The Effect of Cannabinoids on Cytokine Evoked Human Colonic Mucosal Damage and Caco-2 Epithelial Permeability

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Abstract

Inflammatory bowel disease (IBD) is a disease characterised by two subtypes, ulcerative colitis (UC) and Crohn's disease (CD). Both conditions can lead to inflammation and ulceration of the gastrointestinal mucosa. Treatments are available for IBD, however they can cause severe adverse effects and may not be useful in all patients. As a result, there is still an unmet need for novel IBD treatments. In animal models of colitis, cannabinoid (CB) agonists have shown efficacy in reducing inflammation. To further investigate this, we used a human colonic mucosal explant model to determine if CB agonists could attenuate mucosal damage. To induce damage in colonic mucosa, pro-inflammatory cytokines (that are elevated in IBD patients) were used. These included a combination of TNF-α + IL-1β and in other studies, IL-17A. Furthermore, we also tested if these cytokines modulated biochemical markers of inflammation. Immunohistochemistry was used to determine the identity of immune cells in the lamina propria of the mucosa and also localisation of IL-17A.

Treatment of colonic mucosa with TNF-α + IL-1β induced damage characterised by luminal epithelial loss, crypt destruction and increased lymphocyte density. In addition, elevations in nitrite levels were found in TNF-α + IL-1β treated explants compared to controls. These damage parameters were attenuated by treatment with CB2R agonists. We found that PGE_2 concentration was significantly decreased after TNF-α + IL-1β incubation suggesting reductions in PGE_2 may partially mediate mucosal damage.

IL-17A also induced a course of mucosal damage similar to that observed with TNF-α + IL-1β treatment, however no increase in lymphocyte density occurred. In this study, damage was reduced by the endocannabinoid anandamide as well as cannabidiol. We did not determine whether this effect was CB1R or CB2R mediated. Nitrite concentrations

were not elevated after IL-17A treatment, however increased matrix metalloprotease activity was detected, suggesting this may mediate IL-17A induced mucosal damage. ELISA and western blotting was used to determine if the TNF-α + IL-1β combination we previously studied could influence IL-17A levels. There was no significant change in IL-17A expression, however basal expression of IL-17A was found in human colonic mucosa. This was confirmed by immunohistochemistry, showing extensive expression of IL-17A, particularly at the edge of the lumen. Therefore, IL-17A may also play a homeostatic or protective role against micro-organisms in the human colon.

Cell culture studies examined the effects of cytokines and cannabinoids on Caco-2 epithelial permeability. In IBD, it has been established that increased mucosal permeability contributes to inflammation. TNF-α + IL-1β increased epithelial permeability; however this was not attenuated by CB ligands. IL-17A did not induce any significant increases in permeability.

In conclusion, this thesis demonstrates that CB2R agonists may be useful in attenuating damage in human colonic mucosa induced by cytokines. Therefore, CB2R agonists may have utility as novel therapeutics in IBD. In addition, IL-17A which can be damaging in this model is also expressed in healthy human colonic mucosa, suggesting a homeostatic or protective role. It may be the case that excessive expression of IL-17A in IBD contributes to inflammation.

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Statements of Authorship

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Author Contributions

By signing the statement of Authorship, each author certifies that their stated contribution to the publication is accurate and permission is granted for the publication to be included in the candidate's thesis.

Mr. Benjamin Harvey conducted experiments, interpreted and analysed data, prepared graphical representations of data and wrote the draft manuscript for each section.

Signature …… Date……

Ms. Lauren Nicotra conducted experiments, analysed data and assisted with manuscript evaluation.

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Ms. Megan Vu conducted experiments and assisted with manuscript evaluation.

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Dr. Scott Smid supervised the development of work and experimental design. Assisted in data interpretation and critical manuscript evaluation. Acted as the corresponding author.

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By signing the statement of Authorship, each author certifies that their stated contribution to the publication is accurate and permission is granted for the publication to be included in the candidate's thesis.

Mr. Benjamin Harvey performed all experiments and analysed and interpreted data. Wrote the draft manuscript for each section and assisted with preparation of the revised manuscript.

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Dr. David Wattchow performed the colorectal surgery to obtain mucosal samples and assisted with manuscript evaluation and feedback.

22/2/14

Dr. Tiong Sia assisted with mucosal sample collection and assisted with manuscript evaluation and feedback.

Signature Date 22/2/14

Dr. Scott Smid supervised development of work and experimental design, assisted in data interpretation and manuscript evaluation. Acted as the corresponding author

Signature …… Date 25/3/14

Abbreviations

ABC	avidin biotin complex
AEA	anandamide
ACEA	arachidonoyl 2'-chloroethylamide
AIN-457	secukinumab
2-AG	2- arachidonoylglycerol
5-ASA	5-aminosalicyclic acid (mesalazine)
BCA	bicinchoninic acid
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CAC	colitis associated cancer
CD	Crohn's Disease
CBD	cannabidiol
COX	cyclooxygenase
CB1R	cannabinoid 1 receptor
CB2R	cannabinoid 2 receptor
CNS	central nervous system
DAB	3,3-diaminobenzidine tetrachloride
DAN	2,3-diaminonapthaline
DNBS	2,4 dinitrobenzene sulphonic acid
DSS	dextran sodium sulphate
EC	enterochromaffin
EFS	electrical field stimulation
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
EGC	enteric glial cell
FAAH	fatty acid amide hydrolase
FCS	foetal calf serum
GALT	gut associated lymphoid tissue
GI	gastrointestinal
GPR	G-protein coupled receptor
GWAS	Genome wide association studies

H&E	haematoxylin and eosin
HBD-1	human beta defensin 1
HCAEC	human coronary artery endothelial cells
5-HT	5-hydroxytryptamine, serotonin
ICAM-1	intracellular adhesion molecule 1
IBD	inflammatory bowel disease
IL-1β	interleukin 1 beta
IL	interleukin
IELs	intraepithelial lymphocytes
IFN-γ	interferon gamma
IHC	immunohistochemistry
iNOS	inducible nitric oxide synthase
LPLs	lamina propria lymphocytes
LPS	lipopolysaccharide
MMP	matrix metalloprotease
mRNA	messenger ribonucleic acid
MLCK	myosin light chain kinase
MPO	myeloperoxidase
mAb	monoclonal antibody
MAGL	monoacylglycerol lipase
NO	nitric oxide
NOD2	nucleotide-binding oligomerisation domain 2
NF-κB	nuclear factor kappa B
NSAID	non-steroidal anti-inflammatory drug
OM	oil of mustard
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPAR-γ	peroxisome proliferator-activated receptor gamma
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophil granulocytes
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
RA	rheumatoid arthritis
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

TLR-4	toll like receptor 4
Δ^9-THC	Δ^9-tetrahydrocannabinol
TNF-α	tumour necrosis factor alpha
Th	T-helper cell
TNBS	2,4,6 trinitrobenzine sulphonic acid
TEER	trans epithelial electrical resistance
TGF	transforming growth factor
TBST	tris buffered saline with Tween- 20
TLC	thin layer chromatography
UC	ulcerative colitis
ZO-1	Zonula occludens 1

Chapter 1 : Introduction

1.1 Inflammatory Bowel Disease

1.1.1 Overview

Inflammatory bowel disease (IBD) is comprised of two subtypes, Crohn's disease (CD) which can affect any part of the gastrointestinal (GI) tract or ulcerative colitis (UC) which is confined largely to the colon and mucosa. (Xavier and Podolsky, 2007, Akobeng, 2008). The major feature of both conditions is inflammation and ulceration of the gastric mucosa. A study by Farrokhyar et al. (2001) has shown that the incidence of CD appears to be increasing in northern European industrialised countries while the incidence of UC appears stable. In the US, it is believed that about one million patients have IBD with about thirty thousand diagnosed each year (Hanauer, 2006a). In Australia, a recent study found that the incidence of CD is one of the highest in the industrialised world while the incidence of UC is lower compared to other westernised countries (Wilson et al., 2010) (Table 1.1). The mortality rate for both conditions is low and most patients can be expected to have normal life expectancy with appropriate treatment, however both of these conditions can have serious effects on quality of life (Farrokhyar et al., 2001, Cohen, 2002).

Table 1.1: Incidence of IBD (CD and UC) per 100,000 individuals determined in a range of studies of western countries. Adapted from (Wilson et al., 2010)

	Incidence per 100,000		
Region	IBD Overall	Crohn's Disease (CD)	Ulcerative Colitis
Australia 2008	29.6	17.4	11.2
Canada, 2000	29.2	16.3	12.9
New Zealand 2004	25.2	16.5	7.6
Denmark 2005	23.1	8.6	13.4
United Kingdom 2000	22.2	8.3	13.9

1.1.2 Histological Changes

In both CD and UC, significant changes in the normal morphology of the bowel can occur (Table 1.2). In both forms of IBD the major changes include: increased immune cell infiltrate, ulceration and erosion of the epithelium, distorted crypt architecture and increased numbers of lymphoid aggregates (Le Berre et al., 1995). Major histological differences between CD and UC occur. In CD, the entire thickness of the bowel can be involved (transmural) with the presence of granulomas while in UC, the inflammation is confined mainly to the mucosa (Bouma and Strober, 2003).

Table 1.2: Various differences between clinical CD and UC. Adapted from: (Roberts-Thomson et al., 2011)

	Crohn's Disease (CD)	**Ulcerative Colitis (UC)**
Site	Commonly small and large bowel	Large bowel
Distribution	Segmental	Continuous with rectum
Mucosal surface	Cobblestone, aphthous and deeper ulcers	Granular, superficial ulcers
Inflammation	Transmural, eccentric	Mucosal, circumferential
Pseudopolyps	Uncommon	Common
Thickening of bowel wall	Usually prominent	Mild
Colonic shortening	Yes, fibrosis	Yes, muscle hypertrophy
Strictures	Common	Uncommon
Perianal disease	Common	Rare
Fistulas	Common	Rare
Abscesses	Common	Rare
Recurrence after surgery	Common	Rare
Oral lesions	Common	Rare
Risk of malignancy	Low	Moderate

1.1.3 Symptoms and Complications

The symptoms of both CD and UC are somewhat similar and include largely digestive symptoms which range from diarrhoea to abdominal pain and tenderness. In CD, abdominal pain has been suggested to occur in up to 80% of patients (Munkholm et al., 1992). Other symptoms of CD include bloody diarrhoea, development of perianal or anal abscesses, rectal bleeding, weight loss, fever, oral ulceration and night sweats (Rao et al., 1988, Gibson and Iser, 2005, Sands and Siegel, 2010).

In UC, symptoms include severe diarrhoea, weight loss, dehydration and loss of peristaltic function (Bouma and Strober, 2003, Osterman and Lichtenstein, 2010). Some patients (up to 30%) may present with constipation rather than with diarrhoea (Ferri, 2011). Other issues that can arise in IBD include a lower quality of life (particularly in active CD), lost productivity and stress (Cohen, 2002, Morrison et al., 2009).

Although mortality rates for appropriately treated IBD are low, complications can occur that can be life threatening. These include malnutrition and growth failure, particularly in paediatric CD patients caused by excessive diarrhoea and bleeding resulting in loss of nutrients and electrolytes (Akobeng, 2008). Toxic megacolon, in which inflammation is extremely severe and that affects underlying muscle layers, may cause the colon to rupture and can be fatal (Carter et al., 2004). It is likely the major cause of mortality from IBD is the significantly higher risk of colorectal cancer (colitis associated cancer, CAC) compared to patients without IBD (Bernstein et al., 2001). The risk increases, especially if IBD is diagnosed at a younger age. Appropriate treatment of IBD appears to reduce the incidence of CAC (Foersch et al., 2012).

1.1.4 Current Treatments for IBD

There are currently a range of pharmacological interventions available for the treatment of IBD. These drug classes include aminosalicylates, immunosuppressants and monoclonal antibodies (mAbs) that target TNF-α. Generally, these treatments are well tolerated, reduce inflammation and therefore provide relief from symptoms. However, these treatments do not cure the disease and inflammation and symptoms can reoccur. Some treatments can have serious adverse effects, including increased risk of infection when using immunosuppressants and anti TNF-α antibodies (Stallmach et al., 2010, Mowat et al., 2011).

1.1.4.1 Aminosalicylates

The current first line treatment for managing UC and preventing relapse are aminosalicylates including sulfasalazine and mesalazine (Carter et al., 2004). The mechanism of action of these drugs is likely to be inhibition of immune function including cytokine activity and also by scavenging reactive oxygen species (Hanauer, 2004). These

drugs work most effectively if applied to the inflamed colon through the use of foams or enemas. These compounds are recommended for all patients as they can reduce the chance of relapse by one third and are generally well tolerated (Gibson and Iser, 2005, Hanauer, 2006b). For management of CD, aminosalicylates do not show such clear efficacy and are usually only effective in maintaining remission in 40-55% of mainly mild cases of CD (Hanauer, 2004, Akobeng, 2008). Adverse effects of these medications include nausea, headache and rash. Sulfasalazine has additional adverse effects which can include vomiting or mild haemolysis (Scribano, 2008).

1.1.4.2 Corticosteroids

Corticosteroids such as hydrocortisone are very effective agents in reducing inflammation, but can have serious side effects. These agents are used when rapid remission of inflammation is necessary (such as failure of aminosalicylate treatment) but cannot be used long term (Gibson and Iser, 2005). Side effects of corticosteroid use include hypertension, increased risk of infection and weight gain. As a result of this they are not ideal for long term use in CD or UC. One corticosteroid, budesonide has shown utility in treating CD located in the small intestine and proximal colon. Budesonide also has a lack of systemic adverse effects, allowing it to be used for longer periods than traditional corticosteroids (Akobeng, 2008).

1.1.4.3 Immunosuppressants and Anti TNF-α Antibodies

These agents are useful in CD, where inflammation cannot be controlled effectively or relapse occurs despite treatment with aminosalicylates. Examples of these compounds include azathioprine or methotrexate, which work by inhibiting cell proliferation and reducing the immune response (Mowat et al., 2011). As a consequence of this the adverse effects of these compounds are leukopenia and hence an increased risk of infection

(Scribano, 2008). Monoclonal antibodies (mAbs) inhibiting the activity of TNF-α (such as infliximab) are another treatment available to reduce inflammation in IBD (Schreiber et al., 1999). This mAb is effective in CD patients where traditional therapies have failed (Akobeng and Zachos, 2009). The major drawbacks of using this antibody are the high cost of treatment (Akobeng and Zachos, 2009), risk of serious infections (such as tuberculosis) or development malignancy (Keane et al., 2001, Stallmach et al., 2010) and the possibility of causing apoptosis in activated immune cells (Brown and Mayer, 2007).

1.1.4.4 Surgical Interventions for IBD

Pharmacological treatments are not always completely successful in managing colitis in severe UC and CD cases. Approximately 30% of UC patients and up to 75% of CD patients (who have had CD for greater than 10 years) will require surgical removal of inflamed tissue (Mowat et al., 2011). Generally the colon (colectomy) or part of the colon and the rectum (proctocolectomy) is removed in UC patients and this can prevent further inflammation and can be considered curative (Osterman and Lichtenstein, 2010). Surgery is complex in CD patients, as the risk of severe complications is present and inflammation is likely to reoccur elsewhere (Sands and Siegel, 2010). The main drawbacks of colectomy are an increase in bowel movements and management of ileostomy by the patient (Gibson and Iser, 2005, Osterman and Lichtenstein, 2010). In the case of proctocolectomy (with ileal pouch anal anastomosis), possible complications include pouchitis or small bowel obstruction (Osterman and Lichtenstein, 2010). As a result of these factors, there is still a largely unmet need for new pharmacological treatments for the management of IBD, to reduce the need for invasive surgical intervention in severe cases.

1.1.5 Causes of Inflammatory Bowel Disease

The exact causes of CD and UC are not entirely clear; however it is proposed that a range of abnormal immune responses, environmental factors and genetic factors may predispose to the development of IBD. The human colon is a vast reservoir of various and normally innocuous bacteria which play a critical role in aiding digestion and maintaining GI homeostasis (Guarner and Malagelada, 2003, Hviid et al., 2011). The colon and other areas of the gut can be colonised by harmful bacteria and if this occurs the immune system responds appropriately to prevent disease. In contrast, the immune system is required to tolerate innocuous bacteria that normally reside in the colon. If an immune response occurs to these bacteria, inflammatory pathology can result.

1.1.5.1 Involvement of Environmental and Genetic Factors in IBD Development

In developed countries the incidence of CD appears to be increasing (Farrokhyar et al., 2001, Bouma and Strober, 2003, Hanauer, 2006a, Wilson et al., 2010) and this may be in part due to increased hygiene levels and hence lack of exposure to antigens during development (Rook, 2011). This may lead to an excessive immune response (lack of immune tolerance) to the normal bacteria found in the colon and the occurrence of inflammation (Okada et al., 2010). Use of medications, especially non-steroidal anti-inflammatory drugs (NSAIDs) appear to increase the risk of relapse of IBD and worsen symptoms (Halter et al., 2001). One factor strongly associated with development of CD is tobacco use. Paradoxically, in UC, tobacco use may help to relieve symptoms of the condition, which highlights possible differences in the immune component of both conditions (Lindberg et al., 1988, Vatn, 2009). The mechanism for this may be due to alterations in immune function caused by nicotine.

Studies of identical twins have suggested a role for genetics in the development of IBD. A range of possible gene variants including those involved in innate immunity may be involved (Hanauer, 2006a). One particular gene suspected to be involved in the development of CD is nucleotide-binding oligomerisation domain 2 (*NOD2)*, which is involved in sensing of the muramyl dipeptide component of bacteria, subsequent activation of NF-κB and release of IL-1β (Hugot et al., 2001, Maeda et al., 2005). Variations in this gene may lead to inappropriate activation of the NF-κB pathway in monocytes and an increase in inflammation. Other possible genetic polymorphisms include those in intracellular adhesion molecule 1 (ICAM-1), toll like receptor 4 (TLR4) and the interleukin 23 (IL-23) receptor (Brown and Mayer, 2007). Genome wide association studies (GWAS) have been utilised to identify additional susceptibility loci in IBD. In terms of CD, 71 susceptibility loci have been identified including those involved with autophagy (dysfunctional microbial processing) and the IL-23 receptor which is involved in the in the Th17 pathway (Lee and Parkes, 2011).

1.1.5.2 Immune Factors Involved in IBD

The human gut contains a vast array of defensive (immunocompetent) components that protect the body from harmful pathogens. These include components such as the gut epithelial barrier and mucosal immune system. The gut associated lymphoid tissue (GALT) contains intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) including T-cells, both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) and a range of regulatory T-cells (Brown and Mayer, 2007). Previously, CD was considered a T-helper 1 (Th1) mediated inflammatory process with expression of interferon gamma (IFN-γ) and in contrast, UC was considered a T-helper 2 (Th2) inflammatory process with production of interleukin 5 (IL-5) and interleukin-13 (IL-13) cytokines (Brown and Mayer, 2007) (Figure 1.1). More recently, a critical role for T-helper 17 (Th17) cells, which are a subclass of

helper T cells, has been found in both CD and UC (Weaver et al., 2012, Hundorfean et al., 2012).

Both UC and CD may be caused by a lack of regulation of the normal immune response. The array of environmental and genetic factors that can lead to the development of IBD are often those involved with the immune system. The human colon contains large amounts of non-pathogenic or commensal micro-organisms and the immune system must recognise these antigens as benign and tolerate them without activating an immune response. Conversely, it must also be able to recognise micro-organisms which are pathogenic and generate an immune response to neutralise them. In patients with IBD, this tolerance is absent or deficient and the immune system becomes activated in the presence of these commensal microorganisms leading to a release of inflammatory mediators such as reactive oxygen species, causing inflammation and damage (Brown and Mayer, 2007, Kaser et al., 2010).

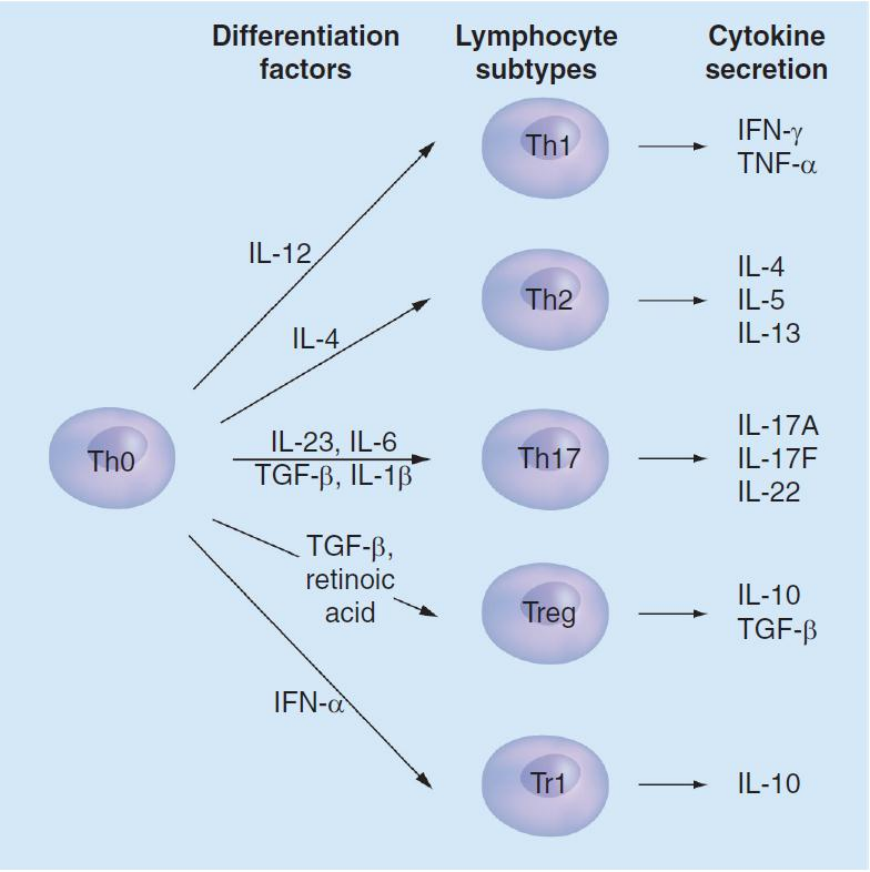

Figure 1.1: Differentiation of helper T-cells under the influence of cytokines into various subtypes. Various T-cell subtypes also produce a range of unique cytokines with various immune functions. From (Roberts-Thomson et al., 2011)

1.1.5.3 Adaptive Immune Response in IBD

Defects may occur in the adaptive immune response of patients with IBD, leading to a lack of immune response to pathogenic bacteria when necessary. One study measured levels of IFN-γ and interleukin-4 (IL-4) secreted from cloned and stimulated $CD4^+$ T lymphocytes from patients with CD, UC or non-inflammatory gut diseases (including colon cancer). It was shown that T-cells from CD patients produced high levels of IFN-γ and low levels of IL-4 while patients with UC or non-inflammatory gut diseases produced both IFN-γ and IL-4. In CD, it was also noted macrophages secreting IL-12 were present. This data suggests that in CD, IL-12 may play a role in development of the disease by stimulating

release of IFN-γ and IL-4 from T cells and a Th1 predominant response (Parronchi et al., 1997). This study was performed before the role of Th17 cells in CD had been determined and this important lymphocyte subclass was not discussed. It is now known that Th17 cells play a critical role in CD development and maintenance.

1.1.5.4 The Innate Immune Response in IBD

A number of components of the innate system can be affected in IBD. These defects can result in increased inflammation due to activation of the innate immune system by commensal bacteria in the gut (Gersemann et al., 2012). These defects include reduced expression of genes that are involved in mucus production (mucin genes) in ileal CD patients compared to healthy controls (Buisine et al., 1999). Mucus is a first line barrier that protects the underlying luminal epithelium from pathogens. Defects in the integrity of luminal epithelium can also occur in CD patients, leading to infiltration of both pathogens and commensal bacteria, leading to inflammation (Muise et al., 2009). This is discussed in further detail in section 1.3.9 of this introduction. In the colon, β-defensins (a class of anti-microbial peptide) are produced by colonic mucosa. In both CD and UC, there is reduced expression of β-defensin 1 (HBD-1) mRNA compared to healthy controls in inflamed biopsies (Wehkamp et al., 2003). The lack of expression of HBD-1 may partially facilitate inflammation in IBD as β-defensins are important in protecting the colonic mucosa against bacterial infiltration.

In terms of innate immune cells, it has been established that large quantities of polymorphonuclear neutrophil granulocytes (PMN) from the blood are found localised in inflamed lesions of the gut. These PMNs can release cytokines including tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) (especially in CD) which can cause tissue damage (Nikolaus et al., 1998). This was demonstrated *in vivo* by stimulating PMNs

taken from patients with IBD with lipopolysaccharide (LPS) (Nikolaus et al., 1998). It has also been suggested that a lack of neutrophil apoptosis and subsequent overactivity of neutrophils may underlie part of the pathology of IBD (Brannigan et al., 2000). In addition, macrophages and dendritic cells play a role in IBD. In IBD patients, macrophages have increased density of activating receptors and dendritic cells can produce greater amounts of pro-inflammatory cytokines (Steinbach and Plevy, 2014). These changes highlight the role of these innate cells types in the development and maintenance of IBD.

1.1.5.5 Interleukin 17 Family Cytokines in IBD

In IBD, one of the key cytokines involved in development of this condition is interleukin 17 (IL-17). The gene for this cytokine was first discovered in rodents where it was named CTLA-8 and it has shared homology to a viral gene found in *herpesvirus Saimiri* (Rouvier et al., 1993). The same authors also found the gene could be mapped on human chromosomes. At that point however, it was not known what function this gene possessed. It was subsequently discovered that CTLA-8 (now termed IL-17) could induce the production of IL-6 and cause proliferation of T-cells (Yao et al., 1995a). In the same study a unique receptor for IL-17 was also found to be expressed on T-cells, B-cells and fibroblasts. IL-17 mRNA was subsequently found in human peripheral blood T cells, but only when they were stimulated (Yao et al., 1995b).

Further studies revealed that various subtypes of IL-17 are produced in humans. The first subtype of IL-17 discovered is now known as IL-17A (often referred to as IL-17 in the literature). In addition, other members of the IL-17 family of cytokines include IL-17B, IL-17C, IL-17D, IL-17E and IL-17F (Kolls and Lindén, 2004). In terms of IBD, the main IL-17 family members involved appear to be IL-17A, IL-17C and IL-17F, and these will be discussed in further detail in this thesis.

The exact IL-17 subtype under investigation is not often described in the literature (particularly early literature) and the variable functions and roles of these cytokines remain undetermined. Much of the literature refers simply to IL-17 and it is therefore assumed that IL-17A is being described (unless otherwise stated). It is possible that other IL-17 family members, particularly IL-17F may cross react with antibodies commercially available (Yapici et al., 2012). This highlights the importance of further study in this area and explicit clarification of which IL-17 subtype is being investigated.

1.1.6 Interleukin 17A (IL-17A) in IBD

IL-17A can be produced by a subclass of T-helper cells known as Th17 cells, which are located in a high density at mucosal surfaces where these cells play an important role in protecting the mucosa from infection (Guglani and Khader, 2010). IL-17A can also be produced from a range of other immune cells including natural killer cells, macrophages and neutrophils under specific conditions (O'Connor et al., 2010). The Th17 subclass of T-helper cells is distinct from Th1 and Th2 cells. These cells possess the IL-23 receptor and it is known that genetic alterations in this receptor are important in the development of IBD (Duerr et al., 2006). In animal studies, the development of Th17 cells can be increased on exposure to segmented filamentous bacteria (SFB) (Atarashi et al., 2010).

IL-17A plays an important role in inflammation, particularly in recruiting neutrophils to the tissue (Cua and Tato, 2010). However, IL-17A can play a role in tissue homeostasis and maintenance of epithelial barrier function (Kinugasa et al., 2000). This divergence of effects makes characterising the function of IL-17A difficult, but it may have both inflammatory effects and reparative effects. It is hypothesised that IL-17A may induce inflammatory damage indirectly by causing the production of IFN-γ (Annunziato et al., 2007). In human macrophages, stimulation with IL-17A can cause the production of TNF-

α and IL-1β which are also important cytokines in the pathogenesis of IBD (Jovanovic et al., 1998). Measurement of stromelysin, a matrix metalloprotease (MMP) was also performed and it was found that IL-17A induced large increases in expression of this enzyme, which facilitates the breakdown of extracellular matrix (Jovanovic et al., 1998).

In patients with IBD, changes in the expression of IL-17A have been reported. Using immunohistochemistry (IHC), expression of IL-17A was observed in both CD and UC patient colonic tissue, but not in tissue from control patients (Fujino et al., 2003). The source of IL-17A appeared to be T-cells and monocytes. The same authors also found elevated IL-17A in the serum of CD patients, but this was less pronounced in UC patients. Expression of IL-17A mRNA was also elevated in the inflamed mucosa of CD and UC patients. The use of multiple techniques to quantify IL-17A levels provides substantial evidence of elevations of IL-17A in IBD patients. An additional study cultured tissue from IBD patients and healthy controls and measured IL-17A in the tissue media (Rovedatti et al., 2009). Similar to the previous study, elevations in IL-17A were found in inflamed CD and UC tissues, with lower quantities in the uninflamed tissues. In contrast to the study by Fujino et al. (2003), this study by Rovedatti et al. (2009) demonstrated expression of IL-17A in healthy patient tissues.

A recent clinical trial in CD patients attempted to use a specific neutralising antibody against IL-17A, secukinumab (AIN-457) (Hueber et al., 2012). The result of this clinical trial demonstrated that the antibody was not only ineffective at reducing disease severity but actually worsened disease. This contrasts with much of the animal data available which suggests IL-17A is pro-inflammatory. The adverse effects in the trial were due to an increase in fungal infections occurring in secukinumab-treated patients compared to placebo controls. This result suggests IL-17A (in humans) also plays a critical role in protection against various fungi and reducing or eliminating activity leads to infection.

Interestingly, treatment of rheumatoid arthritis (RA) patients with secukinumab showed some beneficial effects at reducing disease severity (Hueber et al., 2010). In this trial no significant increase in infection was noted, unlike in the CD trial. In both trials participants were given 10 mg/kg secukinumab doses twice (over the duration of the study), so it is unlikely that increased infection risk is dose related, but rather due to a distinctive feature or differential role of IL-17A in CD patients not present in RA patients. Pre-clinical data demonstrating the true selectivity of secukinumab does not appear to be available; although it is suggested by the study authors it is highly selective for IL-17A (Hueber et al., 2010). Without publishing the actual data on selectivity, it is not possible to conclude it is entirely selective for IL-17A. The study by Hueber et al (2010) also showed decreased IL-17F activity when secukinumab was administered. In addition secukinumab may inhibit the activity of other forms of IL-17, such as IL-17C involved in providing mucosal protection and immunity.

1.1.6.1 Interleukin 17C (IL-17C) in Colitis

IL-17C was first identified as a unique member of the IL-17 family, with 23% homology to IL-17A (Li et al., 2000, Kolls and Lindén, 2004). IL-17C can be produced from the epithelium with less expression from other cell types usually associated with IL-17A expression, such as Th17 cells (Ramirez-Carrozzi et al., 2011). The receptor for IL-17C has been identified as IL-17RE and in animal knockout models (IL-17RE$^{-/-}$) increased mortality from *Citrobacter rodentium* infection occurs, suggesting that IL-17C plays a role in protection against this bacterium (Song et al., 2011). In a human monocyte cell line (THP-1) IL-17C can induce the release of pro-inflammatory cytokines such as TNF-α and IL-1β (Li et al., 2000) and IL-17C production can worsen collagen induced arthritis (Yamaguchi et al., 2007). IL-17C can also induce production of IL-6 (critical to differentiation of Th17 cells) in human sub-epithelial myofibroblasts, which are cells involved in repair during inflammatory damage (Yagi et al., 2007). This activity is similar to IL-17A, although a lesser amount of IL-6 is produced. Additionally, MMP-3 production is induced by IL-17C, but to a lesser extent than IL-17A. The implications of this are not discussed by Yagi et al. (2007), but these results together with those of Kolls et al. (2000) and Yamaguchi et al. (2007) indicate that IL-17C may also have pro-inflammatory ability.

In the colon however, it appears that IL-17C as well as providing protection against *Citrobacter rodentium* may have protective and restorative roles in maintaining epithelial integrity. This has been demonstrated in a mouse colitis model where IL-17C$^{-/-}$ mice had worsened DSS induced colitis compared to wild type controls (Ramirez-Carrozzi et al., 2011, Reynolds et al., 2012). In addition, Reynolds et al. (2012) showed that IL-17C can increase expression of tight junction proteins such as occludin and claudins, indicating IL-17C can improve the integrity of epithelial tight junctions. To date, there are no studies examining the role of IL-17C in human IBD, however it could be predicted that it has a

protective role against certain bacteria or yeast strains (similar to IL-17A) and provide epithelial protection by enhancing barrier function (Hueber et al., 2012).

1.1.6.2 Tumour Necrosis Factor Alpha (TNF-α) and Interleukin 1β (IL-1β)

TNF-α is a pro-inflammatory cytokine that can be produced in the gut mainly from macrophages but also lymphocytes (Th1 lineage). In patients with IBD, increases in the number of macrophages that produce TNF-α (and also IFN-γ) occur and tissue TNF-α concentration correlates with disease severity (Olsen et al., 2007, Leon et al., 2009). Studies have highlighted the importance of IL-1β and TNF-α in the risk of relapse of IBD (Schreiber et al., 1999) and the clinical effectiveness of anti-TNF-α antibodies demonstrates that this cytokine likely plays a key role in the pathology of IBD (Kaser et al., 2010). TNF-α recruits leukocytes to the area, is able to increase vascular permeability and induce vasodilation (Bradley, 2008, Roberts-Thomson et al., 2011). TNF-α can also increase gut epithelial permeability. Studies in a Caco-2 cell model of an epithelial barrier have shown that TNF-α is able to alter expression of the tight junction protein zonula occludens 1 (ZO-1) resulting in increased permeability (Ma et al., 2004).

The cytokine IL-1β has been shown to be elevated in IBD tissue (both UC and CD) in a number of studies and is likely to be mainly produced from macrophages (Mahida et al., 1989, Ligumsky et al., 1990, Raddatz et al., 2005). Elevations of IL-1β even in un-inflamed areas of mucosa in CD patients compared to controls have been reported, which is similar to observed elevated expression of TNF-α (Reimund et al., 1996).

1.2 Experimental Models of Colitis

1.2.1 Animal Models

There are over 50 different animal models of colitis, which can be used to study varying features of clinical colitis and IBD (Hoffmann et al., 2002). These models have provided great insight into the pathogenesis and mechanisms underlying IBD, in particular the key importance of T-cells (Blumberg et al., 1999, Neurath, 2012). In addition, these models provide a ready means of investigating the efficacy of compounds that may be used as treatments for IBD.

Animal models can be ordered into a number of categories including the commonly used chemical colitis models, genetic models (developed over the past 20 years), antigen induced colitis, adoptive transfer models and spontaneous models, where colitis develops without intervention (Hoffmann et al., 2002). As chemical induced models of colitis are used commonly in literature relevant to this thesis, additional detail in respect of these models will be provided.

Dextran sodium sulphate (DSS) is used in a simple model used to develop colitis in rodents. Acute DSS administration induces a form of colitis by activation of the innate immune system (Wirtz et al., 2007). This form of colitis tends to resemble some features of UC (Gaudio et al., 1999). DSS is dissolved in the drinking water of mice at a concentration of up to 5%. The water is consumed by the mice for seven days causing colitis (Massa et al., 2004). This treatment causes an increase in damage to the mucosa and also an increase in MPO levels (Massa et al., 2004, Kimball et al., 2006).

2,4 dinitrobenzene sulphonic acid (DNBS) is used to cause a form of colitis in mice and rats. This form of colitis is generally Th1 mediated and infiltration of macrophages and lymphocytes occur (Wirtz et al., 2007). Mice are starved for 36 hours before intrarectal

administration of 5 mg of DNBS dissolved in 50% ethanol. This is done with light isoflurane anaesthesia (Massa et al., 2004). For studies of colitis, mice can be sacrificed three days after DNBS treatment when inflammation has occurred. DNBS causes significant damage to colonic mucosa and increases in levels of MPO (Massa et al., 2004). 2,4,6 trinitrobenzine sulphonic acid (TNBS) administration varies slightly to DNBS administration as it is given by enema of 30 mg in 50% ethanol. Colitis takes about one week to establish in the TNBS model (D'Argenio et al., 2006). In this particular model a number of histological changes including severe ulceration occur after twenty four hours (Kruschewski et al., 2001). The same study also found an increase in blood flow which decreased below control levels after three days.

Croton oil is an irritant compound that can be used in rodents as an acute or chronic colitis model. To initiate colitis, mice are fasted for eighteen hours before being given a single 0.05 mL oral dose of croton oil. In order for colitis to become chronic, a second 0.05 mL dose is given twenty-four hours after the first dose (Puig and Pol, 1998). The result of croton oil administration is weight loss of up to three grams within three hours and diarrhoea. Disruption of small intestine mucosa and lymphocyte infiltration also occurs (Puig and Pol, 1998). This model has been used by other research groups where is was found to be similarly effective in causing colitis (Izzo et al., 2001).

1.2.2 Limitations of Animal Models and Conclusions

Animal models do have limitations, as they do not fully replicate all the features of IBD. In rodent models (the most common colitis model), the colitis usually heals over time and spontaneous flares of inflammation do not occur (Grisham, 1993, Hoffmann et al., 2002, Neurath, 2012). In addition, major differences in the activity of transforming growth factor beta (TGF-β) on T helper 17 (Th17) cells, which are involved in IBD, exist between mouse

models of colitis and IBD (Chen and O'Shea, 2008). These differences make translating conclusions from mouse models of colitis to IBD more complex. These models also can have a number of ethical considerations as the colitis in these models can be severe, leading to morbidity or mortality (Dothel et al., 2013). This contrasts with IBD, where mortality is uncommon.

As a result of these factors, animal models play an important role in the study of IBD. These models do however have limitations and the similarity of these models to human IBD is not ideal. As a result, other models of colitis that can model different aspects of IBD are worthwhile investigating. The main focus of this thesis will be the use and investigation of a model of colitis using *ex vivo* human colonic mucosa (explant model).

1.2.3 *Ex vivo* Human Colonic Explant Models

The use of animal or human tissue *ex vivo* in explant culture is a useful technique that can provide controlled experimental conditions compared to *in vivo* models (Randall et al., 2011). Explant culture also allows experimentation with human tissue (collected by biopsy or during surgical procedures) rather than animal tissue, which has advantages in terms of improved translation to human disease. In addition, explant experiments can be ethically acceptable as no animal use is required. In terms of explant culture of GI tissues, this has been attempted with duodenum, jejunum, ileum and colon from both humans and animals (Randall et al., 2011).

Explant culture has generally been technically difficult as the tissue needs to be perfused with oxygen to survive. An early and successful attempt at this technique demonstrated human small intestinal tissue could be cultured for 24 hours under explant conditions (Browning and Trier, 1969). In addition this study showed the tissue was viable at 24 hours demonstrated by active cell proliferation and fat absorption. In addition, electron

microscopy revealed similar structural morphology to control (immediately processed) tissue. Time in culture can be extended with optimised methods and media supplementation, for example rat colon has previously been cultured for up to 14 days with intact morphology (Autrup et al., 1978).

The explant model utilised in our laboratory uses human colon tissue taken from patients undergoing surgical resection to remove colorectal cancer. The tissue to be used under explant conditions is taken at a distance from the tumour site, near the surgical margins. The tissue is histologically normal and free from malignancy or dysplasia. The explant conditions used in the model are based on previous methodologies that have been successfully used (Jarry et al., 2008a).When this tissue is treated with exogenous inflammatory cytokines such as IL-1β and TNF-α, inflammatory damage to the tissue can be induced resembling features of colitis (Nicotra et al., 2013). This model has some advantages over animal models as the inflammation and mucosal damage caused by inflammatory cytokines may more closely replicate the inflammation found in IBD.

1.2.4 Cell Culture Models of Epithelial Permeability

As previously described, changes in epithelial barrier function can occur during IBD. As a result, there is benefit in developing *in vitro* models where these permeability changes can be investigated and interventions to reduce permeability tested. There are a range of colonic epithelial cell lines that can be cultured *in vitro* and used to investigate mechanisms of altered epithelial permeability in altered inflammatory settings. In these models, functional measurement of permeability can be obtained by measurement of trans epithelial electrical resistance (TEER) or movement of molecules such as large molecular weight dextrans across the monolayer. TEER gives a measurement of paracellular permeability, which is permeability between cells. In addition, an isolated cellular model

allows molecular studies of tight junction expression and localisation of chemokine or cytokine origins.

In order to carry out these studies, colonic epithelial cells are grown on a permeable support (such as a Corning Transwell®) to induce monolayer formation (Hubatsch et al., 2007). Cell lines which have been successfully utilised for studies of colonic epithelial permeability in the literature include HT-29 (Bucker et al., 2011), T-84 (Fischer et al., 2013) and Caco-2 (Ma et al., 2004).

1.2.4.1 Caco-2 Cell Culture

For our studies of epithelial permeability, we have elected to use the well characterised Caco-2 cell line. This cell line is an epithelial cell line derived from a human colorectal carcinoma which differentiates to form a functional monolayer of intestinal epithelium. This cell line is also used extensively in pharmacokinetic studies of drug transport across intestinal epithelium and allows predictions of the bioavailability of drugs (Artursson, 1990, Hilgers et al., 1990). This cell line has some advantages over HT-29 and T-84 cells even though all are derived from a colon carcinoma and have an epithelial phenotype. These advantages include spontaneous differentiation in culture (compared to HT-29 cells) and a developed brush border and epithelial phenotype (compared to T-84 cells) (Meunier et al., 1995). In addition, the Caco-2 line has been used previously to examine effects of cytokines and other compounds on epithelial permeability (Ma et al., 2004, Amin et al., 2009, Al-Sadi et al., 2010, Van De Walle et al., 2010). Other applications of the Caco-2 culture are measuring increased levels of formed reactive oxygen species, cytokines and other inflammatory mediators which can be produced during colitis (Borrelli et al., 2009). The Caco-2 model can also be used to determine if compounds can repair the epithelium when it is damaged by physical trauma (Wright et al., 2005).

1.3 The Function of the Endocannabinoid System in the Gut

The G-protein coupled cannabinoid receptors are key components of the endocannabinoid system. First cloned in 1990 and named CB1 receptor (CB1R), further studies determined that plant cannabinoids, such as Δ^9- tetrahydrocannabinol (Δ^9-THC) (the main psychoactive constituent of marijuana) bind to this receptor (Devane et al., 1988, Matsuda et al., 1990). The CB2 receptor (CB2R) was first cloned in 1993 (Munro et al., 1993) and was also found to be a G-protein coupled receptor, with 48% homology to the CB1R (Storr et al., 2008b).

1.3.1 Endogenous Cannabinoid Ligands

Two major endogenous ligands for the CB receptors are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). AEA was initially discovered in the porcine brain shortly after the CB1R was discovered (Devane et al., 1992). Both of these compounds are synthesised as required from arachidonic acid via phospholipases when triggered by calcium influx into cells (Klein et al., 2003). AEA is relatively non selective for CB1R and CB2R (Klein et al., 2003). Endocannabinoids are rapidly metabolised, suggesting they act near to their site of synthesis (Piomelli et al., 2000). The action of AEA and 2-AG are terminated when the compounds undergo hydrolysis by fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) respectively (Pertwee, 2005, Long et al., 2009). In addition to these metabolic pathways, AEA can be metabolised by COX-2 forming prostaglandin ethanolamides (prostamides) (Fowler, 2007). These compounds have unique biological activity as they interact with a unique set of prostaglandin receptor heterodimers (Kozak et al., 2002, Liang et al., 2008). This is detailed further in section 1.4.10 of this thesis.

1.3.2 Cannabinoid Receptor Localisation

CB1R are located extensively on neurones in the CNS, however they are also localised on neurones of the human enteric nervous system (ENS) and intestinal crypt epithelium (Wright et al., 2005). The cannabinoid 2 receptor (CB2R) is located mainly on immune cells (such as macrophages and less densely on plasma cells) and also on some neurones of the enteric nervous system (Wright et al., 2005, Izzo, 2007, Storr et al., 2008b). In the human colon, expression of CB1R occurs on the epithelial cells of crypts with low levels on the smooth muscle. CB2R have also been localised in the human colon, with high density on nerves of the myenteric plexus, epithelial cells of crypts and low levels of expression on smooth muscle (Marquez et al., 2009).

1.3.3 General Overview of the Endocannabinoid System in the CNS and GI Systems

The main role of the endocannabinoid system in the CNS is control of motor functions, emotional response and cognition (Pandey et al., 2009). In addition, the endocannabinoid system also plays a role in the GI motility. Activation of the CB1R modulates peristalsis and reduces intestinal motility (Izzo et al., 2001, Hinds et al., 2006). Incubation of human ileum with the CB1R agonist WIN-55,212-2 inhibits the twitch response induced by electric field stimulation (EFS) (Croci et al., 1998). Using human colonic tissue, the EFS twitch response can also be reduced by addition of ACEA, a selective CB1R agonist (Hinds et al., 2006). Reduction of intestinal motility appears to occur primarily by CB1R activation, but in some experimental pathological conditions such as administration of LPS to rats, the CB2R can play a role in modulating motility (Mathison et al., 2004). Other GI disorders reduced by activation of the CB1R receptor include visceral pain, emesis, and diarrhoea (Sanger, 2007). These studies suggest that there is therapeutic potential in utilising the endocannabinoid system to treat these conditions.

1.3.4 The Endocannabinoid System in Colitis

The general immune suppressing effects of cannabinoids suggest they may have beneficial effects in the treatment of IBD, as they can suppress the excessive activation of the immune system in the gut and reduce inflammation (Kunos and Pacher, 2004, Klein, 2005). Some patients have attempted to use cannabis as an adjunct treatment for IBD; however data on the use and efficacy of this is limited. In a Canadian population with CD, 15.9% of patients currently use cannabis to treat CD, while 11.6% of UC patients use cannabis (Lal et al., 2011). The incidence of cannabis use increased if the patient has used analgesics chronically for GI pain or had previously undergone abdominal surgery (Lal et al., 2011). This provides some insight that cannabinoids may alleviate visceral pain in humans and warrants further exploratory and mechanistic studies into the therapeutic potential of cannabinoid ligands as anti-colitis treatments.

1.3.5 Animal Colitis Models and Cannabinoids

Many studies have demonstrated changes in the state of the endocannabinoid system in animal models of colitis which suggest the endocannabinoid system has a protective role. In a model of small intestinal inflammation induced by administration of croton oil, there was a strong up-regulation of CB1R in the jejunum detected with western blotting (Izzo et al., 2001). It was noted that levels of AEA and 2-AG were not altered. Reductions in motility were greater in croton oil-treated mice compared to controls during administration of CP-55,940 (Izzo et al., 2001). This suggests that cannabinoids can reduce intestinal motility with greater effect during inflammation, when CB1R expression is up-regulated. Another study used CB1R knockout mice ($CB1R^{-/-}$) to highlight the importance of the CB1R during inflammation. Using two models of colitis induction by DNBS or DSS, it was found that mice lacking CB1R had significantly worse scoring of colitis parameters. In

addition, it was also demonstrated that significant up-regulation of CB1R mRNA occurred in mice treated with DNBS compared to control, which further reinforces that CB1R was up-regulated during colitis (Massa et al., 2004). Administration of the CB1R antagonist SR141716A also worsened colitis parameters (such as decreased body weight), highlighting the critical importance of the endocannabinoid system and the CB1R in these models (Massa et al., 2004). Other parameters measured were body weight and myeloperoxidase (MPO) activity, which is a marker of neutrophil activity. In both cases a lack of CB1R resulted in a reduction in body weight of about 15% at day 3 of treatment and an increase in MPO activity of up to 300% (Massa et al., 2004).

Direct treatment with CB receptor agonists is also beneficial in the oil of mustard (OM) and DSS colitis model (Kimball et al., 2006). After treatment with OM, an increase in colitis parameters (such as colon shrinkage and inflammatory lesions) occurred. Treatment of mice with 2.5 mg/kg of ACEA (a selective CB1R agonist) reduced all colitis parameters. Treatment with JWH-133 (a selective CB2R agonist) also reduced colitis parameters to a slightly lesser extent. Similar results were obtained with the DSS model of colitis, although cannabinoid treatment was less effective.

The levels of AEA increase in the colonic sub-mucosa of animals treated with TNBS suggesting up-regulation, possibly as a mechanism to reverse inflammatory damage (D'Argenio et al., 2006). The same authors were able to show that increasing endocannabinoid levels by inhibiting reuptake, using VDM-11, (an AEA transport inhibitor) was able to return tissue damage scores to near control levels. This same effect was observed with the conventional treatment for colitis, mesalazine (5-ASA). Interestingly, 5-ASA treatment resulted in increased AEA levels, but not via modulating FAAH or AEA reuptake. The authors speculated that 5-ASA blocks cyclooxygenase 2 (COX-2) preventing metabolism of AEA and resulting in increased AEA levels, which

may in part explain the indirect mechanism of 5-ASA in the treatment for IBD (D'Argenio et al., 2006).

Massa et al. (2004) found that FAAH knockout mice ($FAAH^{-/-}$) had improved colitis parameters in a DSS and DNBS model of colitis, again indicating that elevated endocannabinoid levels are beneficial in reducing colitis (Massa et al., 2004). FAAH inhibitors have been tested in the TNBS model of colitis and increasing endocannabinoid levels had a beneficial effect in this study. The use of URB597 to inhibit FAAH and subsequent AEA metabolism improved colonic morphology after inflammation, reduced MPO activity and also abrogated the reduction in colon length observed in this model (Storr et al., 2008a). A unique component of this study was testing the effects of a FAAH inhibitor on CB2R knockout mice ($CB2R^{-/-}$). Interestingly, it was found that these mice did not show an equivalent improvement in colitis parameters, suggesting the CB2R also plays a critical role in modulating inflammation.

In relation to studies manipulating the activity or expression of the endocannabinoid system, the role of 2-arachidonylglycerol (2-AG) cannot be discounted. In the TNBS model of mouse colitis for example, increasing levels of 2-AG by inhibiting the enzyme MAGL with JZL184 demonstrated improvement in colonic histology scoring compared to vehicle-treated animals, as well as reductions in plasma pro-inflammatory cytokine concentrations, such as IL-1β, TNFα and IL-6 (Alhouayek et al., 2011). Blocking the beneficial effect of 2-AG using the CB1R antagonist SR141716A or the CB2R antagonist AM630 reversed the beneficial effects of JZL184. This indicates that activation of the endocannabinoid system by 2-AG is beneficial in reducing colitis and that the effect is both CB1R and CB2R-dependent (Alhouayek et al., 2011). The levels of 2-AG are higher in the healthy colon than AEA levels; however these levels do not change significantly during inflammation compared to AEA levels (D'Argenio et al., 2006).

1.3.6 Effects of Novel CB Ligands in Animal Models

In terms of unusual or novel CB ligands and purported CB-sensitive orphan GPRs such as GPR55 and GPR19, there is evidence of a wide-ranging degree of anti-inflammatory effects in colitis models. The phytocannabinoid, CBD has recently shown promise as a potential intervention for colitis. The advantage of this cannabinoid is that it is devoid of psychoactive effects, making it an attractive potential therapeutic compound. It has an unusual mechanism of action as it does not directly activate CB1R or CB2R and it may have activity as a FAAH inhibitor or PPAR-γ activator (Esposito et al., 2012, Izzo et al., 2009). CBD also reduced pro-inflammatory cytokine and iNOS expression in a murine DNBS colitis model and attenuated lipid peroxidation and reactive oxygen species in a colonic cell line (Borrelli et al., 2009). Studies in a mouse model of TNBS colitis showed that CBD, when administered either intraperitoneally or rectally, reduced colon damage (Schicho and Storr, 2012). The biochemical marker of neutrophil infiltration, MPO, was also reduced in CBD treated mice. While oral administration of CBD had no significant effect on colitis or MPO activity, this study suggests that rectal administration of CBD may be a potential treatment option for inflammation in IBD, as many currently available IBD treatments such as mesalazine (5-ASA) are administered in this way.

1.3.7 Effects of Cannabinoids on Immune Modulation

Cannabinoids can directly influence the function of the immune system, as both CB1R and CB2R have been located on immune cells, with a highest density on B-cells (Bouaboula et al., 1993). Further studies using PCR revealed a 10 to 100 times higher gene expression of CB2R mRNA on immune cells compared to CB1R (Galiegue et al., 1995). There is little acute effect of smoking cannabis on immune function, but immune cells such as alveolar macrophages directly exposed to cannabis smoke can be suppressed, resulting in a reduced

ability to kill tumour cells, inability to use nitric oxide to kill bacteria and reduction in expression of TNF-α (Baldwin et al., 1997). Inflammatory cytokines produced by Th1 cells (IL-12 and IFN-γ) were also reduced in mice treated with Δ^9-THC (Klein et al., 2000). This study also showed that treatment with CB antagonists reduced the fall in Th1 activity, which directly implicates CB1R and CB2R in this effect (Klein et al., 2000). Cannabinoids also appear to modulate the immune system by playing a role in migration of immune cells, suppression of lymphocyte proliferation (including T and B cells) causing apoptosis and inhibition of antibody production (Kaminski et al., 1994, Schwarz et al., 1994, Derocq et al., 2000)

Cannabinoids have other immunosuppressive functions in a range of cells, including increasing production of the immunosuppressive cytokine, transforming growth factor beta (TGF-β) in human peripheral blood lymphocytes via activation of CB2R (Gardner et al., 2002). It was also shown that CB2R expression can be reduced by TGF-β production in an autocrine manner. Recently it has been established that both CBD and Δ^9-THC can reduce mRNA expression of IL-17 in a model of multiple sclerosis, although via a CB receptor-independent mechanism (Kozela et al., 2013). As IL-17 is also involved extensively in IBD, these cannabinoids may also reduce or modulate IL-17 expression in clinical colitis.

Cannabinoids also affect migration of T-cells. Primary $CD4^+$ and $CD8^+$ lymphocyte migration induced by the chemokine CXCL12 was reduced by synthetic cannabinoids and the CB2R selective agonist JWH-015 (Ghosh et al., 2006). Similar studies with T-lymphocytes also showed that cannabinoid ligands, such as JWH-133 and 2-AG could reduce migration that occurred under the influence of CXCL12 (Coopman et al., 2007). Expression of CB2R on immune cells appears to be variable. For example, in the study by Ghosh et al. (2006), expression was present in T-lymphocytes; however, Coopman et al. (2007) found expression only occurred after stimulation with an antigen. In both of these

studies, measurement or CB2R expression varied and it may be possible that these methodological differences account for differences in the localisation of CB2R on these cells. These studies point to a crucial role for the endocannabinoid system, particularly the CB2R receptor in modulation and activity of the immune system. Thus, there is strong potential for the development of cannabinoid pharmacological interventions that target the immune system, particularly in the treatment of colitis.

1.3.8 Human GI studies of the Endocannabinoid System, Inflammatory Bowel Disease and Immune modulation

In human UC colonic biopsies, treatment with CBD reduced the production of nitrite and inhibited S100B protein expression (a glial activation marker), suggesting CBD can reduce inflammation in both the acute and remission phase of the disease (De Filippis et al., 2011). This study suggests that CBD may provide anti-inflammatory activity via a mechanism of modulating enteric glial cells (EGCs) in addition to other anti-inflammatory pathways suppressed by CBD.

A study in human colonic tissue demonstrated increased expression of CB2R during active UC and a decrease in receptor expression during quiescence (Marquez et al., 2009). In addition, CB1R levels also decreased during quiescence. These effects were only seen in mild to moderate cases of colitis and not in those classed as severe. In a similar study examining CD and UC colonic tissues, elevations in CB1R expression were observed in inflamed tissue compared to quiescent areas and control patient samples. In contrast, no changes in CB2R expression were detected (Di Sabatino et al., 2011). Elevations in CB1R mRNA expression and lack of CB2R expression have also been detected in a similar study, but only in CD patients (Stintzing et al., 2011).

Changes in endocannabinoid levels have been demonstrated with decreased AEA and elevated FAAH levels in inflamed UC and CD colonic mucosal tissues compared to uninvolved areas and control biopsies (Di Sabatino et al., 2011). This indicates reduced AEA levels in IBD affected colon, possibly from reduced production of endocannabinoids but also potentially an elevated breakdown via FAAH. This study indicates changes in the endocannabinoid system occur during both forms of IBD and that a lack of AEA expression may exacerbate or facilitate inflammation.

The study by Marquez et al. (2009) demonstrated a dramatic increase in FAAH occurred during acute inflammation in UC-affected colonic tissue. These levels of FAAH remained high in quiescent tissue and did not return to control levels. This may indicate that high FAAH activity may contribute to UC and that blocking FAAH activity may be beneficial. This would result in higher tissue concentrations of endocannabinoids and potentially offer protection to colonic tissue during inflammation.

The ability of AEA to supress immune function in human T-lymphocytes without causing cytotoxicity has also been demonstrated. Treatment with AEA can supress proliferation and inhibit release of inflammatory cytokines such as TNF-α (Cencioni et al., 2010). The same study also showed T-lymphocytes produce IL-17 under PMA (phorbol 12-myristate 13-acetate) and ionomycin stimulation, which was slightly reduced with AEA treatment. This study did not focus specifically on IL-17 production, but given the role of this cytokine in IBD it would be beneficial to investigate this further.

1.3.9 Cannabinoids and Epithelial Barrier Function

Epithelial cells form an essential barrier between the intestinal lumen and the underlying lamina propria. Damage to the epithelial barrier can allow luminal antigens and food particles to enter the lamina propria and induce inflammation. Patients with CD have been

shown to have increased gut permeability (Muise et al., 2009). Previous studies have shown that TNF-α causes increased permeability in an *in vitro* model of epithelial paracellular barrier function (Ma et al., 2004). This is believed to occur via increased expression of NF-κB and resulting disruption of important tight junction proteins such as zonula occludens 1 (ZO-1). Similarly, IL-1β has been shown to increase paracellular permeability in an *in vitro* model by downstream activation of myosin light chain kinase (MLCK) and cytoskeletal contraction, which unseals tight junctions (Al-Sadi et al., 2010). In other models of permeability it was shown that TNF-α and IFN-γ can disrupt barrier function of T84 cells via an apoptosis-independent mechanism (Bruewer et al., 2003).

With increased epithelial permeability in IBD and protective effects of cannabinoids on inflammation, it would be beneficial to investigate the effects of cannabinoids on epithelial permeability. Before examining the effects of cannabinoids on gut permeability, earlier studies investigated other endothelial barriers. One such study showed treatment of human coronary artery endothelial cells (HCAECs) with high glucose induced an increase in endothelial permeability (possibly via cadherin disruption) that was reversed by treatment with the cannabinoid, CBD (Rajesh et al., 2007). Interestingly, this effect appeared to be CB1R and CB2R independent, as a range of CB receptor antagonists was not able to abolish this effect. It is not surprising that this occurred however, as CBD has only a weak affinity for CB1R or CB2R (Borrelli et al., 2009).

The first study of the effect of cannabinoids on gut permeability showed selected phytocannabinoids such as Δ^9-THC and CBD may have benefit in a colonic epithelial Caco-2 cell line, where EDTA was used to increase permeability (Alhamoruni et al., 2010). The same authors then expanded on this work and examined the effects of a combination of TNF-α and IFN-γ induced paracellular permeability increases in Caco-2 cells over 72 hours. Δ^9-THC and CBD were able to increase the rate of recovery caused by

cytokine-induced increases in permeability in this model (Alhamoruni et al., 2011). The effects of cannabinoids varied depending if they were applied apically or basolaterally to the Caco-2 monolayers. Recently, it has been demonstrated that activation of the CB1R is protective in a model of blood brain barrier disruption in mice (Chi et al., 2012). In stressful circumstances mice develop increases in intestinal permeability. If $CB1R^{-/-}$ mice were stressed in the same way as wild type mice (via immobilisation and acoustic stress) they have significantly higher increases in intestinal permeability (Zoppi et al., 2012). This study shows the CB1R plays an important role in modulating gut permeability during stress. This data ties in with studies showing $CB1R^{-/-}$ mice have worsened colitis, perhaps due in part to epithelial barrier disruption (Massa et al., 2004).

1.3.10 Alternate Metabolic Pathways for Endocannabinoids: Role and Expression in Colitis

Alternate metabolic pathways exist for endocannabinoids aside from the conventional FAAH conversion to arachidonic acid. Endocannabinoids may also be acted upon by cyclooxygenases, lipoxygenases and cytochrome P450 oxidases, all of which may produce biologically active lipid mediators acting on pathways divergent to the endocannabinoids themselves (Fowler, 2007).

The cyclooxygenase 1 (COX-1) and COX-2 enzymes are expressed in the human GI tract and likely play a role in modulating colonic contractility (Fornai et al., 2005). COX-1 is expressed in the neurones of myenteric ganglia and muscle layers and is constitutively active while COX-2 expression is inducible and it is expressed in longitudinal muscle with some expression on myenteric ganglia (Fornai et al., 2005). In murine models similar expression of COX-1 and COX-2 is seen and inhibiting these enzymes with indomethacin (a COX-1 and COX-2 inhibitor) produces a reduction in contractility (Porcher et al., 2004). In UC and CD patients, the levels of COX-2 in the neural cells of the myenteric plexus are

increased up to eightfold higher than control levels in all UC patients studied and 83% of CD patients (Roberts et al., 2001).

In patients with colitis, inhibition of COX-2 may be detrimental. One study found that a 67% of patients using a COX-2 inhibitor had increased symptoms of UC, including rectal bleeding (Matuk et al., 2004). In this study withdrawing use of the COX-2 inhibitor improved symptoms, suggesting a causative effect. The mechanisms behind increased symptoms may be inhibition of production of prostaglandins (such as $PGF_{2\alpha}$ or PGE_2), which can also have anti-inflammatory effects (Matuk et al., 2004). Studies in mice have also highlighted the importance of COX-2 in colitis; when it is inhibited, mice have a worsened form of colitis that can lead to perforation of the bowel and death after one week (Reuter et al., 1996). Gene knockout studies in animals lacking COX-2 or COX-1 have shown an exacerbation of colitis induced by DSS, further supporting the notion that these enzymes play a critical role in protection against inflammation (Morteau et al., 2000). In contrast to the above studies, it was found that the COX-2 inhibitor, etoricoxib appeared to be well tolerated in patients with colitis with only 11% of patients showing a worsening of colitis symptoms (El Miedany et al., 2006). It is therefore not entirely clear if inhibition of COX-2 is detrimental in colitis, or whether animals are generally more susceptible to inflammatory damage from COX-2 inhibition than humans.

The COX-2 enzyme has a connection to the endocannabinoid system which may be protective in colitis. As previously mentioned, AEA can be metabolised by COX-2, forming prostaglandin ethanolamides or 'prostamides'. The corresponding pathway for 2-AG metabolism by COX-2 results in the production of prostaglandin glycerol esters (Kozak et al., 2002). These alternative metabolic pathways were first shown using a recombinant human COX-2 enzyme system (Yu et al., 1997). The major product formed from AEA oxygenation was prostaglandin E_2 (PGE_2) ethanolamide (PGE_2-EA). The same

researchers also showed that COX-1 does not convert AEA to prostamides. Subsequent studies have confirmed that COX-2 can convert AEA to prostamides (Kozak et al., 2002).

Analogues of these compounds currently have utility as effective agents to treat glaucoma, with a synthetic analogue of prostamide $F_{2\alpha}$ marketed as bimatoprost (Woodward et al., 2004, Woodward et al., 2008). Bimatoprost was shown to be highly effective in reducing intraocular pressure in normotensive beagle dog eyes (Woodward et al., 2004). These effects can be reversed by a specific prostamide receptor antagonist, AGN204396, which suggests prostamides may act at unique prostaglandin receptor heterodimers (Fowler, 2007, Woodward et al., 2008). As described previously, a number of studies found worsening of symptoms when COX-2 was inhibited and this has been attributed this to a lack of prostaglandin production (Morteau et al., 2000, Matuk et al., 2004). GI endocannabinoid expression and constitutive COX-2 expression may result in a basal level of prostamide production. Moreover, elevations in endocannabinoid expression and COX-2 induction occurring in colitis and IBD may potentially elicit significant increases in prostamides that have to date been overlooked in research in this field. Given there is recognised cross reactivity with prostaglandins and prostamides in commercial ELISA assays and the significant contribution of prostamides in inflammatory conditions, studies where prostalglandins have shown protective effects in colitis may be attributable to prostamides (Glass et al., 2005).

1.4 Research Aims

Studies using animal models of colitis have demonstrated that therapy with CB agonists or increasing the activity of the endocannabinoid system (such as via FAAH inhibition) provides a protective effect against inflammation. Considering this and the fact that biochemical changes occur in the endocannabinoid system during IBD, it is logical to

investigate cannabinoid interventions as novel treatments for IBD. The major deficiency in knowledge to date is whether cannabinoid treatment would actually be beneficial in IBD. To address this, we will use a human colonic mucosal explant model to investigate the effect of cannabinoid interventions against inflammatory cytokine-induced damage. This will give some insight as to whether cannabinoid interventions would be beneficial in the inflamed human colonic mucosa. In addition, mucosal explants allow for the study of cytokine effects on colonic mucosa, which may yield insights into how cytokines generate inflammatory damage in the human colon.

Research aims addressed in publication 1 (Chapter 2):

1. Investigate whether CB1R, CB2R and mixed CB1R/CB2R agonists can reduce mucosal tissue damage (luminal epithelial damage, crypt damage and lymphocyte infiltration) induced by TNF-α + IL-1β incubation.
    a. If mucosal damage is reduced by these CB agonists, determine if CB1R or CB2R receptor activation is involved in damage reduction (by use of CB1R or CB2R receptor specific antagonists.
2. Determine if TNF-α + IL-1β incubation elevates inflammatory mediators such as nitric oxide (measured by stable nitrite concentration) which may be involved in causing mucosal damage.
3. Establish if increased cell proliferation is responsible for the increased number of lymphocytes we have previously found in mucosal explant sections incubated with TNF-α + IL-1β.

4. Determine if the cytokine combination (TNF-α + IL-1β) increases epithelial permeability in Caco-2 monolayers

    a. If this is found to be the case, test CB agonists to determine if permeability increases can be reversed or reduced

Research aims addressed in publication 2 (Chapter 3)

1. Determine if the cytokine IL-17A can cause mucosal damage in colonic explants by scoring damage to luminal epithelium, crypt epithelium and measuring lymphocyte density.
    a. If mucosal damage occurs, can this be reduced by treatment with cannabinoids (endocannabinoids or phytocannabinoids).
2. If mucosal damage occurs due to IL-17A treatment, determine if increases in matrix metalloprotease (MMP) are involved in mediating this damage
3. Determine if TNF-α + IL-1β treatment of mucosal explants influences expression of IL-17A and if IL-17A is expressed in colonic mucosa under baseline (un-incubated conditions)
4. Test whether IL-17A can influence paracellular permeability in Caco-2 cells

Research aims addressed in Chapter 4

1. Further investigate biochemical changes that may occur in colonic mucosal explants after cytokine (TNF-α + IL-1β or IL-17A) incubation. The purpose of this is to further understand how these cytokines cause damage and to find suitable biomarkers of damage that can be measured in this model.

Chapter 2 : Cannabinoid CB2 Receptor Activation Attenuates Cytokine Evoked Mucosal Damage in a Human Colonic Explant without Changing Epithelial Permeability

Harvey, BS, Nicotra, LL, Vu, M and Smid, SD (2013) 'Cannabinoid CB2 receptor activation attenuates cytokine-evoked mucosal damage in a human colonic explant model without changing epithelial permeability', *Cytokine*, 63(2): 209-217.

The main purpose of this study was to determine if various cannabinoid ligands could reduce mucosal damage parameters (luminal epithelial damage, crypt destruction and lymphocyte density) mediated by TNF-α and IL-1β treatment in a human colonic explant model. Additional aims of this study were to determine if TNF-α and IL-1β could modify nitrite levels (a marker of inflammatory damage) in mucosal explants, determine if lymphocytes proliferate in mucosal explants when treated with TNF-α and IL-1β and establish if TNF-α and IL-1β could modulate epithelial permeability in Caco-2 cells, a human colonic epithelial cell line.

This study builds on our previous work published in Nicotra et al. (2013) where we first showed that TNF-α and IL-1β can induce damage in a human colonic explant model and that the endocannabinoid (anandamide) is protective in this model. In many animal colitis models, activation or up-regulation of the endocannabinoid system is protective and this study extended these findings to human colonic mucosa.

This study showed in terms of scoring of damage parameters, TNF-α and IL-1β induced a significant increase in crypt destruction, luminal epithelial damage and lymphocyte

density. This effect was reduced by the immunosuppressant hydrocortisone, a standard treatment for flares of IBD. We also aimed to determine if proliferation was responsible for the increase in lymphocytes observed. However, TNF-α and IL-1β did not cause increased proliferation of lymphocytes as measured by BrdU immunohistochemistry (IHC). We did show that there was a change in the pattern of BrdU staining, whereby many epithelial cells along the entire crypt length showed a proliferative phenotype under TNF-α and IL-1β treatment compared to control mucosa (treated with vehicle), where only epithelial cells at the base of crypts showed positive BrdU staining. This finding alone may be significant in terms of inflammation or cytokines rendering the epithelium susceptible to genotoxic damage in different scenarios.

Measurement of nitrite concentration showed that TNF-α and IL-1β induced a significant elevation in nitrite levels compared to incubation control, suggesting nitrite precursors (such as nitric oxide) may partially mediate damage induced by cytokines.

The critical findings of the study were that anandamide reduces all parameters of mucosal damage and this effect is reversed (except for lymphocyte density which remained unchanged) by JTE-907, a selective CB2R receptor inverse agonist. Damage parameters were not reversed by ACEA, a selective CB1R agonist which indicates that the protective effect of anandamide is mediated via CB2R. To confirm this finding a CB2R selective agonist, JWH-015 was tested and it reduced all damage parameters which were elevated when mucosa was treated with TNF-α and IL-1β. The ability of JWH-015 to reverse damage parameters was inhibited by JTE-907, providing evidence that activation of the CB2R reduces mucosal damage.

In addition, we investigated whether CB ligands could reduce paracellular epithelial permeability increases induced by TNF-α and IL-1β. However, we found CB ligands,

including anandamide, cannabidiol, ACEA or JWH-015 had no significant effect on restoring epithelial permeability to control levels. It was noted that none of these ligands had any detrimental effects on Caco-2 paracellular permeability when tested on their own.

Western blotting showed a strong expression of CB2R receptors, but not CB1R in colonic mucosa, which may explain why CB2R activation produced an effect on damage parameters. CB receptor expression in Caco-2 cells was also measured to determine if these cells can respond to the CB ligands. Both CB1R and CB2R were found in Caco-2 cells (CB1R to a lesser extent.)

Overall this study provides evidence that CB2R ligands in particular can reduce cytokine-mediated damage in human colonic mucosa. This is significant as it extrapolates the findings of many animal colitis studies to humans. There are other critical cytokines involved in IBD, including interleukin 17 (IL-17) and these cytokines are worthy of investigation in this model. Whether IL-17 can cause damage to human colonic mucosa or alterations in epithelial permeability restored by CB ligands is unknown at present. The subsequent study in Chapter 3 of this thesis explores this question further by examining the effect of IL-17 treatment in the colonic explant model and in Caco-2 epithelial cells.

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Cannabinoid CB2 receptor activation attenuates cytokine-evoked mucosal damage in a human colonic explant model without changing epithelial permeability

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ABSTRACT

Cannabinoid receptor activation is protective in animal colitis models. We sought to investigate if cannabinoids attenuated colitis-like tissue damage in human colonic specimens, with the hypothesis that cannabinoids would be protective in a cytokine-driven model of human colonic mucosal damage. Healthy human colonic mucosa was incubated with pro-inflammatory cytokines TNF-α and IL-1β to elicit colitis-like tissue damage. The cytokine-driven increase in scored crypt and mucosal damage and lymphocyte density was attenuated with concomitant hydrocortisone pretreatment. The cannabinoid receptor 2 (CB2) receptor-selective agonist JWH-015 significantly reduced colitis scores following cytokine incubation, as evidenced by a reduction in mucosal crypt and luminal epithelial damage and lymphocyte density in the lamina propria. The effect of JWH-015 was reversed in the presence of the CB2 receptor inverse agonist JTE-907. Anandamide was also protective in the cytokine-incubated explant colitis model in a manner reversible with JTE-907, while CB1 receptor agonism with ACEA was without effect. TNF-α and IL-1β together evoked an increase in paracellular epithelial permeability in Caco-2 cell monolayers over 48 h of incubation. However, neither CB2 nor CB1 receptor activation altered the cytokine-evoked increase in permeability. These findings support a discrete role for CB2 receptors in the attenuation of detrimental pro-inflammatory cytokine-mediated mucosal damage in the human colon without directly affecting mucosal epithelial barrier function.

1. Introduction

Cannabinoids (CBs) have demonstrated anti-inflammatory effects in the gastrointestinal tract in animal models of colitis [1]. Cannabinoid receptor 1 (CB1) knockout mice develop a significantly worsened form of experimental colitis when compared to wild type mice, indicating an acute requirement of CB1 receptors for protection from colitis [2]. Activation of cannabinoid receptor 2 (CB2) also inhibits both the extent of inflammatory damage and the accelerated gastrointestinal transit that occurs in experimental colitis [3,4]. Endocannabinoids also reduce pro-inflammatory cytokine expression in immune cells and colonic biopsies from inflammatory bowel disease (IBD) patients [5], suggesting a direct immune-modulatory role in this anti-inflammatory effect. However, the cannabinoid pharmacology underlying such an anti-inflammatory action has yet to be clearly established in the human colon, which is relevant when considering the many possible non-cannabinoid receptor-mediated actions of endocannabinoids in the gut [6]. In addition, the role of endocannabinoids in modulating epithelial barrier function and mucosal integrity is uncertain, as endocannabinoids and other cannabinoid ligands may have opposing effects on epithelial permeability *in vitro* [7,8].

In this study we developed a colitis explant model using healthy human colonic mucosal tissue incubated with the major proinflammatory cytokines TNF-α and IL-1β, known to be elevated in IBD with a capacity to induce mucosal chemokine expression in human colonic mucosa [9]. In this setting we measured indices of epithelial and crypt damage and lymphocyte numbers and investigated the effects of anandamide (AEA) and cannabinoid receptor-selective ligands on cytokine-evoked damage. In addition, we investigated whether pro-inflammatory cytokines such as TNF-α and IL-1β may mediate direct effects on epithelial permeability and if so, whether cannabinoid ligands may alter such changes in epithelial barrier function.

2. Materials and methods

2.1. Human colonic tissue collection

Experiments were performed using human colonic tissue taken from patients admitted to the Royal Adelaide Hospital with

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colorectal carcinoma as the primary indication for surgery. Approval was provided by the Royal Adelaide Hospital Human Research Ethics Committee for the study and informed patient consent given in all cases. Full-thickness specimens from descending and sigmoid colon were taken from up to 23 consenting patients (14 male, 9 female). Resections were transferred to pathology services on ice with no more than 30 min elapsing between resection removal and specimen collection. Specimens were placed in ice-cold carbogenated (95% O_2, 5% CO_2) Krebs solution and rapidly transferred on ice to the laboratory for further dissection. Only specimens obtained from macroscopically healthy tissue within the colon were utilised and confirmed via subsequent histological examination as free from neoplasia, fibrosis or inflammation.

2.2. Explant colitis model

Full-thickness colonic specimens were pinned in Sylgard-coated Petri dishes containing carbogenated Krebs solution at 4 °C. Mucosa and submucosa was dissected away from the muscularis propria. Colonic mucosal specimens containing intact submucosa were placed into separate Petri dishes containing 5 mL cell culture medium (RPMI 1640), bovine serum albumin (BSA) (0.01%), penicillin (100 U/mL), and streptomycin (100 μg/mL). Media was perfused with carbogen (95% O_2, 5% CO_2) via manifold inlets into each well. Mucosal specimens were then incubated at 37 °C for 20 h on an orbital mixing platform in a temperature-controlled chamber (OM11, Ratek Instruments, Victoria, Australia), alone or with cytokine and/or drug interventions. At the completion of the incubation period, tissue was immediately placed into neutral buffered formalin for histological processing and assessment and scoring of mucosal damage and lymphocyte numbers. Media was also retained for subsequent measurement of nitrite concentration, a biochemical marker of nitric oxide production accompanying inflammatory states.

2.3. Epithelial permeability measurements

Caco-2 cells (passage 40–70) derived from human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in 75 cm^2 tissue culture flasks (Corning Life Sciences, Lowell, MA, USA) at 37 °C with 5% CO_2. Cell culture media was DMEM (Invitrogen, Mulgrave, VIC, Australia) supplemented with 10% foetal calf serum (Sigma Aldrich, Castle Hill, NSW, Australia), 1% penicillin/streptomycin solution, 1% non-essential amino acids solution and 1% sodium pyruvate (complete DMEM) (Invitrogen, Mulgrave, VIC, Australia).

When cells reached 80% confluence cells were washed in phosphate buffered saline (PBS) pH 7.4 and trypsin EDTA (Invitrogen, Mulgrave, VIC, Australia) was added and incubated with the cells for 5 min to detach cells. Cells were then centrifuged and resuspended in complete DMEM. Experiments were carried out using Corning Costar Transwell Inserts (6.5 mm diameter, 0.4 μm pore size, tissue culture treated polyester membrane) (Corning Life Sciences, Lowell, MA, USA). The basolateral compartment was filled with 0.6 mL of complete DMEM containing 1% amphotericin B (Sigma Aldrich, Castle Hill, NSW, Australia). All subsequent media used in the Transwell plate was supplemented with 1% amphotericin B to avoid fungal contamination. Cells were seeded at a density of 16 500 cells per insert in 0.1 mL of media in the apical compartment. An insert on the plate was left blank and contained media only. The Transwell plate was then incubated at 37 °C and 5% CO_2 for 6 h before having the media in the apical compartment removed and replaced with 0.1 mL of fresh media, to prevent excessive clumping of cells [10]. Media in the apical and basolateral well was replaced every 2–3 days for up to 25 days as the cells reached confluence and differentiated. During the period of cell growth the trans-epithelial electrical resistance (TEER) was measured using the EVOM2 epithelial voltohmeter with chopstick electrode (World Precision Instruments, Sarasota, FL, USA) to determine the integrity of the monolayer. The formula: *TEER Monolayer* (cm^2) = [*TEER total* (Ω) − *TEER blank* (Ω)] × 0.33 (cm^2) was used to calculate TEER. Inserts with TEER values over 500 Ω/cm^2 were used in experiments. Before TEER measurements, media was replaced with complete DMEM without amphotericin B. TEER was measured to obtain baseline TEER values after allowing the plate to equilibrate to room temperature for 5 min.

2.4. Cytokine and cannabinoid treatments

Human colonic mucosal explant samples were incubated with pro-inflammatory cytokines TNF-α and IL-1β (both at 10 ng/mL) for 20 h. ELISA assays for both TNF-α and IL-1β in media following incubation indicated less than 20% loss of cytokines over 20 h. Cytokine-incubated samples were compared against incubation controls consisting of adjacent tissue sections minus cytokine and drug incubations and including vehicle (0.1% ethanol) where appropriate. Incubation controls were also compared with non-incubated mucosal specimens retained in Krebs solution at 4 °C for 20 h, to indicate overall integrity of mucosal sections as they were obtained from resective procedures. Colonic mucosal explant specimens were removed from the incubation media at 20 h post-incubation and then fixed in 10% neutral-buffered formalin solution overnight prior to processing for histological assessment.

Mucosal specimens incubated with cytokines (TNF-α and IL-1β (10 ng/mL), 20 h) were compared with cytokine-incubated mucosa concomitantly treated with the following cannabinoid ligands: the endocannabinoid anandamide (10^{-6} M) or JWH-015 (CB2 receptor-selective agonist, 10^{-7} M) ± JTE-907 (CB2 receptor-selective inverse agonist, 10^{-7} M), or arachidonoyl chloroethylamide (ACEA, CB1 selective agonist, 10^{-7} M). Drug concentrations were chosen based on previous functional studies using human colonic tissue [11]. Additional studies using lipopolysaccharide (LPS, 1 μg/mL) or hydrocortisone (10^{-7} M) were performed to investigate additional potential inflammatory and anti-inflammatory stimuli on cytokine-treated mucosal samples. Appropriate incubation controls (minus cytokines) and drug controls were also performed.

Confluent Caco-2 cells were treated with both human recombinant TNF-α and IL-1β each at a concentration of 100 ng/mL, which produced optimal and significant TEER reductions in preliminary studies when compared to 10 ng/mL cytokine concentrations. All cytokine treatments and vehicle (PBS) were applied to the basolateral compartment of the Transwell. TEER measurements were taken at the time points of 5, 24 and 48 h. The TEER of each well was measured after allowing the temperature of the Transwell plate to equilibrate to room temperature for 5 min. For cannabinoid drug interventions, Caco-2 cells were treated basolaterally with either anandamide (AEA; 10^{-5} M), ACEA (10^{-6} M), JWH-015 (10^{-6} M) or the phytocannabinoid cannabidiol (CBD; 10^{-5} M), utilising concentrations based on previous studies in either colonic tissue [12] or Caco-2 cells [7]. Caco-2 cells were pretreated with CB ligands for 15 min prior to cytokine additions to allow for drug equilibration. Each CB ligand was also tested without cytokines to control for any direct effects on epithelial permeability.

2.5. Assessment of mucosal damage and inflammation

Mucosal damage and lymphocyte numbers in the lamina propria were determined via histological analysis following haematoxylin and eosin staining of paraffin-embedded sections. Scoring was performed by the same assessor in order to maintain consistency and the investigator was blinded to the sample groups.

Explant tissue and non-incubated control samples were examined under light microscopy at low power and scored for crypt destruction, luminal epithelial damage and lymphocyte density in the lamina propria. The scoring parameters and the methods of assessment chosen to evaluate inflammatory damage were adapted from previous methodologies [3,13] and performed within two distinct fields of view; at 10× magnification for luminal epithelial damage, 20× magnification for crypt damage and 40× magnification for lymphocyte counting. Crypt destruction was measured as the average percentage loss of columnar epithelium from within each crypt, based on samples of all crypts within the fields of view. Luminal epithelial damage was assessed as the average percentage loss of luminal epithelial cells from surface epithelial layers (i.e., excluding crypts). Lymphocyte density was determined by the counting of lymphocytes in lamina propria per 20 cells.

Explant media was utilised for the measurement of nitrite concentration, a marker of nitric oxide production in control and cytokine-treated mucosal samples. For samples, 50 μL of media was added to 50 μL of Griess reagent in a microplate incubated at room temperature for 30 min, whereupon absorbance was read at 540 nm in a Synergy MX microplate reader (Biotek Instruments, Bedfordshire, UK). Nitrite concentration was extrapolated from the absorbance standard curve prepared from nitrite ion solution in RPMI-1640 media.

2.6. 5-Bromo-2′-deoxyuridine (BrdU) immunohistochemistry

These experiments were performed on incubation control and cytokine-incubated mucosal samples (conditions as per explant studies), to assess the effects of cytokine treatment on immune cell and epithelial proliferation. Freshly dissected human colonic mucosal tissue was incubated with 40 μg/mL solution of BrdU for 20 h in RPMI media. Samples were then fixed in 10% neutral-buffered formalin, embedded in paraffin and 7 μm sections cut and mounted on silane-coated slides. Sections then underwent antigen retrieval via 3 washes of xylene, slowly rehydration and rinsing in PBS. Slides were immersed in 10 mM citrate buffer (pH 6.0) and microwaved at 800 W for 4 min, then at 160 W for 10 min. Slides were then cooled, washed in PBS and immersed into 2 M HCl for 45 min. Slides were then rinsed in PBS prior to antibody incubation.

For antibody binding, slides were dehydrated via sequential water to alcohol washes, immersed in 3% hydrogen peroxide in methanol, rehydrated and washed in PBS. Slides were then treated with 0.1% Triton-X100 in citrate buffer, washed again in PBS and covered with 50% horse serum in PBS for 30mins. Following rinsing in PBS, sections were incubated with Avidin Biotin Blocking kit as per manufacturer's instructions. Primary BrdU antibody (Abcam ab2284, diluted 1:200) was applied with 5% horse serum in PBS to each section and then incubated at 4 °C for 16 h in a humidified chamber. Vectastain ABC reagent was then applied to each section for 30 min followed by rinsing in PBS. Antibody was visualised with 3,3-diaminobenzidine (DAB) tetrachloride at room temperature for 2 min, washed in distilled water and counter-stained with Lillie Mayer hematoxylin for 2 min.

2.7. Mucosal cannabinoid receptor expression

Western blotting was performed to analyse the presence of cannabinoid receptors in the human colonic mucosa. Fresh non-incubated colonic mucosal tissue was lysed with ice-cold RIPA buffer containing protease inhibitors (Aprotinin 2 μg/mL, Pepstatin A 1 μg/mL, PMSF 1 mM and EDTA 5 mM). Homogenates were stored on ice for 30 min prior to centrifugation at 12 000 rpm. Supernatant protein concentrations were determined by a BCA assay (Thermo Fisher Scientific, Scoresby, VIC, Australia) as per the

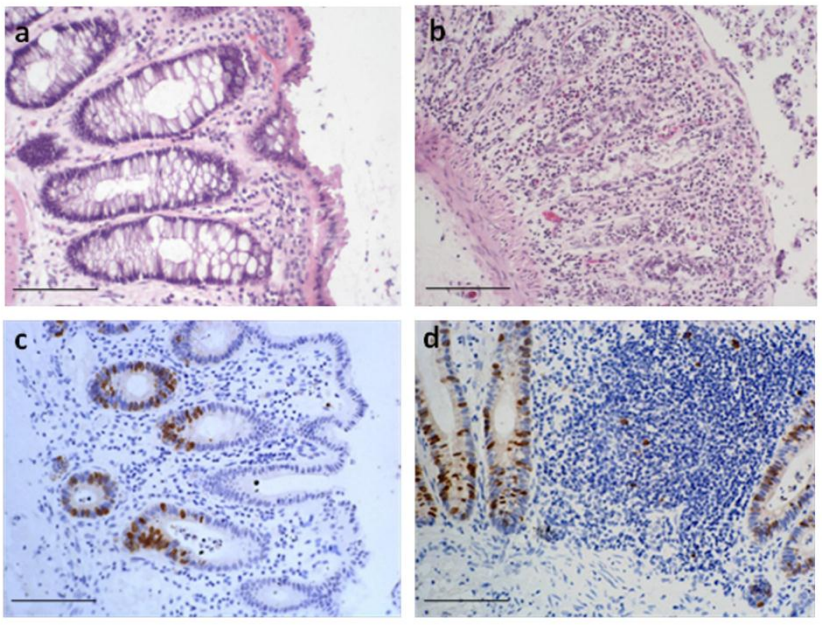

Fig. 1. Top panels: haematoxylin and eosin stained human colonic mucosal explant specimens. (a) Incubation control (no cytokines added) demonstrating intact crypts and luminal epithelium. (b) Pro-inflammatory cytokine TNF-α and IL-1β (10 ng/mL, 20 h)-incubated specimen, demonstrating crypt and luminal epithelial damage and increased lymphocyte numbers in lamina propria. Bottom panels: Proliferative marker (BrdU) staining in human colonic mucosal explant specimens. (c) Incubation control and (d) cytokine TNF-α and IL-1β (10 ng/mL)-incubated. Epithelial cells in crypts took up BrdU along their entire length following cytokine incubation, indicating enhanced proliferative activity compared to the base of germinal crypts in controls. Selected cells in the lamina propria were also BrdU positive, but lymphocytes did not exhibit BrdU labelling. Scale bar, 20 μm.

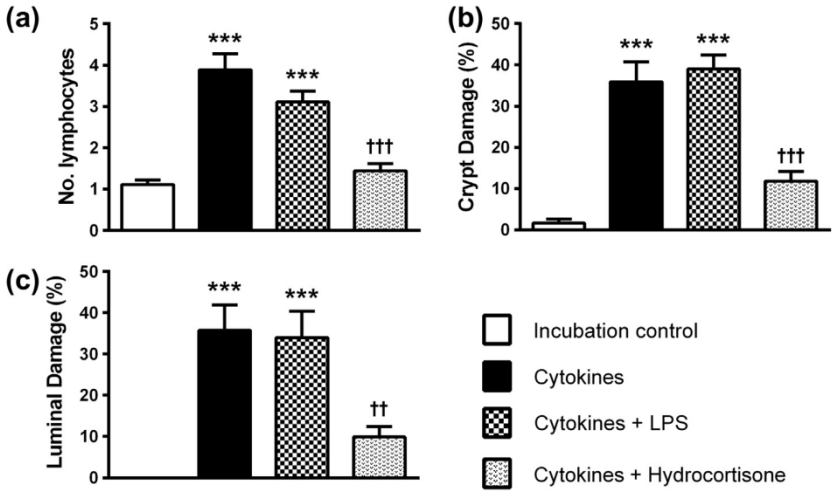

Fig. 2. Scored damage and lymphocyte density in human colonic mucosal explant tissue following cytokine treatment and either lipopolysaccharide (LPS) or hydrocortisone co-incubation. Incubation of human colonic mucosa under tissue culture conditions together with pro-inflammatory cytokines TNF-α and IL-1β (10 ng/mL, 20 h) evokes damage to the luminal and crypt epithelium and increased lymphocyte numbers in the lamina propria (per 20 cells). Concomitant hydrocortisone treatment (10^{-7} M) inhibited cytokine-evoked damage. Lipopolysaccharide (LPS; 1 μg/mL) treatment did not further alter cytokine-evoked damage. $^{***}p < 0.001$ vs. Control; $^{†††}p < 0.001$, $^{††}p < 0.01$ vs. cytokines; $n = 9$.

manufacturer's directions and supernatants stored at −70 °C until required.

Samples were prepared by mixing with 2× Laemmli's sample buffer, placed in a boiling water bath for 3 min, and cooled on ice. Protein samples (20 μg) were then loaded onto precast mini-gels (Mini-Protean anykD, Bio-Rad, CA, USA) and run at 200 V. Proteins were then transferred to a nitrocellulose membrane in Towbin blotting buffer (25 mM Tris-base, 192 mM glycine, 20% methanol) at 100 V for 70 min. Afterwards, the membrane was blocked in 5% filtered skim milk powder in Tris buffered saline with 0.05% Tween 20 (TBST) for 1 h. The primary antibody used was the anti-cannabinoid receptor CB1 (1–77) rabbit anti-human pAb (Calbiochem: 209550 at 1:1000) or the rabbit anti-human anti-cannabinoid receptor 2 antibody (Abcam; ab3561 at 1:1000). The membranes were incubated with their respective antibody over night at 4 °C under agitation, washed repeatedly with TBST followed by incubation with appropriate secondary antibody (Dylight 800 IgG) with a dilution of 1:10 000 in TBST for 1 h at room temperature. The membrane was washed repeatedly with TBST, dried, and directly visualised under 800 nm using a LiCor Odyssey Imaging System (Li-Cor Biosciences, Cambridge, UK).

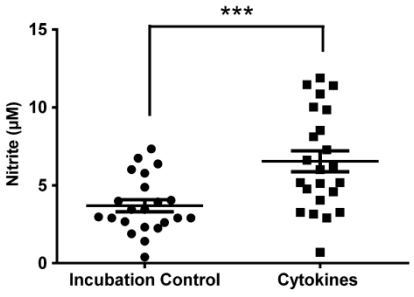

Fig. 3. Nitrite concentrations (μM) in explant media from incubation control and cytokine-treated mucosal tissue (TNF-α and IL-1β, 10 ng/mL, 20 h). Pro-inflammatory cytokine treatment resulted in a significant elevation in mucosal nitrite production. $^{***}p < 0.001$; $n = 23$.

2.8. Chemicals

All chemicals were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia) unless otherwise stated. Human recombinant TNF-α and IL-1β were obtained from Symansis Cytokines (Timaru, NZ). Arachidonyl-2′-chloroethylamide (ACEA) and JTE-907 were obtained from Tocris Cookson Ltd. (Bristol, UK). Cannabidiol was provided by Cayman Chemicals (Ann Arbor, MI, USA). Anti-BrdU primary antibody (ab2284) and rabbit anti-human anti-cannabinoid receptor 2 antibody (ab3561) were sourced from Abcam (c/o Sapphire Biosciences, Waterloo NSW, Australia). The anti-cannabinoid receptor CB1 (1–77) rabbit anti-human primary antibody was sourced from Calbiochem (c/o Merck Pty Ltd., Kilsyth, Victoria). Avidin–Biotin kits and Vectastain were obtained from Vector Laboratories (Burlingame, CA, USA). DAB-Plus Reagent Set was obtained from Invitrogen (Mulgrave, VIC, Australia).

2.9. Statistical analysis

Scoring indices for control, cytokine and/or endocannabinoid or cannabinoid-treated explant mucosal tissue and TEER values were analysed using repeated measures analysis of variance (ANOVA) and Bonferroni's post hoc test. A student's *t*-test was used for the analysis of nitrite concentrations in control and cytokine-treated explant media. A p value obtained for all data of $\leqslant 0.05$ was considered significant. Analysis and production of graphs was performed in GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Characterisation of mucosal damage following cytokine incubation and effects of cannabinoids in human colonic explants

Pro-inflammatory cytokine incubation in healthy human colonic mucosal and submucosal specimens *ex vivo* induced a course of tissue damage resembling some features of colitis, such as mucosal sloughing and epithelial denudation, crypt loss and increased lymphocyte numbers in the lamina propria (Fig. 1b) vs.

incubation control explant specimens (Fig. 1a). This was reflected in the group data by a significant increase in lamina propria lymphocyte number (Fig. 2a: 3.9 ± 0.4 vs. 1.1 ± 0.3), crypt epithelial damage (Fig. 2b: 35.9 ± 4.8% vs. 1.67 ± 0.9%) and mucosal luminal damage (Fig. 2c: 35.8 ± 6.2% vs. 0%) when compared to incubation controls ($n = 9$). Such damage was also associated with a corresponding increase in nitrite production from sampled media of cytokine-treated explant tissue when compared to incubation control (Fig. 3: 6.5 ± 0.6 μM vs. 3.7 ± 0.4 μM; $n = 23$). The increase in scored mucosal damage and lymphocytes was reduced following hydrocortisone (10^{-7} M) co-incubation, where lymphocyte number (Fig. 2a: 1.4 ± 0.2 vs. 3.9 ± 0.4), crypt damage (Fig. 2b: 11.8 ± 2.4% vs. 35.9 ± 4.9%) and luminal epithelium damage (Fig. 2c: 9.9 ± 2.5% vs. 35.8 ± 6.2%) was significantly reduced vs. incubation control ($n = 9$). Lipopolysaccharide (LPS; 1 μg/mL) together with cytokine incubation did not further affect lymphocyte number and mucosal damage scores compared to cytokine incubation alone (Fig. 2a–c: lymphocytes: 3.1 ± 0.3 vs. 3.9 ± 0.4, crypt damage: 39.0 ± 3.4% vs. 35.9 ± 4.8%, luminal damage: 34.0 ± 6.4% vs. 35.8 ± 6.2%; $n = 9$). Additional representative histological images of hydrocortisone and LPS-treated, in addition to cannabinoid-treated mucosal explant specimens are also included (Fig. 1, Supplementary data).

BrdU staining was employed to determine whether increased lamina propria lymphocyte numbers could be accounted for via enhanced cell proliferation. In incubation control mucosal explants, BrdU staining was localised to proliferative germinal zones in crypts, with negligible BrdU uptake in lamina propria cells or luminal enterocytes (Fig. 1c). By contrast, cytokine-incubated colonic mucosa crypt BrdU staining often extended along the length of crypts where epithelium remained intact (Fig. 1d). However, in lymphocyte clusters within the lamina propria there was no BrdU staining, indicating a lack of proliferation in the presence of TNF-α and IL-1β (Fig. 1d). Cells within the lamina propria that displayed positive BrdU staining were not positively identified, but were excluded as lymphocytes based on morphological characteristics.

Cytokine-evoked tissue damage was also significantly reduced in the presence of the major endocannabinoid anandamide (AEA) (Fig. 4a–c: lymphocytes: 1.5 ± 0.2 vs. 3.8 ± 0.3; crypt damage: 14.5 ± 3.6% vs. 59.4 ± 6.2%; luminal damage: 13.8 ± 3.6% vs. 59.2 ± 6.4% vs. cytokines; $n = 8$). The protective effects of anandamide on luminal and crypt epithelium were significantly reversed in the presence of the CB2 receptor inverse agonist JTE-907; however lymphocyte numbers in the lamina propria remained fewer (Fig. 4a–c: lymphocytes: 2.0 ± 0.2 vs. 1.5 ± 0.2; crypt damage: 59.3 ± 10.1% vs. 14.5 ± 3.6%; luminal damage: 35.4 ± 4.5% vs. 13.8 ± 3.6% vs. cytokines + AEA; $n = 8$). This indicates that a substantive part of the mucosal protective effects of anandamide in the explant colitis model occur through activation of the CB2 receptor, but that the anandamide-mediated suppression of lymphocyte movement or aggregation may be CB2-independent. In contrast, the CB1 receptor-selective agonist ACEA did not significantly alter lymphocyte density (Fig. 4a) or cytokine-evoked mucosal damage (Fig. 4b and c). Incubation of explant mucosal tissue with cannabinoid agonists AEA or ACEA alone (minus cytokines) did not alter mucosal damage scores or lymphocyte numbers when compared to incubation control (data not shown).

The CB2 receptor-mediated protective activity was further supported via the actions of the CB2 receptor-selective agonist JWH-015, which reduced the cytokine-evoked increase in luminal mucosal and crypt damage scores together with lamina propria lymphocyte density (Fig. 5a–c: lymphocytes: 1.5 ± 0.2 vs. 3.8 ± 0.3; crypt damage: 12.5 ± 3.3% vs. 37.7 ± 4.7%; luminal damage: 12.0 ± 3.2% vs. 32.5 ± 6.8% vs. cytokines; $n = 7$). The protective effect of JWH-015 also occurred in a reversible manner, as it was negated in the presence of the CB2 receptor-selective inverse agonist JTE-907 (Fig. 5a–c: lymphocytes: 3.3 ± 0.2 vs. 1.5 ± 0.2; crypt damage: 32.2 ± 6.1% vs. 12.5 ± 3.3%; luminal damage: 31.3 ± 6.9% vs. 12.0 ± 3.2%, JTE-907 + JWH-015 vs. JWH-015; $n = 7$). JWH-015 in the absence of cytokines did not alter colitis scores (not shown) and there was no direct pro-inflammatory or otherwise damaging effect of JTE-907 in the explant mucosa in the absence of cytokines (Fig. 5a–c).

3.2. Cannabinoid effects on cytokine induced changes in epithelial permeability

A combination of TNF-α and IL-1β evoked a time-dependent increase in Caco-2 monolayer permeability, as demonstrated by a significant overall decrease in trans-epithelial resistance (TEER) over 48 h (Fig. 6a–b: 901.0 ± 98.3 Ω/cm^2 at 0 h vs. 690.6 ± 50.5 Ω/cm^2 at 48 h; $n = 4$). Addition of anandamide (AEA; 10^{-5} M) did

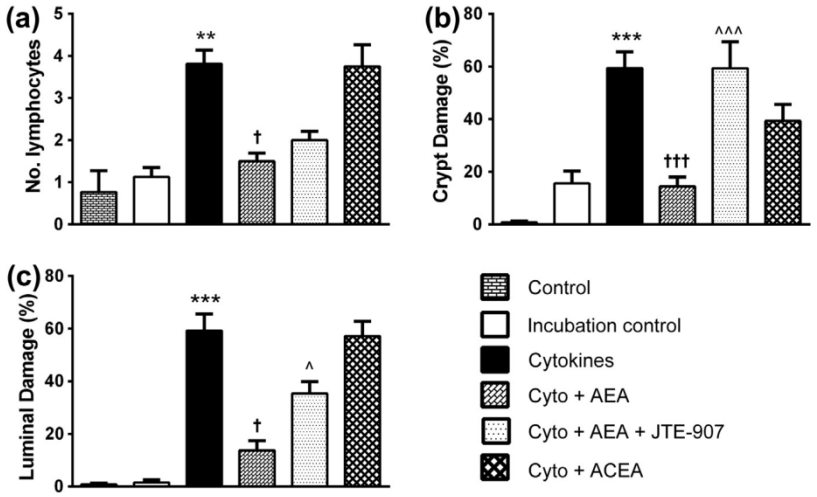

Fig. 4. The anandamide (AEA; 10^{-6} M)-mediated reduction in (b) mucosal crypt and (c) luminal epithelial damage in cytokine-treated human colonic mucosal explant tissue was significantly reversed in the presence of the CB2 receptor inverse agonist JTE-907 (10^{-6} M). However the anandamide-mediated reduction in (a) lymphocyte numbers was not reversed by JTE-907. The CB1 receptor-selective agonist (ACEA; 10^{-6} M) did not significantly alter cytokine-evoked colitis scores. $n = 8$. Cyto = Cytokines. $^{***}p < 0.001$, $^{**}p < 0.01$ vs. Incubation control, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger}p < 0.05$ vs. Cytokines, $^{\wedge\wedge\wedge}p < 0.001$, $^{\wedge}p < 0.05$ vs. Cyto + AEA.

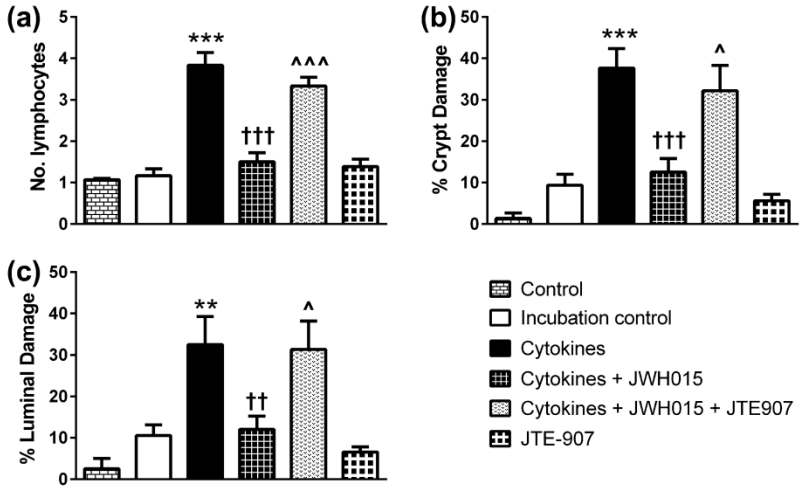

Fig. 5. The CB_2 receptor agonist JWH-015 (10^{-7} M) decreased (a) lymphocyte number (per 20 cells), (b) crypt and (c) luminal epithelial damage in cytokine-incubated human colonic mucosal explants. This was reversed in the presence of JTE-907 (CB_2 receptor inverse agonist; 10^{-6} M). JTE-907 in the absence of cytokines had no effect on mucosal scored damage or lymphocyte numbers. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Incubation control; $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger}p < 0.01$ vs. Cytokines; $\wedge\wedge\wedge p < 0.001$, $\wedge p < 0.05$ vs. Cyto + JWH-015. Cyto = Cytokines. $n = 7$.

not reverse the cytokine-induced increase in permeability (Fig. 6a: 733.5 ± 37.2 Ω/cm^2 vs. 690.6 ± 50.5 Ω/cm^2 at 48 h; $n = 4$). When anandamide was tested alone, it did not have any influence on paracellular permeability compared to the vehicle control (Fig. 6a). There was also no significant change in permeability induced by the phytocannabinoid cannabidiol (CBD; 10^{-6} M), when compared to either vehicle controls or cytokine treatment (Fig. 6b: 740.5 ± 24.5 Ω/cm^2 vs. cytokines 690.6 ± 50.5 Ω/cm^2 at 48 h; $n = 4$). While CBD treatment of Caco-2 cells resulted in a trend towards slightly higher TEER values against both vehicle and cytokine treatment groups, these were not significantly different (Fig. 6b).

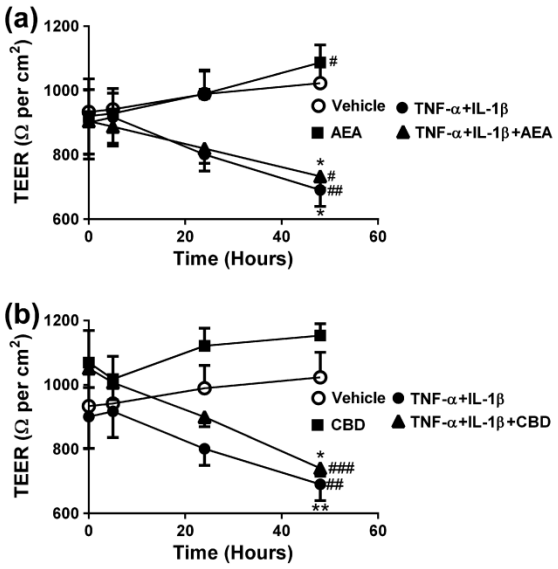

Fig. 6. Effects of (a) the endocannabinoid, anandamide (AEA), or (b) the phytocannabinoid, cannabidiol (CBD) on cytokine-evoked trans-epithelial electrical resistance (TEER) reductions as a measure of Caco-2 paracellular permeability over 48 h. (a) Treatment with AEA (10^{-5} M) did not alter the decrease in TEER due to combined treatment with 100 ng/mL TNF-α and IL-1β. AEA alone did not cause any significant change in TEER compared to vehicle. (b) Treatment with CBD (10^{-6} M) did not alter the decrease in TEER due to combined treatment with 100 ng/mL TNF-α and IL-1β. CBD alone did not cause any significant change in TEER compared to vehicle. $n = 4$ independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. vehicle; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. time 0.

To determine if activation of CB1 or CB2 receptors plays a role in modulating colonic epithelial permeability, the receptor-selective agonists ACEA (CB1 agonist >1400 fold selectivity over CB2) and JWH-015 (CB2 receptor-selective agonist) were used in the presence of TNF-α and IL-1β in Caco-2 monolayers over 48 h of incubation. ACEA (10^{-6} M) had no effect on the decrease in TEER induced by cytokines (Fig. 7a; 699.2 ± 27.2% vs. 690.6 ± 50.5 Ω/cm^2 at 48 h; $n = 4$) and no significant effect on TEER in the absence of cytokines (vs. vehicle). JWH-015 (10^{-6} M) similarly had no effect on cytokine-induced changes in paracellular permeability (Fig. 7b; 678.6 ± 43.6% vs. cytokines 690.6 ± 50.5 Ω/cm^2 at 48 h; $n = 4$) in addition to no significant effect on permeability when tested alone.

3.3. Cannabinoid receptor expression in human colonic tissue and Caco-2 cells

Western blotting for the CB1 receptor revealed a faint 65 kDa band in the human mucosa and a distinct band of expression in Caco-2 cells, with prominent immunoreactivity (IR) in human colonic muscularis (Fig 8a). CB2 receptor immunoreactivity revealed two dense bands in the 50–70 kDa region in both human colonic muscularis and mucosal tissue (Fig. 8b). There was comparatively a much higher expression of CB2-IR in the colonic mucosa than CB1-IR. In addition, faint bands of CB2 receptor immunoreactivity were observed in Caco-2 cells (Fig. 8b).

4. Discussion and conclusions

Results indicate that proinflammatory cytokine treatment in healthy human mucosal specimens generated mucosal damage and lymphocyte infiltration in the lamina propria in a form resembling acute colitis in some of its histological features. This corresponded to an increase in nitrite production in the explant media, which has previously been shown to be indicative of active inflammation in human gastrointestinal mucosal explant

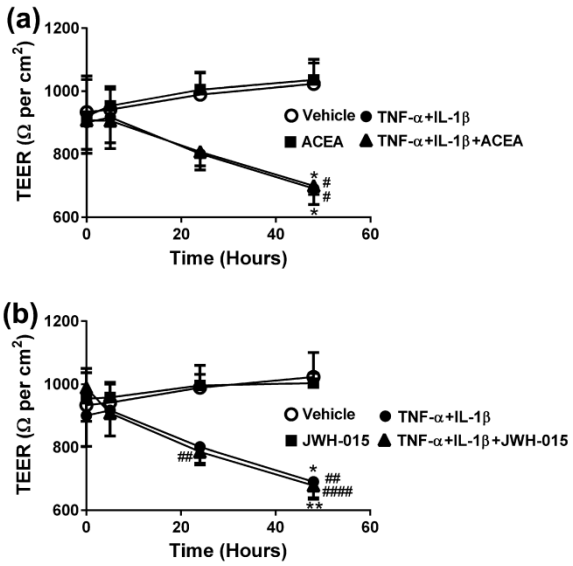

Fig. 7. Effect of (a) the selective CB1 agonist, arachidonyl-2′-chloroethylamide (ACEA) or (b) the selective CB2 receptor-selective agonist, JWH-015 on cytokine-evoked trans-epithelial electrical resistance (TEER) reductions as a measure of Caco-2 paracellular permeability over 48 h. (a) Treatment with ACEA (10^{-6} M) did not reduce the decrease in TEER from combined treatment with 100 ng/mL TNF-α and IL-1β. ACEA alone did not cause any significant change in TEER compared to vehicle. (b) Treatment with JWH-015 (10^{-6} M) did not alter the decrease in TEER due to combined treatment with 100 ng/mL TNF-α and IL-1β. JWH-015 alone did not cause any significant change in TEER compared to vehicle. $n = 4$ independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. vehicle; $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ vs. time 0.

specimens [14]. Cytokines such as TNF-α produced by T-cells and intraepithelial lymphocytes stimulate immune cell chemotaxis, increase epithelial permeability and stimulate epithelial chemokine secretion [15,16]. Our results indicate proliferation does not account for the increased lymphocyte number, suggesting migration from extensive lymphoid aggregates in the human colon may be responsible. We find these abundant in normal human submucosal tissue which is retained in the explant specimens. The precise role of lymphocytes in the cytokine-evoked damage is not yet defined, but their involvement was inferred through the suppression of colitis following hydrocortisone co-incubation. Corticosteroids directly suppress T cell activation but also reduce pro-inflammatory cytokines and chemotactic factors arising from the epithelium [17,18], so their influence may be pleitropic in the mucosa. Interestingly, increased BrdU labelling occurred in the mature crypt epithelium following cytokine treatment, possibly indicative of altered replication signalling preceding apoptosis or necrosis, or an early or overlaying repair response to the initiating stages of tissue damage. This is concordant with a previous study demonstrating TNF-α initiated mitogenesis in human duodenal mucosal explant tissue [19].

The major findings in the present study show that the endocannabinoid anandamide decreased both the cytokine-evoked tissue damage and ensuing increased lymphocyte numbers in the human explant mucosa. The crypt epithelial integrity afforded by anandamide was lost in the presence of the CB2 receptor inverse agonist JTE-907, indicating a component of mucosal protection occurring via anandamide arising from actions at the CB2 receptor. However, luminal epithelial integrity was only partially restored and lymphocyte infiltration unaltered following CB2 receptor antagonism, suggesting potentially other pathways of endocannabinoid-mediated protection. This is unlikely to be mediated by CB1 receptor activity of anandamide, as the CB1 receptor-selective specific agonist ACEA did not inhibit any of the cytokine-evoked changes in mucosal structure or lymphocyte density. In addition, separate studies in our laboratory suggest that CB1 receptor blockade was unable to prevent the reductions in scored mucosal damage and lymphocyte number arising from anandamide co-incubation (unpublished results). In addition to the comparatively low expression of CB1 receptor in the human mucosal tissue, these findings collectively indicate negligible input for CB1 receptors in providing mucosal protection in this setting. Additional pathways for protection from anandamide may possibly lie in diverging metabolic pathways or non-cannabinoid receptor activity for this endocannabinoid. This includes COX-2 metabolites such as prostaglandin ethanolamides, which may have protective or restorative roles in human gastrointestinal tract [6]. Relative to CB1 receptors, CB2 receptor expression was also comparatively much stronger than the CB1 receptor in human colonic mucosal tissue, supporting a more prominent role for CB2 receptors in mucosal protection. The presence of multiple bands of CB2 receptor immunoreactivity has been previously shown in brain and spleen in regions corresponding to our banding in colon tissue in the 50–70 kDa range [20], and most likely represents post-translational modifications of the native receptor.

These findings also underpin studies in clinically relevant settings such as in IBD-affected mucosa, where an anandamide analogue was found to inhibit the elevated secretion of proinflammatory cytokines IFN-γ and TNF-α from explant biopsies [5]. While anandamide displays a partial agonist efficacy at CB2 receptors, *in vitro* studies using high concentrations have found this sufficient to demonstrate a direct effect of anandamide on suppressing human T cell chemotaxis and proliferation via a CB2 receptor-mediated action [21]. In humans, CB2 receptors are found in highest numbers in B lymphocytes and NK cells, although they can variably be found on neutrophils and monocytes [22]. CB2 receptor activation has been shown to suppress human T-cell and monocyte migration [23,24] and may directly suppress lymphocyte activity through the enhanced auto-production of TGF-β [25]. A direct role for CB2 receptors in human explant colitis was confirmed via the reversible reduction in colitis scores using a CB2 receptor-selective agonist JWH-015. The CB2 receptor has shown a emerging role in modulation of gastrointestinal disorders [26] and CB2 receptor expression is elevated in the ulcerative colitis-affected bowel [27]. CB2 receptor activation has also been shown to be effective

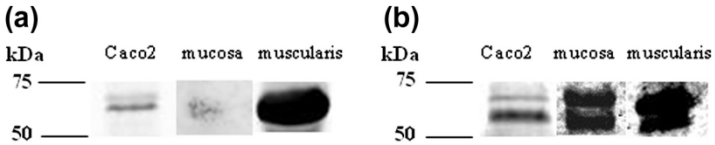

Fig. 8. Western blot labelling of (a) CB1 and (b) CB2 receptor immunoreactivity from human mucosal and muscularis colonic homogenates, in addition to Caco-2 epithelial lysates. Specific labelling occurred in the 65 kDa region corresponding to the CB1 receptor in Caco-2 cells, with faint expression in human mucosal epithelium but prominent muscularis labelling. CB2 receptor labelling indicated multiple dense banding in the 55 kDa and 60 kDa region prominent in human colonic muscularis and mucosal tissue, in addition to labelling in Caco-2 cells. Protein loading per well = 20 μg.

in reducing the extent and severity of colitis in animal models [3,28], and the findings of this study now extend this property into human colonic tissue. In this regard, pharmacological targeting of the CB2 receptor may provide a selective therapeutic modality for the treatment of colitis, especially where CB2 receptors are upregulated in IBD [27]. However, CB2 receptor expression occurs in other tissues and immune cells including microglia, and CB2 receptor activation alters a range of immune cell functions and nociceptive signalling. Further developments are therefore needed in addressing the selectivity and off-target actions of CB2 ligands before the therapeutic potential of this receptor in colitis can be reasonably evaluated [29].

To explore in isolation the functional effects of cytokines on the epithelium and to dissect out possible pathways for cannabinoid-based protective effects in mucosal tissue, the Caco-2 intestinal epithelial cell line was used. The combination of pro-inflammatory cytokines TNF-α and IL-1β directly inhibited epithelial resistance over 48 h of incubation in Caco-2 cells. However, neither anandamide nor the CB1 and CB2 receptor-selective cannabinoid ligands altered the effects of cytokines on epithelial permeability. Additional studies with the phytocannabinoid cannabidiol also failed to reveal an influence of this compound on permeability. Cannabidiol has unusual pharmacology in that it is largely not active at conventional cannabinoid receptors but has a range of non-cannabinoid and other non-receptor mediated actions [30], including antioxidant and PPAR-γ activity in colitis models [31,32]. However we found it lacked any protective effects on epithelial permeability.

These results together with the findings from the explant studies suggest the cannabinoids attenuate human colonic mucosal damage without affecting permeability directly. Previous studies using either chemical agents or cytokines have shown variable effects of endocannabinoids on epithelial permeability changes *in vitro*, dependent on the type of cannabinoid ligand used (endocannabinoid or phytocannabinoid) and whether application to Caco-2 cells was apical or basolateral [7,8]. In the latter study, apical application of phytocannabinoids significantly inhibited permeability reductions caused by cytokine application, whereas endocannabinoid application actually worsened the enhanced permeability evoked by cytokines [8]. We utilised basolateral application in the present study, however we did not see any protection against permeability changes from anandamide, CB1 or CB2 receptor selective ligands or cannabidiol, similarly to the previous study [8]. This occurred in spite of the demonstrated expression of both CB receptor subtypes receptors in the Caco-2 cell line. Our findings are concordant with studies showing no direct effects of non-selective cannabinoid receptor agonists on mucosal short circuit current [33,34] and collectively suggest that cannabinoid receptors do not modify mucosal permeability directly, although studies utilising human mucosal strips to measure permeability, such as Ussing chamber studies, are warranted to test this assertion conclusively. The gastrointestinal epithelium can secrete many cytokines, chemokines and lymphocyte chemo-attractants which act to facilitate inflammation [35,36], so it is more likely that CB2 receptors modulate this action without altering epithelial permeability directly. For example, interleukin 8 secretion from human colonic epithelial cells is inhibited via a CB2 receptor-mediated pathway [37], indicating that epithelial cannabinoid receptors act to limit the inflammatory cascade though which barrier function may subsequently be perturbed.

In conclusion, pro-inflammatory cytokines evoke a form of colitis-like damage in human colonic explant tissue that is ameliorated primarily by CB2 receptor activation. The major endogenous cannabinoid anandamide also mediates a protective effect in-part via this pathway. In the absence of a direct functional role on epithelial permeability, such protection may occur from modulation of immune cell activation or immune cell chemotaxis. These results highlight a role for CB2 receptors in affording mucosal protection against the damaging effects of proinflammatory cytokines in the human colon.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.04.032.

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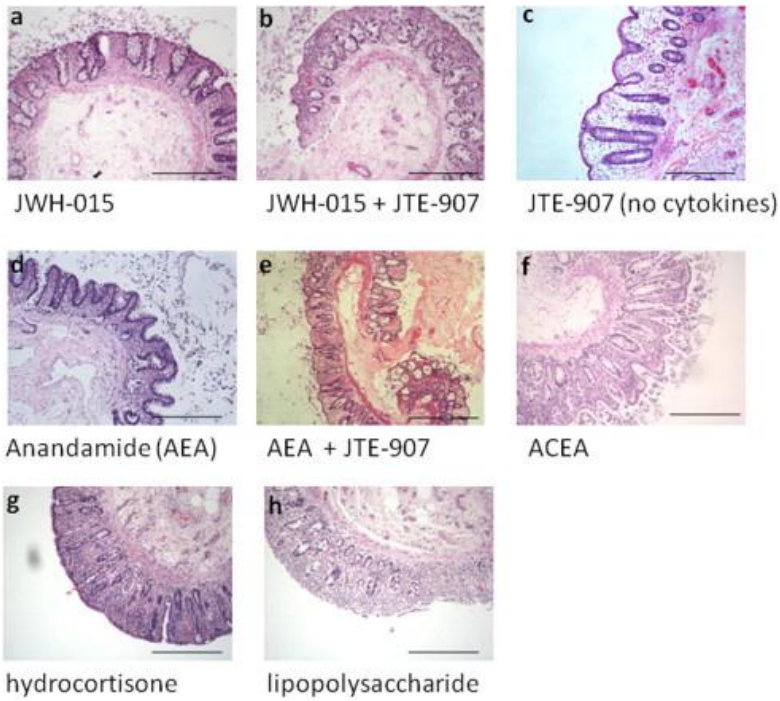
2.1 Publication 1: Appendix A. Supplementary Material

Supplementary figure available online at: http://dx.doi.org/10.1016/j.cyto.2013.04.032



Supplementary Figure S1.

Representative histological images of human colonic mucosal specimens following cytokine and drug incubations. All specimens except for (c) incubated in TNF-α and IL-1β (10 ng/mL, 20 h): (a) CB2 receptor agonist JWH-015 (10^{-7} M); (b) JWH-015 + CB2 receptor inverse agonist JTE-907 (10^{-6} M); (c) JTE-907 (minus cytokines); (d) anandamide (AEA; 10^{-6} M); (e) AEA + JTE-907 (10^{-6} M); (f) CB1 receptor agonist ACEA (10^{-7} M); (g) hydrocortisone (10^{-7}M); (h) lipopolysaccharide (1 μg/mL). Scale bars *a*, *b*, *d*, *e* = 100 μm; *c*, *f*, *g*, *h* = 50 μm.

Chapter 3 : Interleukin 17A Evoked Mucosal Damage is Attenuated by Cannabidiol and Anandamide in a Human Colonic Explant Model

Harvey, BS, Sia, TC, Wattchow, DA and Smid, SD (2014) 'Interleukin 17A evoked mucosal damage is attenuated by cannabidiol and anandamide in a human colonic explant model', *Cytokine*, 65(2): 236-244.

This series of experiments was conducted to continue from the previous chapter, where the effect of cytokines, TNF-α and IL-1β and cannabinoid ligands were studied in explant mucosa. In this study, the effects of IL-17A on mucosal damage parameters including luminal epithelial damage, crypt destruction and lymphocyte density was examined. In addition, the endocannabinoid, anandamide (AEA) and the phytocannabinoid, cannabidiol (CBD) were tested to determine any protective effect against IL-17A induced mucosal damage. In addition, to determine a biochemical mechanism of action for IL-17A induced damage, matrix metalloprotease (MMP) activity was measured in IL-17A and control treated mucosal explants.

Previous studies have demonstrated that increases in TNF-α and IL-1β levels in colitis could be linked with an increase in IL-17A expression. To determine if in the mucosal explants, TNF-α and IL-1β cause changes in IL-17A levels, western blotting, ELISA and immunohistochemistry (IHC) was used to measure IL-17A expression. Finally, as we have previously shown in Chapter 2, TNF-α and IL-1β can cause increases in Caco-2 epithelial

permeability; we therefore tested if IL-17A also has similar effects on epithelial permeability as other pro-inflammatory cytokines.

The first major finding of this study was that IL-17A caused a course of mucosal damage similar to that seen with TNF-α and IL-1β treatment. This damage included luminal epithelial loss and crypt destruction, but not an increase in lymphocyte density. In addition, treatment with AEA, CBD or hydrocortisone was able to reverse luminal epithelial damage and crypt destruction (although AEA did not affect crypt destruction).

Treatment with IL-17A also caused increased MMP enzyme activity, suggesting a possible mechanism for facilitating the damage produced by IL-17A. Incubation with TNF-α and IL-1β did not show any significant change in IL-17A expression as measured by ELISA, western blotting or IHC. We did find, however that IL-17A was expressed extensively in human colonic mucosa under basal conditions. This expression was particularly evident in the luminal epithelium and in a range of various cell types (possibly macrophages) found in the lamina propria. As the antibody we used had not been tested in this application previously, we performed a neutralisation control, whereby the IL-17A antibody was combined with recombinant IL-17A, thereby neutralising activity. This study showed the antibody was fairly specific for IL-17A, but could possibly be binding to other IL-17 isoforms. To explore this further we also used an IL-17C antibody in mucosal explant sections to localise the expression of this cytokine. We found extensive expression (similar to that of IL-17A) in the luminal epithelium and on a range of cell types, but noticeable differences in the pattern of expression in the lamina propria that suggested the antibodies were discrete and therefore relatively selective.

In epithelial permeability experiments, IL-17A treatment of Caco-2 cells did not cause any significant changes in permeability as measured by TEER. Two concentrations of IL-17A

(10 and 100 ng/mL) were tested, but neither caused any changes in TEER. We did find however by western blotting that Caco-2 cells do express IL-17A, and that this expression is not affected by prior TNF-α and IL-1β treatment.

In conclusion, this study provides evidence that IL-17A, although expressed endogenously in colonic mucosa, has the ability to cause damage to the mucosa. The localisation of IL-17A (by IHC) to the mucosal epithelium is a novel finding. In addition, IL-17C is expressed extensively in colonic mucosa. In clinical colitis, this balance of IL-17A and IL-17C expression could be perturbed, leading to inflammation and mucosal damage. In addition, we showed AEA and CBD can reduce indices of mucosal damage. This compares with the beneficial effect of cannabinoid ligands on TNF-α and IL-1β induced damage (detailed in Chapter 2 of this thesis). No significant changes in IL-17A expression following TNF-α or IL-1β treatment appear to occur in this model and IL-17A does not appear to affect epithelial permeability.

In the next chapter of this thesis, further mechanistic studies in mucosal explants will be described. The previous studies in this thesis have been largely observational, therefore the next step is to characterise biochemical and other changes (such as identification of immune cell types) that may occur in the explant model after cytokine incubation. Revealing these changes may assist in explaining how cytokines, including IL-17A, TNF-α and IL-1β cause mucosal damage in human tissue.

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Interleukin 17A evoked mucosal damage is attenuated by cannabidiol and anandamide in a human colonic explant model

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ABSTRACT

Interleukin 17A (IL-17A) is a cytokine linked to inflammatory bowel disease. We investigated IL-17A expression in human colonic mucosa, whether IL-17A can elicit colonic mucosal damage in a human explant model and modulate gastrointestinal epithelial permeability in cell culture. We also tested if select cannabinoid ligands, shown to be protective in colitis models could attenuate damage caused by IL-17A. In addition, the ability of pro-inflammatory cytokines TNF-α and IL-1β to modulate levels of IL-17A in the explant colitis model was also explored. IL-17A incubation caused significant mucosal epithelial and crypt damage which were attenuated following hydrocortisone treatment, and also reduced following anandamide or cannabidiol incubation. IL-17A-evoked mucosal damage was also associated with an increase in matrix metalloprotease activity. However, IL-17A did not induce any significant changes in epithelial permeability in confluent Caco-2 cell monolayers over a 48 h incubation period. IL-17A was located predominantly in human mucosal epithelium together with IL-17C, but both IL-17A and IL-17C were also expressed in the lamina propria and submucosa. Incubation of human colonic mucosal tissue or Caco-2 cells with pro-inflammatory cytokines TNF-α and IL-1β however did not alter IL-17A expression. These results indicate IL-17A has a widespread distribution in the human colon and the capacity to elicit mucosal damage which can be attenuated by cannabinoid ligands.

1. Introduction

Inflammatory bowel disease (IBD) encompassing both Crohn's disease (CD) and ulcerative colitis (UC) share common features of inflammation and ulceration of the gastrointestinal mucosa [1]. The incidence of CD particularly in western developed countries is increasing [2,3]. In IBD, damage to the intestinal epithelial barrier allows luminal antigens to enter the underlying lamina propria (LP) and initiate or facilitate further inflammation [4–6]. In CD, pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1β are present in the gut [7] and elicit increased epithelial paracellular permeability [8–10] and mucosal damage [11–13].

One of the critical cytokines more recently linked with IBD pathogenesis and expression is interleukin 17 A (IL-17A). IL-17A is produced by a subclass of T-helper cells known as Th17 cells, located at high density at mucosal surfaces where they play a role in protecting the mucosa from infection [14]. IL-17A can also be produced from a range of other immune cells including natural killer cells, macrophages and neutrophils [15,16]. Elevated expression of IL-17A has been observed in both CD and UC patient colonic mucosal tissue and in the serum of CD patients [17,18]. However, the precise functional role of IL-17A in the mucosal inflammatory response is not clear. Colonic epithelial IL-17A expression can initiate neutrophil chemotaxis [19], while inflammatory cytokines such as TNF-α and IL-1β and inflammatory mediators such as PGE_2 can further augment IL-17A expression and activity, providing a potentially reinforcing stimulus for colitis [20–22]. However, IL-17A also has demonstrated protective and restorative roles in the gastrointestinal tract, down-regulating the expression of inflammatory mediators and bolstering epithelial barrier function [23–25]. The direct effects of IL-17A on human colonic mucosal integrity have not yet been explored.

Additional members of the IL-17 family of cytokines include IL-17C and IL-17F. IL-7C is produced primarily by epithelial cells and may have a role in immune response to bacteria [26]. In a mouse colitis model, lack of IL-17C expression was shown to be detrimental, suggesting IL-17C may play a protective role in the colon [27]. In humans, elevated IL-17C gene expression is found in UC but not CD patients [28], so whether IL-17C plays a pathological or protective role in colitis is unclear. IL-17F can form a heterodimer with IL-17A and it has been suggested that IL-17F has a similar

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role to IL-17A in inflammation [29]. In animal colitis studies, neutralisation of both IL-17A and IL-17F simultaneously was found to be efficacious in reducing colitis parameters [30].

In animal models of colitis, cannabinoid (CB) ligands are effective in reducing inflammatory tissue damage [31,32]. We have previously demonstrated that anandamide can ameliorate tissue damage evoked by TNF-α and IL-1β in a human explant colitis model [11]. Furthermore, IL-17A expression in lymphocytes is reduced by anandamide, strongly implicating the endocannabinoid system in the regulation of Th17 development and function [33]. The phytocannabinoid, cannabidiol (CBD) also attenuated colonic mucosal damage and reduced markers of tissue damage in rodent colitis models [31,34] and inflammatory indices in human colonic UC-affected biopsies [35].

In the present study we utilised IL-17A incubation in healthy human colonic mucosal explant tissue to determine effects on mucosal integrity, alone or in the presence of the cannabinoid ligands anandamide and cannabidiol. In addition, *in situ* mucosal IL-17A expression and matrix metalloprotease (MMP) enzyme activity were measured following incubation with pro-inflammatory cytokines TNF-α and IL-1β, as elevated MMP levels may play a role in mediating tissue damage in colitis [36]. The immunohistochemical expression of IL-17A was also compared with that of IL-17C in human colonic mucosa, where IL-17C expression is more commonly identified in the epithelium [37]. Previously, we have shown increased colonic epithelial permeability occurs in the presence of proinflammatory cytokines TNF-α and IL-1β [38], so a comparison of the effects of IL-17A on functional epithelial permeability changes in Caco-2 cells was also investigated, together with the measurement of IL-17A expression in Caco-2 cells following TNF-α and IL-1β incubation.

2. Materials and methods

2.1. Human colonic explant tissue: cytokine and cannabinoid treatments

Specimens of human colonic mucosa (14 patients) were obtained from consenting patients undergoing surgery for colonic malignancy at Flinders Medical Centre, Adelaide, under approval from the Flinders University Human Research Ethics Committee. The full thickness tissue obtained was located at margins distant from the tumour site. The tissue was then transferred to the laboratory within 1 h in ice cold carbogenated Kreb's solution. Tissue was then dissected and the mucosal layer removed from the underlying muscularis. The submucosa was left attached to the mucosal layer. The section of mucosa was further dissected into sections approximately 4 mm wide by 8 mm long. One section of mucosa was immediately placed in 10% neutral buffered formalin to serve as a 'non-incubated control' Remaining sections were then placed in culture media (RPMI-1640 with 1% penicillin–streptomycin solution and 0.01% bovine serum albumin (BSA)) with drug treatments.

Treatments included: vehicle control, IL-17A (10 ng/ml), hydrocortisone (1 μM) + IL-17A, anandamide (10 μM) + IL-17A and cannabidiol (10 μM) + IL-17A. Drug concentrations were chosen based on optimal concentrations in previous studies [11,38]. Tissues were incubated at 37 °C in borosilicate glass vials to prevent media evaporation and gassed with carbogen (95% oxygen and 5% CO_2) to oxygenate the tissue and balance pH for 20 h. Tissue sections were then removed and placed in 10% neutral buffered formalin followed by processing and embedding into blocks for cutting. Sections of 7 μm were cut and placed onto albumin coated slides, allowed to dry overnight and stained routinely by haematoxylin and eosin (H&E) stain for histological assessment. Tissue slides stained with H&E were photographed using an Olympus BH-2 microscope (Olympus Corporation, Tokyo, Japan) with a Nikon digital sight DS-5MC camera and captured using Nikon NIS-Elements BR software (Nikon Corporation, Tokyo, Japan). Scoring was performed by the same investigator blinded to the treatment for each tissue section using methodology we have previously used [11,38] and based on methodology adapted from other studies [39,40]. Images taken at 10× magnification were used to score crypt and luminal epithelial damage while 40× images were used to score lymphocyte density. The total length of the luminal epithelium and sections of damage were measured in arbitrary units and then the damaged sections where epithelium appeared abnormal (compared to un-incubated control mucosa) or missing measured, allowing a percentage of damage calculation. Crypts were assessed in a similar fashion. As we have previously found increases in lymphocyte density when explant mucosa is treated with TNF-α and IL-1β [11,38] we also counted lymphocyte density per 20 cells in the lamina propria in this study.

2.2. Matrix metalloprotease (MMP) enzyme activity assay

We aimed to investigate if IL-17A or TNF-α and IL-1β altered MMP enzyme activity in the colonic explant mucosal tissue. A generic MMP enzyme activity assay was performed using a SensoLyte Generic MMP assay, (Anaspec, Fremont, CA, USA). Homogenised explant mucosa prepared as described in Section 2.4 was assayed for MMP according to the manufacturer's directions. Concentration of protein in samples was determined by BCA assay (Thermo Fisher Scientific, Scoresby, VIC, Australia). The activity of MMP enzyme was expressed as pmol of reference standard/mg mucosal protein/minute.

2.3. Interleukin 17A ELISA

To quantitate the expression of IL-17A in human colonic mucosa and Caco-2 cells, alone and following pro-inflammatory cytokine incubation with TNF-α and IL-1β, an enzyme linked immunosorbent assay (ELISA) was used to determine the concentration of IL-17A in media obtained from explant incubations after 20 h and in media from Caco-2 cells. Media was centrifuged at 1500g for 10 min at 4 °C to remove any cell debris. The concentration of IL-17A in media supernatants was quantified using a Legend Max Human IL-17A ELISA Kit (BioLegend, San Diego, CA, USA) according to the manufacturer's directions.

2.4. Western blotting for IL-17A in human colonic mucosa

To determine if the cytokines TNF-α and IL-1β induced changes in IL-17A expression in explant mucosal tissue, western blotting was used to detect expression of IL-17A protein. Colonic mucosal samples treated with TNF-α and IL-1β (10 ng/ml) or control samples were homogenised and sonicated in lysis buffer (150 mM NaCl, 1% Triton X-100 and 50 mM Tris) with protease inhibitors, pepstatin A (1 μg/ml), aprotinin (2 μg/ml), antipain (1 μg/ml) and PMSF (1 mM). Samples were then centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant collected and stored at −70 °C until needed. The protein concentration of each sample was determined using a BCA assay kit (Thermo Fisher Scientific, Scoresby, VIC, Australia). Samples were then diluted further in lysis buffer as necessary and mixed with Laemmli loading buffer (62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% β-mercaptoethanol.) Samples were boiled at 100 °C for 5 min, placed on ice, vortexed then 20 μg of protein loaded onto a Mini Protean TGX precast (any kD) gel (Bio-Rad, Gladesville, NSW, Australia). Molecular weight markers (Precision Plus Dual Colour, Bio-Rad) was loaded and electrophoresis performed at 200 V for 35 min in SDS–PAGE running buffer (25 mM Tris,

192 mM glycine, 1% SDS at pH 8.3.) After electrophoresis, proteins were transferred onto a PVDF membrane (wet transfer, 100 V for 70 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol)). The membrane was then blocked in 5% BSA for 1 h at 4 °C and then primary antibodies applied, IL-17A antibody (Abcam #9565) diluted 1:1000 and β-actin antibody (BioLegend #622101) diluted according to the manufacturer's directions and incubated overnight at 4 °C. The membrane was then washed in TBS-T and incubated with the secondary antibody, goat anti rabbit IgG-DyLight 800 conjugate (Thermo Fisher Scientific #35571) at room temperature for 1 h. The membrane was then washed and air dried before detection at 800 nm using a Li-COR Odyssey infrared imaging system (Li-Cor Biosciences, Cambridge, UK). Density of protein bands was determined using Li-COR Image Studio Lite. IL-17A protein was expressed relative to β-actin banding density.

2.5. Immunohistochemical expression of IL-17A and IL-17C in human colonic mucosa

Immunohistochemistry (IHC) for IL-17A and IL-17C was performed on non-incubated control, incubation control and TNF-α + IL-1β-treated mucosal explant samples to determine the tissue distribution of IL-17A and IL-17C. Samples were prepared as described in Section 2.1. Following this, 5 μm sections were cut and mounted on silane-coated slides. Slides were de-waxed with xylene and dehydrated through graded ethanol solutions. Heat mediated antigen retrieval was performed in 10 mM citrate buffer (10 mM tri-sodium citrate, 10 mM citric acid, pH 6.0) with slides microwaved at 800 W for 4 min, then at 160 W for 10 min. Slides were then rinsed in PBS and dehydrated via increasing concentrations of ethanol before being immersed in 3% hydrogen peroxide in methanol for 1 min to block endogenous peroxidase activity. Slides were rehydrated and washed in PBS before being treated with 0.1% Triton X-100 in citrate buffer, to facilitate antibody penetration, washed again and treated with 50% goat serum in PBS for 30 min. Following rinsing in PBS, sections were incubated with an Avidin Biotin Blocking kit as per manufacturer's instructions. To determine if binding of the primary IL-17A antibody (Abcam #9565, diluted 1:200 in 5% goat serum) was specific, the antibody was blocked with immunising peptide (recombinant IL-17A) for 30 min at room temperature with agitation. Alongside this control, primary IL-17A antibody was also diluted in 5% goat serum without immunising peptide and incubated for 30 min at room temperature. Primary IL-17A antibody, IL-17A antibody neutralised with IL-17A or 5% goat serum alone (primary antibody omission control) was applied with 5% goat serum in PBS to each section and then incubated at 4 °C for 20 h in a humidified chamber. To detect IL-17C, the primary IL-17C antibody (Abcam #153896) was diluted 1:250 in 5% goat serum and applied to additional mucosal sections on different slides. Slides were then thoroughly washed in PBS and the secondary antibody biotinylated goat anti-rabbit IgG (Vector BA-1000, diluted 1:200) diluted in 5% goat serum was applied and incubated for 30 min at room temperature. The secondary antibody was washed off in PBS and avidin–biotin complex reagent (Vectastain ABC) prepared according to the manufacturer's directions was then applied to each section for 30 min followed by rinsing in PBS. Antibody was visualised with 3,3-diaminobenzidine tetrachloride (DAB) at room temperature for 2 min, washed in distilled water and counter-stained with Lillie Mayer haematoxylin for 2 min. Slides were then dehydrated and de-waxed in xylene before being sealed with a coverslip for microscopy as described (Section 2.1).

2.6. Caco-2 cell culture and treatment

Caco-2 cells (passage 40–70) derived from human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in 75cm^2 tissue culture flasks (Corning Life Sciences, Lowell, MA, USA) at 37 °C with 5% CO_2 in DMEM supplemented with 10% foetal calf serum and 1% penicillin/streptomycin solution (complete DMEM). Cells were passaged every 3–4 days when at approximately 80% confluence. Experiments were carried out using Corning Costar Transwell Inserts (6.5 mm diameter, 0.4 μm pore size, tissue culture treated polyester membrane) (Corning Life Sciences, Lowell, MA, USA). The basolateral compartment was filled with 0.6 mL of complete DMEM containing 1% amphotericin B. All subsequent media used in the Transwell plate was supplemented with 1% amphotericin B to avoid fungal contamination. Cells were seeded at a density of 16500 cells per insert in 0.1 mL of media in the apical compartment. An insert on the plate was left blank and contained media only. The Transwell plate was then incubated at 37 °C and 5% CO_2. Media in the apical and basolateral well was replaced every 2–3 days for up to 25 days as the cells reached confluence and differentiated. During the period of cell growth the transepithelial electrical resistance (TEER) was measured using a EVOM2 epithelial voltohmeter with chopstick electrode (World Precision Instruments, Sarasota, FL, USA) to determine the integrity of the monolayer. The formula: TEER Monolayer (cm^2) = [TEER total (Ω) − TEER blank (Ω)] × A (cm^2) was used to calculate TEER (where *A* is the area of the insert 0.33cm^2). Only inserts with TEER values over 500 Ω per cm^2 indicative of optimal monolayer confluence were used in experiments. Before treatment, media was replaced with complete DMEM without amphotericin B. TEER was measured to obtain baseline TEER values after allowing the plate to equilibrate to room temperature for 5 min, to minimise temperature induced fluctuations in TEER. Cells were treated with recombinant human interleukin 17 A (IL-17A) at concentrations of 10 or 100 ng/ml applied to the basolateral well. At the time points of 5, 24 and 48 h when testing cytokine effects, the TEER of each well was measured.

2.7. Chemicals and reagents

All chemicals were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia), unless otherwise specified. DAB-*plus* reagent set, RPMI-1640, DMEM and penicillin/streptomycin solution was purchased from Invitrogen (Mulgrave, VIC, Australia). Cannabidiol was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Tumour necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) were obtained from Symansis (Timaru, New Zealand). Precision Plus Molecular weight standards were obtained from Bio-Rad (Gladesville, NSW Australia). Rabbit anti-IL-17 antibody (ab9565) and rabbit anti IL-17C antibody (ab153896) were obtained from Abcam (Cambridge, UK). Rabbit anti β-actin antibody (#622101) was obtained from BioLegend (San Diego, CA, USA). Secondary goat anti rabbit IgG DyLight 800 conjugate (#35571) was obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Biotinylated goat anti-rabbit IgG (Vector BA-1000), Avidin Biotin blocking kit and Vectastain ABC Kit were purchased from Vector Laboratories (Burlingame, CA, USA.)

2.8. Statistical analysis

Mucosal damage scoring from explant studies and MMP activity was compared using a one way ANOVA with a Holm–Šídák post hoc test. TEER data was analysed by a two way analysis of variance (ANOVA). A Bonferroni's post hoc test was used to compare all values to vehicle treated Caco-2 cells. Western blot band density and ELISA data was compared with a paired *t*-test. A p value of $p < 0.05$ was accepted as significant. All graph production and statistical analysis was performed using GraphPad Prism 6.02 for Windows (GraphPad Software, San Diego, USA).

3. Results

3.1. Effect of IL-17A and cannabinoid ligands on human colonic mucosal damage

Human colonic mucosal explants were treated with IL-17A (10 ng/ml) for 20 h before scoring for damage to luminal epithelium and crypts in addition to the measurement of lamina propria lymphocyte density. The non-incubated control mucosa appeared normal with no damage to luminal epithelium or crypts (Fig. 1a). A 20 h incubation period did not induce significant tissue damage to the incubation control (Fig. 1b), however small amounts of epithelial exudate or sloughing were noted compared to non-incubated specimens (Fig. 1a). Addition of IL-17A caused a significant increase in damage to the luminal epithelium (Fig. 2a: 38.4 ± 5.8% vs. 6.3 ± 0.8%), crypts (Fig. 2b: 29.8 ± 5.4% vs. 8.4 ± 2.0%) and considerable epithelial sloughing (Fig. 1c). Treatment with hydrocortisone significantly reduced luminal epithelial damage (Fig 2a: 9.0 ± 1.5% vs. 38.4 ± 5.8%) and crypt damage (Fig. 2b: 12.9 ± 4.0% vs. 29.8 ± 5.4%) in response to IL-17A and reduced visible sloughing (Fig. 1d). Colonic mucosal explant specimens treated with IL-17A together with anandamide (AEA: 10 μM) demonstrated significantly reduced luminal epithelial damage compared to incubation control mucosa (Fig. 2a: 17.1 ± 4.9% vs. 38.4 ± 5.8%) but not crypt damage (Fig. 2b: 17.5 ± 6.3% vs. 29.8 ± 5.4%) and showed reduced levels of luminal sloughing (Fig. 1e). Treatment with cannabidiol also significantly reduced luminal epithelial damage (Fig. 2a: 14.5 ± 6.1% vs. 38.4 ± 5.8%) and crypt damage (Fig. 2b: 12.9 ± 6.0% vs. 29.8 ± 5.4%) in response to IL-17A, with negligible sloughing and retention of goblet cells (Fig. 1f). Despite a trend towards elevated lamina propria lymphocyte density following IL-17A treatment, there was no significant change following either IL-17A or drug incubations compared to incubation control tissue (Fig. 2c).

3.2. IL-17A and TNF-α/IL-1β effects on MMP enzyme activity

Activity of matrix metalloprotease (MMP) enzymes was measured in human mucosal colonic explant homogenates treated with IL-17A (10 ng/ml) or TNF-α + IL-1β (10 ng/ml each) for 20 h as per explant conditions. Compared to incubation control, IL-17A treatment induced a statistically significant increase in mucosal MMP enzyme activity (Fig. 3: 6.23 ± 0.6 vs. 2.87 ± 0.7 pmol/mg/min). In contrast to IL-17A, treatment with TNF-α and IL-1β did not induce a significant change in MMP activity (3.20 ± 0.8 vs. 2.87 ± 0.7 pmol/mg/min).

3.3. IL-17A and IL-17C Expression in Human Colonic Mucosa and Epithelial Cells

Measurable concentrations of IL-17A (2.0 ± 0.10 pg/ml) in human colonic mucosal explants were detected by ELISA (Fig. 4a). However, treatment with pro-inflammatory cytokines TNF-α + IL-1β (10 ng/ml each) did not induce any change in the concentration of IL-17A in colonic explant media (2.1 ± 0.11 pg/ml), while cannabidiol treatment (10 μM) also did not further alter IL-17A concentrations in the presence of pro-inflammatory cytokines (2.1 ± 0.11 pg/ml). In untreated Caco-2 cells measurable IL-17A concentrations were found in cell culture media (4.0 ± 1.0 pg/ml: Fig. 4b). However, there was no significant difference in IL-17A concentrations between untreated cells (vehicle) and cells treated with TNF-α + IL-1β (3.53 ± 0.90 pg/ml).

To quantitate IL-17A expression in human colonic tissue, western blotting in mucosal homogenates was also undertaken. The results showed an observable band at 15 kDa for both control and TNF-α + IL-1β-treated samples (Fig. 4c: representative bands shown) corresponding to the IL-17A homodimer. Recombinant human IL-17A run in the same blot showed a band at an identical molecular weight (Fig. 4e), concordant with expression of IL-17A at 15 kDa in mucosal samples. Group data however revealed no significant changes in IL-17A expression between the control and pro-inflammatory cytokine (TNF-α + IL-1β)-treated groups relative to β-actin (Fig. 4d).

Immunohistochemical studies revealed a widespread pattern of IL-17A labelling in human mucosa (Fig. 5a and b). Immunoreactivity was strong in both luminal and crypt epithelium especially (Fig. 5a), but was also evidenced in an array of cell types in the lamina propria (Fig. 5c and d), with dense labelling in macrophages and plasma cells (Fig. 5c: arrow). Pro-inflammatory cytokine incubation (TNF-α + IL-1β) did not substantively alter the epithelial expression of IL-17A in human colonic mucosa, despite an increased infiltration of cells in lamina propria (Fig. 5b). Exudate

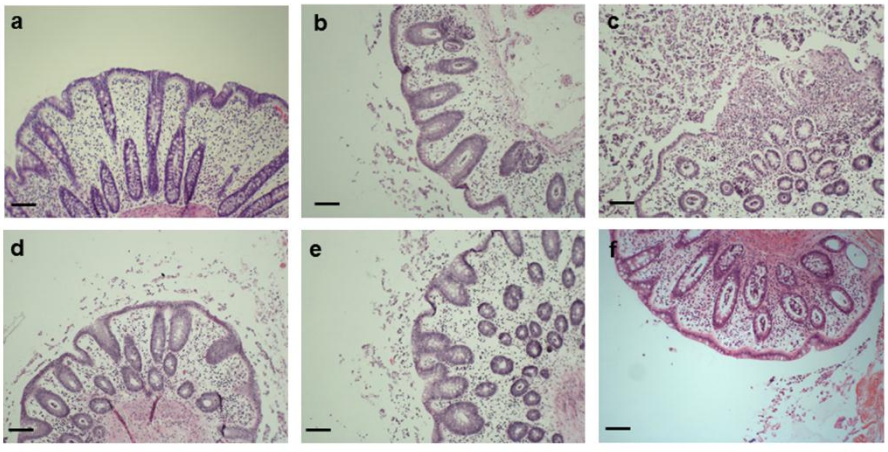

Fig. 1. Representative H&E stained human colonic mucosal explant samples. (a) Colonic mucosa prior to explant incubation (non-incubated control). (b) Colonic mucosa following incubation for 20 h (incubation control). Integrity of the luminal epithelium is retained overall and crypts appear healthy, with slight epithelial sloughing and loss of goblet cells. (c) Treatment with IL-17A (10 ng/ml) for 20 h. Much of the epithelium is denuded and luminal cell debris is evident. Many crypts are damaged but some remain intact. (d) Treatment with IL-17A plus hydrocortisone (1 μM), (e) anandamide (10 μM) or (f) cannabidiol (10 μM). Following each of the drug treatments, mucosal architecture is similar to incubation control, with varying degrees of preservation of luminal and crypt epithelium and minimal sloughing. Scale bar, 100 μm.

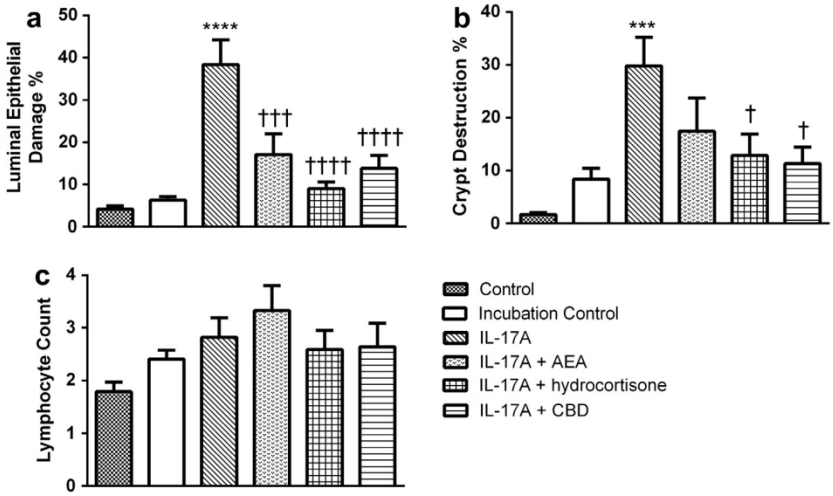

Fig. 2. Measurement of (a) luminal epithelial damage, (b) crypt destruction and (c) lamina propria lymphocyte number (per 20 cells) in human colonic explant tissue following IL-17A and drug treatments. IL-17A (10 ng/ml) caused a significant increase in luminal epithelial damage ($^{****}p < 0.0001$ vs. incubation control, $n = 14$) which was reduced by anandamide (AEA; 10 μM: $^{†††}p < 0.001$, $n = 7$), hydrocortisone (1 μM: $^{††††}p < 0.0001$, $n = 6$) and cannabidiol (CBD 10 μM: $^{††††}p < 0.0001$, $n = 7$). IL-17A caused significant damage to crypt epithelium ($^{***}p < 0.001$ vs. incubation control, $n = 14$). Both hydrocortisone and cannabidiol prevented IL-17A induced damage to crypts ($^{†}p < 0.05$, $n = 6–7$). No significant change was seen in lamina propria lymphocyte density (per 20 cells) following IL-17A (10 ng/ml) or IL-17A plus drug treatments ($n = 14$).

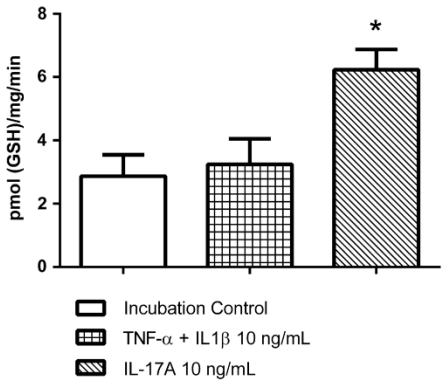

Fig. 3. Enzyme activity of matrix metalloprotease (MMP) in explant mucosa treated with IL-17A (10 ng/ml) or TNF-α and IL-1β (each at 10 ng/ml for 20 h). IL-17A induced a significant increase in MMP activity compared to incubation control ($^{*}p < 0.05$; $n = 7$). Combined TNF-α and IL-1β treatment did not significantly alter MMP activity ($n = 8$).

associated with intraepithelial lymphocyte clusters positively labelled for IL-17A in the lamina propria in control (untreated) colon specimens (Fig. 5e), suggesting a degree of constitutive expression. A series of pre-adsorption and omission control experiments precluded non-specific antibody binding (Supplementary Fig. S1). IL-17C immunoreactivity was also prevalent in the human colonic mucosal epithelium but also included widespread labelling in the lamina propria (Supplementary Fig. S2).

3.4. IL-17A did not affect epithelial permeability in Caco-2 cells

Exogenous IL-17A treatment produced no significant change in trans-epithelial electrical resistance (TEER) in Caco-2 cells at any time points over 48 h of incubation, at either 10 ng/ml or 100 ng/ml concentrations (Fig. 6a and b).

4. Discussion and conclusions

An aim of this study was to determine the effects of IL-17A on human colonic mucosal structure and composition in an explant model, in addition to determining any functional influence of IL-17A on intestinal epithelial paracellular permeability *in vitro*. Furthermore, IL-17A expression was characterised in human colonic mucosa under pro-inflammatory conditions with TNF-α and IL-1β co-incubation. IL-17A is an important cytokine in the pathology of IBD, where elevated expression is seen in inflamed colonic tissue compared to healthy controls [17,18]. The results indicate that exogenous IL-17A damages human colonic mucosal luminal epithelium and crypts, but also that IL-17A has a widespread tissue distribution in the healthy human colonic mucosa. In this context it may not only play an important role in the pathogenesis or facilitation of colitis, but may have more widespread roles in intestinal homeostasis, immune surveillance and response to injury that is becoming increasingly recognised [16]. In a clinical setting, immunoneutralisation of IL-17A in IBD has been shown clinically to increase infection risk, suggestive of a mucoprotective role of IL-17A [41].

To test the role of resident immune cells in cytokine-evoked damage we used hydrocortisone incubation, which has potent immunosuppressive effects and can inhibit T-lymphocyte proliferation and promote apoptosis [42]. Hydrocortisone treatment inhibited luminal epithelial damage and crypt destruction arising from IL-17A treatment. While these results suggest that IL-17A may mediate its effects on tissue damage via immune cell recruitment and activation, hydrocortisone may also directly influence epithelial cell signalling and homeostasis [43], so its protective effect may be pleiotropic in the mucosa. IL-17A did not cause any change in the lamina propria lymphocyte density as we have previously shown with TNF-α and IL-1β incubation in human colonic explant tissue [11], where the source of lymphocytes is most likely from sub-mucosal lymphoid aggregate migration rather than proliferation. In the present study, it may be that IL-17A does not stimulate lymphocyte chemotaxis but rather activation, where IL-17A receptors are expressed on innate T cell lineages such as LTi-like cells and other innate lymphoid cells common to the lamina propria

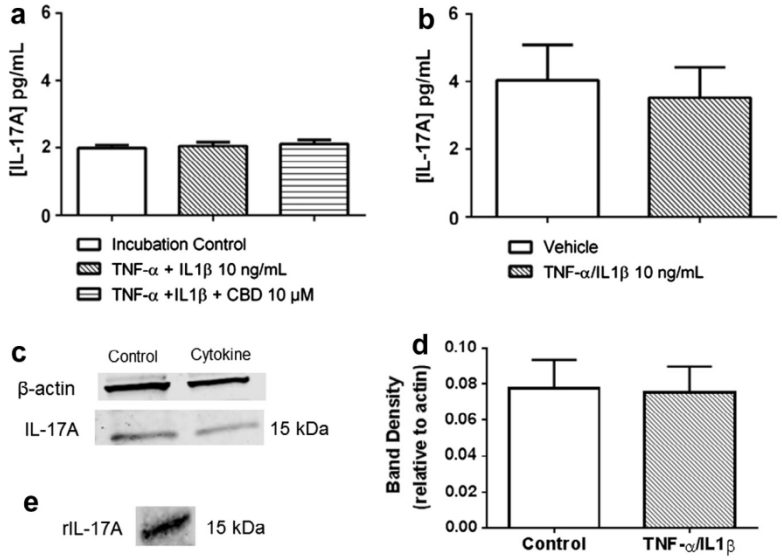

Fig. 4. Western blotting and measurement of IL-17A concentrations in media (by ELISA) from (a) human colonic mucosal explant tissue and (b) Caco-2 cells. No significant elevation in IL-17A was observed following treatment with TNF-α + IL-1β (10 ng/ml) compared to the incubation control tissue or in the TNF-α + IL-1β (10 ng/ml) + cannabidiol group (a). $n = 9$ for incubation control, $n = 8$ for TNF-α + IL-1β (10 ng/ml) and $n = 7$ for TNF-α + IL-1β (10 ng/ml) + cannabidiol. Caco-2 cells treated with vehicle or TNF-α + IL-1β (10 ng/ml) did not show any significant change in IL-17A concentration between treatment groups (b). $n = 6$ for vehicle treated and $n = 5$ for TNF-α + IL-1β (10 ng/ml, 20 h) Caco-2 groups. (c) Western blot for IL-17A and β-actin in human colonic mucosal explants. Cytokine indicates treatment with TNF-α + IL-1β (10 ng/ml, 20 h). Banding for β-actin at approximately 48 kDa can be seen for each sample, while IL-17A banding appears at 15 kDa (c). Densitometry for IL-17 relative to β-actin for mucosal samples demonstrates no change in IL-17A expression following TNF-α + IL-1β (10 ng/ml) treatment (d; $n = 4$ samples). Recombinant human IL-17A (rIL-17A) also produced a distinct band at 15 kDa on western blot (e).

[16]. In regards to effects of IL-17A on other immuno-competent cell types, IL-17A can stimulate macrophage release of TNF-α and IL-1β [44], while TNF-α can itself amplify intestinal IL-17A production and release [45], so such cytokines can act co-operatively to augment each other's expression. However, we did not find measurable levels of TNF-α production in the human explant colonic mucosa following IL-17A incubation (not shown). This may be because of an insufficient incubation time course for induction of TNF-α, or lack of TNF-α sources such as macrophages *ex vivo* or Paneth cells [45], which are sparse in the colon.

The effects of IL-17A on epithelial damage were attenuated by concomitant treatment with the endocannabinoid anandamide, similar to our previous findings using anandamide in an explant colitis model generated via TNF-α and IL-1β treatment [11]. In studies using the DNBS (2,4-dinitrobenzene sulfonic acid) animal model of colitis, increasing anandamide levels also protects against tissue damage and reduces myeloperoxidase activity [46]. In this study we did not determine which of the CB receptors is involved in mediating this effect, as anandamide can activate both CB1 and CB2 receptors in addition to forming an array of pharmacologically active mediators [47]. However, our previous studies indicate this effect is most likely to occur from activation of the CB2 receptor [11,38]. Other studies support a role for the CB2 receptor in the suppression of proliferation of human T-lymphocytes and cytokine release, where anandamide reduced the production of IL-17A from stimulated $CD4^+$ T-lymphocytes via an action at the CB2 receptor [33]. The phytocannabinoid, cannabidiol also had a protective effect in the model, reducing luminal epithelial damage and crypt destruction. The pharmacology underlying cannabidiol's actions however is less clear, as it has low affinity for CB1 or CB2 receptors and it may activate other receptors such as PPAR-γ, leading to an anti-inflammatory effect [48]. In animal models of colitis, cannabidiol reduces colonic damage and inflammatory marker expression [31,34] and lowers reactive oxygen species levels in Caco-2 cells [31], indicative of potentially multiple pathways of anti-inflammatory activity in colitis. Collectively our findings indicate a modulatory role for cannabinoids and the endocannabinoid system in the control of colonic inflammation.

In IBD, levels of matrix metalloprotease enzymes (MMPs) are elevated compared to healthy controls [36,49], so we examined MMP activity to determine if IL-17A elevated overall MMP activity compared to TNF-α and IL-1β in colonic explant mucosa. We found elevated MMP enzyme activity in IL-17A treated samples, but not following TNF-α and IL-1β incubation. The assay used does not discern which MMP enzymes are elevated, but previous studies have shown that IL-17A can increase macrophage stromelysin (MMP-3) that degrades extracellular matrix [44]. Enhanced MMP activity may therefore facilitate tissue damage to the luminal epithelium and crypts following IL-17A incubation in the human colonic mucosa. That the TNF-α and IL-1β cytokine combination did not induce a similar change in MMP activity in explant mucosa highlights a biochemical distinction between the possible mediators of damage between respective pro-inflammatory cytokines. This was also supported by a lack of nitrite production following IL-17A incubation (not shown), whereas we have previously shown that TNF-α and IL-1β increase nitrite production in this model [38]. The time course of explant mucosal incubation may also be a factor, where elevations in active MMPs occur after 72 h of incubation with TNF-α [50,51] and with T cell involvement being critical to MMP activation [51,52]. Such extended incubation times were not possible in the present study due to the likelihood of mucosal degradation in organotypic culture.

We also examined if incubation of colonic explant tissues with TNF-α and IL-1β could alter expression of IL-17A, given evidence that both of these cytokines can augment IL-17A production [21,22]. In addition, TNF-α can induce significant production of IL-17A from Paneth cells, which can be found in the small intestine but to a lesser extent in the colon [45]. IL-17A was measured in

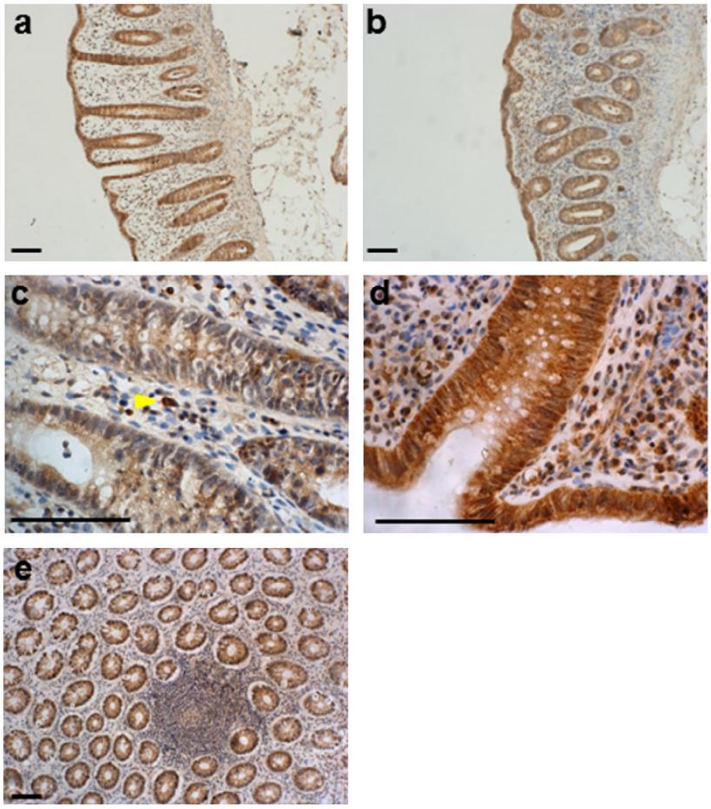

Fig. 5. IL-17A immunolabelling in the human colonic mucosa. Control (a), (e) and cytokine (b and d) (TNF-α + IL-1β, 20 h) treated tissue. IL-17A was strongly expressed in the mucosal epithelium (a), along both luminal and crypt enterocytes (d). There was a similar level of IL-17A expression following TNF-α + IL-1β incubation (b). Various cell types in lamina propria also densely labelled IL-17A immunoreactivity, particularly macrophages (c: arrow). Exudate within lymphocyte clusters also demonstrated concentrated IL-17A immunoreactivity (e).

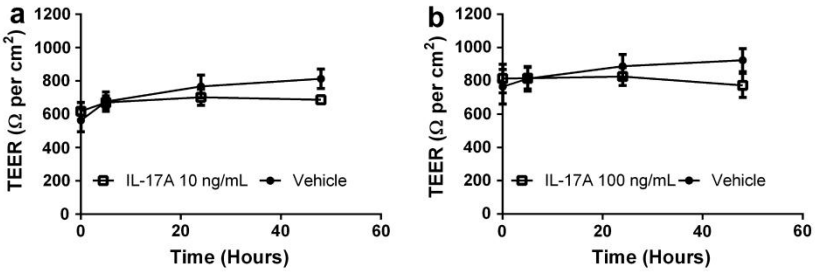

Fig. 6. Effect of IL-17A on permeability of Caco-2 monolayers as measured by trans-epithelial electrical resistance (TEER) at 5,24 and 48 h. (a) IL-17A 10 ng/ml applied basolaterally showed no significant effect on TEER at any time point tested. (b) IL-17A 100 ng/ml applied basolaterally showed no significant effect on TEER at any time point tested. $n = 3$ for 10 ng/ml IL-17A and $n = 6$ for 100 ng/ml IL-17A.

both media supernatants from explant cultures and in mucosal homogenates. There were no significant changes in IL-17A levels in the media or the mucosal homogenate between untreated control samples and cytokine-treated mucosa (TNF-α and IL-1β), nor any effect of cannabidiol treatment. Similar results were noted for Caco-2 cell culture media, whereby no change in IL-17A concentrations occurred following TNF-α and IL-1β incubation. These findings were also supported by immunohistochemical evidence for a relative lack of change of IL-17A expression or distribution in colonic mucosa following TNF-α and IL-1β incubation. The immunohistochemistry also revealed a widespread expression of IL-17A in human colonic mucosal tissue, with particularly strong labelling in the epithelium. This was surprising as limited epithelial expression has been previously noted [17], however other studies have found elevations in IL-17A expression in the mouse small intestine after TNF-α treatment [45].While questionable specificity of IL-17A antibodies is a consideration in immunohistochemical studies [28], neutralisation (pre-absorption) with IL-17A indicated relatively specific binding of the polyclonal IL-17A antibody used in the present study (Supplementary Fig. S1), thus supporting discrete epithelial expression of this cytokine.

We also noted strong epithelial IL-17C expression in human colon, in addition to widespread expression in the lamina propria (Supplementary Fig. S2). IL-17C is a cytokine previously shown to

be expressed in epithelial cells in the colon [37] and our immunohistochemical evidence supports such expression in the human colon. Our findings suggest that both IL-17A and IL-17C may be found in the human colonic epithelium. IL-17C is suggested to play a protective or homeostatic role in the gut epithelium [37]. Future studies determining the balanced expression of IL-17 isoforms in different gastrointestinal pathologies may yield more discriminative information on their functional roles in the human colonic mucosa.

An additional aim of this study was to determine if IL-17A can influence epithelial barrier function. Previous studies have shown that cytokines including TNF-α, IL-1β and IFN-γ can directly increase epithelial permeability [8,9,53] but that IL-17A decreases epithelial permeability in T84 cells [25]. This study did not find any significant effect of IL-17A at either 10 or 100 ng/ml on epithelial permeability. IL-17A was applied only to the basolateral side of the Caco-2 monolayer, while in Kinugasa et al. [25]. study it was applied basolaterally and apically. IL-17 may have variable effects on epithelial permeability depending on cell type or tissue, the subtype of IL-17 or whether it is applied apically or basolaterally. Caco-2 cells produce mRNA for the IL-17A receptor (IL-17RA) [54] and we were able to show that IL-17A is directly produced by this cell line. Even though the pro-inflammatory cytokine combination of TNF-α and IL-1β is known to reduce Caco-2 permeability [38], it did not alter IL-17A expression. Caco-2 cells can however up-regulate gene expression of IL-17A when incubated with bacterial fucose [55], so perhaps IL-17A production may be dependent on bacterial signalling molecules conducive to a surveillance or homeostatic role of this cytokine.

In conclusion, this study demonstrates that IL-17A can induce epithelial and crypt damage in human colonic mucosal explant tissue, without directly affecting epithelial permeability in Caco-2 cells. The cannabinoids, anandamide and cannabidiol reduce IL-17A mediated mucosal damage, providing evidence that endocannabinoids or phytocannabinoids may offer mucosal protection in inflammation. The widespread expression of IL-17A and IL-17C in the human colonic epithelium may also support a wider role for IL-17 family members in innate immune function in the human colon.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.10.006.

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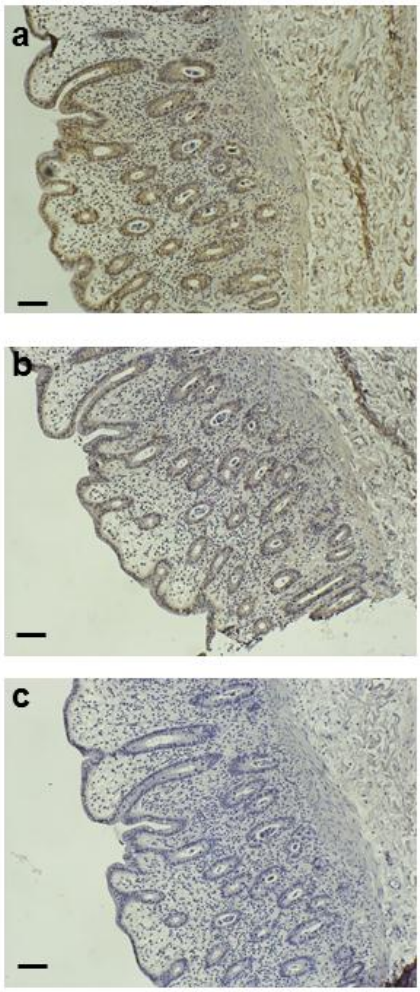
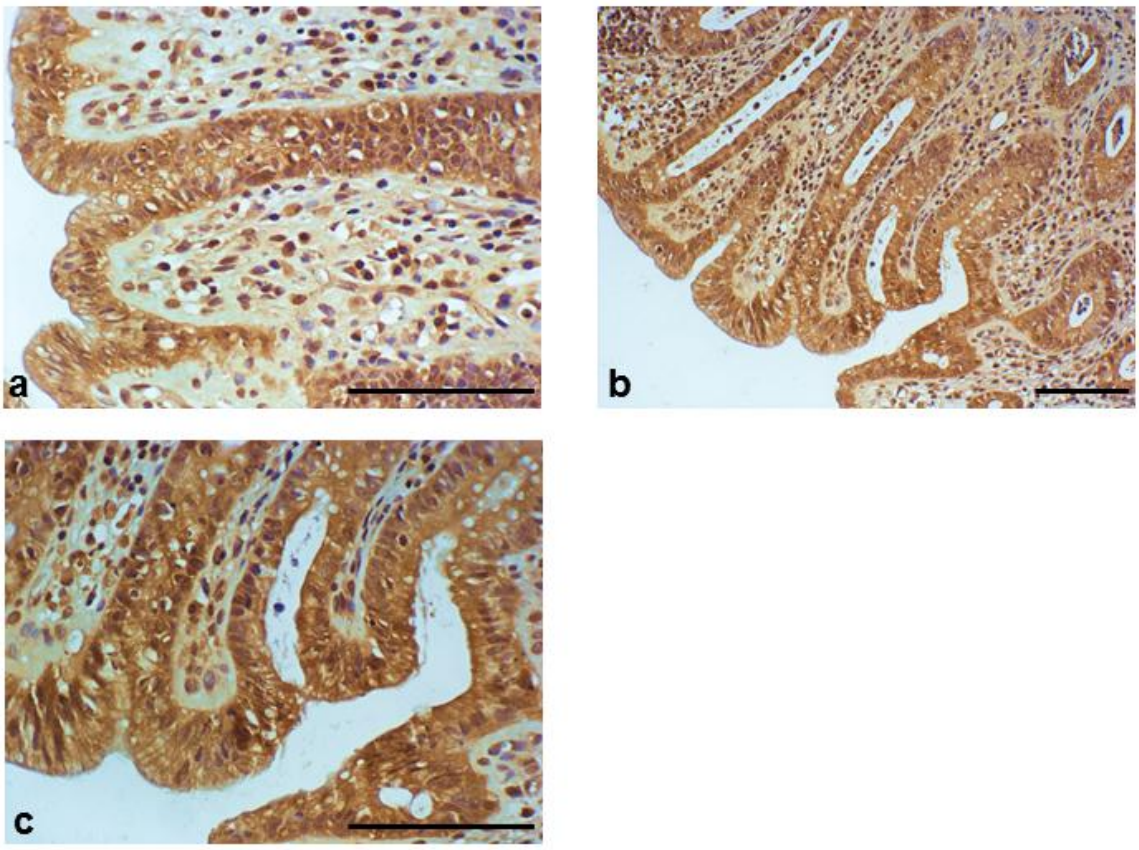
3.1 Publication 2: Appendix A. Supplementary Material

Supplementary figures available online at: http://dx.doi.org/10.1016/j.cyto.2013.10.006



Supplementary Fig. S1.

Controls for IL-17 antibody specificity for immunohistochemistry (IHC) in human colonic mucosa. (a) Labelling for IL-17 in colonic mucosa, primary antibody dilution 1 in 200 without immunising peptide (IL-17). Distinct brown DAB staining is observed in the luminal epithelium, crypt epithelium and in the lamina propria. (b) Labelling for IL-17 where the primary antibody was incubated for 30 min with the immunising peptide (IL-17). A strong reduction in brown DAB staining compared to (a) is observed, indicating specificity of the antibody for IL-17. (c) Omission control; no DAB staining is observed when the primary antibody is omitted indicating the secondary antibody is specific does not react with any proteins in the mucosa. Scale bar, 100 μm.



Supplementary Fig. S2.

IL-17C immunohistochemistry in human colonic mucosa (a-c; untreated, non-incubated). Strong IL-17C immunoreactivity was found in the colonic epithelium, but also widespread labelling occurred in lamina propria enterocytes and immune cells. Scale bar, 100 μm.

Chapter 4 : Further Characterisation of the Effects of Cytokines in the Human Colitis Explant Model and the Effects of Cannabidiol on Biochemical Markers

4.1 Introduction

The purpose of this series of experiments was to further characterise the effects of TNF-α + IL-1β and IL-17A on mucosal damage in the explant model. In particular, these experiments in-part aimed to determine mechanisms that may explain how these cytokines cause luminal epithelial damage, crypt destruction and increases in lymphocyte density observed in the model. A number of biochemical markers were investigated including nitrite concentrations and comparative prostaglandin and prostaglandin ethanolamide (prostamide) concentrations.

Previous studies have shown that anandamide (AEA) is also a substrate for cyclooxygenase 2 (COX-2) and is converted by COX-2 into prostamides. Another major endocannabinoid, 2-arachidonyl glycerol (2-AG) is also converted by COX-2 into prostaglandin glycerol esters (PG-GEs) (Kozak et al., 2002); however, we have not examined their role in our studies to date. These compounds have distinct biological activity compared to prostaglandins, likely due to the fact that they bind to unique prostamide receptor heterodimers, in which there are specific antagonists available (Liang et al., 2008, Woodward et al., 2008).

As AEA levels are altered in animal colitis models, there may be changes in levels of prostamide production in IBD (D'Argenio et al., 2006). This is largely unrecognised, as conventional ELISA for prostaglandins cross react with prostamides (Glass et al., 2005). In addition, COX-2 is elevated in IBD, which could dramatically increase prostamide levels above baseline (Roberts et al., 2001). Previously, we have shown that TNF-α + IL-

1β induced damage in mucosal explants can be attenuated with a synthetic prostamide, bimatoprost (Nicotra et al., 2013).To explain how this may occur, we studied expression of PGE_2 and $PGF_{2\alpha}$ and the associated prostaglandin ethanolamides (PGE_2-EA and $PGF_{2\alpha}$-EA) in explant colonic mucosa. Prostaglandins and prostamides in explant media samples treated with TNF-α + IL-1β, vehicle or anandamide (AEA) were extracted and then separated by thin layer chromatography (TLC). This was to prevent cross reactivity in the subsequent ELISA and allow separate measurement of prostaglandin and prostamide concentrations. As we had initially studied the effect of TNF-α + IL-1β only, it was not a primary focus of these studies to explore the effect of IL-17A on prostamide concentrations, therefore these experiments were not carried out.

In ulcerative colitis (UC), production of nitric oxide (NO) can occur in inflamed tissue, which may lead to tissue damage by peroxynitrite formation (Kimura et al., 1998, Linehan et al., 2005). In addition, the concentration of nitrite (a stable breakdown product from NO production, which can be quantified) can increase following cytokine treatment. In lung epithelial cells, cytokine treatment (to simulate inflammation, such as occurs in asthma) can also elevate nitrite levels (Robbins et al., 1994). In Chapter 2 of this thesis we observed nitrite elevations in response to TNF-α + IL-1β treatment in colonic mucosa. As a result, we wanted to determine if IL-17A, which also causes damage to colonic mucosa as described in Chapter 3, could also cause elevations in nitrite levels. This would provide an additional potential mechanism for the damage seen by IL-17A treatment in the explant model.

IL-17A can induce the expression of TNF-α from Paneth cells and this partially drives a potent, systemic inflammatory response (Takahashi et al., 2008). Other studies have also shown IL-17 can promote TNF-α and IL-1β production by macrophages (Jovanovic et al., 1998). As IL-17A may increase production of TNF-α, we aimed to test whether IL-17A

treatment in the explant model has any influence of the levels of TNF-α, which can be measured by ELISA. It could be possible that IL-17A production may mediate some tissue damage by subsequent production of TNF-α, which we have shown can cause mucosal damage in the explant model in previous chapters.

In Chapter 2, we showed that the number of lymphocytes increased in mucosal explants treated with TNF-α + IL-1β compared to incubation control. In contrast, IL-17A treatment did not cause this increase in lymphocyte density. The exact identity (or type) of these lymphocytes is not yet known. As a result we proposed to use immunohistochemistry (IHC) to determine the identity of these lymphocytes. As a preliminary determination of immune cell identities, mucosal explants and positive controls (human tonsil) were stained for CD3, a common immune cell marker for T-cells (Chetty and Gatter, 1994). In addition, we also wanted to quantify if any changes in macrophage expression occurred in mucosal explants under the influence of TNF-α and IL-1β treatment. Studies have shown that in clinical IBD and animal colitis, increases in the number of macrophages producing inflammatory cytokines occurs (Yan et al., 2012, Cader and Kaser, 2013).

Previously in Chapter 3, we performed IHC for IL-17A in explant mucosal tissues. In addition to the staining patterns described previously in this thesis, we also identified cell types located at the edge of crypts with intense IL-17 immunoreactivity. The morphology and location of these cells suggests they may be enterochromaffin cells (EC cells). EC cells are a major source of 5-HT in the gut and can be detected with anti-5-HT antibodies (Cremon et al., 2011). To make a preliminary determination if these IL-17A expressing cells are in fact EC cells, we performed 5-HT IHC on mucosal explant sections to identify EC cells in the mucosa.

4.2 Materials and Methods

4.2.1 Explant Colitis Model

The methodology for preparation, incubation and processing of colonic mucosa in the explant colitis model is described in Chapters 2 and 3 of this thesis.

4.2.2 Thin Layer Chromatography

The separation and quantitation of prostanoids and prostamides was based on a method from a previous study (Glass et al, 2005). To calculate the concentration of prostaglandin E_2 or $F_{2\alpha}$ and the respective ethanolamides, it was first necessary to separate these two compounds by thin layer chromatography (TLC). This was due to cross reactivity of the respective ethanolamide with the prostaglandin when using ELISA (Glass et al., 2005). To separate PGE_2 from PGE_2-EA, 200 µL of explant media was treated with 1 mL of 90:10 chloroform:methanol. To separate $PGF_{2\alpha}$ from $PGF_{2\alpha}$-EA, 80:20 chloroform:methanol with citric acid added (pH 3.5) was used. Samples were then centrifuged and the aqueous layer removed before being left overnight at room temperature to allow the solvent to evaporate. Samples were then reconstituted with 20 µL of ethanol and spotted onto a glass silica TLC plate (Merck-Millipore, Kilsyth, VIC, Australia). A lamp was used to heat the sample and allow solvent evaporation. In addition to explant samples, standards for PGE_2, PGE_2-EA, $PGF_{2\alpha}$ and $PGF_{2\alpha}$-EA were also spotted onto the plate. Compounds were separated on the plate using 90:10 ethyl acetate:methanol. The location of each sample on the plate was visualised using iodine vapour and compared to the standard prostamide. The silica containing sample was scraped off and stored at -20°C. For ELISA, samples were reconstituted in RPMI-1640 (as used in the explant incubation). Samples of RPMI-1640 containing known concentrations of prostanoid standards were assayed to determine the percentage of sample recovered from extraction. The study by Glass et al. (2005) using

the method of extraction described above showed approximately 60% of prostamide is recovered(Glass et al., 2005). Recovery percentages for prostamides in this study were as follows: PGE_2; 81%, PGE_2-EA; 34%, $PGF_{2\alpha}$; 52% and $PGF_{2\alpha}$-EA; 69%.

4.2.3 Prostaglandin E_2 and Prostaglandin $F_{2\alpha}$ ELISA

To measure the concentration of PGE_2 and PGE_2-EA, a prostaglandin E_2 ELISA kit was used. $PGF_{2\alpha}$ and $PGF_{2\alpha}$-EA were measured using a prostaglandin $F_{2\alpha}$ ELISA Kit. For each kit, the manufacturer's directions were followed. At the conclusion of the assay, the absorbance of the wells on ELISA plates was read at 405 nm using a PolarStar Galaxy microplate Reader (BMG Labtech, Durham, NC, USA.). To calculate the concentrations of prostanoid in each sample and prepare the standard curve (using a 4 parameter logistic fit), online analysis software recommended by the manufacturer of the ELISA kit was used. This software was located at:

http://www.myassays.com/kit.aspx?id=514010&manu=caymanchem&referral=caymanchem

http://www.myassays.com/kit.aspx?id=516011&manu=caymanchem&referral=caymanchem

Prostanoid concentrations were expressed relative to wet tissue weight.

4.2.4 Nitrite (NO_2^-) Assay (Colourmetric: Griess' Reagent)

To measure nitrite concentrations in explant media, a standard curve was first prepared by serially diluting a 10 µM nitrite ion solution in RPMI-1640 media and plating 50 µL of each standard in a 96 well microplate. For experimental samples, 50 µL each was placed in the microplate and to each sample and standard, 50 µL of Griess' reagent was added. The plate was incubated at room temperature for 30 min and absorbance was read at 540 nm in a Synergy MX microplate reader (Biotek Instruments, Bedfordshire, UK). The

standard curve was plotted in Graphpad Prism 6.02 (Graphpad Software, San Diego, USA.) and the unknown sample values interpolated from the curve.

4.2.5 Nitrite (NO_2^-) Assay (Fluorometric: DAN Reaction)

To detect the concentration of nitrite in explant samples, media obtained from explant incubations (explant media) was tested for nitrite using the Nitrite/Nitrate Assay Kit (06239) (Sigma Aldrich, Castle Hill, NSW, Australia.) with a modified method. Explant media was centrifuged at 1500 rpm for 10 min at 4°C then placed into a black fluorescence microplate in appropriate wells. A standard curve for nitrite (1, 2.5, 5 and 10 µM) was also prepared. Media samples and standards were then mixed with the fluorescence reagent solution, 2,3-diaminonapthaline (DAN) and incubated at room temperature for 15 min. Following this, NaOH was placed into each well and fluorescence read at excitation of 360 nm and emission 425 nm using a Synergy MX microplate reader (Biotek Instruments, Bedfordshire, UK).

4.2.6 TNF-α ELISA

A TNF-α ELISA was used to determine if treatment of human colonic mucosa with IL-17A or pharmacological interventions could influence the expression of TNF-α. Media supernatants collected in explant studies were used for this experiment. Samples included incubation control, IL-17A (10 ng/mL), IL-17A + anandamide (10 µM), IL-17A + hydrocortisone (1 µM) and IL-17A + cannabidiol (10 µM)-treated mucosa. Media was stored at -20°C before the assay. Media was thawed and centrifuged at 1500g for 10 min at 4°C to remove debris.

A standard for TNF-α was prepared by adding assay buffer to the lyophilised TNF-α powder to prepare a 20 ng/mL solution. This was diluted to the final concentrations of

1000, 500, 250, 125, 62.5, 31.3 and 15.6 pg/mL. The pre-coated ELISA plate was washed four times with wash buffer and then 50 µL of assay buffer added to each well containing the standards and samples. Following this, 50 µL of sample and each standard (in duplicate) was added to the appropriate wells.

The plate was incubated at room temperature for 2 hours on an orbital shaker with gentle agitation. Contents of the plate were then discarded and the plate washed four times with wash buffer to remove residual sample and standard. 100 µL of the human TNF-α detection antibody was added to each well and the plate sealed and incubated for 1 hour at room temperature with gentle agitation on an orbital shaker. Contents of the plate were discarded and the plate washed four times with wash buffer. Following this, 100 µL of avidin-HRP solution was added to each well and the plate sealed and incubated at room temperature for 30 min with gentle agitation. Contents of the wells were again discarded and the plate washed thoroughly five times before 100 µL of substrate solution was added to each well to develop colour for 15 min. Stop solution (sulphuric acid) was added to each well to stop the colour reaction. The microplate was read at 450 nm with the wavelength at 560 nm subtracted from this reading using a Fluostar Galaxy microplate reader (BMG Labtech, Durham, NC, USA.). A standard curve to TNF-α was prepared in GraphPad Prism and unknown TNF-α concentrations interpolated from this curve.

4.2.7 CD3 Immunohistochemistry

Immunohistochemistry (IHC) in order to locate the distribution of $CD3^+$ immune cells (helper and cytotoxic T-lymphocytes) in human colonic mucosa was performed on un-incubated human colonic mucosa, incubation control tissue and cytokine (TNF-α + IL-1β 10 ng/mL)-treated mucosa. Human tonsil tissue was stained for CD3 as a positive control recommended by the manufacturer (Abcam). Archived human tonsil tissue was kindly

supplied by Dr. Rachel Gibson (School of Medical Sciences, The University of Adelaide). Colonic mucosal samples were prepared as described in section 4.2.1. Following this, 4 µm sections were cut and mounted on silane-coated slides. Slides were dewaxed with xylene and dehydrated through graded ethanol solutions. Heat-mediated antigen retrieval was performed in 10 mM citrate buffer (10 mM tri-sodium citrate, 10 mM citric acid, pH 6.0) with slides microwaved at 800W for 4 min, then at 160W for 10 min. Slides were then rinsed in PBS and dehydrated via increasing concentrations of ethanol before being immersed in 3% H_2O_2 in methanol for 1 minute to block endogenous peroxidase activity. Slides were rehydrated and washed in PBS before being treated with 0.1% Triton-X100 in citrate buffer, to facilitate antibody penetration and washed again and treated with 50% goat serum in PBS for 30 min. Following rinsing in PBS, sections were incubated with an Avidin Biotin Blocking kit as per manufacturer's instructions. Primary anti-CD3 antibody (Abcam ab5690) was also diluted 1:100 in 5% goat serum and applied to tissue sections and then incubated at 4°C for 20 hours in a humidified chamber.

Slides were then thoroughly washed in PBS and the secondary antibody, biotinylated goat anti-rabbit IgG (Vector BA-1000 1:200) diluted in 5% goat serum was applied and incubated for 30 min at room temperature. The secondary antibody was washed off in PBS and avidin-biotin complex reagent (Vectastain ABC) prepared according to the manufacturer's directions was then applied to each section for 30 min followed by rinsing in PBS. Antibody was visualised with 3,3-diaminobenzidine tetrachloride (DAB) at room temperature for 2 min, washed in distilled water and counter-stained with Lillie Mayer haematoxylin for 2 min. Slides were then dehydrated and dewaxed in xylene before being sealed with a coverslip for microscopy as described in section 4.2.1.

4.2.8 Macrophage Immunohistochemistry

Immunohistochemistry (IHC) was used to locate the distribution of macrophages in human colonic mucosa. It was performed on un-incubated human colonic mucosa, human tonsil tissue and rat spleen tissue in order to determine the suitability and optimise the antibody for this application (Table 4.1). Human spleen is the recommended positive control for this antibody; however this tissue was not available at the time of performing these experiments, so rat spleen was used. Samples were prepared as described in section 4.2.1. Following this, 4 µm sections were cut and mounted on silane-coated slides. Slides were dewaxed with xylene and dehydrated through graded ethanol solutions. Heat mediated antigen retrieval was performed in 10 mM citrate buffer (10 mM tri-sodium citrate, 10 mM citric acid, pH 6.0) with slides microwaved at 800W for 4 min, then at 160W for 10 min. Slides were then rinsed in PBS and dehydrated via increasing concentrations of ethanol before being immersed in 3% H_2O_2 in methanol for 1 minute to block endogenous peroxidase activity. Slides were rehydrated and washed in PBS before being treated with 0.1% Triton-X100 in citrate buffer, to facilitate antibody penetration and washed again and treated with 50% rabbit serum in PBS for 30 min. Following rinsing in PBS, sections were incubated with an Avidin Biotin Blocking kit as per manufacturer's instructions. Primary anti-macrophage (MAC387) antibody (Abcam ab22506) was diluted at a range of concentrations in order to optimise the primary antibody concentration in 5% rabbit serum and applied to tissue sections and then incubated at 4°C for 20 hours in a humidified chamber.

Table 4.1 Macrophage immunohistochemistry optimisation in colonic mucosa, tonsil and spleen and the concentrations of primary antibody (MAC387) tested.

Tissue Type	Species	Tested Dilutions (Primary antibody)
Colonic Mucosa	Human	1:100
Tonsil	Human	1:100, 1:500, 1:1000 and omit primary
Spleen	Rat	1:100 and omit primary

Slides were then thoroughly washed in PBS and secondary antibody biotinylated rabbit anti-mouse IgG (Abcam ab6727 diluted 1:200) diluted in 5% rabbit serum was applied and incubated for 30 min at room temperature. The secondary antibody was washed off in PBS and Vectastain ABC prepared according to the manufacturer's directions was then applied to each section for 30 min followed by rinsing in PBS. Antibody was visualised with DAB at room temperature for 2 min, washed in distilled water and counter-stained with Lillie Mayer haematoxylin for 2 min. Slides were then dehydrated and dewaxed in xylene before being sealed with a coverslip for microscopy as described in section 4.2.1.

4.2.9 IL-17A Immunohistochemistry

IHC for IL-17A was performed using a previously developed method which is detailed in Chapter 3 of this thesis.

4.2.10 Serotonin (5-HT) Immunohistochemistry

Immunohistochemistry (IHC) to locate EC cells via the presence of 5-HT located within these cells was performed on human colonic mucosa previously obtained from explant studies. Additional 4 µm sections were cut from archival blocks and mounted on silane-

coated slides. Preparation of slides for IHC was performed as described in section 4.2.7. Following this, primary anti-serotonin [5-HT-H209] antibody (Abcam ab16007) was diluted 1:20 in 5% rabbit serum and applied to tissue sections and then incubated at 4°C for 20 hours in a humidified chamber.

Slides were then thoroughly washed in PBS and the secondary antibody, biotinylated rabbit anti-mouse IgG (Abcam ab6727 1:200) diluted in 5% rabbit serum was applied and incubated for 30 min at room temperature. The secondary antibody was washed off in PBS and Vectastain ABC was then applied to each section for 30 min followed by rinsing in PBS. Antibody was visualised with DAB at room temperature for 2 min, washed in distilled water and counter-stained with Lillie Mayer haematoxylin for 2 min. Slides were then dehydrated and dewaxed in xylene before being sealed with a coverslip for microscopy as described in section 4.2.1.

4.2.11 Chemicals and reagents

All chemicals and reagents were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia), unless otherwise specified. DAB-plus reagent set, RPMI-1640 was purchased from Life Technologies (Invitrogen) (Mulgrave, VIC, Australia). Rabbit anti-CD3 antibody, mouse monoclonal anti-MAC387 antibody, mouse monoclonal anti-5-HT, rabbit anti-mouse IgG secondary antibody and TNF-α human ELISA Kit (Item #100654) were purchased from Abcam (Cambridge, UK). Cannabidiol, prostaglandin E_2 EIA kit (#514010) and prostaglandin $F_{2\alpha}$ EIA Kit (#516011) were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Biotinylated goat anti-rabbit IgG secondary antibody (Vector BA-1000), Avidin Biotin blocking kit and Vectastain ABC Kit were purchased from Vector Laboratories (Burlingame, CA, USA.)

4.2.12 Statistical Analysis

Statistical analysis for prostamide ELISA and nitrite concentrations was performed using a one way ANOVA with a Holm–Šídák post hoc test. A p value of $p<0.05$ was accepted as significant. All statistical analysis and graph preparation was performed in GraphPad Prism 6.02 (GraphPad Software, San Diego, USA).

4.3 Results

4.3.1 Prostaglandin E_2 and Prostaglandin E_2 ethanolamide ELISA

Following TLC separation of PGE_2 and the respective ethanolamide (PGE_2-EA), ELISA was performed to quantify the concentration of these prostanoids in media from explant studies. Concentrations of PGE_2 and PGE_2-EA were compared between incubation control, TNF-α + IL-1β (10 ng/mL) and TNF-α + IL-1β (10 ng/mL) + anandamide (AEA, 10 µM) treated mucosal explants. Concentration data is presented as pg/mL of prostanoid per mg of wet mucosa weight. The concentration of PGE_2 in incubation control mucosa was 6.69 ± 2.19 pg/mg, TNF-α + IL-1β treated: 1.99 ± 0.39 pg/mg and TNF-α + IL-1β + AEA treated: 1.95 ± 0.36 pg/mg (Fig. 4.1a). There was a statistically significant decrease in PGE_2 concentration between incubation control mucosa and TNF-α + IL-1β treated mucosa (6.69 ± 2.19 vs. 1.99 ± 0.39 pg/mg). There was no significant difference in PGE_2 concentration when TNF-α + IL-1β treated mucosa was also treated with AEA.

In terms of quantification of prostaglandin E_2 ethanolamide (PGE_2-EA) concentration, the mean concentration of PGE_2-EA in incubation control tissue was 3.41 ±1.22 pg/mg, TNF-α + IL-1β treated: 2.03 ± 0.48 pg/mg and TNF-α + IL-1β + AEA treated: 1.25 ± 0.20 pg/mg (Fig. 4.1b). There was no statistically significant difference between any of the

treatment groups in terms of PGE_2-EA concentration.

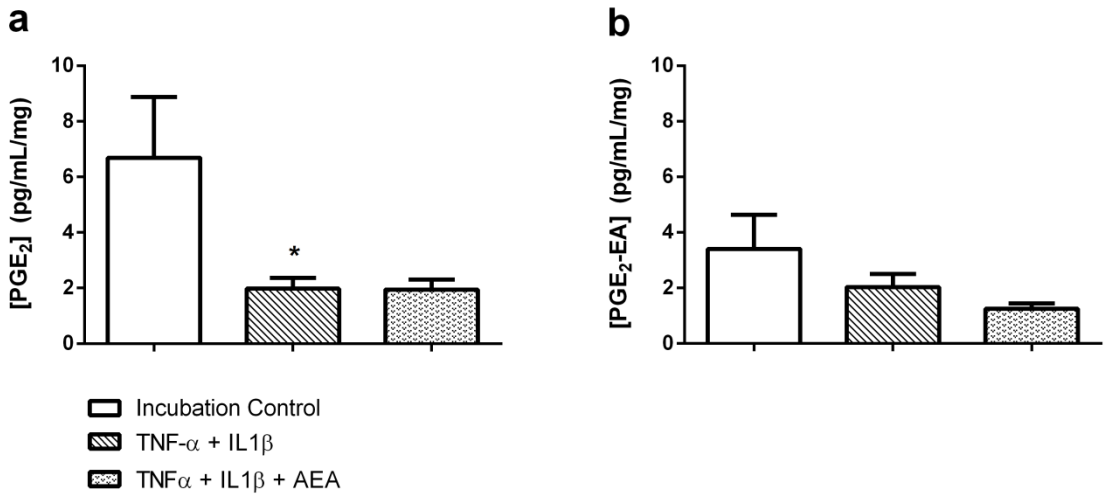


Figure 4.1. Effect of TNF-α + IL-1β and anandamide (AEA) on prostaglandin E_2 (PGE_2) and prostaglandin E_2 ethanolamide (PGE_2-EA) concentration in mucosal explants.

Treatment of mucosal explants with TNF-α + IL-1β (10 ng/mL) produced a statistically significant reduction in PGE_2 levels (*=p<0.05). The addition of anandamide (AEA; 10 μM) to TNF-α + IL-1β treatment did not have any effect on PGE_2 concentration (**a**). n=9 for incubation control, n=10 for TNF-α + IL-1β and n=5 for TNF-α + IL-1β +AEA. PGE_2-EA concentration was not significantly altered from incubation control concentration with TNF-α + IL-1β (10 ng/mL) or TNF-α + IL-1β + AEA (**b**). n=10 for incubation control, n=10 for TNF-α + IL-1β and n=6 for TNF-α + IL-1β+AEA.

4.3.2 Prostaglandin $F_{2\alpha}$ and Prostaglandin $F_{2\alpha}$ ethanolamide ELISA

Separation of $PGF_{2\alpha}$ and the respective ethanolamide by TLC was performed and followed by ELISA to measure the concentration of these prostanoids in explant media. Mucosa was treated with the same concentrations of cytokine and AEA as described in section 4.3.1. $PGF_{2\alpha}$ concentrations in mucosal explant media were 0.54 ± 0.09 pg/mg in incubation control mucosa, 0.81 ± 0.31 pg/mg in TNF-α + IL-1β treated mucosa and 0.40 ± 0.10 pg/mg in TNF-α + IL-1β + AEA treated mucosa (Fig. 4.2a). There were no statistically significant differences between these treatment groups. Similar measurements were carried out for $PGF_{2\alpha}$-EA; 1.22 ± 0.41 pg/mg in incubation control, 0.53 ± 0.16

pg/mg in TNF-α + IL-1β treated mucosa and 0.70 ± 0.32 pg/mg in TNF-α + IL-1β + AEA treated mucosa (Fig. 4.2b). Like $PGF_{2\alpha}$, there was no significant difference between treatment groups in $PGF_{2\alpha}$-EA concentrations.

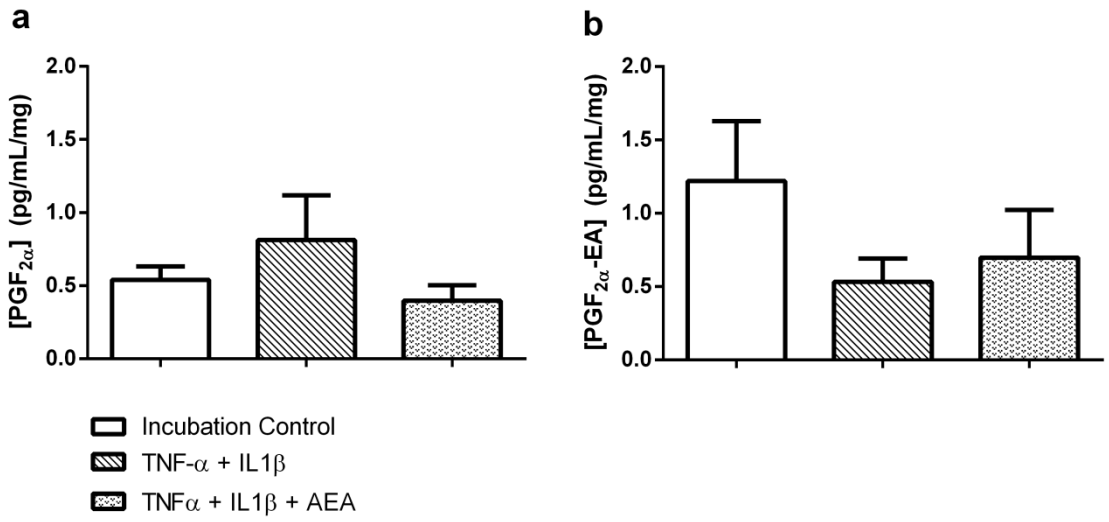


Figure 4.2. Effect of TNF-α + IL-1β and anandamide (AEA) on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin $F_{2\alpha}$ ethanolamide ($PGF_{2\alpha}$-EA) concentration in mucosal explants.

No significant change in concentration of $PGF_{2\alpha}$ was detected in explant mucosa treated with TNF-α + IL-1β (10 ng/mL) or TNF-α + IL-1β + anandamide (AEA; 10 μM) (**a**). n=9 for incubation control and TNF-α + IL-1β, n=5 for TNF-α + IL-1β + AEA. No significant change was also observed for $PGF_{2\alpha}$-EA concentration in mucosal explants treated with TNF-α + IL-1β (10 ng/mL) or TNF-α + IL-1β + anandamide (AEA; 10 μM) (**b**). n=11 for incubation control, n=10 for TNF-α + IL-1β and n=4 for TNF-α + IL-1β + AEA.

4.3.3 Nitrite (NO_2^-) Assay (Colourimetric)

Measurement of nitrite concentration in explant media was used as an end point marker of the production of compounds such as nitric oxide. Nitrite concentration in incubation control mucosa media was 4.57 ± 1.15 μM (Fig 4.3a). No statistically significant change in nitrite levels occurred in explant mucosa when treated with IL-17A (6.71 ± 1.94 μM) compared to incubation control (Fig 4.3a). Nitrite was also measured in treatment groups used in the tissue damage scoring studies. There was no significant effect of any treatment

(IL-17A+ AEA (5.33 ± 1.94 µM) or hydrocortisone (2.46 ± 0.83 µM) or CBD (10.7 ± 2.66 µM)) on nitrite concentrations (Fig. 4.3a). In addition, AEA, hydrocortisone and CBD alone were tested to determine if they affected nitrite levels in mucosal explants. None of these interventions significantly elevated nitrite levels compared to incubation control tissue (AEA, 1.78 ± 0.47 µM; hydrocortisone, 2.84 ± 1.16 µM and CBD, 8.28 ± 2.69 µM) (Fig 4.3b).

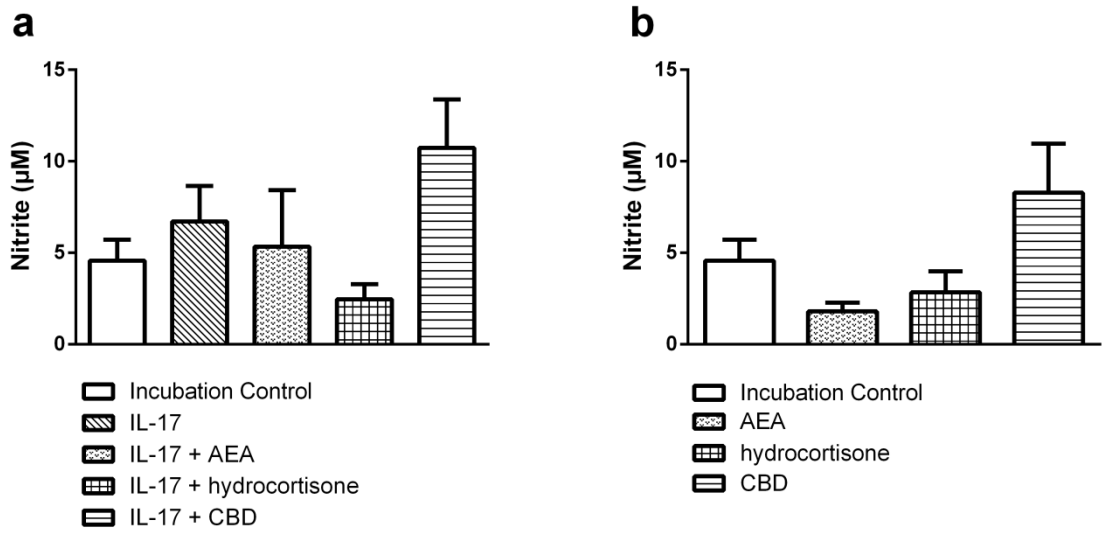


Figure 4.3. Effect of Interleukin 17A (IL-17A) and pharmacological interventions on nitrite concentration in explant mucosa measured by Griess' reagent.

Using a colourimetric nitrite assay, no significant changes in concentration of nitrite in explant media were detected when mucosa was treated with IL-17A (10 ng/mL) or IL-17A + anandamide (AEA; 10 µM), hydrocortisone (1 µM) or cannabidiol (CBD; 10 µM) (**a**). n=10 for incubation control and IL-17A, n=6 for IL-17A + AEA and IL-17A + hydrocortisone and n=4 for IL-17A + CBD. These interventions were also tested without IL-17A in explant studies as a control to determine if they have any effect on nitrite concentrations. No significant effect of these interventions was found on nitrite levels compared to incubation control (**b**). n=10 for incubation control, n=6 for AEA and hydrocortisone and n=4 for CBD.

4.3.4 Nitrite (NO_2^-) Assay (Fluorometric)

A fluorometric nitrite assay was also performed to compare the measurements obtained with colourimetric nitrite assay. This assay is more sensitive and can detect lower concentrations of nitrite compared to colourimetric methods such the commonly used Griess' Reagent. The same samples were analysed using this method (except for control samples containing only AEA, hydrocortisone or CBD). Incubation control media had a nitrite concentration of 2.97 ± 1.05 µM (Fig 4.4). This value was less than that obtained using the colorimetric method. IL-17A-treated media had a nitrite concentration of 2.44 ± 0.65 µM which was not significantly different to incubation control. Treatment with IL-17

+ AEA (1.42 ± 0.31 µM) or hydrocortisone (1.17 ± 0.27 µM) or CBD (2.64 ± 1.29 µM) did not produce a significant change in nitrite levels compared to IL-17A treated mucosal explants (Fig. 4.4).

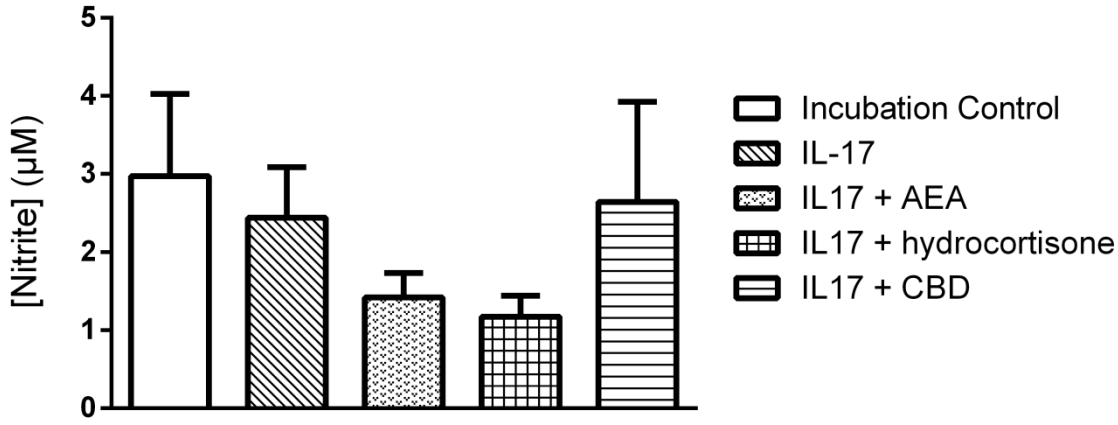


Figure 4.4. Concentration of nitrite in mucosal explant media treated with IL-17A and pharmacological interventions measured by fluorometric assay.

Using a fluorometric nitrite assay (DAN reaction), with increased sensitivity, did not show any significant changes in concentration of nitrite in explant media when mucosa was treated with IL-17A (10 ng/mL) or IL-17A + anandamide (AEA; 10 µM), hydrocortisone (1 µM) or cannabidiol (CBD; 10 µM). n=10 for incubation control and IL-17A, n=6 for IL-17A + AEA and IL-17A + hydrocortisone and n=4 for IL-17A + CBD.

4.3.5 TNF-α ELISA

The TNF-α ELISA was performed on explant media samples from the following treatment groups: incubation control, IL-17A, IL-17A + AEA, IL-17A + hydrocortisone and IL-17A + CBD. The aim was to determine if IL-17A treatment influenced the levels of TNF-α in explant mucosa and if so, did cannabinoid interventions or hydrocortisone affect TNF-α concentrations. Although the sensitivity of this ELISA kit was < 10 pg/mL, no quantifiable amount of TNF-α was detected in the vast majority of the samples (37 samples). The assay however, was reliable, as the standard curve was acceptable with a strong linear regression (r^2=0.9962) (Fig.4.5). In addition, the TNF-α standard included

with the kit (manufacturers assessed concentration: 468 ± 117 pg/mL) was estimated to contain 514.6 ± 15.5 pg/mL from this standard curve which is within the actual concentration range of the standard.

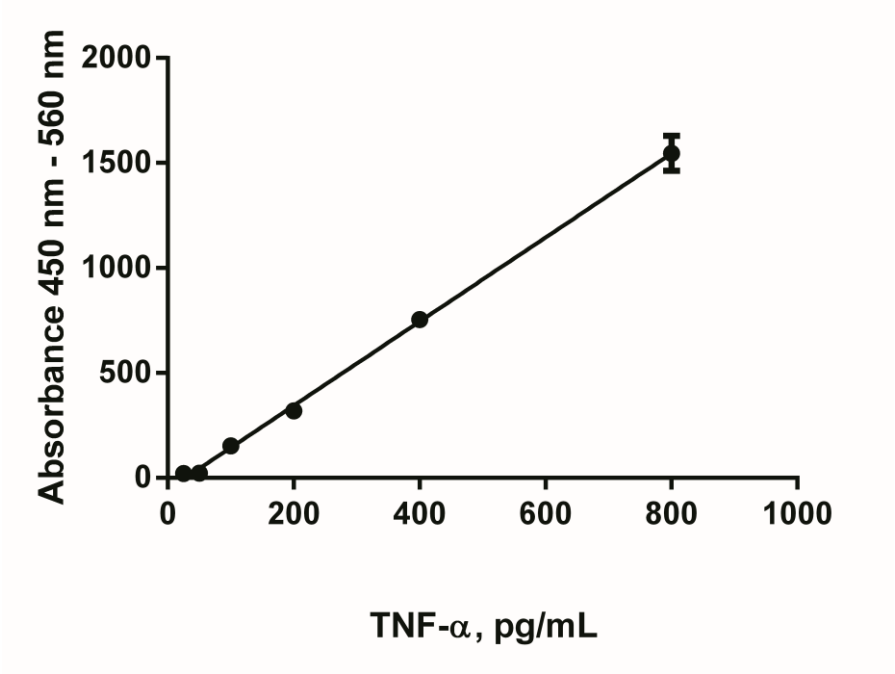


Figure 4.5. Standard curve for TNF-α ELISA assay.

The standard curve was prepared over the TNF-α concentration range of 25-800 pg/mL with each concentration assayed in duplicate. Blank absorbance was subtracted from each concentration. The standard curve was acceptable with an r^2value of 0.9962. The equation for the linear regression was y=2.002 ± 0.039-56.4 ± 14.7

4.3.6 CD3 Immunohistochemistry

Immunohistochemistry (IHC) for CD3 was carried out to make a qualitative assessment as to whether the number of CD3 positive lymphocytes changes under the influence of cytokine (TNF-α + IL-1β) treatment. As previously shown in our explant model, an increase in lymphocyte number occurs when mucosa is treated with cytokines (Chapter 2) and Nicotra et al. (2013). While the morphology of lymphocytes is readily discernible with H&E staining, CD3 labelling would provide a more definitive identification of the lymphocytes observed in explant mucosa compared to using H&E staining alone.

Staining for CD3 in human tonsil using a primary CD3 antibody dilution of 1 in 100 as a starting concentration produced a well-defined immunoreactive labelling pattern of $CD3^+$ T-cells, with distinct bands of cells which did not stain for CD3 (black arrow) (Fig. 4.6a). This result suggests the CD3 antibody was functioning correctly and staining only specific cell types expressing CD3. A negative control, where the primary antibody was omitted, was also performed in the tonsil tissue and showed a complete absence of CD3 staining indicating selectivity of the secondary antibody (Fig. 4.6b). In human colonic mucosa, CD3 staining was observed extensively in non-incubated control tissue. Many of the cells located in the lamina propria stained strongly for CD3, indicating they are T-lymphocytes (black arrow) (Fig. 4.6c). Mucosa subject to explant incubation conditions but without any cytokine treatment (incubation control) (Fig. 4.6d) also demonstrated extensive CD3 staining, with intense staining of particular cell types in the lamina propria indicative of T-lymphocytes. There is no obvious qualitative difference in staining patterns between incubated (Fig. 4.6c) an un-incubated control mucosa (Fig. 4.6d).

Mucosa treated with TNF-α and IL-1β (10 ng/mL) also demonstrated extensive CD3 staining and in addition a possible lymphoid aggregate (black arrow) could be seen in this particular tissue section (Fig. 4.6e). Qualitatively, the pattern of staining in cytokine-treated tissue does not appear different from incubation control tissue, with a large number of cells in the lamina propria appearing to be T-lymphocytes.

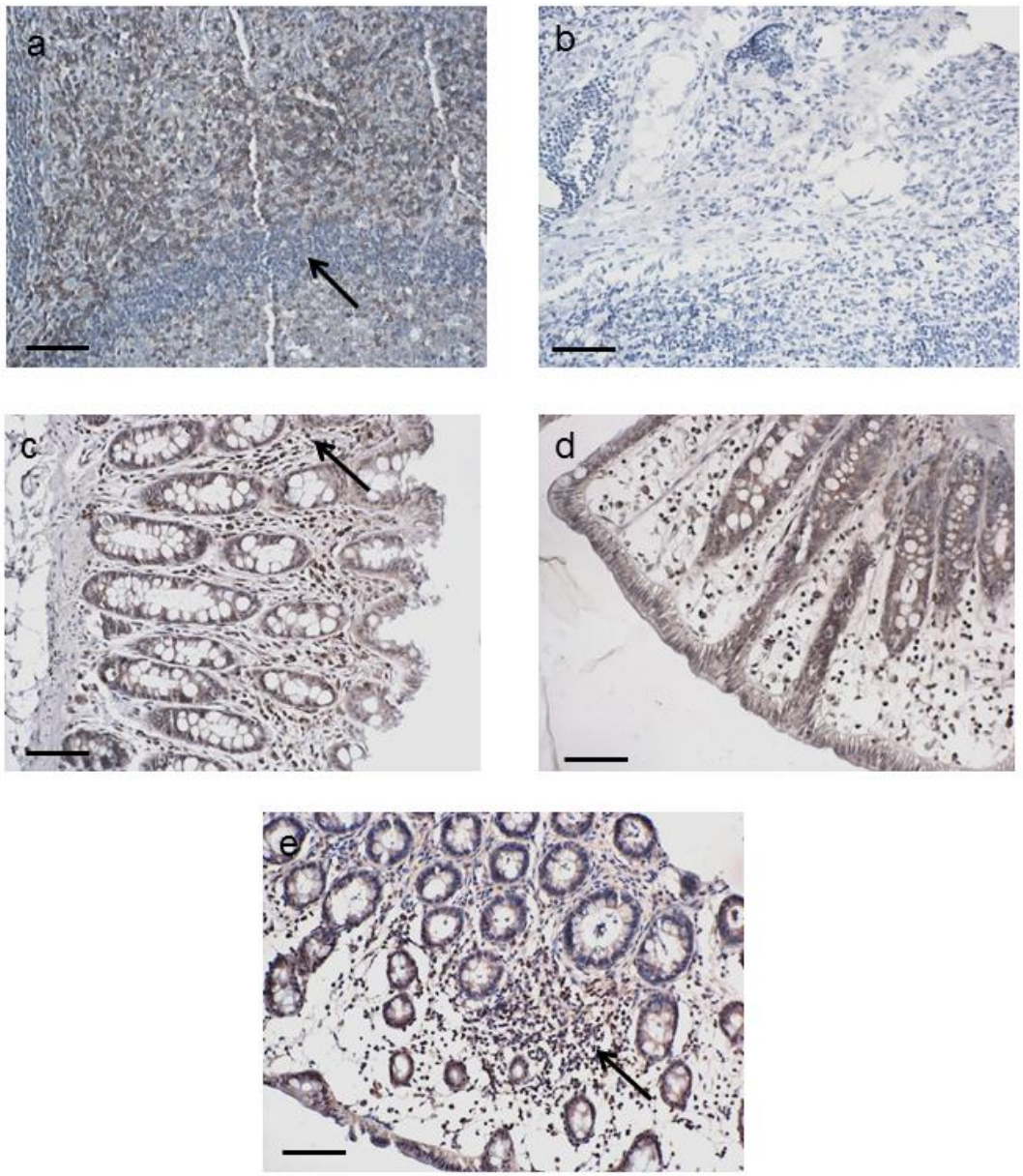


Figure 4.6: Immunohistochemistry for CD3 in human colonic mucosa and human tonsil tissue.

Staining for CD3 (T-lymphocytes) in human tonsil as a positive control, showed extensive staining with distinct bands of unlabelled cells (arrow) (**a**). Omission control without primary CD3 antibody in human tonsil shows a lack of labelling (**b**). Staining for CD3 lymphocytes in un-incubated control colonic mucosa (**c**), arrow highlights staining. Incubation control mucosa stained for CD3 (**d**) and TNF-a + IL-1b (10 ng/mL) treated colonic mucosa stained for CD3 (**e**). All mucosal explant samples are from one patient for comparison. Scale bar, 100 µm.

4.3.7 Macrophage Immunohistochemistry

Immunohistochemistry for macrophages was performed with the aim of determining if there was a change in macrophage number when mucosa was treated with TNF-α + IL-1β, as activation and proliferation of macrophages may be partly responsible for damage observed in mucosa. As the antibody had not been extensively characterised, staining was first attempted in rat spleen and human tonsil. Human spleen is the recommended positive control for this antibody, but was un-available at the time, so rat spleen was used as a substitute. A starting concentration of the primary antibody (1 in 100) was used in human tonsil. Strong staining of an unknown (possibly connective) tissue occurred (black arrow) with some faint staining of certain cell types (Fig. 4.7a). Small, rounded granules appeared in the tissue section, probably paraffin wax not removed during final xylene clearing. In another section of tonsil at higher magnification, many cells stained as possible macrophages with some isolated cells also staining (black arrow) (Fig. 4.7b).

In rat spleen, faint staining for the macrophage marker occurred (Fig. 4.7c), but the staining was weak and not particularly concentrated. In an additional rat spleen section, no apparent staining could be observed even with the relatively high 1 in 100 dilution of primary antibody (Fig. 4.7d). After performing these studies in rat spleen, it was subsequently determined (approximately a year later) by the manufacturer that the MAC387 antibody does not react with rat tissue. In human colonic mucosa, macrophage staining was absent, with a lack of any distinct stain in the section (Fig. 4.7e). Additional sections from other patients also showed a similar pattern, with a lack of any distinct macrophage staining (data not shown).

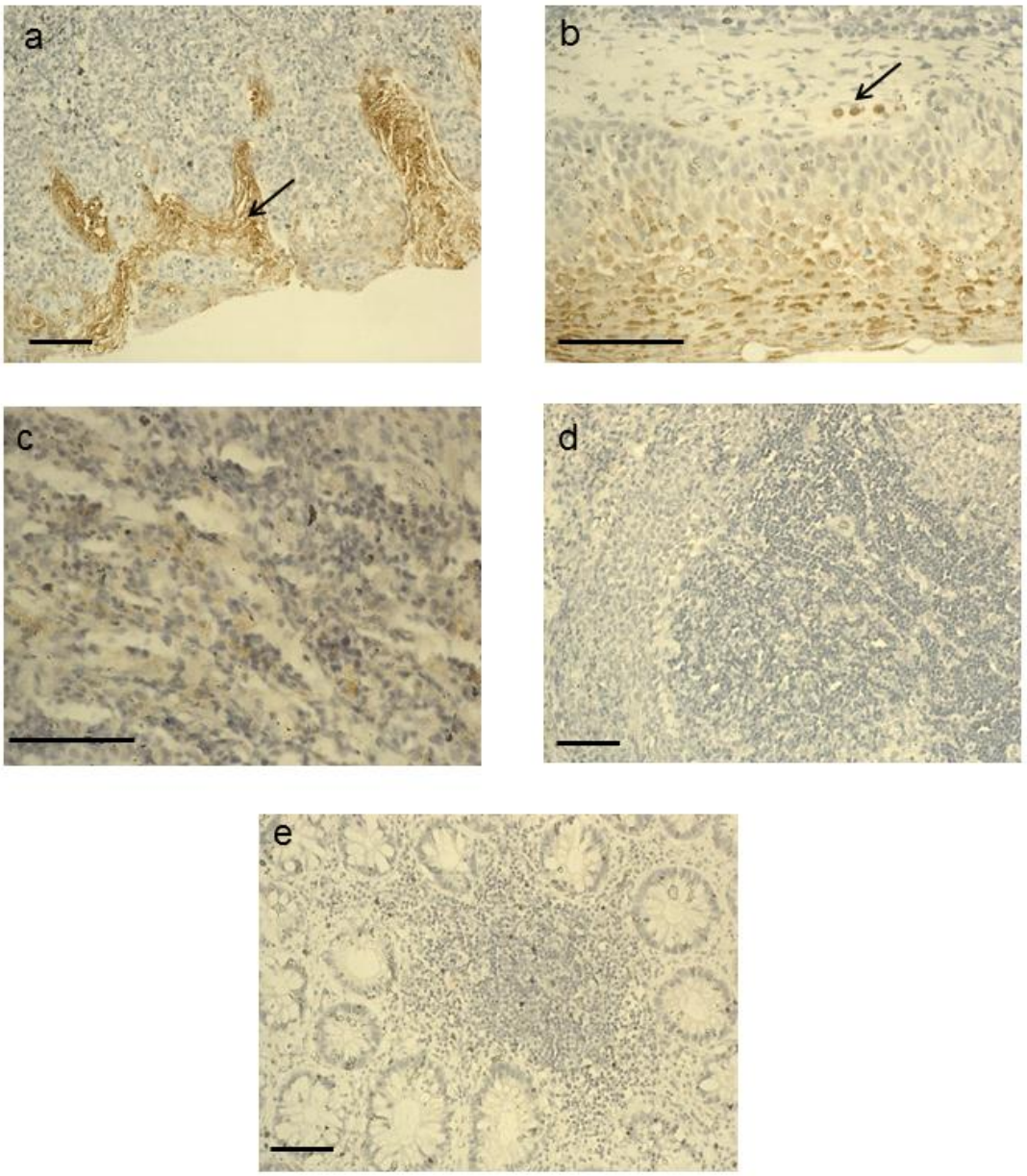


Figure 4.7: Immunohistochemical staining for macrophages using MAC387 primary antibody in human tonsil, rat spleen and human colonic mucosa.

Immunolabelling for macrophages in human tonsil, showed non-specific staining of connective tissue and a lack of staining of any particular cell types (arrow) (a). Higher magnification (40 ×) of human tonsil shows some specific cells labelled (arrow), but many other cell types also labelled (b). In rat spleen, staining was weak (c) and in some cases also absent (d). A lack of staining was also seen in human colonic mucosa (un-incubated control) (e). Small dark spots in this cluster (e) may be a result of paraffin wax not removed during xylene clearing. Scale bar, 100 µm.

4.3.8 IL-17A and 5-HT Immunohistochemistry

Immunohistochemistry for IL-17A and serotonin (5-HT) was performed as an initial study in an attempt to determine if intensely labelled IL-17A positive cells are actually EC cells containing 5-HT. Staining for IL-17A using the method previously described in Chapter 3, showed small, rounded cells (arrows) with intense staining for IL-17A (Fig. 4.8a). In Fig. 4.8b these particular cells are magnified and highlighted (arrows). In addition, staining for IL-17A appeared in goblet cells and also in select cells of the lamina propria, as we have previously seen in Chapter 3. Staining for 5-HT is shown in Fig 4.8c,d. This antibody produced a very specific stain of cells at the edge of crypts with a similar morphology and localisation to those cells also staining for IL-17A in Fig. 4.8a,b, although these cells appeared larger and granular. Intense staining for 5-HT and the particular distribution of stain strongly suggests these cells are EC cells (Fig. 4.8c,d) (Cremon et al., 2011). Overall, the performance of the 5-HT antibody used in this study was very good with specific labelling and an absence of background staining.

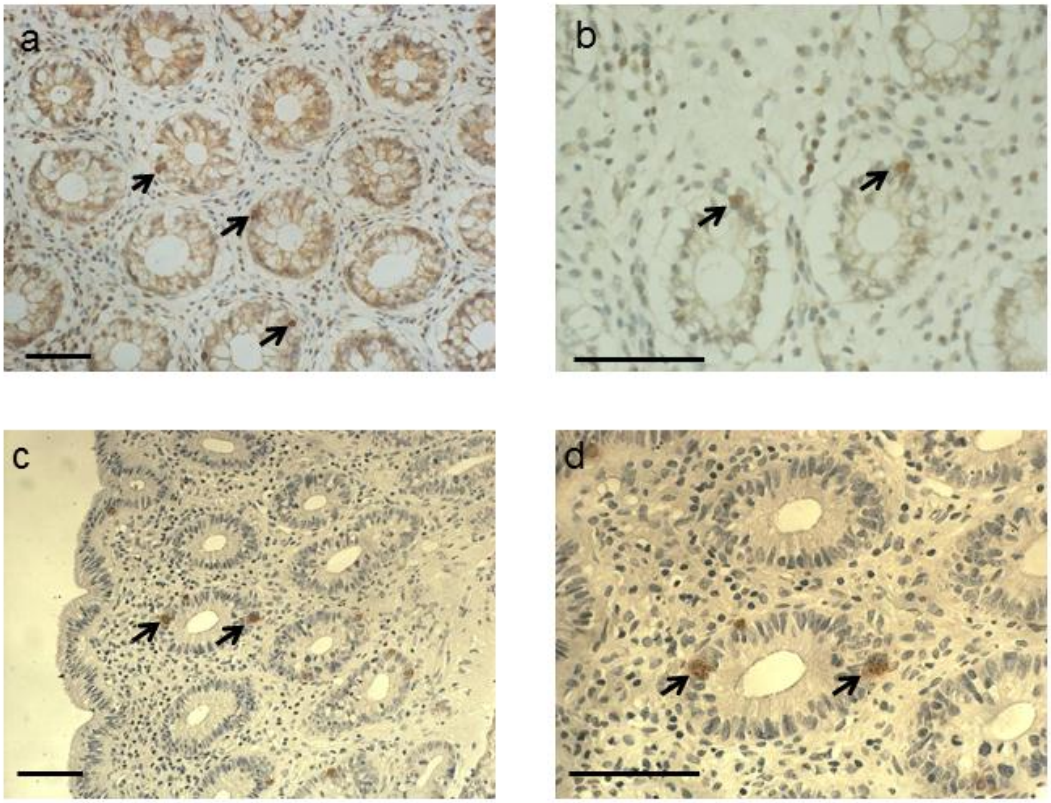


Figure 4.8: Immunohistochemical staining for IL-17A expression and serotonin (5-HT) containing enterochromaffin cells in human colonic mucosal explants.

Immunolabelling for IL-17A and 5-HT expression in human colonic mucosa. IL-17A expression was localised extensively in goblet cells, select cells of the lamina propria and small, intensely labelled cells at the edge of crypts (arrows) (a,b). This is shown at 40 × magnification in (b). Labelling for 5-HT is highly specific and only select cells, likely to be enterochromaffin cells (ECs) at the edges of crypts are labelled (arrows) (c,d). ECs labelling for 5-HT are similar in morphology and location to those cells expressing IL-17A. Scale bar, 100 µm.

4.4 Discussion

The overall aim of this series of experiments was to determine how proinflammatory cytokines involved in IBD such as TNF-α + IL-1β and IL-17A cause damage to human colonic mucosal tissue under explant conditions. To study this, biochemical assays of inflammatory mediators and immunohistochemical assays of select immune cells were carried out to determine their distribution and expression associated with the mucosal damage. Mucosal damage after inflammatory cytokine incubation involved luminal

epithelial loss, crypt destruction and additionally, in the case of TNF-α + IL-1β, an increase in lymphocyte density in the lamina propria. Scoring data for these parameters is presented in Chapters 2 and 3 of this thesis.

The concentration of prostaglandins and prostamides in explant media following incubation with TNF-α + IL-1β and the endocannabinoid, anandamide (AEA) was quantified by ELISA. The significant finding here was a decrease in PGE_2 levels after TNF-α + IL-1β treatment compared to incubation control. Previous studies have suggested that PGE_2 may actually have a protective or restorative role in the gut (Kabashima et al., 2002, Jiang et al., 2010).This is also evidenced by the potential capacity of NSAID treatment to worsen IBD as the conversion of arachidonic acid to PGE_2 is inhibited, although this concept remains controversial (Kefalakes et al., 2009). It may be possible that a decrease in PGE_2 production after cytokine treatment is involved in mucosal damage observed in the explant model. In the case of other prostanoids measured ($PGF_{2\alpha}$, $PGF_{2\alpha}$-EA and PGE_2-EA), there was no significant changes in levels of these prostanoids after cytokine treatment. In addition, the production of prostanoids after anandamide (AEA) treatment was not affected. As AEA is converted by COX-2 into PGE_2-EA and subsequently $PGF_{2\alpha}$,-EA, we hypothesised that AEA treatment may increase the amount of prostaglandin ethanolamides formed. This however, did not appear to occur in these studies, likely because we did not see an induction of COX-2 activity or expression in the cytokine-treated mucosal explants in our previous study (Nicotra et al., 2013). While COX-2 activity can be induced in human colonic mucosal samples, the time course for induction in these studies was a maximum of 8 hours, so our longer duration of incubation (20 hours) may have led to a diminished level of expression (Dai et al., 2012). It was interesting to note that PGE_2-EA and $PGF_{2\alpha}$-EA were found in measurable quantities in human colonic mucosa which to our knowledge has not been detected before.

The detection of prostamide and prostaglandin levels by ELISA is difficult, as ELISA kits used in these studies cross react with both prostamides and prostaglandins. Separation by thin layer chromatography (TLC) is a useful method of first separating these compounds to allow separate ELISAs to be run (Glass et al., 2005). We used this technique in our studies, but it was difficult to perform accurately. In addition, we found poor recovery of PGE_2-EA (34%) compared to other prostanoids in our studies. This may explain why we did not see any significant changes in PGE_2-EA levels in this study. Ideally, detection of prostamides by mass spectrometry may be a useful alternate method of quantifying prostanoids, although this technique also has limitations in terms of sensitivity (Weber et al., 2004) and was not available during these studies.

Previous studies have shown that nitrite levels can be increased when inflammation occurs in the colon, such as during UC (Kolios et al., 1998, Linehan et al., 2005). Nitrite is a stable breakdown product of nitric oxide which can be measured by a number of assays. The use of Griess' reagent is a convenient colourimetric method of measuring nitrite concentrations (Guevara et al., 1998). In this study, nitrite was measured after IL-17A treatment of mucosal explants and in the presence of cannabinoid and hydrocortisone treatment. There was no significant difference in nitrite levels after IL-17A treatment. Although not statistically significant, nitrite levels tended to decrease after hydrocortisone treatment. All of the compounds; AEA, hydrocortisone and CBD were also tested on their own to determine if they affected nitrite levels. None of these compounds significantly increased nitrite; however, cannabidiol tended to show an elevation of nitrite compared to control. The drawback of the Griess nitrite assay is that it is not particularly sensitive at low concentrations of nitrite and can only detect accurately concentrations > 2.5 µM (Promega, 2009). Evidence of this was shown when at low nitrite concentrations < 2.5µM

were trialled in the standard curve, there was increased variability between two replicates of the standard concentration (data not shown).

To address this issue, a fluorometric nitrite assay was trialled to detect nitrite. This assay kit was more sensitive than the colourimetric assay previously used (detection range 1-10 μM) (Sigma-Aldrich, 2013). Therefore, if limited amounts of nitrite were produced by the mucosa, it was predicted it may be more likely to be detected by this assay. The same interventions were tested again in this assay; however IL-17A did not produce a significant elevation in nitrite levels. There was however a trend for AEA and hydrocortisone to reduce overall nitrite levels, but this is not relevant in the context of a lack of IL-17A elevation of nitrite. The overall performance of the assay was reliable, with an accurate standard curve produced even at low nitrite concentrations (data not shown). One major issue with the assay was the possible interference of phenol red found in RPMI-1640 media (Sigma-Aldrich, 2013). The media from the explant studies contained phenol red, and it was not possible to repeat the explant studies without phenol red. To determine if this would actually interfere with the experiment, the standard curve was prepared in RPMI-1640 containing phenol red and it did not appear to affect the assay. However, to confirm that this was not interfering with this study, explant studies could be repeated in phenol red free RPMI-1640 and nitrite measured using this method. As per COX-2 expression described previously, it is possible we may have missed the ideal time for measurement due to the relatively long time course.

Previously it has been shown that IL-17A can influence the production of TNF-α. Mice treated with IL-17A release large amounts of TNF-α from intestinal Paneth cells leading to shock (Takahashi et al., 2008). Although Paneth cells are not extensively found in the colon compared to the small intestine, small amounts of these or other immune cells (such as macrophages and T-cells) may release TNF-α. To test this hypothesis, we attempted to

measure TNF-α levels using ELISA after IL-17A treatment. No detectable concentrations of TNF-α could be found in any of the mucosal explant homogenates tested (data not shown). Therefore, it appeared that IL-17A may not induce an increase in detectable TNF-α in this model. The standard curve for the kit was successfully prepared and suggests the assay was performing as specified. The limit of detection for this ELISA kit was 24.58 pg/mL therefore, it may be possible that the level of TNF-α produced by IL-17A is undetectable using this ELISA. In further studies, another ELISA that has greater sensitivity and is optimised for use with mucosal homogenates could be used. We have also previously observed an increase in TNF-α secretion after T-cell stimulation in human colonic mucosal explants using a combination of the lymphocyte activators, phytohaemagglutinin (PHA) and concanavalin A (Con A) in preliminary experiments (W. Reed, unpublished data). This indicates that selective immune cell activation may be required to reveal significant changes in tissue TNF-α.

In Chapter 2, treatment of explant mucosa with TNF-α and IL-1β resulted in a significant increase in lamina propria lymphocyte density compared to incubation control. The lineage of these lymphocytes was unknown and we attempted to use immunohistochemistry to determine if these lymphocytes were T or B cells. Due to the effectiveness of hydrocortisone in reducing lymphocyte density, it is conceivable these lymphocytes were T-cells that migrate from extensive sub-mucosal lymphoid aggregates. This was inferred as lymphocytes did not proliferate which was determined by BrdU staining experiments. The first antibody we tested was for CD3, a marker common to both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T-lymphocytes (Coico et al., 2003). As we had not previously used this antibody before we carried out optimisation experiments using human tonsil as a positive control.

Staining of $CD3^+$ cells in tonsil was successful and showed distinct areas of staining containing T-cells with surrounding bands of absent staining. From this, an optimal antibody concentration was obtained to test in mucosal explants. Staining of colonic mucosa was not as clear as that in tonsil, with some non-specific staining of luminal epithelium. Many cells stained for CD3, indicating a vast majority of these lymphocytes in the mucosal explants are T-cells. This may explain why hydrocortisone treatment in the model was useful in reversing damage parameters and lymphocyte density. There was not however, any qualitative change in T-cell density seen between TNF-α and IL-1β treated sections compared to incubation control in the limited number of samples we stained for CD3. Using H&E staining we do however notice a significant increase in overall lymphocyte density between control and TNF-α and IL-1β treatment. This result may indicate that the T-cell population in the lamina propria remains stable after TNF-α and IL-1β treatment and other types of lymphocytes are responsible for the increase in lymphocyte density seen with H&E staining. These other lymphocytes could include B-cells or natural killer (NK) cells (Roberts-Thomson et al., 2011). Further IHC studies with other markers such as CD19 for B-cells could be used to determine the identity of these lymphocytes. In addition, flow cytometry (FACS) ideally would give qualitative data of $CD3^+$ cells and other immune cell types in mucosal explants. From this data, we could be more confident that $CD3^+$ lymphocyte density does not actually change.

The other cell type that we aimed to identify was macrophages in mucosal explants. Studies have suggested that macrophage activity can be increased in IBD, for example increased production of pro-inflammatory cytokines (Steinbach and Plevy, 2014). We hypothesised that increased activity of macrophages and subsequent production of downstream mediators, such as nitrite, may contribute to tissue damage observed with TNF-α + IL-1β treatment in mucosal explants. In addition, we also wanted to determine if

macrophage density changed after TNF-α + IL-1β treatment. If we found this to be the case a technique such as BrdU could then be used to determine if macrophages are proliferating. As we had not used this antibody before, we optimised it first in rat spleen. The optimal positive control is human spleen, but this tissue was not available for our studies. Staining of rat spleen was not ideal, with dense staining appearing in pockets of possible connective tissue, rather than labelling specific cell types. In addition, there were some sections of rat spleen that showed only very weak labelling, or what appeared to be non-specific staining. Approximately 1 year after performing this study, the antibody specifications were modified by the manufacturer to indicate the antibody does not react with rat tissue. Therefore, it is not surprising we did not see any immunoreactivity in rat spleen. When we tested this antibody in human colonic mucosa, a lack of consistent staining was seen. As a result, we did not pursue use of this antibody. It is not entirely clear why this antibody did not appear to work, as generally it has given clear results as seen on the manufacturer's website. In future a different macrophage marker could be tested, possibly CD68 which shows specificity only for macrophage lineage cells (Murray and Wynn, 2011). It is likely that immunohistochemistry for immune cells may generally have more utility in studying movement or positioning of immune cells within mucosa and the relationship to mucosal damage, rather than measuring changes in cell number.

After performing IL-17A IHC, presented in Chapter 3, we noticed small, densely labelled cells located at the basolateral aspect of the crypts. Due to their location and morphology it is possible these cells may be an enterochromaffin cells (EC) or other enteroendocrine cells (Cremon et al., 2011, Gershon, 2013). To begin the process of determining the identity of these cells, we used tissue sections from mucosa stained for IL-17A and then stained for 5-HT. The results showed that EC cells were present in these tissue sections at a similar location and with similar morphology to cells expressing IL-17A It was difficult

however to find the exact locations in the tissue to match IL-17A expression with 5-HT expression. Therefore, we cannot confirm that these EC cells also contain IL-17A without performing a co-localisation experiment and staining for 5-HT and IL-17A on the same tissue section. Previous studies have not identified if EC cells contain or produce IL-17A, however previously it has been shown that an isolated enteroendocrine cell line can produce pro-inflammatory cytokines and other chemokines (Selleri et al., 2008). In addition, as described previously, Paneth cells can produce IL-17A (Takahashi et al., 2008). EC cells are related to Paneth cells as they share a common stem cell precursor (van der Flier and Clevers, 2009). Therefore, it could be speculated that if the ability to produce IL-17A is common to the stem cell precursor it may be retained in the EC cell (as well as the Paneth cell).

In conclusion, a range of biochemical markers including nitrite, TNF-α and prostanoid concentrations were tested in this series of studies. It appeared that these markers did not show any major significant changes in mucosal explants when treated with IL-17A. This could be due to an actual lack of biochemical changes in these markers, or in some cases further optimisation of these assays being required. In terms of the immunohistochemistry studies, no significant changes appeared to occur between TNF-α and IL-1β treated explants compared to incubation control. Reasonable optimisation of CD3 staining was performed and this could be further pursued. In the case of the macrophage (MAC387) antibody, this probably is not a very useful antibody and an alternative marker could be used. We also showed that EC cells may possibly contain IL-17A in the colon, although further studies would be required to confirm this. Although these studies did not provide significant insight into immune cells and inflammatory mediators potentially expected to be altered in the model, they provide a guide to possible studies that could be conducted to determine how inflammatory cytokines cause damage in colonic mucosa, as well as

potential limitations in *ex vivo* studies of mucosal inflammation. If any of these markers showed clear changes with cytokine treatment, the next logical study would be to determine if cannabinoid treatments modulate these markers. This would then provide a mechanism for how these compounds reverse the mucosal damage demonstrated in this model.

Chapter 5 : Discussion and Conclusions

As many previous animal studies of colitis have shown, treatment with cannabinoids appears to reduce inflammation and mucosal damage in these models. This provides evidence that cannabinoids have potential immune suppressant and muco-protective effects. Although these models have been extremely useful in studying aspects of human IBD, they do not model all aspects of the disease. Questions regarding the efficacy of cannabinoids in treating human IBD remain unanswered. In addition, treatments for IBD are available, but many can show a lack of efficacy or development of adverse effects, particularly immunosuppressants used to treat CD.

The primary aim of this thesis was to determine if cannabinoids provide mucosal protection against pro-inflammatory cytokine induced damage in human colonic mucosa. To study this, a human colonic mucosal explant model was used. The pro-inflammatory cytokines used to produce tissue inflammation included TNF-α, IL-1β and IL-17A. All of these cytokines play an important role in the pathology of IBD (Fujino et al., 2003, Olsen et al., 2007, Roberts-Thomson et al., 2011). Previously in our laboratory, we have demonstrated that a combination of TNF-α and IL-1β induces damage in human colonic mucosa. This damage consists of luminal epithelial loss, crypt destruction and lymphocyte infiltration in the lamina propria (Nicotra et al., 2013). We used this model extensively in this thesis and further characterised biochemical changes that occur after cytokine treatment.

An additional aspect of this thesis was to further characterise the effects of these cytokines on GI epithelial barrier permeability in Caco-2 monolayers. If these cytokines had any detrimental effect on permeability, cannabinoid interventions could be tested in this model to determine if they can restore permeability. This, in combination with data from the

human mucosal explant studies, may then demonstrate cannabinoids can restore epithelial permeability as well as protect tissue from inflammatory damage.

Throughout this thesis a number of different techniques have been used including histological scoring, biochemical assays (i.e. ELISA and western blotting), immunohistochemistry and cell culture. All of these techniques aimed to firstly determine the effects of either TNF-α + IL-1β or IL-17A on human colonic mucosal explants or Caco-2 monolayers. Secondly, to determine the effects of cannabinoids on cytokine induced mucosal damage, biochemical changes and Caco-2 monolayer permeability. This discussion will therefore be structured in terms of the different experimental techniques used in order to clearly explain the links between the two publications and conventional chapter that compose this thesis.

5.1 Histological Scoring of TNF-α + IL-1β and IL-17A Induced Colonic Mucosal Damage

In this study, both the cytokine combination TNF-α + IL-1β, or IL-17A induced a form of mucosal damage which has some features of clinical colitis. This damage was measured by blinded scoring of damage parameters using H&E stained slides for each treatment group. Both cytokine treatments caused significant damage to the luminal epithelium and crypt destruction. We did not directly compare whether IL-17A or TNF-α + IL-1β causes greater luminal epithelial damage or crypt destruction, as this was not one of the aims of this study (although this could be considered in the future). TNF-α + IL-1β treatment induced an increase in lymphocyte density while IL-17A did not demonstrate this. Increased lamina propria lymphocyte density is a feature of IBD and it appears that IL-17A alone (in this model) may not be sufficient to induce this. In IBD, all three of the cytokines studied in

this thesis (and many others such as IFN-γ) would be present together, which may explain why some of the features of IBD are not present.

5.2 Pharmacological Interventions to Attenuate Mucosal Damage

A typical pharmacological intervention in the treatment of IBD is the immune-suppressant, hydrocortisone. We tested hydrocortisone in mucosal explants treated with TNF-α + IL-1β and IL-17A and found it reduced lymphocyte density (not applicable to IL-17A treatment), luminal epithelial damage and crypt destruction. This result was anticipated and helps support the validity of cytokine-induced damage in mucosal explants as a viable model of colitis. As hydrocortisone has a relatively well understood mechanism of action we can infer that activation of T-lymphocytes may mediate the mucosal damage observed in explants (Newton, 2000). Less is known about effects of hydrocortisone on modulating IL-17A activity, although in mice induction of sepsis produced increases in IL-17A which were further increased with removal of adrenal glands (which produce hydrocortisone, cortisol) (Bosmann et al., 2013). Therefore it may be possible that hydrocortisone supresses IL-17A production.

We also tested if cannabinoid agonists could also reduce mucosal damage. In animal studies, cannabinoids reduce mucosal inflammation and other indices of damage in chemical models of colitis, and this is well established (Massa et al., 2004, D'Argenio et al., 2006, Kimball et al., 2006, Borrelli et al., 2009, Schicho and Storr, 2012). We found the endocannabinoid, AEA produced significant reductions in damage parameters which were reversed by JTE-907 (CB2R inverse agonist). In addition, the CB1R selective agonist ACEA did not reduce indices of mucosal damage after TNF-α + IL-1β treatment. These results all suggest it is the CB2R mediating these protective actions. Treatment of explants with JWH-105 (a selective CB2R agonist) and subsequent reversal or protective effects

with the CB2R inverse agonist JTE-907 strengthen the evidence for the involvement of the CB2R. These results tie in with the fact that CB2R are expressed mainly on immune cells and supress their function, which is described in detail in the introduction of this thesis. These results are supported by animal studies, where CB2R activation is protective. Interestingly, CB1R knockout animals have worsened colitis (Massa et al., 2004); however in our human mucosa, the CB1R does not seem to be involved. This may be due to differences between humans and rodent models or differences in the way colitis is induced (cytokine treatment vs. chemical induction).

We also tested if select cannabinoids could reduce IL-17A-induced mucosal damage. Treatment with AEA also reduced luminal epithelial damage, as it also did in TNF-α + IL-1β treated explants. It did not significantly reduce crypt destruction, but with additional explant studies this may be statistically significant. We did not test if the effects of AEA are mediated by CB1R or CB2R (using selective CB1R/CB2R agonists) as we did with TNF-α + IL-1β treated explants, but this would be a valuable future study to further determine how AEA protects against IL-17A induced damage. In the IL-17A studies we also tested if, the non-psychoactive phytocannabinoid, CBD reduced mucosal damage. Studies in animal models have demonstrated its protective effects in colitis models, with relative lack of behavioural or toxic effects (Cunha et al., 1980, Borrelli et al., 2009, Esposito et al., 2012). As this compound is devoid of these effects it may make it an attractive compound to use in the treatment of IBD. Recent studies have however suggested CBD may have some pro-inflammatory properties in rodents, although this was a high dose (75 mg/kg, by oral gavage) so it is not entirely clear whether this would be a beneficial treatment (Karmaus et al., 2013). Previously, CBD has been administered at 20 mg/kg by oral gavage, but this had no effect on colitis parameters (Schicho and Storr, 2012). To our knowledge CBD has not been tested in human colonic mucosa specifically

against IL-17A induced mucosal damage, however it has shown benefit against inflammation in human UC biopsies stimulated with LPS + IFN-γ (De Filippis et al., 2011). This result provides additional evidence from human tissue that CBD may be an effective treatment against inflammation. We did not test CBD in TNF-α + IL-1β treated explants, but this would be useful to perform in the future. As CBD has unusual pharmacology and can bind to a range of receptors (for example, PPAR-γ (Ryberg et al., 2007, Esposito et al., 2012)), further understanding of its mechanism of action is required.

5.3 Biochemical Markers Involved in Mucosal Damage

Following on from histological scoring of mucosal damage, we also wanted to determine which markers of inflammation may be changed in the presence of cytokines. For many of these markers, only the effect of cytokine treatment compared to untreated controls was studied. Nitrite is a useful marker for tissue damage that can be readily measured by a simple assay. Nitrite was measured by colourimetric Griess' reagent in mucosa treated with TNF-α + IL-1β. In this study, the cytokine treatment caused a significant increase in nitrite levels compared to incubation control. We did not further determine if cannabinoid interventions or hydrocortisone reduced nitrite levels. If this was tested and found to be the case, it would provide some explanation as to how these compounds reduced mucosal damage in explants. In Chapter 4, we also tested if IL-17A could increase nitrite, with the hypothesis that this may be involved in IL-17A induced mucosal damage. No significant elevation in nitrite occurred, although only a smaller sample size than the TNF-α + IL-1β treated explants was available. This result suggests IL-17A may not cause mucosal damage by increasing NO (measured by nitrite concentration), in contrast to TNF-α + IL-1β where elevations of nitrite appear to occur. This demonstrates differences in the way in which these cytokines appear to exert mucosal damage. Our results correlate with other studies showing nitrite elevations (Kimura et al., 1998, Linehan et al., 2005); however studies

examining IL-17A effects on nitrite levels in colon have not been published. It is possible however for IL-17 to increase nitrite production in cartilage (Attur et al., 1997). As discussed in Chapter 4 there were some methodological issues with the nitrite assay overall, and to overcome this refinements of the assay or increased sample size would be required to draw definite conclusions regarding IL-17A effects on nitrite production in colonic mucosa. In addition, nitrite measurement does tend to have a high background (especially in the colourimetric assay) therefore, it may not be a particularly specific marker of inflammatory damage.

An additional biochemical marker that we studied was enzyme activity of matrix metalloprotease (MMP). MMP activity was elevated in mucosal explants treated with IL-17A suggesting MMP enzyme activity may be responsible for mucosal damage observed in the scoring data. This result is consistent with other literature where IL-17A can induce expression of MMP activity (Jovanovic et al., 1998). The assay used did not differentiate between different sub-types of MMP and this would be useful in future to determine. It would have been informative to measure MMP activity in IL-17A + CBD, AEA and hydrocortisone treated explants. This would have provided a mechanism for how these compounds reduce mucosal damage. It has been reported that CB agonists can reduce MMP activity (Blazquez et al., 2008). We also measured MMP activity in TNF-α + IL-1β treated explants and this data are included in Chapter 3. No significant elevations in MMP activity were observed which contrasts with the literature, which suggests that in skin, TNF-α can increase MMP-2 activity (Han et al., 2001). In the colon, it is inferred that TNF-α could increase MMP activity as CD patients treated with the anti-TNF-α antibody (infliximab) have reduced MMP expression (Di Sabatino et al., 2009).

As we had extensively studied TNF-α and IL-1β in explants and found that IL-17A could cause a similar form of mucosal damage, we considered whether the TNF-α + IL-1β

combination may induce expression of IL-17A which may form part of the mechanism of mucosal damage. In one study, TNF-α was shown to induce IL-17A release from Paneth cells (Takahashi et al., 2008). Conversely, another study suggested IL-17A could increase expression of TNF-α (Jovanovic et al., 1998). Because of this, we attempted to measure IL-17A expression in mucosal explants via ELISA, western blotting and immunohistochemistry. The ELISA (of media supernatant) revealed expression of IL-17A under basal conditions (incubation control). Treatment with TNF-α + IL-1β and TNF-α + IL-1β + CBD (in a limited number of samples) did not show any change in IL-17A expression. The western blot revealed the same results with no change in expression, although this was in homogenised mucosa. This indicated the IL-17A was not bound to the tissue as we thought which may have explained why the ELISA did not show a significant result. This data shows it is unlikely (in this model) that TNF-α + IL-1β influences expression of IL-17A and elevations in IL-17A expression (after TNF-α + IL-1β treatment) do not explain mucosal damage in the model. Again, it is difficult to draw direct comparisons with the literature as the model has not been extensively studied. Conversely, we also found IL-17A treatment did not induce TNF-α expression as measured by ELISA of the media supernatant (Chapter 4). We did not however perform western blotting of the mucosal homogenate, so it is possible the TNF-α remained bound to the mucosal tissue rather than being secreted into the media.

As described in Chapter 4, we also measured prostaglandins and prostaglandin ethanolamides by ELISA. These experiments were conducted to further expand on a previous study we performed where prostaglandin ethanolamides (specifically, bimatoprost) was shown to be protective in the mucosal explant model (Nicotra et al., 2013). The general aim was to determine if TNF-α + IL-1β treatment modified overall prostamide levels in mucosal explants. Overall, we found a significant reduction in PGE_2

concentration in TNF-α + IL-1β treated explants. Other prostanoids measured showed no significant changes in concentration. As detailed in Chapter 4 (discussion), reasons why these outcomes may have occurred are provided. It is possible that PGE2 production may have occurred earlier in the explant incubation and decline by 20 hours (Dai et al., 2012). A future study could include sampling the media and assaying prostanoids earlier time points. Interestingly, PGE_2 treatment was protective in our mucosal explants (but only against luminal epithelial damage) (Nicotra et al., 2013) and treatment with TNF-α + IL-1β reduced levels of PGE_2. Therefore cytokines may influence prostaglandin levels in the colon and this may play a role in mucosal damage. A study by Kabashima et al., (2002) also suggested PGE_2 can be protective in colitis and this hypothesis was discussed in Chapter 4 of this thesis.

5.4 Immunohistochemical Studies of Immune Cell Class, IL-17 Family Cytokine Expression, 5-HT Expression and Cell Proliferation

Throughout this project a range of immunohistochemical studies were performed to determine the location (and type) of immune cells in mucosal explants, IL-17A/IL-17C expression, 5-HT expression in enterochromaffin cells and BrdU immunohistochemistry to locate cells undergoing proliferation. The purpose of this was to elucidate immune cell or other cell changes that may be associated with tissue damage in the explant model.

5.4.1 BrdU Cell Proliferation Immunohistochemistry

The purpose of this experiment was to determine a mechanism for the observed increase in the number of lymphocytes seen with TNF-α + IL-1β treatments. Proliferation was proposed as a possible mechanism and this was measured by BrdU immunohistochemistry. This assay was chosen as it was relatively accurate and specific. BrdU immunohistochemistry showed extensive cell proliferation along the length of the crypts,

but no significant proliferation of lymphocytes. These results suggested lymphocytes were likely entering the mucosa from areas such as sub-mucosal lymphoid aggregates (which were retained with the mucosa) in response to TNF-α + IL-1β, rather than from actual proliferation. It is also possible that TNF-α + IL-1β could cause a reduced rate of apoptosis of lymphocytes and this could be measured in future studies by a TUNEL assay for example. Changes in cell proliferation patterns were observed suggesting TNF-α + IL-1β has the ability to modulate cell proliferation. Previous studies have suggested that this is possible in duodenal mucosal explants (Zachrisson et al., 2001). We did not perform BrdU immunohistochemistry with IL-17A treated tissue as we did not observe any increase in lymphocyte density in these experiments. In retrospect, it would be interesting to see if IL-17A can also alter cell proliferation patterns. In addition, an experiment where the sub-mucosa was completely removed may reveal a lack of lymphocyte density increases, as this is possibly the source of lymphocytes. Alternatively, a time course study where immunohistochemistry for lymphocytes *in situ* was performed may show the migration of these cells.

5.4.2 Immunohistochemistry for Immune Cell Subtypes

Immunohistochemistry was performed to determine the type of immune cells found in human colonic explants and changes from interventions. Specifically, $CD3^+$ expressing T-cells (encompassing both $CD4^+$ "helper" and $CD8^+$ "cytotoxic") and macrophages were labelled. Overall, this was largely unsuccessful as it took some time to optimise the antibodies sufficiently. In addition, any major changes in the number of $CD3^+$ cells between TNF-α + IL-1β-treated and untreated mucosal explants were not evident. As discussed in Chapter 4, the use of flow cytometry may be a more reliable technique to determine which immune cells are present in the explant. Following this, immunohistochemistry could then be used to determine the location of these immune cells

in the mucosa. Because we did not have any major success with these antibodies, we did not attempt to use them in IL-17A-treated mucosa.

5.4.3 IL-17A and IL-17C Expression in Human Colon Explants

Following on from studies examining if TNF-α + IL-1β could induce IL-17A expression; we used immunohistochemistry to localise where IL-17A was expressed in the mucosa. These results were quite surprising as there appeared to be extensive expression of IL-17A in untreated control tissue as well as TNF-α + IL-1β treated tissue. Much weaker expression had been previously shown by Fujino et al. (2003), but this was in inflamed (IBD) tissue. These studies were carried out before the paper by Hueber et al. (2012) was published showing the failure of the anti-IL-17A antibody, secukinumab in CD clinical trials. As such extensive expression of IL-17A appears to occur even in healthy mucosa it is perhaps not surprising in retrospect that neutralising its activity may have detrimental effects. We did have some concern that our IL-17 antibody may actually be non-specific (for IL-17A) and labelling other IL-17 family cytokines i.e. IL-17C. Neutralisation controls described in Chapter 3, showed that the antibody was fairly specific, but it has been reported that many do cross-react and this is important when studying IL-17 family cytokines (Yapici et al., 2012). We also performed immunohistochemistry for IL-17C (Chapter 3) and found extensive expression in the lamina propria as well as epithelium. It would have been interesting to determine if TNF-α + IL-1β reduces expression of IL-17C and this may explain in part its damaging effects on epithelium. Overall, the actions of IL-17 family cytokines are only beginning to be clearly understood and may play may varying (protective or damaging) roles in the human colonic mucosa. As evidenced by the secukinumab clinical trial, IL-17A may actually have some protective active in IBD patients. In contrast, before this it was believed to have an inflammatory role as evidenced by increased expression during IBD (Fujino et al., 2003, Pène et al., 2008).

Another novel aspect of the IL-17A studies was the possibility that EC cells may produce IL-17A. We were able to locate cells with similar morphology to those cells expressing IL-17A by staining for 5-HT. It should be noted that this is a preliminary finding and further studies (such as immunofluorescence double labelling) would be required to confirm this. If found to be the case, it would be novel and indicate EC cells probably also play an important role in immune modulation which recent studies suggest may be possible (Selleri et al., 2008, Cremon et al., 2011).

5.5 Caco-2 *in vitro* Model of Paracellular Permeability

Another aspect of IBD pathology is increased paracellular permeability in the gut facilitating bacterial translocation and leading to an enhanced immune response and further inflammation. As a result of this, we wanted to study if cytokines such as TNF-α, IL-1β and IL-17A can directly induce changes in Caco-2 permeability. We found that the combination of TNF-α + IL-1β (which we investigated as this combination was used in mucosal explant studies) induced significant increases in paracellular permeability. This result was expected as previous studies (using either TNF-α or IL-1β alone) showed increases in permeability (Ma et al., 2004, Al-Sadi et al., 2010). It may be possible that permeability increases account for some of the mucosal damage observed under explant conditions. To confirm this, a technique such as the use of Ussing chambers to measure permeability in human colonic mucosa would be useful. In contrast, IL-17A did not appear in our studies to have any effect on permeability. In previous studies IL-17 has been shown to actually decrease permeability, although this was in a different cell line (T-84) with the IL-17 added to the apical and basolateral compartments of the Transwell (Kinugasa et al., 2000). It is suspected that TNF-α + IL-1β likely disrupts tight junctions in the Caco-2 monolayer. To confirm this, immunofluorescent staining for tight junctions, such as occludin could be performed. Differences in the effect of cannabinoids on epithelial

permeability between this study and those by Alhamoruni et al. (2010) and Alhamoruni et al. (2012) in Caco-2 cells occurred. In contrast, we found no significant effect of cannabinoid agonists on epithelial permeability after increases in permeability were induced by TNF-α + IL-1β treatment. The possible reasons for this are discussed in further detail in Chapter 2 of this thesis (page 48).

5.6 Study Limitations and Future Directions

The human colonic explant model is a useful tool for studying interactions between cytokines, mucosal damage and pharmacological interventions. The model does however have some disadvantages compared to *in vivo* animal models. The main disadvantage of the model is that the mucosal tissue is separated from the blood supply and therefore the complete adaptive (and portions of the innate) immune response, with migration of immune cells from the blood cannot occur. As a result, this model is best suited to studying aspects of the innate immune response in colitis. The immune response and subsequent mucosal damage likely occurs in the explant mucosa due to immune cells, such as lymphocytes, macrophages and mast cells which are already resident in the tissue (i.e. in the lamina propria and the retained sub-mucosa). In addition, other factors such as availability of patient tissue and the heterogeneous human population make obtaining samples and statistical power challenging. As these explant models are not extensively used (likely due to the many of the factors described above) there is also a lack of relevant literature in which to compare findings. Aside from these limiting factors, as discussed in this thesis there are many advantages of using human mucosal tissue, which make these studies worthwhile, particularly clinical translation to IBD. The other possible application of human explant tissue would be in an Ussing chamber system where epithelial permeability before and after inflammatory cytokine treatment could be measured. This would expand greatly on studies performed in Caco-2 cell monolayers.

Aside from the general limitations of the model described above, these studies did not completely answer all of the aims of the project. These remaining aims lead to future directions and additional studies that are required. Overall, the mechanisms for cytokine induced mucosal damage are not entirely clear. Some mechanisms have been identified in this study such as MMP elevation, increases in nitrite production or reductions in PGE_2 expression, but there are likely other mechanisms of mucosal damage that remain unknown. Further study of these mechanisms is useful to identify possible targets for IBD therapies.

The effect of cytokines on mucosa also should be further characterised in terms of concentration-dependence of mucosal damage and testing of TNF-α and IL-1β individually. In addition, much of the damage may occur earlier than 20 hours and may even peak before this, therefore scoring damage at earlier time points would be useful. Other cytokines such as IFN-γ also play a major role in IBD and it would be interesting to test these cytokines in the explant model (Roberts-Thomson et al., 2011).

Studies of IL-17A were not as clear cut as expected, as IL-17A was extensively expressed by the normal colonic mucosa and there were some issues with antibody quality (i.e. possible minor cross-reaction with IL-17C or F). It appears that excessive IL-17A is damaging when incubated exogenously with colonic mucosa, but there is basal expression of IL-17A which if neutralised (in clinical studies) is detrimental (Hueber et al., 2012). Cannabinoids such as AEA and CBD appear to protect the mucosa from IL-17A induced damage, as described in Chapter 3. It is not clear however if this effect may be detrimental in IBD, if the protective ability of IL-17A against infections is lost.

5.7 Conclusion

The first aim of this thesis was to investigate whether CB agonists can reduce parameters of mucosal damage induced by TNF-α + IL-1β incubation in human colonic explant mucosa. We found that mucosal damage can be successfully reduced by treatment with cannabinoid agonists. Further investigation revealed that CB2R activation is critical in mediating this effect. The second aim asked whether nitrite levels increased when mucosa was treated with TNF-α + IL-1β. This was found to be the case and it is likely that this may be a mechanism for TNF-α + IL-1β mediated mucosal damage in inflamed human colon. Increased lymphocyte density is observed when mucosa is incubated with TNF-α + IL-1β. The third aim was to determine whether proliferation is responsible for this. We found this not to be the case, as BrdU immunohistochemistry revealed lymphocyte proliferation does not occur, however altered patterns of crypt epithelial cell proliferation do occur after TNF-α + IL-1β treatment. It may also be possible that increases in proliferation of epithelial cells induced by cytokines may perturb barrier function which may further exacerbate colitis. In Caco-2 monolayers (a model of isolated colonic epithelium) TNF-α + IL-1β can induce increases in permeability, which addresses Aim 4. In our study, however these permeability increases could not be reversed with either AEA, CBD or the selective CB1R and CB2R agonists ACEA or JWH-015 respectively. This indicates a lack of direct activity of CB ligands on epithelial permeability.

The second set of aims addressed the effects of IL-17A on human colonic mucosa and Caco-2 cells. The first aim was to determine if IL-17A could induce mucosal damage similarly to that observed by TNF-α + IL-1β incubation. We found that IL-17A could also induce a similar course of mucosal damage, however a lack of lymphocyte infiltration occurred in contrast to TNF-α + IL-1β treatment. Subsequent incubation of explants with AEA or CBD reduced mucosal damage parameters (Aim 1a), but we did not investigate in

this study which CB receptor (or other receptor for CBD, such as GPR55 or PPAR-γ) is responsible for mediating this effect and this remains to be elucidated. We also sought to find a biochemical marker that was altered after IL-17A incubation (Aim 2). In the case of IL-17A incubation, MMP was elevated and it is very likely this enzyme mediates mucosal damage as it breaks down extracellular matrix. To address Aim 3 we measured IL-17A expression in mucosal explants (via western blot, ELISA and immunohistochemistry) to determine if TNF-α + IL-1β incubation influences concentrations of this cytokine in the mucosal explant model. This was not found to not occur and the findings of the western blot, ELISA and immunohistochemistry results corroborated a lack of change. We did observe that IL-17A (and subsequently IL-17C) is endogenously expressed in human colonic mucosa. In Caco-2 monolayers we found IL-17A did not cause any significant change in paracellular permeability, contrasting with TNF-α + IL-1β treatment (Aim 4).

The fourth chapter of this thesis generally aimed to find other suitable biomarkers and changes of expression of these markers (including the identification of immune cell types) that occurred after cytokine incubation. The major significant finding here was a reduction in PGE_2 levels after TNF-α + IL-1β incubation. Other biochemical studies such as nitrite expression after IL-17A incubation, TNF-α expression after IL-17A incubation and measurement of other prostanoids did not yield significant results. In terms of immunohistochemical studies we did not observe major changes in $CD3^+$ T-cell expression or have success with the MAC387 macrophage antibody. We did find possible expression of IL-17A within enterochromaffin (EC) cells, which were identified by 5-HT immunoreactivity.

In conclusion, this thesis demonstrates for the first time using an explant model of human colonic mucosa that mucosal damage induced by TNF-α + IL-1β (both critically involved in IBD) can be reduced by CB2R agonists. In addition, IL-17A, a relatively recently

discovered cytokine can also induce mucosal damage which is attenuated by either AEA or CBD. IL-17A is not only pro-inflammatory and damaging, but also has basal expression in colonic mucosa that may play a protective or homeostatic role against pathogens. This paradoxical situation raises additional questions about the activity and nature of IL-17 family of cytokines in humans and warrants further study. The mechanism for cytokine-induced mucosal damage appears to vary by cytokine, but may involve elevations of nitric oxide, MMP or reductions in the production of protective prostaglandins. Additionally, TNF-α + IL-1β has the ability to increase epithelial permeability *in vitro* and this may also explain how these cytokines contribute to the pathology of IBD. This thesis provides additional evidence in human colonic mucosa that CB receptor agonists may have therapeutic potential in the treatment of IBD.

Chapter 6 : Bibliography

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