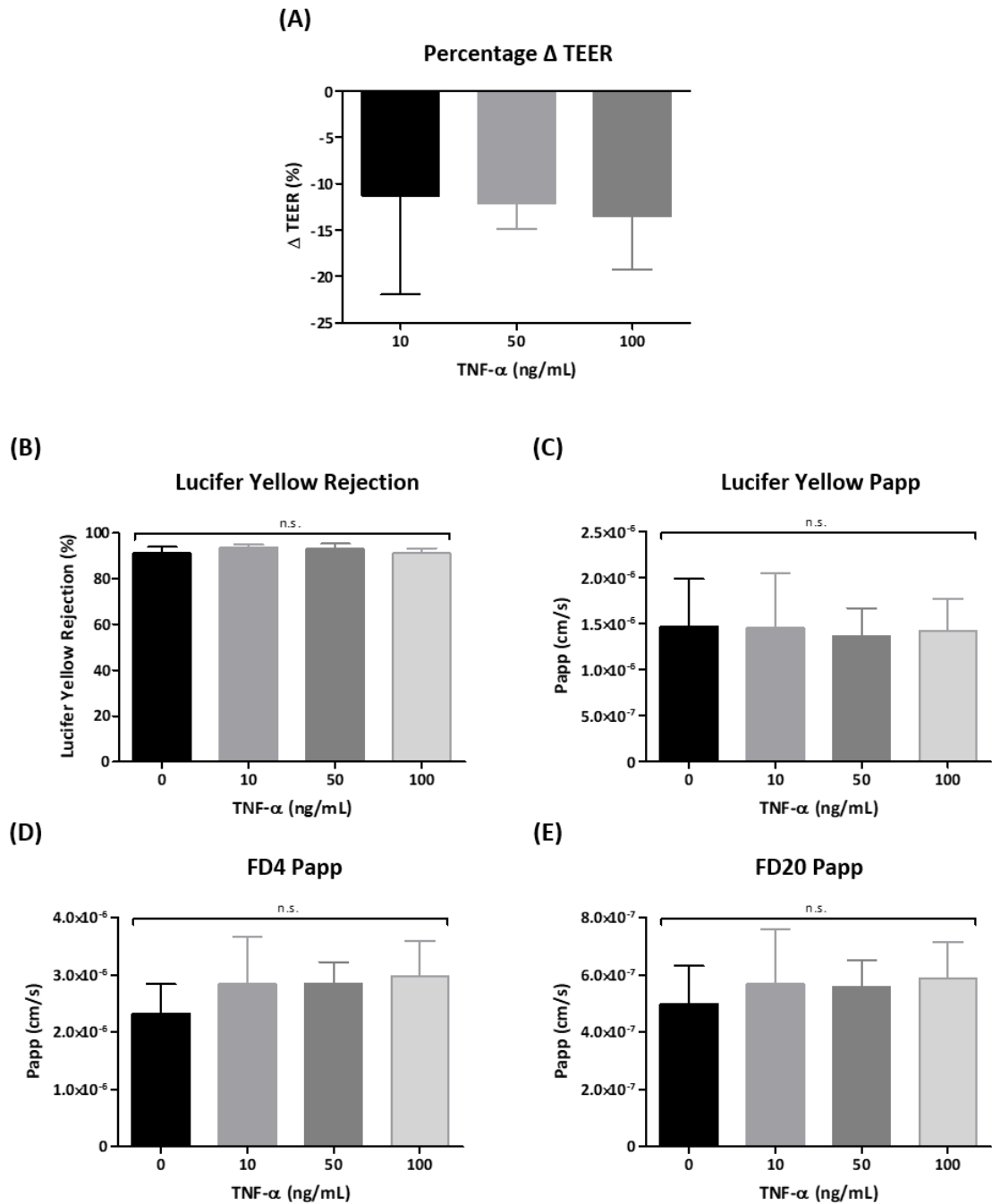


Figure 6.63. TNF- α does not induce a significant alteration in the function of the epithelial barrier. Immune-competent 3D co-cultures were established as defined in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where they were incubated with 0-100 ng/mL TNF- α for 24 hours before TEER measurements were taken and the percentage delta TEER was calculated against unstimulated control models (A). A lucifer yellow rejection assay performed in the apical-basolateral direction revealed no significant differences in the ability of the models to exclude the fluorescent dye (B). The apparent permeability coefficients of lucifer yellow (C), FD4 (D), and FD20 (E) were also calculated and revealed no differences in transport in the apical-basolateral direction. Data represent mean $\pm$ SD, total $n=6$ from 2 independent experimental repeats, each with 3 models cultured as technical repeats. No significant (n.s.) differences were determined by one-way ANOVA with Tukey's post-hoc test.

Once the optimal concentration of TNF- α had been determined, the next pilot study considered the co-stimulation of IFN- γ and TNF- α and the effect of IFN- γ priming of the *in vitro* models prior to TNF- α stimulation. When co-stimulating, 100 IU/mL IFN- γ and 10 ng/mL TNF- α were simultaneously applied to the media of the basolateral compartment. For priming conditions, 100 IU/mL IFN- γ was applied to the basolateral media for 24 hours followed by 10 ng/mL TNF- α to the same compartment for a further 24 hours. Models were then harvested for downstream analysis.

Treatment of models with both stimulants caused structural alterations within the epithelium; enterocytes lost their distinctive columnar architecture and, instead, gained a cuboidal arrangement indicating that IFN- γ is able to enhance the structural response of the cells to TNF- α . (Figure 6.64). This was evident when IFN- γ was utilised as both a co-stimulant and a priming agent, though the phenotype was more striking when co-stimulated with TNF- α where the epithelial cells had a more prominent flattened appearance (highlighted by the arrows). Regarding the structure of the lamina propria, high numbers of macrophages were observed throughout the Alvetex® Scaffold membrane in all stimulated models, while fewer cells were visible in untreated controls suggesting that the immunocytes have responded to the proinflammatory stimuli by either migrating or proliferating.

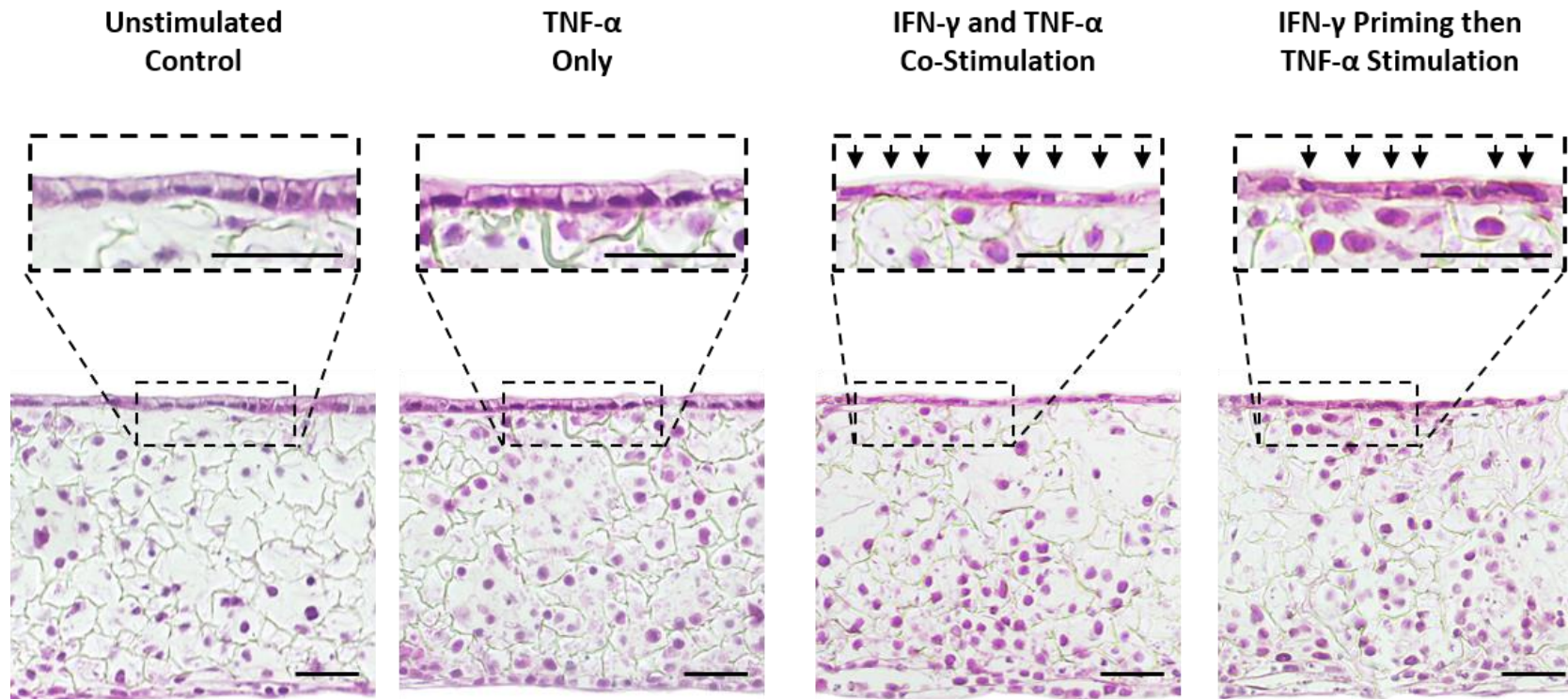


Figure 6.64. IFN- γ and TNF- α have a greater effect on epithelial structure than TNF- α alone. Immune-competent 3D co-cultures were established as defined in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where they were incubated with either: media alone; 10 ng/mL TNF- α for 24 hours; 100 IU/mL IFN- γ and 10 ng/mL TNF- α for 24 hours; or 100 IU/mL IFN- γ for 24 hours followed by 10 ng/mL TNF- α for 24 hours. Models were then harvested for wax embedding and histological analysis; representative H&E-stained images of 6 models are shown. Combined IFN- γ and TNF- α applications induced a flattened morphology across the epithelial monolayer with little evidence of cytoplasmic staining above the nuclei as indicated by the arrows. Scale bars: 50 μ m.

Finally, the functional properties of these models were assessed. Calculation of the percentage change in TEER from control models revealed that TNF- α had little effect on the electrical resistance across the models, while both IFN- γ co-stimulation and IFN- γ priming induced a significant reduction in TEER from both control models and those treated with only TNF- α (Figure 6.65A). Co-stimulation with IFN- γ and TNF- α caused TEER to reduce by 40 % while IFN- γ priming induced a greater reduction by almost 50 %, suggesting that priming may have a greater effect on the epithelial barrier than concurrent treatment. Furthermore, application of both stimulants induced a greater reduction in TEER than IFN- γ alone, which has previously been demonstrated to reduce TEER by approximately 30 %.

Although TEER was reduced, lucifer yellow rejection remained high in the models, with no obvious differences between the different conditions and control models (Figure 6.65B). When the permeability of the standard transport compounds utilised within this chapter were assessed, IFN- γ priming prior to TNF- α treatment was able to induce a significant increase in the absorptive permeability of FD4. Although no other significant differences were found, a general trend was identified and replicated across the models, where TNF- α alone induced minimal changes in permeability but co-stimulation and priming increased the permeability of all tested compounds (Figure 6.65C-E).

The results presented within Section 6.4.5.2. comprise a series of pilot studies focused on the characterisation of model structure via histological staining, and characterisation of the epithelial barrier functional properties. Treatment of models with both IFN- γ and TNF- α were found to induce greater differences in both the structure and function of the *in vitro* triple co-culture models than TNF- α alone. However, no investigations into the immune response generated by the stimulants were conducted, thus future studies would prioritise this aspect to determine which stimulatory condition induces the greatest inflammatory response.

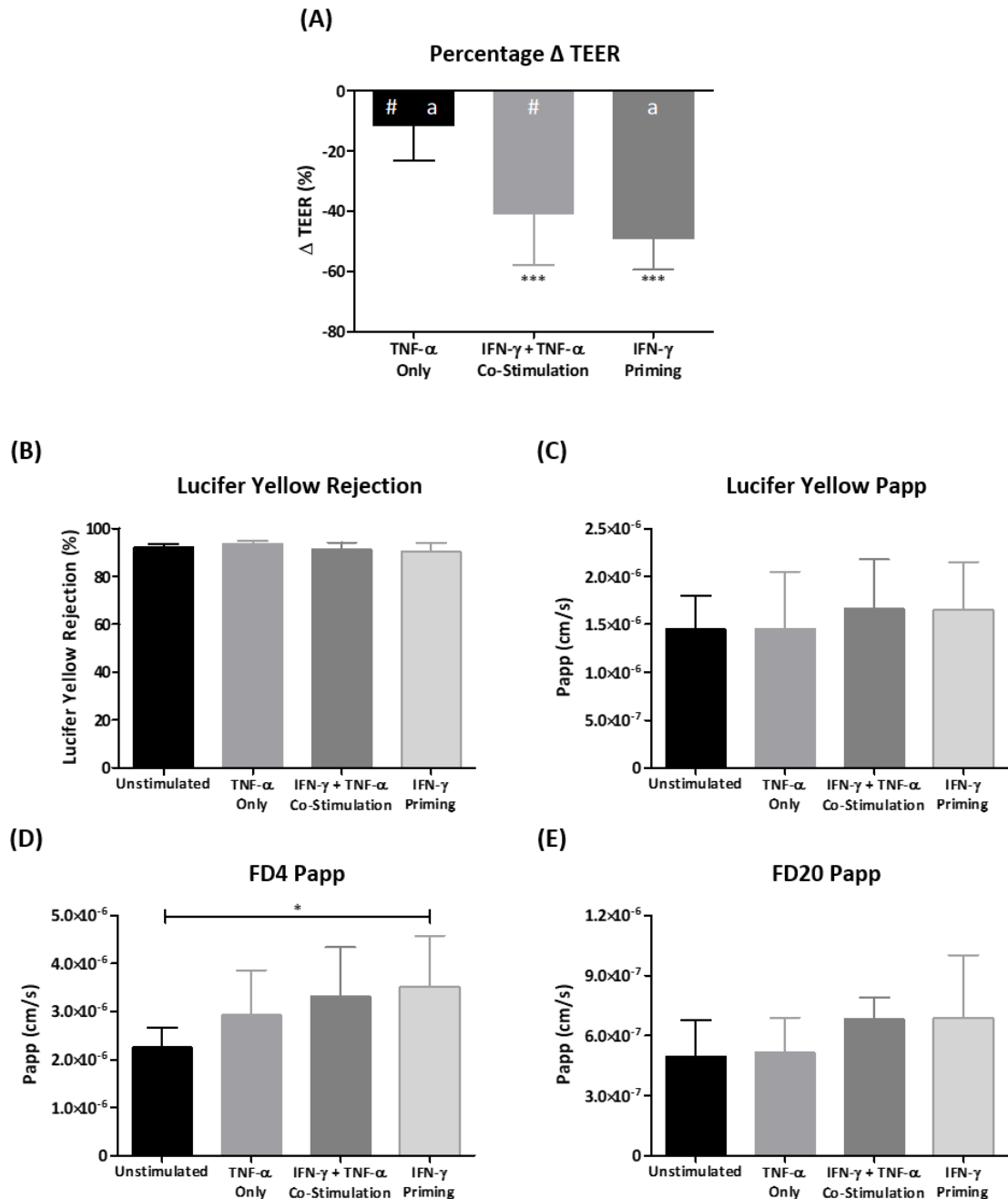


Figure 6.65. Stimulating 3D triple co-cultures with IFN- γ and TNF- α appears to have a greater effect on epithelial barrier function than TNF- α alone. Immune-competent 3D co-cultures were established as defined in Chapter 5. Following epithelial differentiation, models were transferred to a 12-well plate where they were incubated with either: media alone; 10 ng/mL TNF- α for 24 hours; 100 IU/mL IFN- γ and 10 ng/mL TNF- α for 24 hours; or 100 IU/mL IFN- γ for 24 hours followed by 10 ng/mL TNF- α for 24 hours. TEER measurements were taken and the percentage delta TEER was calculated against unstimulated control models (A). A lucifer yellow rejection assay performed in the apical-basolateral direction revealed no significant differences in the ability of the models to exclude the fluorescent dye (B). The apparent permeability coefficients of lucifer yellow (C), FD4 (D), and FD20 (E) were also calculated and highlighted that IFN- γ and TNF- α caused a minor increase in apical-basolateral permeability than TNF- α alone. Data represent mean $\pm$ SD, total n=6 from 2 independent experimental repeats, each with 3 models cultured as technical repeats. * $P \leq 0.05$, *** $P \leq 0.0001$, # $P \leq 0.0001$ and ^a $P \leq 0.0001$ as determined by one-way ANOVA with Tukey's post-hoc test. *** symbolises significant changes from unstimulated control models, while # and ^a represent significant differences between stimulatory conditions.

6.4.6. Summary of Experimental Findings

This chapter has focused on inducing a proinflammatory response in the immune-competent *in vitro* models developed in Chapter 5; this was achieved through introducing various stimulatory molecules and proteins to the culture media. Models were then characterised for any structural changes, with a particular focus on the epithelial monolayer, and functional analysis of the epithelial barrier properties was performed. Additional characterisation of the immune responses generated within the 3D triple co-culture was assessed, primarily through qPCR techniques. The main findings are summarised below:

- Diseased tissue samples exhibit altered structural morphology compared to control biopsies.
- Differentiated U937 macrophages are able to induce a proinflammatory response following stimulation with the bacterial endotoxin, LPS.
- The structure and barrier function of the epithelium of Caco-2-U937 co-cultures and 3D triple co-cultures is partially compromised following acute and repeated LPS treatment.
- A strong proinflammatory response is induced within the triple co-culture when models are subject to repeated LPS applications.
- IFN- γ alone does not cause significant alterations in epithelial structure or function, nor does it induce a strong inflammatory response when applied to *in vitro* models.
- Priming models with IFN- γ before further treatment with either LPS or TNF- α results in greater changes to the model structure and barrier function than IFN- γ alone.

6.5. Discussion

The tissue constructs optimised in the previous chapter sought to establish co-culture models of the intestinal mucosa that incorporated an immune cell component, namely macrophages. Differentiated U937 macrophages were either indirectly combined with Caco-2 enterocytes using the Transwell® cell culture platform or were in direct co-culture within an Alvetex® Scaffold membrane that had been pre-seeded with fibroblasts to represent a lamina propria compartment with an epithelial monolayer cultured on the apical surface. Inclusion of macrophages within the co-culture models revealed no significant differences to the structure or function of the epithelial barrier in an unstimulated state, however, the ability of these immune cells to drive inflammation within the models remained unconfirmed. Thus, this chapter set out to introduce proinflammatory stimuli into the *in vitro* co-culture systems in an attempt to provoke an inflammatory response within the macrophages and determine the effects of proinflammatory stimuli on the epithelial barrier structure and function.

6.5.1. LPS Induces a Proinflammatory Immune Response within Differentiated Macrophages

Lipopolysaccharide is a bacterial endotoxin present in the outer membrane of Gram-negative bacteria. It is known to be a potent activator of both monocytes and macrophages through binding to the cell surface receptor CD14 whereupon it elicits a broad, non-specific immune response (583,614). LPS is widely used *in vitro* to stimulate and characterise the immune response of macrophages including U937, THP-1 and the murine lineage of RAW 264.7 macrophages. It is known to induce a classically activated, M1 phenotype within the macrophages initiating the release of many proinflammatory cytokines including TNF- α , IL-1, and IL-6 that proceed to further provoke inflammatory responses (583,615–618). However, a recent study using murine bone marrow derived macrophages has shown that LPS has a biphasic effect and drives the differentiation of macrophages in a time-dependent manner; short-term LPS stimulation (~3 hours) was found to upregulate the T-cell immunoglobulin mucin 3 (Tim-3)/ galectin-9 (Gal-9) signalling pathway that resulted in an inhibition of M1 polarisation, yet long-term stimulation (12-24 hours) inhibited the same signalling pathway promoting M1 polarisation. Therefore, LPS was incubated for a minimum of 24 hours in the studies presented within this chapter to ensure that the M1 phenotype was present within the cells (619).

Within the context of this study, differentiated macrophages maintained in traditional 2D cell culture and exposed to LPS, exhibited an increased gene expression of TNF- α , IL-6, and IL-8, all of which have been demonstrated to increase following LPS treatment in previous studies (583,615–618,620,621). The increase in TNF- α mRNA is of interest for co-culture studies as TNF- α has been demonstrated to be partially responsible for reducing epithelial barrier integrity in IBD patients and in *in vivo* and *in vitro* intestinal models (298,560,622–625), however the increase in TNF- α expression (2-5 % increase) was much lower than IL-6 (250-900 % increase) and IL-8 (10-25 % increase) suggesting that there may be limited biological effects elicited by the increase in TNF- α in comparison to the other cytokines within this model system.

Also of note, was the increase in CD14 gene expression following a single dose of LPS, correlating with greater positive staining for the protein indicative of an upregulation of the LPS co-receptor. Furthermore, CD14^{hi} monocytes and macrophages migrate to the intestinal mucosa and outnumber the normal M2 tissue resident macrophages in both CD and UC resulting in increased levels of proinflammatory cytokines encouraging pathogenic T cell function (174,626–629). This would suggest that incorporation of these differentiated macrophages into the *in vitro* intestinal mucosa and treating the models with LPS would recapitulate some phenotypes observed within IBD.

Interestingly repeated doses of LPS also induced an increase in the expression of IFN- γ mRNA, a cytokine not generally associated with secretion by macrophage lineages, however it is important to note that these cells are of cancerous origin and therefore may not respond in the same manner as macrophages derived from healthy individuals. Additionally, not all immune cells present within the 2D culture may be fully differentiated into macrophages, thus monocyte contamination may induce the increase in IFN- γ gene expression (630). However, it is also possible that any IFN- γ protein produced by the U937 cells is not secreted by the cells and instead acts in an autocrine, intracellular manner, this is further supported by evidence from RAW 264.7 cells stimulated with LPS that were shown to produce IFN- γ mRNA and stained positively for expression of IFN- γ within the cell cytoplasm (630–633). Furthermore, when examining the secreted IFN- γ protein levels in the culture media collected from triple co-cultures repeatedly stimulated with LPS, limited IFN- γ was found, yet significant increases in the expression levels of MIG and IP-10 (Figure 6.43) that are induced by IFN- γ were identified, lending further support to the hypothesis that IFN- γ is actively signalling intracellularly but may not be secreted.

In addition to an increase in the expression of proinflammatory cytokines, the results presented within demonstrated that the mRNA levels of the anti-inflammatory cytokine, IL-10, were also increased following one or multiple doses of LPS. IL-10 functions as part of an immunoregulatory feedback loop to prevent excessive production of proinflammatory cytokines, suggesting that adjunctive to the initial production of proinflammatory cytokines, LPS induces an immunosuppressive response within the U937 macrophages. Previous studies have highlighted the role of type I interferons, such as IFN- α , in stimulating the production of IL-10 in response to LPS in murine bone marrow derived macrophages (167,634–637). Type I interferons are typically proinflammatory, yet their requirement in the induction of IL-10 indicates a novel immunosuppressive role that requires further exploration. It would therefore be of interest to determine the levels of type I interferons and IL-10 expressed at different time points within the macrophages to confirm that this mechanism of IL-10 stimulation is active within this culture system.

LPS has previously been shown to induce structural changes in PMA-differentiated U937 macrophages, following exposure to 0.1 $\mu\text{g/mL}$ LPS for 12 hours, this change in cellular shape may induce epigenomic and subsequent functional changes within the cells (584,638). This effect was not replicated in this study when macrophages were treated with either 1 or 10 $\mu\text{g/mL}$ LPS for 24 hours - the cells retained their rounded morphology; however, the cellular structure was not examined at earlier time points. Spindle-shaped and rounded macrophages have been shown to exhibit distinct proteomes with Rab family proteins being more

abundant in spindle macrophages and proteins involved in lipid handling are dominant in rounded macrophages (639). Rab family proteins are known to be heavily involved in the control of phagocytosis while lipid handling proteins have crucial roles in metabolism and lysosomal degradation (640–643). Within the present study, LPS incubation period or concentration was not found to significantly effect cellular metabolic activity. Additionally, LPS increased the phagocytosis of the differentiated U937 macrophages in a dose-dependent manner highlighting that although the cells did not acquire a spindle-shaped morphology in response to LPS treatment with a concurrent increase in Rab protein levels, they retained their phagocytic ability. It would thus be interesting to quantify the morphological shape of differentiated U937 cells when exposed to varying concentrations of LPS for multiple incubation periods and relate the cellular shape back to the overall function of the macrophages.

6.5.2. Stimulation of Immune-Competent *In Vitro* Intestinal Models to Provoke an Inflammatory Response and Reduce Epithelial Barrier Integrity

6.5.2.1. LPS stimulation reduces epithelial barrier integrity in *in vitro* models of the intestinal epithelium

LPS levels are greatly enhanced within the intestinal tissue and serum of IBD patients, as such LPS is considered to have important roles in initiating and propagating the intestinal inflammatory response in IBD. This is due to a concurrent increase in the expression of proinflammatory cytokines and circulating endotoxins within the blood, known as endotoxemia (580,644–647). Furthermore, enhanced LPS levels correlate with disease activity while normal LPS levels are recovered following disease treatment, however, recovered CD patients still exhibit slightly higher levels of LPS than healthy individuals (644). Removal of circulating LPS has been demonstrated to enhance the clinical improvement of IBD, further highlighting the important contribution of LPS to disease pathology (644,648). LPS is therefore a physiologically relevant stressor to utilise within *in vitro* intestinal models, thus, along with being used to stimulate an inflammatory response in macrophage cell lines, LPS has also been applied to *in vitro* models of the intestinal mucosa to investigate its effects on the functional properties of the epithelial barrier.

LPS utilised in the experiments presented within was derived from *E. coli*, the specific serotype of *E. coli* was O55:B5 due to its ability to induce endotoxemia *in vivo*, a phenotype associated with elevated LPS levels in IBD (649). Additionally, *E. coli* derived LPS is frequently used to stimulate intestinal *in vitro* cultures. A recent study has shown that proinflammatory cytokine induction remained relatively consistent between LPS derived from different bacterial species, however the production of anti-inflammatory cytokines was more variable (650). Furthermore, although all LPS stimulations examined were able to reduce TEER and increase FITC-dextran permeability across Caco-2 monolayers, LPS purified from *E. coli* and *Serratia marcescens* (*S. marcescens*) elicited the greatest effects on monolayer permeability (650). As *E. coli* derived LPS is widely used, in addition to its ability to induce damage to the epithelial barrier, a serotype of *E. coli* LPS was utilised in this study. Future studies could focus on comparing the effects elicited by LPS derived from different bacterial species and serotypes on the intestinal mucosa models.

Various concentrations of LPS have been utilised in studies with the intestinal epithelial cell lines, ranging from 0.1 ng/mL to 100 µg/mL, however physiologically relevant LPS concentrations are considered to reach a maximum level of 1 ng/mL (651–654). Application of 1 ng/mL LPS to Caco-2 monolayers cultured in the absence of immunocytes has been shown to increase the tight junction permeability, as assessed by TEER measurements and inulin paracellular flux, without resulting in cell death (651). However, these results contradicted numerous other papers which have demonstrated that intestinal epithelial cell lines, including Caco-2, HT29-MTX and T84 cells, are in fact hyporesponsive to LPS, thus require higher concentrations to elicit significant effects (484,569,611,652,655). This is reminiscent of the normal *in vivo* origin of the Caco-2 cells where hyporesponsiveness to LPS is a desirable phenotype to allow the establishment of tolerance within the intestinal mucosa and to prevent chronic inflammation. Therefore, the concentrations of LPS utilised within this chapter are higher than physiologically relevant to provoke an immune response.

Culture of Caco-2 cells using traditional tissue culture plastic revealed that 10 µg/mL LPS treatment accelerated cell proliferation via induction and activation of c-SRC (cellular proto-oncogene tyrosine-protein kinase) and ERK (extracellular-signal-related kinase) (656). Although the proliferative capacity of Caco-2 or HDFn cells was not assessed within this study, no notable differences in the degree of cellular confluence were found by eye when 2D cells were incubated with repeated doses of LPS compared to control cells. Conversely, another study found that application of conditioned media from M1 THP-1 macrophages but not from M2 THP-1 macrophages inhibited the proliferation of both Caco-2 and HT29 cell lines (657). Future analysis could therefore quantify the number of Ki67 expressing Caco-2 cells within the Transwell® and Alvetex®-based models to determine if the enhanced proliferative effect is replicated when cells are maintained under more *in vivo*-like conditions.

Results presented within this chapter revealed that the gene expression of proinflammatory cytokines could be enhanced following repeated 1 µg/mL LPS doses in 2D cultured Caco-2 cells, however the relative fold changes remained low. Although the immune responsiveness of the Caco-2 monolayer or Caco-2 U937 co-culture to LPS was not assessed within the present study, it is hypothesised that the presence of U937 macrophages would enhance the immune response induced in the epithelium. Interestingly, co-culture of Caco-2 cells and PBMC macrophages has proven an enhanced production of TNF-α and IL-1β mRNA in response to LPS compared to Caco-2 monolayers alone (484). A similar increase in IL-8 was identified when HT29-MTX cells were co-cultured with PBMC cells, highlighting the importance of including an immunocyte cell line to replicate more physiological responses to proinflammatory stimuli (569).

One of the primary effects of LPS treatment on the *in vitro* models demonstrated within Sections 6.4.2 and 6.4.3 was a reduced epithelial barrier integrity; this effect was most noticeable following repeated doses of LPS to the immune-competent triple co-culture. LPS treatment of Caco-2 U937 co-cultures resulted in a reduction in TEER by almost 30 % with a simultaneous minor increase in the permeability of FD20 (Figure 6.13). Comparable results were also found in a microfluidic co-culture of Caco-2 and U937 cells which showed a decreased TEER and increased permeability of FD4 following a 24-hour incubation period with 1 µg/mL LPS (300). Furthermore, the Transwell®-based co-culture presented within demonstrated that LPS treatment

induced a discontinuous, dotted, filamentous structure of tight junction proteins, rather than the intact filaments as observed in controls. This discontinuous phenotype observed in tight junction proteins is in agreement with other studies conducted by Ramadan and Jing and Leonard et al., (300,485). Interestingly, this altered protein structure was not observed in the expression profile of E-cadherin suggesting that the adherens junctions are not as prone to alterations induced by LPS as tight junctions. This could explain why the permeability of lucifer yellow was not altered in stimulated Caco-2 U937 co-cultures as adherens junctional components are required to recruit further tight junctional proteins and stabilise the overall structure of the apical junctional complex; intact adherens junctions would suggest that the epithelial barrier remains partially intact (658).

LPS consistently induces a reduction in the epithelial integrity of Caco-2 monolayers when co-cultured with immune cells. A possible explanation for this has been related to the activation MLCK mediated by TLR4/MyD88 signalling pathways that exert detrimental effects on intestinal tight junctions by inducing occludin endocytosis (659,660). This could explain why the mRNA of occludin increased following LPS treatment in both 2D Caco-2 cells and triple co-cultures; a compensatory mechanism may be working to replace the occludin lost through MLCK induced occludin removal from the apical junctional complex. However, this requires further investigation to confirm the role of MLCK within the culture systems. Additional mechanisms have been proposed to explain the increased paracellular flux through epithelial barriers following proinflammatory stimulation. TNF- α is one such cytokine that been postulated as a mechanism for inducing epithelial barrier damage to *in vitro* models of the intestine and to IBD patients. It could be partially responsible for the increased epithelial permeability observed within this chapter as the gene expression of TNF- α was found to be increased following LPS treatment of the triple co-cultures and a significant increase in the overall TNF- α cytokine concentration was detected in the cell culture supernatant following repeated LPS exposure (298,560,622–624).

6.5.2.2. The use of IFN- γ to enhance the proinflammatory response and to exacerbate epithelial damage induced by LPS

IFN- γ is frequently utilised in combination with LPS to induce an M1 phenotype in anergic, M0 macrophages and has also been employed in *in vitro* macrophage cultures to enhance the immune response to LPS. Due to the hyporesponsive nature of intestinal epithelial cells to LPS, IFN- γ can be used to further exacerbate LPS-induced epithelial damage *in vitro*. IFN- γ alone induced minimal changes in the integrity of the epithelial barrier of the immune-competent Caco-2 U937 co-culture and the 3D triple co-culture. Interestingly, IFN- γ treatment alone has been demonstrated to increase the paracellular permeability of Caco-2 monolayers to compounds of high molecular weight due to changes associated with occludin and claudin-1 expression, thus further studies could focus on determining the overall expression levels of both occludin and claudin-1 proteins in the models in response to IFN- γ (661). It is thought that the selective increase in the paracellular flux of larger molecules is part of an early inflammatory response that facilitates the passage of larger antigenic molecules without affecting the overall integrity of the epithelial barrier to small molecules (661).

This phenotype was also observed when triple co-cultures were stimulated with three doses of LPS; the paracellular flux of the large FD4 and FD20 molecules increased while no change was observed in the permeability of the smaller lucifer yellow compound (Figure 6.30). Overall, the results presented in this chapter did not reveal a significant role of IFN- γ treatment alone in inducing epithelial damage and IFN- γ also did not amplify the gene expression of inflammatory cytokines within the triple co-culture, thus Section 6.4.5.1 focused on utilising IFN- γ as a priming agent prior to LPS stimulation.

Caco-2 cells are thought to be hyporesponsive to LPS due to a diminished expression of the LPS receptor, TLR4, requiring a concentration of over 1 $\mu\text{g/mL}$ LPS to successfully induce an increase in the production of IL-8 cytokines (611). On the other hand, HT29 cells contain large quantities of TLR4 but it is present within the cytoplasm of the cell rather than being located at the plasma membrane. Priming of HT29 cells with IFN- γ prior to LPS treatment has been shown to increase the responsiveness of the cell line to LPS, augmenting its uptake and increasing the production of IL-8, while no enhanced response to LPS was identified in the Caco-2 cell line (611,662). However, the cultures developed within Chapter 5 also contain immunocytes which are able to respond to LPS and initiate an immune response which could then affect the structure and function of the epithelium. It would also be interesting to add the U937 macrophages to the HT29-MTX HDFn co-culture and to the Caco-2, HT29-MTX, HDFn co-culture to determine if the epithelial barrier is further diminished with IFN- γ pre-exposure.

Assessment of the epithelial apical junctional complex revealed severe disruption following stimulation of the Transwell® co-culture with IFN- γ and LPS, with areas of discontinuous tight and adherens junction staining across the epithelial monolayer. However, this apparent damage was not reflected in the paracellular flux which remained unaltered from control co-cultures. Regarding the triple co-culture, IFN- γ and LPS induced structural damage to the epithelium and reduced TEER but similarly did not induce major changes in the permeability of the models though increased trends with stimulation were observed. One possible explanation for the structural alterations and the reduction in TEER of triple co-cultures without a concurrent increase in paracellular flux could be due to the fluorescent dyes being sequestered within the lamina propria compartment and not entering the saline solution at the basolateral side of the Alvetex® Scaffold membranes. Although this was not confirmed by immunofluorescent staining, it is likely that any retained fluorescent dye would have been subject to bleaching during the processing of the samples and subsequent immunofluorescent staining techniques.

Though the present study identified no significant alterations to paracellular flux in response to IFN- γ and LPS, numerous other studies have demonstrated an increase in permeability. A study published by Kämpfer et al., (2017) developed an immune-competent intestinal model using Caco-2 cells indirectly co-cultured with differentiated THP-1 macrophages using the Transwell® cell culture platform (301). To stimulate their model and mimic the inflamed intestine, IFN- γ was introduced to the apical compartment where the Caco-2 monolayer was located to prime the cells while THP-1 cells in the basolateral compartment were pre-stimulated with LPS and IFN- γ . Other stimulatory conditions, including LPS treatment alone, did not induce a strong immune response. A temporary reduction in epithelial barrier integrity was identified along with the

production of proinflammatory cytokines following these conditions. Similar to the results presented within this chapter, the network of tight junctions was disrupted following IFN- γ priming and nuclear fragmentation indicative of cell death was observed, though the cell death in the Caco-2 THP-1 co-culture was evident in the epithelium (301).

The role of the fibroblasts and ECM in potentiating the immune response and epithelial damage to IFN- γ and LPS was not fully investigated within the context of this study. However, priming with IFN- γ before LPS treatment induced an increase in the gene expression of TGF- β 1 which could have a significant impact on the production of ECM by the fibroblasts, eventually resulting in fibrosis, a phenotype associated with chronic IBD. Additionally, other studies have found IFN- γ to be more effective at eliciting its biological effects on intestinal epithelial cells when bound to ECM proteins rather than in its soluble form. *In vitro* experiments utilising hyporesponsive, primary intestinal epithelial cells isolated from porcine tissue revealed that ECM-bound IFN- γ significantly increased IL-8 production in response to LPS over soluble IFN- γ in cells isolated from the crypt due to enhanced expression of the LPS receptor, TLR4 (663). Interestingly intestinal epithelial cells isolated from the villi did not modify their responses to LPS when treated with either soluble or membrane bound IFN- γ (663). This study highlights the importance of incorporating fibroblasts and ECM components in *in vitro* models of the intestinal mucosa to fully understand inflammatory processes and gain further insights into the mechanisms underpinning IBD pathologies. Further studies could therefore focus on the response of the fibroblasts and the ECM turnover within the 3D triple co-culture, to proinflammatory stimuli.

Overall, the results presented in Section 6.4.5.1. did not reveal many significant alterations to the function of the epithelial barrier, however IFN- γ priming did induce modifications to the gene expression of various cytokines. TNF- α and IL-1 β were both enhanced following IFN- γ then LPS treatment and the fold-changes were potentially great enough to induce biological effects. Additionally, an increase in the mRNA levels of IL-8 were found similar to the studies presented above that typically utilised IL-8 to determine whether IFN- γ significantly enhanced the immune response (301,611,662,663). The gene expression of the anti-inflammatory IL-10 cytokine was not found to be significantly altered in mRNA lysates of whole 3D tissue constructs, however IFN- γ has been shown to increase the IL-10 receptor on T84 intestinal epithelial cells enhancing their response to IL-10 signalling and initiating epithelial repair processes (664). This suggests that in addition to provoking inflammation, IFN- γ also has roles in maintaining epithelial homeostasis and can have immunosuppressive roles, it would therefore be interesting to establish the levels of the IL-10 receptor not only on Caco-2 cells but also on the fibroblasts and U937 macrophages.

6.5.2.3. The synergistic effects of IFN- γ and TNF- α

As discussed previously TNF- α has been postulated to be partially responsible for epithelial damage during inflammation and has a major role in the pathogenesis of IBD. Anti-TNF- α antibodies are a widely used successful therapy to treat some cases of IBD (189,190). TNF- α induced damage is thought to be due to heightened NF- κ B signalling within the epithelium resulting in an altered localisation and overall downregulation of ZO-1 tight junction proteins that are responsible for maintaining the stability of tight

junctions. Inhibiting NF- κ B signalling within Caco-2 monolayers prevented ZO-1 modifications and maintained the tight junction permeability (622). Additional studies of Caco-2 monolayers treated with TNF- α have also revealed that paracellular flux is increased due to reduced expression of occludin (560). MLCK expression and phosphorylation is also correlated with TNF- α induced epithelial damage as is activation of the MAP kinase ERK1/2 pathway that activates MLCK indicating the role of other signalling pathways in driving damage caused by TNF- α (297,298). Pilot studies conducted in this chapter regarding the treatment of the immune-competent triple co-cultures with TNF- α alone did not result in structural or functional changes to the epithelium, yet the macrophage distribution was altered at higher TNF- α concentration. Therefore, alternative methods were considered to augment the response to TNF- α .

IFN- γ and TNF- α are both heightened in the intestinal mucosa of IBD patients, particularly those with CD (665). Not only do they have roles in IBD, synergism between these two proinflammatory cytokines has been proposed to enhance the pathogenesis of other inflammatory diseases including psoriasis and atherogenesis (666). Similar to TNF- α alone, adjunctive application of IFN- γ and TNF- α to Caco-2 monolayers causes an increase in MLCK expression and MLC phosphorylation resulting in cytoskeletal rearrangement through increased contractility of actomyosin (27). IFN- γ has also been demonstrated to increase the expression of the TNF- α receptor to enhance the responsiveness of intestinal epithelial cells to TNF- α , indicating a possible role for priming with IFN- γ (582). During the pilot study using both IFN- γ and TNF- α as proinflammatory stimuli within the triple co-culture, co-stimulation of models with IFN- γ and TNF- α was found to increase epithelial permeability more than TNF- α alone. The greatest increase was detected following IFN- γ pre-exposure in agreement with the aforementioned studies. Furthermore, structural aberrations in the Caco-2 monolayer were observed; the epithelium exhibited an extremely flattened morphology when both compounds were applied to the model. Although no evidence of Caco-2 cell shedding was found when triple co-cultures were stimulated with either TNF- α alone or with IFN- γ and TNF- α , a possible mechanism involving an enhanced rate of epithelial cell shedding induced by excessive TNF- α stimulation resulting in damage to the epithelial barrier has been proposed. Amplified shedding would prevent the reorganisation of tight and adherens junctions at a fast enough rate to repair the gap left after cells are shed, resulting in areas where the epithelium is not fully intact (667). Interestingly, epithelial shedding was observed when triple co-cultures were primed with IFN- γ prior to LPS treatment, thus it would be useful to quantify the secreted levels of TNF- α present within the cell culture media of these models.

Further investigations could focus more on the mechanism behind the changes to the epithelial barrier induced by IFN- γ and TNF- α , in addition to assessment of the proinflammatory cytokine response. Although the expression of tight junction proteins was not examined, previous experiments using HT29 monolayers have highlighted that IFN- γ and TNF- α synergistically downregulated the expression of occludin that subsequently resulted in a reduction in TEER (668). Intestinal epithelial cells are not the only cells that are affected by synergism between IFN- γ and TNF- α . Macrophages are strongly induced by both IFN- γ and TNF- α which are primarily produced by other immune cells, leading to the production of proinflammatory cytokines and chemokines including RANTES (CCL5), MIG (CXCL9), IP-10 (CXCL10), IL-12 and IL-15 (669,670).

It would be valuable to determine whether these secreted proteins were enhanced in the triple co-culture following co-stimulation and IFN- γ priming.

6.5.2.4. The use of alternative effector molecules to induce an immune response in immune-competent models of the intestinal mucosa

Although not investigated within the present study, application of the proinflammatory cytokine IL-1 β to Caco-2 monolayers induced a greater inflammatory response over IFN- γ , TNF- α and LPS as assessed by the production of interleukins 6 and 8 in addition to the production of nitric oxide (559). Furthermore IL-1 β was found to enhance the production of these cytokines when given adjunctively with the other proinflammatory stimuli (559). In addition to Caco-2 monocultures, IL-1 β has also been employed to stimulate intestinal co-cultures. For instance, a co-culture model utilising Caco-2 cells seeded onto collagen gels embedded with THP-1 macrophages and MUTZ-3 dendritic cells revealed that the inflammatory response elicited by IL-1 β in co-cultures was higher than that of monocultures with increased production of IL-8 (531). When applied apically to the co-culture, IL-1 β induced a redistribution of the immunocytes, with their presence being detected between adjacent epithelial cells (531). An alternative co-culture model with Caco-2 cells seeded on the surface of a collagen gel containing macrophages and dendritic cells isolated from periphery blood monocytes also identified that IL-1 β enhanced inflammation as assessed by IL-8 production, more strongly in co-culture rather than monoculture (485). IL-1 β also induced reorganisation of tight junction proteins including ZO-1 and reduced TEER across cultures (485,486). This model was subsequently used to assess different methods of budesonide encapsulation in nanoparticles to inhibit the proinflammatory response induced by IL-1 β and provide insight into appropriate nanoparticle delivery mechanisms of anti-inflammatory drugs (486).

Other co-cultures of the intestinal mucosa incorporating Caco-2 enterocytes, U937 monocytes and fibroblasts have been utilised to assess the immune responses induced by dietary components, namely wheat proteins. It should be noted that the U937 cells within this model were incorporated as undifferentiated monocytes. Higher gluten content was found to reduce Caco-2 proliferation and viability in addition to disrupting tight junctions (310). Furthermore, gluten stimulated the secretion of interleukin-8 resulting in enhanced U937 migration and macrophage differentiation (310). Additional studies by the group utilising the same 3D *in vitro* model and the cell lines individually also assessed inflammation induced by polyphenol food supplements and discovered that toxic effects were induced by polyphenols (311). Combined, the papers discussed in Section 6.5.2.3. highlight the importance of incorporating primary immune cells or cell lines of immunocyte origin within *in vitro* intestinal models to better recapitulate inflammatory processes and reveal the full extent of damage induced within epithelial monolayers following incubation with a proinflammatory stimulus.

6.5.3. Comparison of the Immune Response Induced by Repeated LPS Stimulation to IBD Patients

The concentration of cytokines secreted into the cell culture media by immune-competent 3D triple co-cultures that were either control models or models stimulated three times with 1 µg/mL LPS were quantified via an ALBIA multiplex cytokine assay. The expression of proinflammatory cytokines, chemokines and growth factors generally increased in the *in vitro* model, the exact concentrations for each cytokine assayed for is presented in Appendix 3. The following discussion will focus on comparing some of the cytokine levels present within the models to serum levels in control and IBD patients to determine if any aspects of IBD have successfully been mimicked *in vitro*.

Proinflammatory cytokines can be utilised as biomarkers to predict response to IBD therapeutic treatments (IL-1β, IL-6, IL-8, IL-9, IL-22, IFN-γ, TNF-α and MCP-1 also known as CCL2) and can indicate when patients have suffered a relapse of the disease (IL-2 and IL-6) (671–679). Furthermore, proinflammatory cytokines, namely IL-1β, IL-6 and TNF-α are responsible for inducing the most widely utilised serum biomarker of inflammation in IBD – C-reactive protein (CRP) to be released from hepatocytes during acute inflammation (671,680). CD levels of CRP are higher than that of UC, but its presence cannot be utilised to distinguish between the two diseases (671,680–682). CRP is present in the serum of healthy individuals at a concentration of less than 1 mg/mL but can increase 1000-fold following induction by cytokines during inflammation and a concentration of less than 10 mg/mL is indicative of IBD remission (671,683–685). Within the laboratory, a 3D model consisting of HepG2 liver hepatocytes has been developed, future experiments could therefore focus on determining whether conditioned media collected from stimulated U937 macrophages and from the *in vitro* models could elicit an increase in the levels of CRP within the liver model.

Numerous inflammatory cytokines were not detected at high levels within the control or stimulated *in vitro* models including IL-2, IL-3, IL-4, IL-5, and IL-13, some of which exhibit alterations in concentration between healthy controls and IBD patients, though the concentration differences are not always significant. IL-2 is primarily produced by activated T lymphocytes and increases the production of IFN-γ and TNF-α in other immune cells (686). Regarding paediatric patients presenting with IBD, the serum levels of IL-2 did not alter much from control sera (1.41 pg/mL), CD patients exhibited slightly increased levels of 1.79 pg/mL while the serum isolated from UC patients had lower levels of IL-2 at 1.02 pg/mL (687). Although in adult patients IL-2 levels were higher overall, they displayed a similar trend between healthy (46.7 pg/mL) and diseased states with slightly higher levels expressed in CD patients (53.4 pg/mL) and a minor decrease in UC patients (45.2 pg/mL) (688). 0.21 and 0.26 pg/mL IL-2 was detected in control and stimulated *in vitro* models respectively, the concentrations detected were closer to paediatric patients than to adults, however no T cells were included within the culture to act as a primary source of IL-2. IL-3 and IL-5, also produced primarily by T cells, were not detected at concentrations over 0.01pg/mL within the *in vitro* models, nor have they been demonstrated to play major roles in IBD. IL-4 can be used to induce an M2 phenotype in *in vitro* macrophages and it did not alter significantly in *in vitro* models or in IBD (689); approximately 2.5 pg/mL IL-4 was produced by tissue cultures while paediatric IBD patients have up to 1.86 pg/mL IL-4 in their serum compared to 0.87

pg/mL identified in controls. Adult IBD patients, like with IL-2, exhibited higher concentrations of IL-4 overall but only have a minor increase in overall cytokine levels compared to controls (8.2 pg/mL and 7.8 pg/mL, respectively) (687,688). IL-13 was present in the media collected from unstimulated models at 22.68 pg/mL and at 25.01 pg/mL in stimulated models. IL-4 and IL-13 are closely related and are thought to be functionally redundant with each other, interestingly, while IL-4 increased in adult IBD patients, IL-13 has been demonstrated to decrease (543,688). This is especially unusual as IL-4 and IL-13 have significant roles in propagating the Th2 immune response which UC was originally considered be.

IL-15 is overexpressed in the inflammatory mucosa of IBD patients and although the overall levels of IL-15 secreted by the triple co-culture were less than that of IBD serum, a similar trend was observed, suggesting that the model may be of use in mimicking the role of IL-15 in IBD. Control models produced 5.81 pg/mL IL-15 which almost doubled following repeated LPS stimulations. IL-15 is present in serum from healthy individuals at 28.3 pg/mL which increased to 38.4 pg/mL and 30.6 pg/mL in CD and UC respectively (688).

The IL-17 family of proteins exhibited elevated levels following repeated LPS stimulation of the *in vitro* 3D triple co-culture. IL-17 cytokines are typically produced by Th17 cells which are themselves amplified in patients with IBD (207–210). However, IL-17 and in particular IL-17E/IL-25 can also be produced by other cell lineages including epithelial and macrophages cells; this is further supported by the data presented within where IL17-E is present within the media of stimulated models at 13.55 pg/mL, while the other IL-17 family members do not exceed concentrations of 3 pg/mL (690). IL-17 exerts its effects on myeloid cell lineages and stromal cells inducing them to produce additional proinflammatory cytokines, exacerbating inflammation (210). Paediatric patients with CD have serum IL-17 levels of 26.89 pg/mL while UC patients had a concentration of 30.05 pg/mL, similar levels to the concentration of IL-17 in the stimulated models (687). However, adult patients have been found to express IL-17 at 389.1 pg/mL and 354.0 pg/mL for CD and UC respectively (688).

Some of the most commonly assessed cytokines in IBD are: IFN- γ , TNF- α , IL-1 β , IL-6, IL-8, IL-10. Both IL-6 and IL-8 were produced at extremely high levels within the models with over 3000 pg/mL present in unstimulated models, thus no significant alterations were detected following stimulation though an increase in concentration was detected. Serum levels of IL-6 in healthy controls were found to be 14.4 pg/mL and similar levels were found in UC, however an increase was detected in CD. In a separate study 1.59 pg/mL was detected in serum from healthy subjects and a similar increase to over 8 pg/mL was found in both CD and UC patients (688,691). Interestingly in organ biopsies, the levels of IL-6 were more similar to levels of the *in vitro* model: biopsies from healthy individuals contained 116.4 pg/mg IL-6 which increased drastically to 1776 pg/mg and 2212 pg/mg in CD and UC respectively (692). This indicates that the levels of cytokines produced by the tissue construct may be more representative of those found within the intestinal mucosa itself rather than in the serum. IL-8 or CXCL8 is a chemoattractant produced by immune cells to stimulate the migration of further immune cells. Differential trends in the serum levels of IL-8 have been identified; Martinez-Fierro found that IL-8 decreased in CD and increased in UC compared to healthy individuals while separate studies

by Szkaradkiewicz and Tatsuki individually demonstrated that both diseased states had an increase in IL-8 compared to controls (687,688,691).

IL-1 β secretion significantly increased following repeated LPS stimulations of the *in vitro* tissue culture constructs from 2.79 pg/mL to 69.71 pg/mL. The level of IL-1 β in untreated models was very similar to control subjects in a study carried out by Martinez-Fierro (3.4 pg/mL) who also demonstrated no significant alterations in expression in either CD or UC (688). Furthermore, no differences were found in paediatric patients from healthy controls. However, in a separate study analysing the cytokine levels within serum detected over 100 pg/mL IL-1 β in controls with an increase to over 300 pg/mL in adult IBD patients, yet no distinction between CD and UC was made in this study (693). Similar levels of IL-1 β were detected in mucosal biopsies from patients with less than 10 pg/mg detected in controls and 109.9 pg/mg and 76.06 pg/mg detected in biopsies from CD and UC patients respectively (692). Again, this study highlights that the *in vitro* models display similar trends and concentrations to mucosal tissues rather than serum levels of cytokines.

Both IFN- γ and TNF- α were amplified following stimulation with LPS in the *in vitro* models, and both have been shown to increase in IBD serum compared to controls of adult patients. Conversely, paediatric patients presenting with IBD exhibited lower IFN- γ levels than healthy individuals suggesting that the disease pathology may alter throughout life (687). Different studies have demonstrated different overall concentrations for the two cytokines with IFN- γ being found at approximately 100 pg/mL in IBD in one study while others have found it to be present at levels just under 1000 pg/mL; similarly, TNF- α has been identified at 100 pg/mL in one study, at over 3000 pg/mL in another and at only 3.2 pg/mL in a third study (688,691,693). The final study more closely resembles the levels of TNF- α identified in the *in vitro* models presented within this chapter (691). All studies discussed utilised different types of multiplex immunoassays to quantify serum levels of cytokines, yet all yielded very different concentrations of cytokines, highlighting the need for greater consistency between analytical techniques that have a similar sensitivity to each other.

Although not significant, IL-10 was found to increase following repeated exposure of models to LPS from 25.06 to 43.17 pg/mL. Serum analysis of patients with IBD identified that CD patients retained a similar level of IL-10 expression to unaffected controls, while patients with UC demonstrated heightened levels (688,691). Large discrepancies in overall levels were once again present between the two studies; with over 700 pg/mL IL-10 present in the study carried out by Martinez-Fierro and only 4.4 pg/mL identified in Szkaradkiewicz' study. This further emphasises the need for greater consistency between studies though overall trends in cytokine levels were similar.

In addition to assessing cytokines, the expression levels of chemokines have also been assessed by various studies that have not only assessed cytokine levels in serum but have also quantified mRNA transcript levels from mucosal biopsies. Interestingly MCP-1 and IP-10 RNA transcript levels decreased in areas of inflamed mucosa in both IBD diseased states compared to non-inflamed mucosal areas within the same patients (665). Contrastingly MIG increased in the inflamed intestine of UC patients but decreased in CD (665). However, RNA transcript levels do not directly correlate with protein levels as has been demonstrated within this

chapter when comparing qPCR data of triple co-cultures stimulated repeatedly with LPS to overall secreted cytokine levels. The results presented here demonstrated a significant increase in the levels of all chemokines assayed for, the only exceptions were IL-8 which exhibited high baseline expression as discussed previously and MCP-1 which was present in the culture media at levels beyond the sensitivity of the assay.

Table 6.1. lists the concentrations of chemokines found within the serum of patients identified from the literature. Two studies primarily were utilised: Martinez-Fierro (688) who analysed the serum of adult patients, and Tatsuki (687) who assessed serum collected from paediatric patients. As with the *in vitro* models, all chemokines examined in the literature displayed elevated levels in IBD compared to controls with the exception of MCP-1 which also has reduced gene expression in inflamed tissue (665,687,688). RANTES secreted by the stimulated models exhibited the most *in vivo* like levels with over 1000 pg/mL detected within the collected conditioned media and serum. Furthermore, mucosal biopsies taken from IBD patients contained significantly enhanced RANTES at concentrations of approximately 3000 pg/mg in both UC and CD compared to control biopsies which contained less than 2000 pg/mg (694). Both MCP-1 and RANTES are produced by both macrophages and epithelial cells, explaining why such high levels were present within the models.

Table 6.1. Comparison of chemokine levels secreted by control and models stimulated repeatedly with LPS to *in vivo* serum concentrations. Serum concentrations were found within the literature as determined by immunoassays. OOR indicates when values were out of range of the assay sensitivity.

Chemokine	In Vitro Models (pg/mL)		In Vivo Serum Levels (pg/mL)			Reference
	Unstimulated	Stimulated	Control	UC	CD	
MCP-1	> OOR	> OOR	84.5	90.2	115.5	(688)
			5.04	3.06	2.72	(687)
MIP-1 α	10.21	1092.19	13.1	12.4	9.72	(688)
			1.96	2.01	4.26	(687)
MIP-1 β	9.25	573.14	250.4	258.1	181.0	(688)
			25.34	33.31	40.65	(687)
Eotaxin	3.03	3.83	137.0	166.5	182.0	(688)
			10.84	26.33	29.32	(687)
IP-10	79.10	17158.47	783.5	1016.9	759.6	(688)
			148.96	205.68	380.67	(687)
RANTES	482.20	1376.88	14849.3	16639.3	18574.0	(688)

Finally, the literature was analysed for concentrations of growth factors present within IBD patients; levels of G-CSF, GM-CSF, VEGF and FGF2 could be ascertained. Three of the four growth factors demonstrated significantly amplified levels in *in vitro* models following repeated LPS stimulations, FGF2 on the other hand decreased. FGF2 is normally upregulated in IBD as it promotes fibrostenosis during chronic inflammation –

excessive deposition of ECM that can result in constriction of the intestinal lumen (95,537). Adult IBD patients' serum contains FGF2 at concentrations close to 100 pg/mL while childhood IBD has lower levels of FGF2 by a factor of 10 (687,688). Interestingly, although levels of FGF2 decreased following model stimulation, the levels remained higher than in patients with IBD with over 200 pg/mL being identified within the media indicating that the model may still possess characteristics of fibrosis if maintained for longer periods. G-CSF was present within serum samples at higher concentrations than GM-CSF (with maximal concentrations in IBD serum being of 172 pg/mL to 40 pg/mL respectively), a trait which was also identified within the models (~3000 pg/mL G-CSF and ~500 pg/mL GM-CSF in stimulated models), suggesting that the ratio of the two cytokines is physiologically relevant to the diseased states (688). Lastly VEGF is present at higher concentrations in IBD serum compared to control serum, a trend also observed in control and stimulated models, however the overall levels of VEGF present within the culture media collected from models was over 40X higher than *in vivo* serum levels (687,688).

Overall, levels of cytokines present in IBD has been shown to vary drastically from study to study and large differences were present in adult and paediatric patients, however, overall trends were often similar. Most trends were recapitulated within the immune-competent 3D co-culture upon stimulation, though exact concentrations could be vastly different depending on the cytokine examined.

6.6. Conclusions

This chapter aimed to establish an immune response within the *in vitro* co-culture models developed in Chapter 5. Application of three effector molecules were provided basolaterally to the models, unless otherwise stated, to mimic the location of proinflammatory stimuli and cytokines within the intestinal mucosa. Following stimulation, the structure and function of the epithelial monolayer was assessed in addition to characterising the gene expression levels of pro- and anti-inflammatory cytokines.

Prior to utilising *in vitro* models, IBD intestinal tissue samples were assessed by histological and immunofluorescent staining techniques. Infiltration of immune cells into the lamina propria of the tissues was widespread in both CD and UC, indicative of inflammation. Further differences in the expression of components of the apical junctional complex and of ECM proteins were highlighted.

Initial experiments focused on stimulating *in vitro* models with varying concentrations of LPS for 24 hours before concentrating on repeated stimulation with LPS in an attempt to mimic chronic inflammation more representative of the inflamed IBD mucosa. Epithelial damage was evident following stimulation as alterations to the structure of the epithelium were observed in addition to increased paracellular permeability – a predominant manifestation of IBD pathology. Furthermore, the 3D triple co-culture was demonstrated to have a strong proinflammatory response to repeated LPS stimulation with overall trends in cytokine expression comparable to that of patients with IBD.

Supplementary to LPS stimulation, IFN- γ alone or in synergy with LPS and TNF- α was assessed. When applied to models alone, IFN- γ did not elicit significant effects on the epithelial barrier nor did it induce a strong inflammatory response. However, when models were treated with IFN- γ and an additional effector molecule, greater damage to the epithelial barrier was evident. The overall morphology of the epithelium was altered, and cells did not display the homogenous, elongated columnar phenotype typical of Caco-2 cells in the co-culture model. Furthermore, some alterations in TEER and the paracellular permeability were observed. Although the expression of proinflammatory cytokines was elevated when IFN- γ was employed as a priming agent prior to LPS stimulation, the concentrations of cytokines secreted into the cell culture media was not examined. Additionally, the inflammatory response induced by IFN- γ and TNF- α was not assessed, thus, future experiments would focus on fully characterising the immune response generated by these compounds within the triple co-cultures.

Overall, this chapter has successfully induced an inflammatory response within the immune-competent *in vitro* models concurrent with significant defects to epithelial barrier integrity. Taken together this suggests that the models have the potential to mimic aspects of the pathology of CD and UC and could be utilised to investigate the protective effects of novel chemical compounds.

Chapter 7: General Discussion and Future Directions

7.1. Summary of Key Findings

This project aimed to develop novel *in vitro* models of the intestinal mucosa using standardised cell lines and 3D cell culture techniques, which would be suitable alternatives to current models for use in investigations regarding intestinal transport and inflammation. Preliminary work focused on optimising a simplistic co-culture of the gold standard Caco-2 intestinal epithelial cell line and fibroblasts, to recreate the intestinal epithelium and supporting lamina propria respectively. Further work then enhanced the complexity of the initial co-culture system through the incorporation of different cell lines to recreate the mucus-secreting goblet cells and a constituent of the immune system. The final immune-competent model was then utilised in investigations regarding the generation of an inflammatory response. The key experimental findings and outputs at each stage of model development are outlined below:

- Fibroblasts cultured within Alvetex® Scaffold membranes secrete endogenous ECM proteins that support an overlying epithelium of Caco-2 enterocytes.
- Co-culture of Caco-2 cells with fibroblasts alters the structure and function of the epithelial monolayer to provide a more *in vivo*-like tissue culture platform for further investigations into epithelial barrier function.
- Mucus-producing HT29-MTX cells could be incorporated into the epithelium at a physiologically relevant ratio to represent goblet cells, thereby enhancing the complexity of the overall model.
- Macrophages differentiated from U937 monocytes can be incorporated to the lamina propria component and the resulting lamina propria retained the ability to support the epithelium.
- Addition of U937 macrophages alone, did not compromise the overall structure and function of the *in vitro* model.
- An immune response can be initiated in the immune-competent *in vitro* model by the addition of proinflammatory effector molecules that result in damage to the epithelium.
- The immune-competent model has the potential to mimic aspects of the inflamed mucosa during inflammatory bowel diseases – Crohn's disease and ulcerative colitis.

7.2. Recreating the Structure and Function of the Intestinal Mucosa *In Vitro*

The intestinal epithelium functions as the principal site of nutrient and drug absorption in the body, in addition to providing a barrier to the external environment. Accordingly, *in vitro* models of the intestine have largely focused on the structure and function of the epithelium. However, the crucial role of stromal cells in maintaining homeostasis and providing physical support to the epithelium, in part through the production of ECM, cannot be dismissed. Therefore, initial model development concentrated on incorporating a stromal cell lineage into a co-culture with the gold standard intestinal epithelial cell line.

7.2.1. Bioengineering the Intestinal Microenvironment *In Vitro*

Existing models that incorporate a fibroblast lineage often embed the cells within a hydrogel scaffold, comprising of ECM constituents, alternatively, decellularised scaffolds from intestinal tissues can be utilised to culture fibroblasts and provide the unique crypt-villi architecture native to the intestine (314,319,325,326,347,348). However, these methods have numerous limitations including the animal origin of both tissue segments and hydrogel components, and the lack of evidence of *de novo* ECM synthesis by the stromal cells. Furthermore, the ECM present in hydrogels does not accurately recapitulate the *in vivo* microenvironment; hydrogels typically do not include the full complement of ECM proteins such as elastin and fibronectin. Fibronectin is of particular interest as it has roles not only in wound healing, but also in maintaining epithelial attachment and integrity along with having regulatory roles in epithelial differentiation and polarisation (385–387). On the other hand, the techniques utilised to render tissue segments acellular can disrupt the ECM composition due to their harsh nature, resulting in variability between models (327,328). Unlike the aforementioned techniques used to create a subepithelial lamina propria compartment, the model presented in Chapter 3 exhibited endogenous ECM secretion (including collagens and fibronectin) with indication of continual renewal as evidenced by the presence of active MMP proteins. The structure and function of the epithelium in the co-culture was demonstrated to be more like *in vivo* intestinal tissue than the Caco-2 monolayer, possibly due to the enhanced columnar organisation and straightened structure of the lateral membrane observed via TEM analysis. This altered morphology was not present when Caco-2 monolayers were cultured in fibroblast conditioned media, indicating that direct co-culture of fibroblasts and epithelial cells is crucial for establishing more physiologically relevant tissues (405).

Although enhanced structure and TEER values more similar to that of the *in vivo* intestine were observed in co-culture with fibroblasts, the cells were not of intestinal origin and were therefore a limiting factor in the physiological relevance of the model. The overarching role of fibroblasts is to maintain structural integrity of tissues irrespective of their location, however differential gene expression programmes have been identified based on fibroblast origin in a phenomenon termed global topographic differentiation (404). Intestinal fibroblasts have been found to have distinct populations with unique functions. Fibroblasts at the base of intestinal crypts express low levels of platelet-derived growth factor receptor A (PDGFRA) and secrete canonical Wnt and BMP inhibitor proteins that function to maintain intestinal stem cell proliferation, while fibroblasts at the top of the crypts exhibit high PDGFRA levels and secrete noncanonical Wnt proteins and

BMP ligands to encourage epithelial differentiation (695). Due to these disparities in fibroblast activity and origin, a structural comparison of the dermal fibroblast model to an alternative model developed using CCD-18co colonic fibroblasts was undertaken. Examination of the models by immunofluorescence and histological staining techniques did not reveal significant differences in the composition of the ECM and although the nuclei shape appeared to better resemble the *in vivo* intestine in the CCD-18co co-culture model, the epithelium was not as highly organised and elongated when compared to the HDFn lamina propria component. Furthermore, functional comparison of TEER and lucifer yellow paracellular flux did not reveal alterations to the epithelial barrier integrity as a result of the different fibroblasts (405). This, in combination with other published co-cultures using dermal fibroblasts and Caco-2 enterocytes, indicated that the skin-derived fibroblasts were suitable to use within the model (356,358). Gene expression analysis could be a focus of future experiments to highlight any disparities due to anatomical location. Comparison of the ability a wider array of intestinal-derived fibroblast lineages to form a model could also be undertaken. Potential fibroblasts include adult-derived H-InMyoFib cells or the foetal-derived Hs 1.Int fibroblasts. It would then be possible to determine if the cells induce differential effects within the epithelial monolayer. As these cells have been isolated from individuals of different age groups, it would also be of interest to determine if developmental signalling pathways are altered.

7.2.2. The Importance of Incorporating a Mucus-Producing Cell Lineage in Models of the Intestinal Mucosa

Goblet cells comprise the second largest cell population within the intestinal epithelium, and the mucus produced by these cells has been demonstrated to act as a barrier to absorption and pathogenic infection, as discussed in Chapter 4. Due to the importance of goblet cells in maintaining homeostasis and the overall barrier function of the epithelium, incorporation of mucus-producing cells into the epithelial-lamina propria co-culture was next investigated. Previous studies have demonstrated that adding characteristic goblet-like cells - HT29-MTX cells - into epithelial co-cultures at physiologically relevant levels (~10 % in the small intestine and ~24 % in the large intestine) resulted in an increase in paracellular permeability, particularly regarding the flux of hydrophilic molecules that are underrepresented by Caco-2 monolayers (274,455). Although only a minor increase in permeability was detected with the addition of goblet-like cells to the Caco-2 HDFn co-culture, structural differences could be identified, namely the presence of mucin granules within the cytoplasm of cells within the epithelium. However, evidence of a mucus layer lining the surface of the cells was not easily identified. It would therefore be interesting to determine the extent to which genes involved in the expression of adherent mucins (*MUC1* or *MUC3*) and secreted mucins (*MUC2* and *MUC5AC*) were expressed. To ascertain whether further functional changes were apparent after goblet-like cell inclusion, a set of transport experiments investigating the paracellular permeability of a set of compounds with different affinities for mucus could be conducted. It is hypothesised that hydrophilic compounds would display increased permeability; conversely, lipophilic drugs such as testosterone, would be expected to pass through the models more slowly as has previously been demonstrated (451).

The model has the potential to be utilised to investigate the efficacy and transport of drug formulations and delivery systems such as novel nanoparticles as has been done for epithelial co-cultures (696,697). Additionally, studies have demonstrated that epithelial co-cultures incorporating goblet-like cells can more accurately predict the oral bioavailability of dietary compounds such as iron and other, more toxic metal ions including mercury (698,699). Therefore, the tissue culture platform containing fibroblasts in addition to epithelial cells could be utilised to not only assess absorption, but also to investigate the cytotoxic effects of ingested dietary compounds on stromal cell lineages.

Studies utilising *in vitro* intestinal epithelial models with a mucus-producing component have also examined the role of mucus in bacterial colonisation and pathogenic infection. HT29-MTX monolayers were demonstrated to have higher adhesion of *Salmonella* strains to the models than the non-mucus producing Caco-2 and HT29-H monocultures (452). Furthermore, Caco-2 HT29-MTX co-cultures were found to promote greater adhesion of probiotic bacteria than Caco-2 monocultures (700). The role of mucus in preventing pathogenic infection and promoting commensal bacterial adhesion was not investigated in the context of this thesis. Future experiments could examine the effects of different bacterial strains or metabolites on the model permeability in addition to examining any alterations to the structure of the epithelium, as pathogenic bacterial strains are known to disrupt tight junctions within epithelial models (452,567). Additionally, the interaction between fibroblasts and invasive bacterial strains could be investigated using the immune-competent model developed in Chapter 5 to determine if and how the composition of the ECM is perturbed by the presence of bacteria.

7.2.3. Inclusion of Alternative Epithelial Lineages into the *In Vitro* Co-Culture

Although Caco-2 and HT29-MTX co-cultures are frequently used to represent the absorptive enterocytes and mucus-producing goblet cells of the intestinal epithelium respectively, the cells are of cancerous origin and have numerous differences in gene and protein expression and overall function to the native intestinal cells. To further improve the overall function and enhance the ability of the model to mimic the native human intestine, a further alternative epithelial cell population could be exploited. Lgr5⁺ intestinal stem cells are utilised in organoid cultures of the intestinal epithelium. These primary cells have the unique ability to differentiate into all epithelial cell lineages present within the intestine and have recently been cultured on the surface of decellularised scaffolds resulting in the formation of a more stable barrier than that of the enclosed organoids (352). Crypt segments have been derived from organoid cultures and successfully maintained on Transwell® membranes where they differentiated into all intestinal epithelial lineages, overcoming the enclosed geometry of the organoids and allowing access to the luminal side of the epithelium (316,701,702). Thus, such organoids have the potential to be included into the co-culture model with a lamina propria compartment to better recreate the intestinal microenvironment. This would allow all four intestinal epithelial lineages to be present within the model and would negate the need for cancerous cell lines, enhancing the relevance of the model to the human *in vivo* intestine.

7.3. Recapitulating Inflammatory Processes *In Vitro*

The intestinal mucosa is home to the largest population of tissue-resident macrophages within the entire body and, due to their ability to secrete a wide range of inflammatory cytokines and chemokines, have been implicated in the pathogenesis of IBD where they aggravate intestinal inflammation. Additionally, macrophages are widely used in co-culture models of the intestinal mucosa that incorporate an immune cell lineage. Based on the abovementioned points, Chapters 5 and 6 were focused on the development and application of an immune-competent intestinal model that incorporated macrophages.

7.3.1. Immune-Competent 3D Co-Cultures Exhibit Structural Similarities to *In Vivo* Tissue

Due to their ease of use and reproducibility, U937 monocytes that had been differentiated into cells more representative of macrophages, were selected for the purpose of the co-culture. Unlike THP-1 macrophages, no adverse effects were identified regarding the epithelial barrier structure or function when U937 macrophages were incorporated into models either indirectly at the base of a Caco-2 Transwell® monolayer, or directly when introduced to the fibroblast lamina propria compartment (512,526). This suggests that they are a more advantageous cell line to use to represent healthy intestinal mucosa than the THP-1 lineage. Structurally, models without the introduction of an inflammatory stimulus exhibited a well polarised epithelium like *in vivo* tissue (Figure 7.1). Regarding the distribution of immune cells, previous staining of healthy tissue identified that the majority of CD68⁺ macrophages were primarily located towards the base of the crypts away from the epithelial surface (Figures 6.3); a similar distribution was observed in control models where U937 macrophages are most prevalent at the base of the lamina propria at the opposite side to the epithelium.

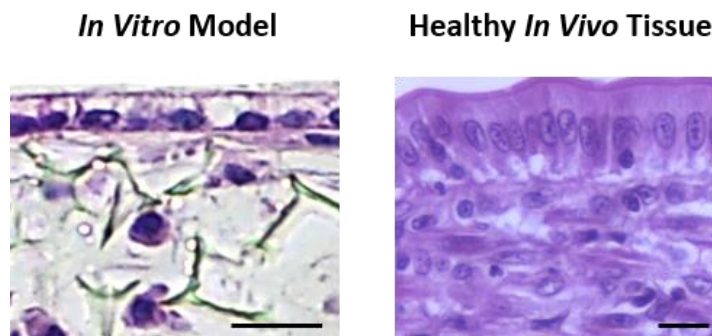


Figure 7.1. Histological analysis of the immune-competent 3D intestinal model reveals structural similarities to healthy intestinal tissue samples. Representative H&E images of the Caco-2, U937, HDFn co-culture model and human colonic tissue. Both epithelial monolayers exhibit a well polarised columnar morphology resting on an ECM-rich lamina propria. However, the overall architecture of the model and tissue is vastly different. Scale bars: 20 μ m.

However, several major differences to native intestinal tissue are apparent, primarily the overall architecture of the intestine which exhibits a distinctive crypt-villi axis in the small intestine and crypt-only anatomy within the large intestine. Various studies have focused on recreating the intestine architecture through the development of organoid cultures or through the use of moulded hydrogel scaffolds, yet both of these approaches have limitations (288,317,318,334,335,703). Organoid cultures are often expensive to maintain

and have highly specific culture requirements, while hydrogel-based scaffolds contract and degrade over time resulting in changes to the structure and physical properties of the epithelial cell culture platform. Conversely, Alvetex® cultures can be maintained for relatively long culture periods with minimal media requirements. Additionally, the membrane orientation allows for comparisons to be made to Transwell® cultures typically employed when modelling the intestine epithelium structure and function. As a polyHIPE, Alvetex® is manufactured via an oil and water emulsion cured in a cylindrical mould; studies utilising polyHIPEs have demonstrated that they can have tunable mechanical properties that can be adapted to best resemble the tissue of interest (704,705). It may therefore be possible that the mould used to create Alvetex® Scaffold could be adapted and tuned to recreate the crypt-villi intestinal architecture more accurately. It would then be interesting to recreate the immune-competent co-culture model and determine how distribution of fibroblasts and immune cells and structure of the epithelium is affected.

A further noticeable difference between the *in vitro* model and *in vivo* tissue is the presence of goblet cells. HT29-MTX cells were successfully incorporated into the epithelium of the Caco-2 HDFn co-culture model as discussed in Chapter 4; future experiments could therefore focus on determining whether the goblet-like cells could be incorporated into the immune-competent 3D culture. HT29 cells have been used in previous co-cultures with U937 macrophages. HT29 organoids were established on U937 macrophages maintained in collagen gels on microcarrier beads; the epithelium displayed apical-basolateral polarity and produced mucins while the macrophages retained their phagocytic ability in co-culture (534). This model has been employed to investigate the attachment of *Salmonella* bacteria to the epithelium and demonstrated that the presence of macrophages reduces bacterial adherence compared to HT29 monocultures (534). As HT29 cells have been successfully maintained in co-culture with U937 cells, it is hypothesised that the derivative HT29-MTX cell line would perform equally as well in co-culture and that their incorporation to the models presented in Chapter 5 would better recapitulate the intestinal mucosa. The model would then have the potential to be used in investigations regarding bacterial colonisation and the role bacteria play in instigating and propagating an immune response.

Additional limitations of the immune-competent triple co-culture include the cancerous origin of the immune cells. The nature of these cells often led to incomplete differentiation into macrophage-like cells and resulted in high numbers of U937 cells falling through the Alvetex® Scaffold membrane throughout the culture period, as such the numbers of cells present within the final model were not fully controllable and differences could be observed across different experimental occasions. Additionally, up to 15 % of these co-cultures failed to form an acceptable epithelium with a columnar epithelium and an appropriate TEER measurement. Further optimisation of the maintenance of these models may therefore be required before wider adoption of the model would be possible. Immune cells isolated from PBMCs may provide a useful alternative within the culture as they can also be derived from IBD patients and would therefore exhibit any abnormalities in gene expression, cellular function or protein activity and secretion observed *in vivo*. However, the ability of these cells to successfully form a co-culture model could be limited as they often have more specific media and nutrient requirements than cell lines.

7.3.2. Application of the Immune-Competent 3D Co-Culture to Mimic Aspects of IBD

Three different proinflammatory effector molecules were applied to the immune-competent co-cultures in an effort to induce an inflammatory response and recreate aspects of IBD *in vitro*. All three stimuli (LPS, IFN- γ and TNF- α) induce an M1 phenotype within macrophages which promotes a proinflammatory response within the cells (540,541). Structural alterations to the epithelium were identified after stimulation and can be observed in Figure 7.2; the epithelial monolayer presented with reduced apical-basolateral polarity as indicated by the flattened morphology of the cells. However, this phenotype is not observed in within the inflamed intestine where the epithelium remains well polarised with a distinctive columnar morphology suggesting that the structural changes *in vitro* are not equivalent to alterations *in vivo*. Although flattened epithelial morphology is not reported within other *in vitro* models mimicking the inflamed intestine, other aberrations to the epithelium are identified including discontinuous expression of tight junction proteins and altered expression of transporter proteins (300,320,485).

Further differences to *in vivo* tissue can be observed, the most striking of which is the density of immune cells (Figure 7.2). Within diseased intestinal tissue, mononuclear cells are visible throughout the lamina propria, and appear to heavily outnumber other cell lineages – these cells are part of the transmucosal inflammatory infiltrate associated with the inflamed intestine in IBD. In the *in vitro* model, macrophages within the lamina propria compartment are not present at such high densities. Surprisingly, higher initial seeding densities did not increase the end numbers of macrophages present in the final model, this may be due to possible migration of immune cells out of the lamina propria and into the cell culture supernatant. The composition of the immune cell infiltration within the lamina propria consists of a wide variety of both innate and adaptive immune cells including neutrophils, dendritic cells, and T lymphocytes, none of which have yet been incorporated into the model, thereby limiting the use of the model in understanding the dynamic interactions of the differing immune cells (706–708).

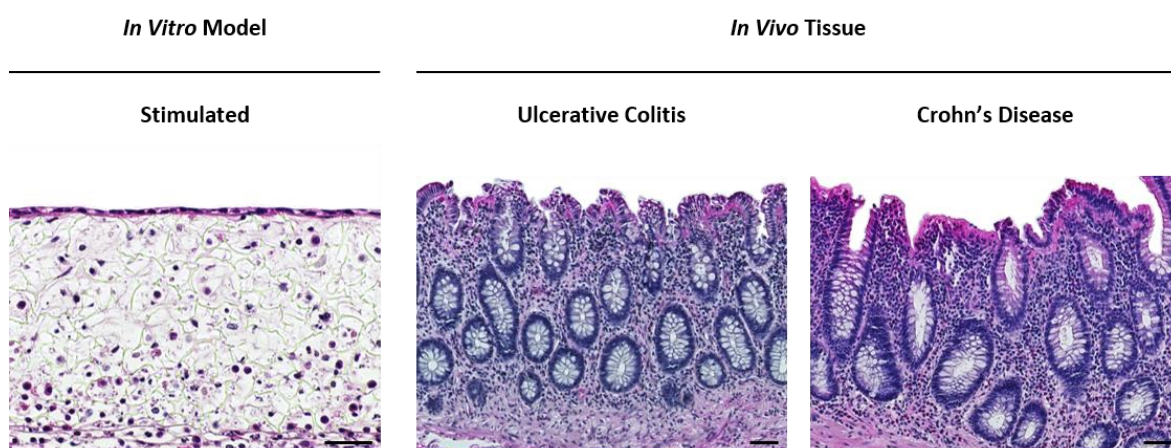


Figure 7.2. Histological analysis of the immune-competent 3D intestinal model stimulated with repeated LPS disease reveals structural disparities to IBD intestinal tissue samples. Representative H&E images of the Caco-2, U937, HDFn co-culture model stimulated with three repeated incubations of LPS treatment and human colonic tissue isolated from UC and CD patients. A flattened epithelial morphology is apparent *in vitro* but epithelial cells *in vivo* display a highly polarised structure. Scale bars: 50 μ m.

Proinflammatory cytokines were found to have higher gene expression upon stimulation of the models and displayed similar trends to cytokine expression profiles found in the serum and biopsies of patients with IBD. Moreover, decreased epithelial barrier function and increased paracellular flux are major factors driving the pathogenesis of IBD leading to higher levels of microbial penetration into the mucosal tissues that then propagate the immune response (536,709,710). A reduction in epithelial barrier integrity was identified within the models, though to varying degrees depending on the stimulatory molecule assessed, suggesting that this key aspect of IBD pathology has been recreated *in vivo*. Numerous studies have related this disruption in barrier function to TNF- α which is primarily produced by intestinal macrophages, thus incorporation of U937 macrophages within the immune-competent triple co-culture acts as a highly relevant source of cytokines involved in IBD (298,560,622–624,659,660). Within the models, TNF- α was demonstrated to be increased following multiple exposures to LPS and the levels of *TNF* gene expression were heightened following all stimulatory conditions assessed. However, the overall levels of TNF- α produced were not comparable to the serum levels of adult IBD patients suggesting that other cytokines may be responsible for the epithelial damage in the *in vitro* model. Irrespective of the concentration of TNF- α produced by the models, a valuable experiment would be to block the activity of TNF- α within the models and following application of a proinflammatory stimulus, characterise the effect on the epithelium. This would confirm whether other cytokines have roles in disrupting epithelial function.

Overall, the immune-competent triple co-culture model has been demonstrated to have the potential to study aspects of IBD *in vitro*, namely the induction of cytokines from macrophages following a proinflammatory insult and epithelial damage. Although not investigated within the context of this study, a key aspect of IBD pathology is disruption to the ECM (711–714). As the model contains both fibroblasts and endogenous ECM, and has been demonstrated to produce MMPs and TIMPs involved in ECM remodelling, the model could also prove useful in investigating aspects of tissue damage and fibrosis that result in fistulisation (ECM degradation) and fibrostenosis (excessive ECM deposition causing stricturing effects) respectively (95).

7.4. Future Applications of the Immune-Competent Intestinal Model

Experiments conducted using the immune-competent models highlighted that structural and functional changes can be induced in the models in addition to the initiation of an inflammatory response. These experiments have formed a foundation on which further experimental parameters can be tested.

7.4.1. Incorporation of HT29-MTX Goblet-Like Cells into the Immune-Competent Co-Cultures

The importance of incorporating a mucus-producing cell lineage in *in vitro* models of the intestinal mucosa was highlighted in various parts of this project. HT29-MTX cells have been successfully integrated into epithelial-fibroblast co-cultures but have not yet been included in the models with immune cells. Inclusion of this lineage would allow for future investigations regarding bacterial adhesion and pathogenesis and their role in propagating an inflammatory response.

7.4.2. Comparison of Different Cell Lines in the *In Vitro* Intestinal Mucosal Model

The cell lines selected for use within the models were chosen due to their widespread use and ease of culture. However, alternative cell lines exist with varied structural and functional properties that could be incorporated into the culture systems.

7.4.2.1. Alternative epithelial lineages

The epithelial cell lineages used in this study are of cancerous origin, thus do not truly reflect the precise function of the *in vivo* enterocytes and goblet cells due to alterations in their tissue origin, expression of transporter proteins, and barrier properties. As discussed in Section 7.2.3 primary cells derived from Lgr5⁺ organoids have been successfully cultured as monolayers on the surface of Transwell® membranes (316,701,702). It is therefore hypothesised that the culture of organoids could be adapted to grow on the surface of a lamina propria compartment cultured in Alvetex® Scaffold and the resulting models would have improved structure, function, and relevance to the *in vivo* intestine due to the presence of all intestinal epithelial cell lineages. Furthermore, the resultant models would have the potential to be adapted to personalised medicine studies where stem cells isolated from IBD patients could be utilised within the model to understand the interaction of the epithelium in response to proinflammatory stimuli.

7.4.2.2. Alternative fibroblast lineages

The importance of using an appropriate fibroblast lineage was discussed above and in Chapter 3. Although the models presented within include fibroblasts of human origin, they are not tissue matched to the intestine. Future experiments could therefore build on the comparisons started in Chapter 3 to additional intestinal stromal cell lineages and investigate alterations to the composition of the lamina propria in addition to identifying any structural and functional changes induced within the epithelium.

7.4.2.3. Alternative macrophage populations

Macrophages utilised in indirect and direct co-cultures within this project were differentiated from U937 monocytes. Within the laboratory an alternative model using THP-1 macrophages has been developed. Future experiments could therefore compare the structure and function of not only unstimulated control models, but also compare the immune response generated upon stimulation within the two models. Although THP-1 cells have been demonstrated to diminish epithelial barrier integrity when co-cultured with Caco-2 cells, while U937 and PBMC cells have not, various studies investigating the cytokine profile of macrophages in response to numerous stimuli have determined that THP-1 cells demonstrate a more similar expression profile to PBMC cells than U937 macrophages (616,715–717). It is therefore plausible that THP-1 macrophages would induce an inflammatory response more representative of the IBD intestine.

7.4.2.4. Alternative immune cell lineages

Macrophages were selected for incorporation into the immune-competent co-cultures due to their widespread presence in the intestinal epithelium and involvement in immune cell regulation and IBD. However, other immune cell lineages could be incorporated into the model.

7.4.2.4.1. Dendritic cells

Dendritic cells are implicated in the pathogenesis of IBD due to their role in microbial sampling and their ability to accumulate at the site of active inflammation in IBD where they recruit further immune cells including neutrophils and T lymphocytes, perpetuating inflammatory processes (718). Further evidence for their role in IBD stems from, murine colitis models that have demonstrated that transfer of bone marrow derived dendritic cells enhances inflammation while depletion of the cells reduces it (719–721). Therefore, inclusion of dendritic cells within the model would enable the study of interactions between the epithelium and dendritic cells in addition to the signalling processes occurring between macrophages and dendritic cells. Dendritic cells can be differentiated from both U937 and THP-1 monocytes and could be included within the model. Alternatively, dendritic cells and macrophages isolated from PBMCs have been incorporated into intestinal models to mimic the inflamed barrier function (485,486). Thus, PBMCs could provide an alternative, non-cancerous option for investigating the role of these two cell lineages within the model and also provide the opportunity to incorporate IBD-patient derived immune cells into the co-culture.

7.4.2.4.2. T lymphocytes

T lymphocytes are prolific in sustaining inflammation during active IBD and are potential therapeutic targets in the treatment of IBD (722,723). One of the key mechanisms thought to be crucial in the development of chronic inflammation is the imbalance of regulatory T cells and Th17 cells (154,220,221,724,725). Therefore, Jurkat T cells, an immortalised T cell lineage, have the possibility to be integrated into the model. Jurkat T cells have previously been utilised in indirect co-cultures with Caco-2 monolayers to investigate the impact of food supplements on overall gut health (529). As Jurkat T cells are a suspension cell lineage, they offer the potential to investigate tissue homing and chemotaxis following treatment of the models with a proinflammatory stimulus. T lymphocytes derived from PBMC also have the potential to be utilised within the model as a suspension cell lineage and would, again, provide a more *in vivo*-like alternative to the immortalised cell line due to the presence of multiple T lymphocyte subtypes including CD4⁺ and CD8⁺ T cells.

7.4.3. Further Characterise the Effects of LPS, IFN- γ and TNF- α and Evaluate the Effects of Alternative Stimulatory Molecules

Instigations regarding immune cell function were primarily focused on LPS and IFN- γ though a pilot study investigating the effects of TNF- α was also conducted. Further experiments could therefore not only focus on further characterising the response induced in the pilot studies but could also examine the effects of other

inflammatory stimuli. Additionally, signalling mechanisms underpinning the observed changes could be investigated by western blot analysis of phosphorylated and non-phosphorylated signalling proteins such as MLCK.

7.4.3.1. Further characterisation of the immune response generated using IFN- γ and TNF- α

Greater structural analysis of models stimulated using TNF- α and IFN- γ individually or adjunctively would be performed. Although a more thorough study investigating the effects of IFN- γ and LPS was conducted, the full complement of cytokines, chemokines and growth factors produced was not investigated. A future study investigating this, in addition to TNF- α , would be conducted using either ELISA analysis or shipment of further samples to EveTechnologies. Only triple co-cultures were examined for their ability to generate an inflammatory response; future studies could therefore investigate how this response is altered using the indirect Transwell®-based co-culture. As the epithelial and immune components are separated in these models, it would be possible to determine which cytokines were being induced by each cell lineage alone.

7.4.3.2. Evaluate the role of the lamina propria upon stimulation

The importance of stromal cells and the lamina propria in maintaining intestinal homeostasis has been discussed during this thesis, yet their role in propagating the inflammatory response was not investigated in the context of this study. Therefore, future experiments would focus on evaluating the ECM composition and remodelling within the lamina propria compartment of the triple co-culture following stimulation. Additionally, investigations could focus on the extent of an inflammatory response generated using only the Caco-2 HDFn co-culture model without the presence of U937 macrophages. This could be accomplished via the western blot analysis and ELISA techniques to investigate the overall levels of secreted ECM and the levels of ECM remodelling proteins, including TIMPs and MMPs, present within the models before and after stimulation. As discussed in Section 7.4.2, alternative fibroblast lineages would be investigated within the Caco-2 HDFn model and further investigations could then assess how inflammatory stimuli would affect different fibroblast populations within the immune-competent triple co-culture.

7.4.3.3. Investigate alternative stimulatory molecules

7.4.3.3.1. Interleukin-1 β

Previous studies have demonstrated the importance of IL-1 β in initiating an immune response and causing epithelial damage *in vitro*; additionally IL-1 β has been shown to amplify the response induced by the three effector molecules assessed within this study (485,486,531,559,570). Therefore, future studies could examine the inflammatory response and epithelial damage caused by IL-1 β alone and in combination with other stimulatory compounds on the models developed within this thesis.

7.4.3.3.2. Transforming growth factor- β 1

The immune responses generated within the models by LPS, IFN- γ and TNF- α were more representative of acute inflammation rather than chronic inflammation even following repeated applications of LPS. Therefore, alternative studies could focus on replicating the chronic immune response within the models. TGF- β 1 is postulated as an effector molecule for this purpose, as it is known to be upregulated within chronic IBD and drives a phenotypic change in fibroblasts to myofibroblasts encouraging the deposition of ECM, leading to fibrosis which is frequently observed in the IBD intestinal mucosa (726–730).

7.4.4. Investigate the Impact of Protective Compounds Following Model Stimulation

In vitro models are frequently used to investigate the protective effects of known and novel IBD therapeutic treatments. For example, three different anti-TNF- α therapies have been investigated using THP-1 cells. All three therapies were found to significantly reduce the levels of bioactive TNF- α following LPS stimulation. However, the antibody therapies etanercept and infliximab did not decrease TNF- α levels to the same extent as the drug pirfenidone that functions by preventing TNF- α synthesis (731). How the three compounds compare when using more complex *in vitro* models has yet to be determined, thus a future experiment could compare the effectiveness of these different treatments following stimulation of the triple co-culture. This would demonstrate the potential use of the model in testing novel therapeutic compounds.

7.4.5. Promotion of the Model and Development of Assays to Enhance Model Uptake

To promote the model and encourage its adoption by other researchers, numerous steps can be taken. Firstly, the model has a direct impact on the principles established by the NC3Rs: replacement, reduction, and refinement – specifically the cellular composition and structure of the model lends itself to investigations of IBD pathologies which could reduce the numbers of animals being utilised in such studies. Although animal systems provide a more holistic method to study all aspects of the disease pathology, they are not truly representative of the human disease phenotypes and their complex nature can impede investigations regarding the impact of individual cell lineages or the roles of specific cytokines. Thus the *in vitro* model offers a more controllable system to investigate IBD pathologies.

The model requires no specialist equipment or knowledge of chemistry or physics to maintain, thus could prove useful for researchers requiring a simple research system. However, one of the limitations of the model is the prolonged culture time, therefore further adaptations to the model could be made to increase throughput. Pilot studies have been conducted to recreate the model using a 96-well Alvetex® Scaffold format, which although would still require a long maintenance period, would enable the production of greater numbers of models. Furthermore, the laboratory currently has an industrial collaboration with Reprocell Europe, a contract research organisation. Utilising this partnership further could enable the model to be commercialised, whereby premade models could be shipped directly to researchers or a service to test potential therapeutics could be provided.

Prior to providing the aforementioned services, stringent assays must be developed to analyse the models following application of a proinflammatory stimulus and potential treatment. Concomitant qPCR and immunoassay techniques such as ELISA or ALBIA would provide detailed information regarding the production and overall secreted levels of cytokines produced in response to stimulation and potential treatments. Furthermore, assessment of junctional markers via western blot analysis and wholemount immunostaining could also be utilised as a proxy for barrier integrity to determine overall protein levels and distribution. The work presented within has described the use of transport assays of a set of standardised compounds including lucifer yellow, rhodamine 123, FD4 and FD20 to assess the functional properties of the epithelial barrier. These could be further expanded to incorporate a wider variety of compounds that are transported both transcellularly and paracellularly. Furthermore, incorporation of other compounds with a wider variety of chemical properties would allow greater understanding of how the pharmacokinetics and pharmacodynamics are affected upon intestinal inflammation. Potential drugs include the poorly permeable atenolol which has an absorption rate of 50 % in humans, and propranolol which is highly absorbed at over 90 % (732,733). Additionally, an Ussing chamber adaptor has been developed within the laboratory to hold Alvetex® inserts. This would allow for model transport to be directly compared to that of both healthy, non-inflamed and active IBD intestinal tissues.

7.5. Conclusions

In conclusion, this thesis has focused on the development, optimisation, and characterisation of a series of novel *in vitro* models of the intestinal mucosa. The research has demonstrated that utilising 3D cell culture techniques, fibroblasts producing *de novo* ECM can support and enhance the differentiation of intestinal epithelial cell lines resulting in more *in vivo*-like functional properties. The initial co-culture was utilised as a foundation to incorporate multiple cell lineages into the model to recreate the structure and function of the intestine more accurately. Furthermore, these models have the potential to be utilised in future studies investigating the transport of novel drug compounds in addition to mimicking facets of IBD pathology. Future work will seek to integrate the four cell lineages utilised within this project into one final model and will focus on further investigations regarding intestinal inflammation and IBD.

Chapter 8: Bibliography

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Chapter 9: Appendices

To supplement the methods and results chapters, additional material has been included within the following appendices, including recipes for buffers used throughout the project and the average cytokine expression levels as determined by the EveTechnologies cytokine assay.

9.1. Appendix 1 – Recipes

The following appendix contains recipes for all the buffers utilised throughout the study in addition to recipes used to cast SDS-page gels used in western blot analysis. Unless otherwise specified 100 mL 10X buffer was diluted in 900 mL dH₂O to give a final 1X buffer solution.

9.1.1. Buffer Recipes

9.1.1.1. 10X Phosphate Buffered Saline (PBS)

NaCl	80 g
KCl	2 g
Na ₂ HPO ₄	11.5 g
KH ₂ PO ₄	2 g

The above components were dissolved in 1 L dH₂O and the pH of the resulting solution adjusted to 7.4.

9.1.1.2. 1X Hanks Buffered Saline Solution (HBSS)

HBSS powder was purchased from Sigma-Aldrich and dissolved in 1 L dH₂O with 25 mM HEPES and 0.35 g NaHCO₃ added to the solution. The pH of the buffer was then adjusted to 7.4.

9.1.1.3. 10X Citrate Buffer

Citric Acid	19.2 g

Dissolved in 1 L dH₂O and the pH was adjusted to 6.0. For use in immunofluorescent staining protocols, 0.5 % Tween 20 is added to the diluted 1X buffer recipe.

9.1.1.4. 10X Tank Buffer

Tris Base 30.28 g
Glycine 144.1 g
SDS 10 g

The above reagents were combined and dissolved in 1 L dH₂O and the pH was adjusted to 8.3.

9.1.1.5. 10X Transfer Buffer

Tris Base 30.28 g
Glycine 144.1 g

The above reagents were combined and dissolved in 1 L dH₂O and the pH was adjusted to 8.3. To make up the 1X buffer solution 100 mL 10X buffer was added to 800 mL dH₂O and 200 mL methanol.

9.1.1.6. 10X Blot Rinse Buffer (BRB-T / TBS-T)

Tris HCl 12.1 g
NaCl 88 g
EDTA 3.27 g

The above reagents were combined and dissolved in 1 L dH₂O and the pH was adjusted to 7.4. 0.1 % Tween 20 is added to the final 1X buffer solution.

9.1.2. Polyacrylamide Gel Recipes

Table 9.1. Recipes for polyacrylamide gels used in SDS page. Volumes are sufficient for one gel. Resolving gels were first cast and left to set, 5 % stacking gels were then cast on the top of the resolving gel.

Component	5 % Stacking Gel	8 % Resolving Gel	10 % Resolving Gel
dH ₂ O	2.8 mL	5.7 mL	5.3 mL
ProSieve® 50 Acrylamide Gel Solution	375 µL	1.6 mL	2 mL
1.5 M Tris pH 6.8	500 µL	-	-
1.5 M Tris pH 8.8	-	2.5 mL	2.5 mL
10 % SDS	37.5 µL	100 µL	100 µL
10 % APS	37.5 µL	100 µL	100 µL
TEMED	3.75 µL	5 µL	5 µL

9.2. Appendix 2 – qPCR Melt Curves

The following appendix contains the melt curves and melt peaks for primers utilised throughout this thesis.

9.2.2. Loading Control Primers

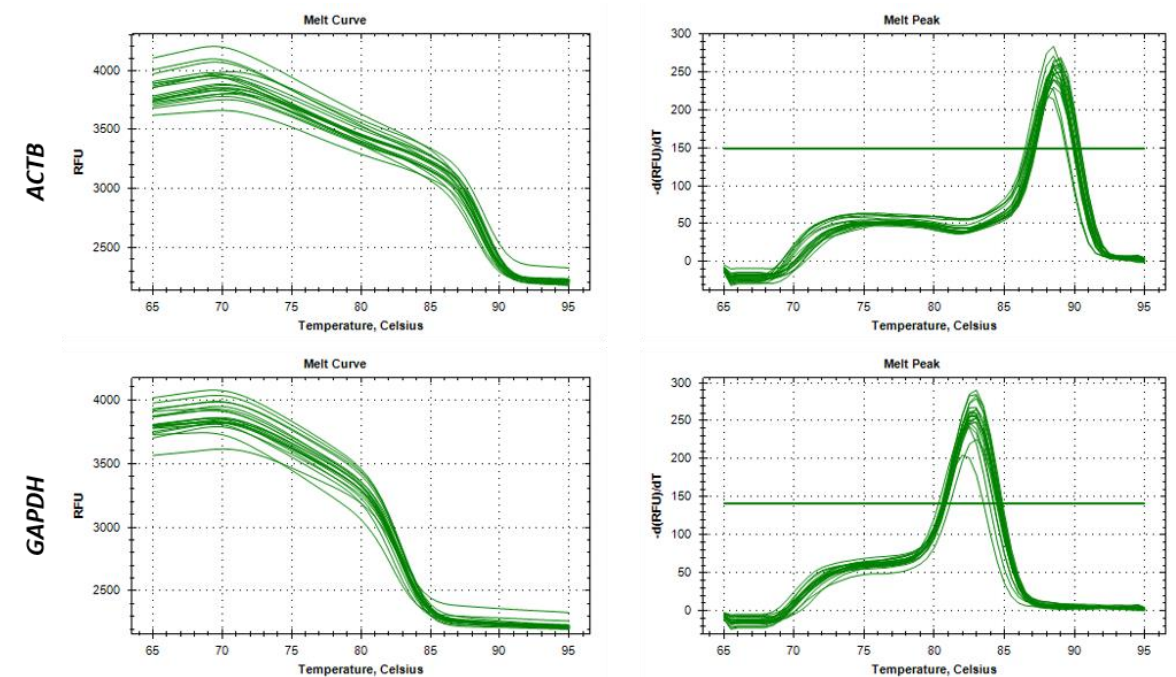


Figure 9.1. qPCR melt curves of loading control primers. The melt curve and melt peak graphs of primers used as loading controls: ACTB (actin) and GAPDH.

9.2.3. Intestinal Transporter Primers

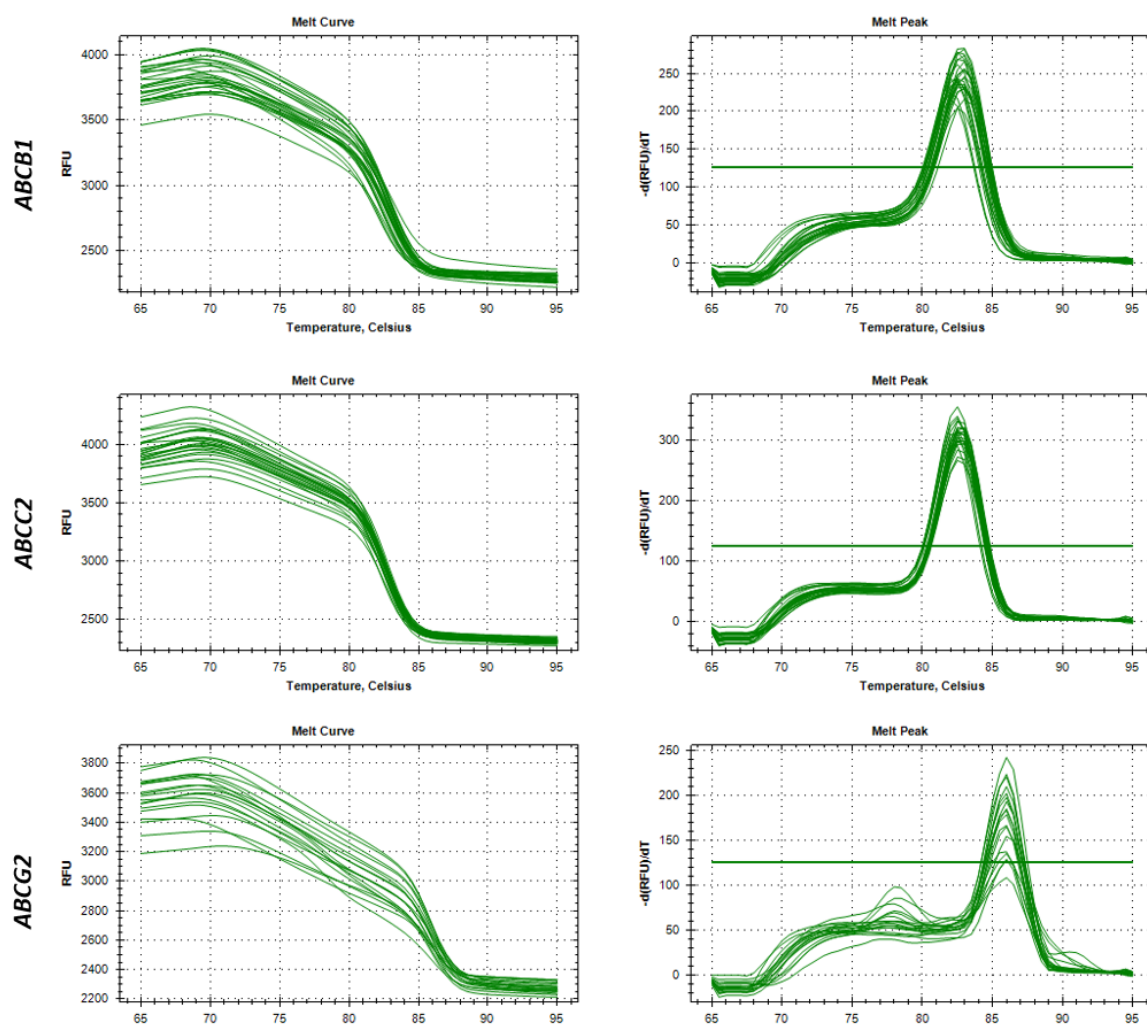


Figure 9.2. qPCR melt curves of primers for the intestinal transporters. The melt curve and melt peak graphs of primers of the intestinal transporter proteins: ABCB1 (P-gp), ABCC2 (MRP2), and ABCG2 (BCRP).

9.2.4. Apical Junctional Complex Primers

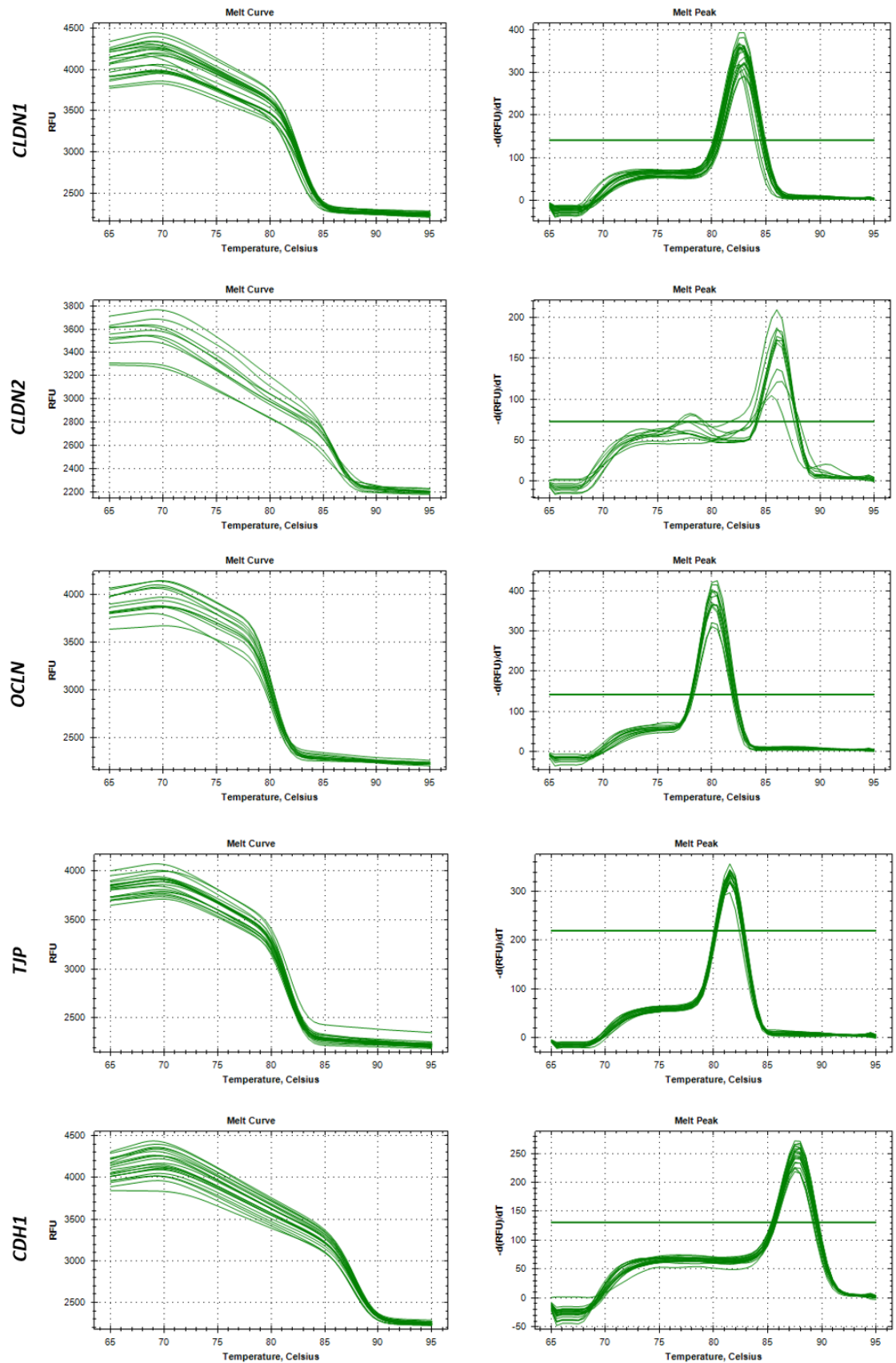


Figure 9.3. qPCR melt curves of primers for components of the apical junctional complex. The melt curve and melt peak graphs of primers of the apical junctional complex constituents: CLDN1 (claudin-1), CLDN2 (claudin-2), OCLN (occludin), TJP (ZO-1), and CDH1 (E-cadherin).

9.2.5. Primers of Inflammatory Cytokines

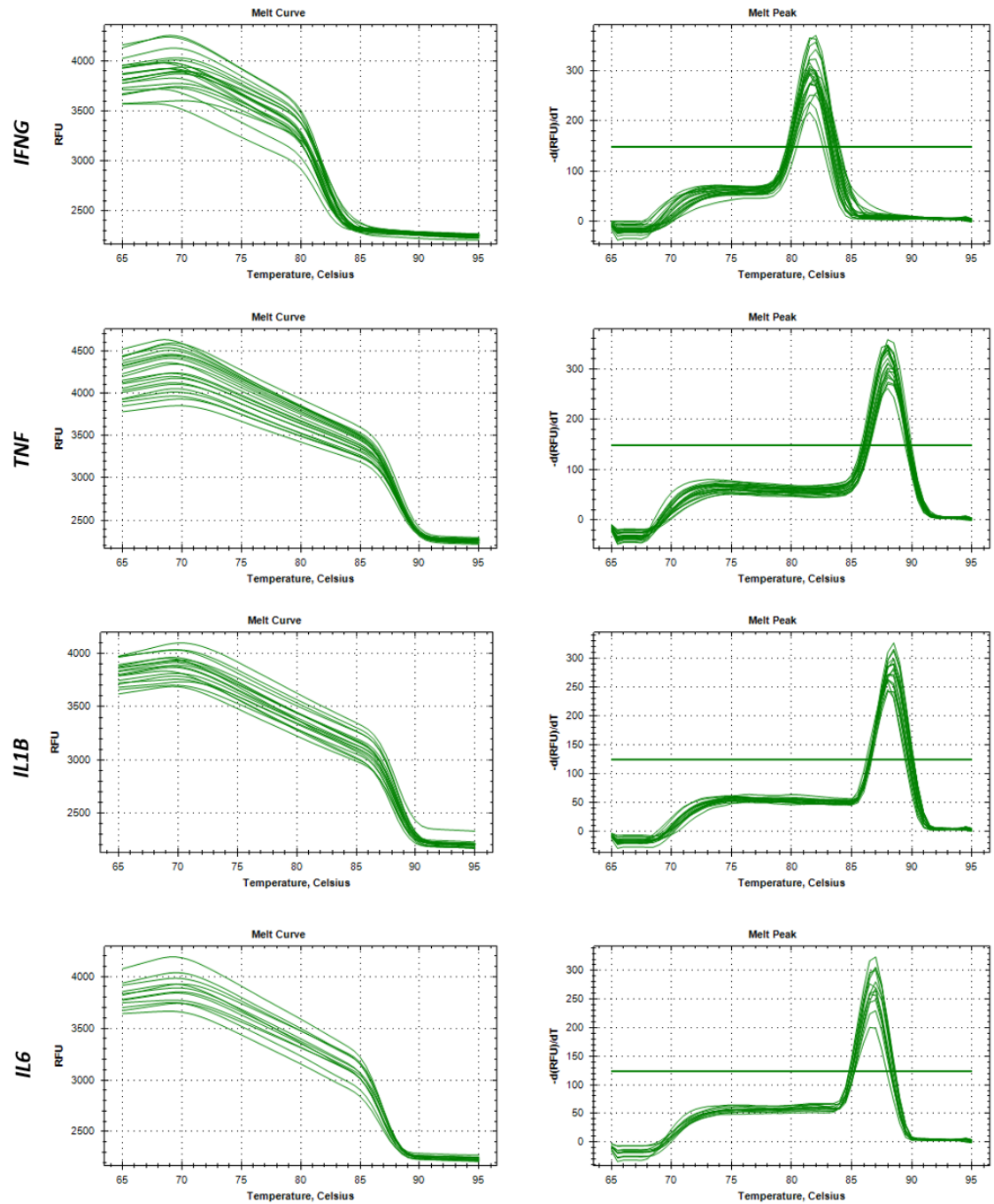


Figure 9.4. qPCR melt curves of primers of inflammatory markers. The melt curve and melt peak graphs of inflammatory primers: IFNG (IFN- γ), TNF (TNF- α), OCLN (occludin), IL1B (IL-1 β), and IL6 (IL-6).

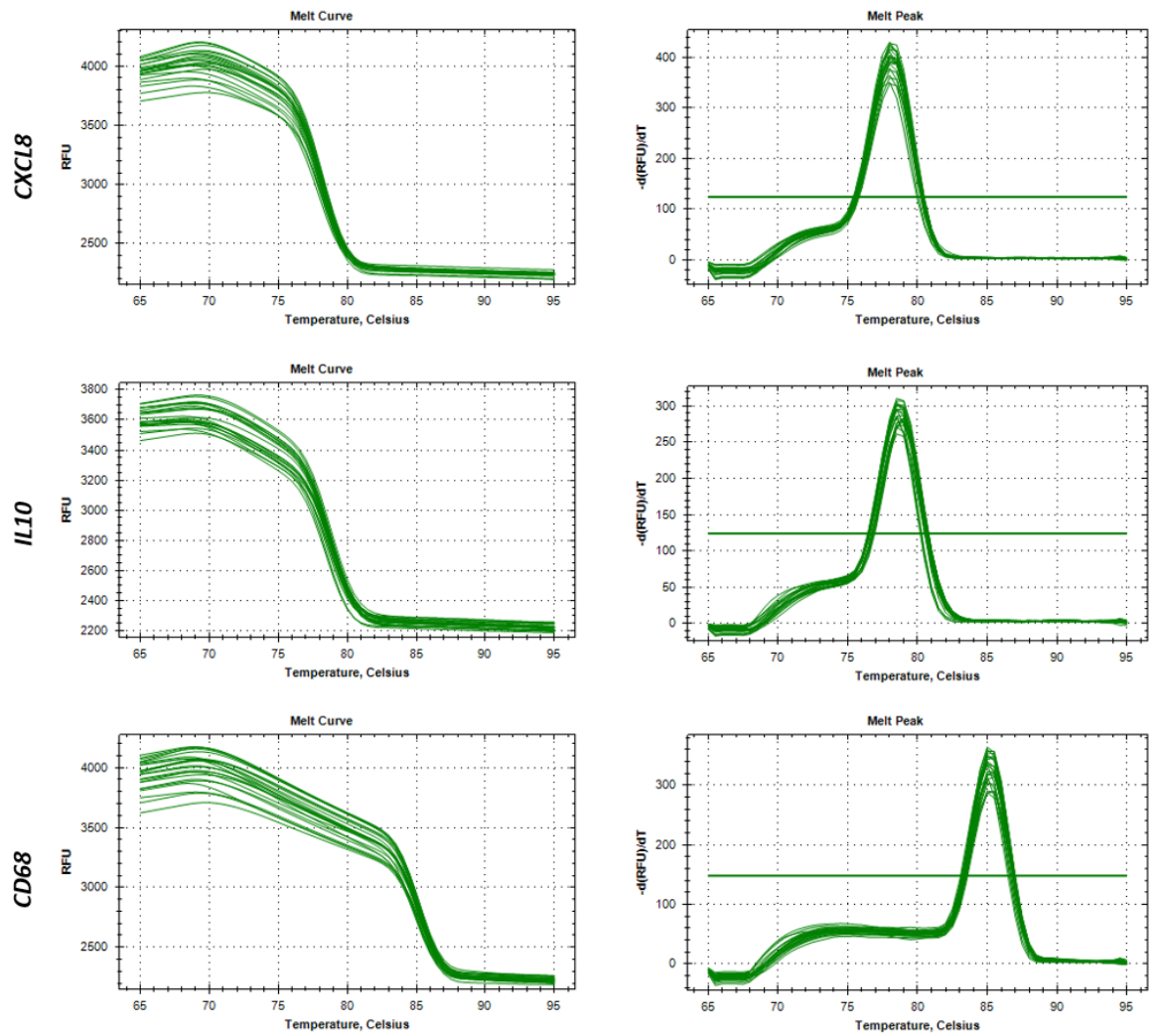


Figure 9.5. qPCR melt curves of primers of inflammatory markers. The melt curve and melt peak graphs of inflammatory primers: CXCL8 (IL-8), IL10 (IL-10), and CD68 (CD68).

9.3. Appendix 3 – Cytokine Concentrations

Table 9.2. Cytokine concentrations from control and stimulated immune-competent triple co-cultures. Stimulated models were given three doses of 1 µg/mL LPS, 24 hours apart. Change indicates whether the expression levels increased, decreased, or remained the same following stimulation. Data represent mean from three independent experimental repeats. OOR indicates when cytokine levels were out of range for the sensitivity of the assay. * $P \leq 0.05$, ** $P \leq 0.001$, and *** $P \leq 0.0001$ as determined by Student's t-test.

Cytokine	Unstimulated Control (pg/mL)	Repeated LPS Stimulation (pg/mL)	Change	P Value
sCD40L	6.18	9.02	–	–
EGF	3.86	5.73	↑	***
Eotaxin	3.03	3.83	↑	***
FGF-2	348.34	238.15	↓	–
FLT-3L	0.76	2.40	↑	***
Fractalkine	15.96	20.80	↑	***
G-CSF	644.05	2815.51	↑	***
GM-CSF	145.20	524.81	↑	***
GROα	1537.33	1903.33	↑	*
IFN-α2	5.43	12.29	↑	***
IFN-γ	0.60	0.69	↑	*
IL-1α	3.41	52.11	↑	***
IL-1β	2.79	69.71	↑	***
IL-1RA	11.50	513.27	↑	***
IL-2	0.21	0.26	–	–
IL-3	<OOR	<OOR	–	–
IL-4	2.44	2.74	–	–
IL-5	0.05	0.08	–	–
IL-6	3708.35	4407.60	↑	–
IL-7	<OOR	<OOR	–	–
IL-8	5486.05	6638.55	↑	–
IL-9	2.32	2.67	–	–
IL-10	25.06	43.17	↑	–
IL-12p40	6.98	16.20	↑	***
IL-12p70	0.19	0.30	–	–
IL-13	22.68	25.01	–	–
IL-15	5.81	10.14	↑	**
IL-17A	0.78	1.38	↑	***
IL-17E/IL-25	6.12	13.55	↑	***
IL-17F	0.94	2.37	↑	***
IL-18	0.96	1.84	–	–
IL-22	42.78	52.34	↑	***
IL-27	28.28	43.30	↑	***
IP-10	79.10	17158.47	↑	*
MCP-1	> OOR	> OOR	–	–
MCP-3	131.85	604.85	↑	***
M-CSF	184.23	216.34	↑	–
MDC	12.13	28.03	↑	***
MIG/CXCL9	5.96	135.90	↑	***
MIP-1α	10.21	1092.19	↑	***
MIP-1β	9.25	573.14	↑	***
PDGF-AA	53.69	58.57	–	–
PDGF-AB/BB	19.59	23.62	–	–
RANTES	482.20	1376.88	↑	*
TGF-α	2.16	3.51	↑	***
TNF-α	14.33	65.26	↑	***
TNF-β	55.68	58.20	–	–
VEGF-A	3449.48	5113.32	↑	*