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The role of novel IL-1 family members in Inflammatory Bowel Disease

2015

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B.A. (Mod.) Biochemistry and Immunology



A thesis submitted to

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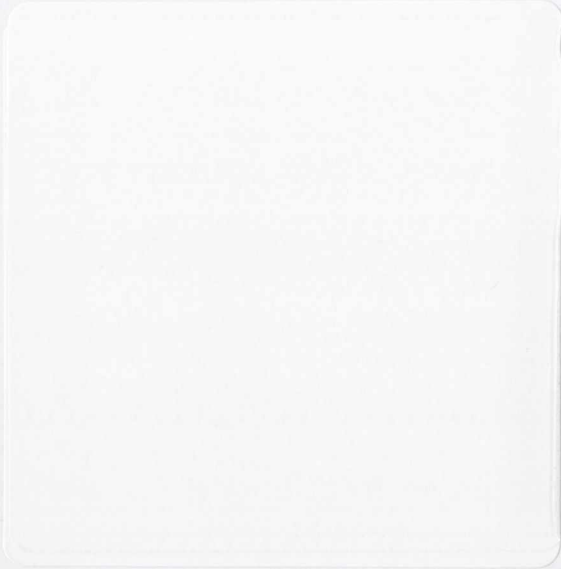
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Abstract

Inflammatory Bowel Disease (IBD) is characterized by an imbalance between effector and regulatory arms of intestinal immunity. Emerging evidence suggests that IL-1 family members play a critical role in the maintenance of gut homeostasis. In this study we have measured expression levels of the novel IL-1 family members, IL-36 and IL-37, in a paediatric IBD cohort. We subsequently used the DSS model of colitis to investigate the role of the IL-36R pathway in intestinal inflammation.

A recent study employing humanized transgenic mouse model demonstrated a protective role of IL-37 in murine colitis. However the role of this cytokine in human disease remains unclear. We found that *IL37* expression was significantly lower in IBD patients. To investigate the significance of these findings we examined signals that regulate IL-37 expression. TLR and NLR stimulation of colonic epithelial cells and primary monocytes leads to enhanced IL-37 expression. We have shown that the expression of IL-37 is inhibited by the Th1 signature cytokine IFN- γ , which is a known pathogenic factor in IBD. Furthermore, over-expression of IL-37 leads to a reduction in TLR and NLR responses.

We have also identified IL-36 α as a pathogenic factor in the context of IBD. We have shown that *IL36A* expression is significantly elevated in UC patients compared to controls. To further investigate the role of the IL-36 pathway in IBD, we have demonstrated that colonic IL-36 expression is also elevated during a DSS-induced model of murine colitis. Furthermore, *il36r*^{-/-} mice are less susceptible to DSS-induced colitis, which is paralleled with a reduction in neutrophil numbers in the Lamina Propria of *il36r*^{-/-} mice. SIGIRR is an orphan receptor of the IL-1R family that has been shown to negatively regulate IL-1R signaling. We have demonstrated that *sigirr*^{-/-} mice are more susceptible to DSS-induced colitis. Interestingly, we have also found that SIGIRR acts as a negative regulator of IL-36 signaling further implicating this pathway in disease pathogenesis.

These observations identify the complex and important roles of novel IL-1 family members in the homeostasis of the intestinal mucosa, which is altered in IBD and reveal their potential as novel therapeutic targets.

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Publications

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Abbreviations

Ab	Antibody
Ag	Antigen
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
Asp	Aspartate
Bcl-2	B-cell lymphoma 2
BCR	B cell receptor
BID	BH3 interacting-domain
BMDC	Bone Marrow-derived Dendritic Cell
BMM	Bone Marrow-derived Macrophage
BSA	Bovine Serum Albumin
C	Celsius
CC	Chemokine (C-C) motif
CCL	CC chemokine ligands
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CGD	Chronic Granulomatous Disease
CXCL	C-X-C motif ligand
d	Day
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid

<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbant Assay
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead winged box transcription factor 3
FSc	Forward Scatter
g	Gravity
GATA-3	GATA-binding protein 3
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
h	Hour
H ₂ O ₂	Hydrogen peroxide
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
IBD	Inflammatory Bowel Disease
ICAM-1	Intracellular Adhesion Molecule- 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
iNOS	inducible Nitric Oxide Synthase
iTreg	inducible regulatory T cell
JAK	Janus Kinase

KC	Keratinocyte chemoattractant
kDa	kilo Daltons
KO	Knockout
LPS	Lipopolysaccharide
M	Molar
MCP1	Monocyte chemotactic protein 1
MDP	Muramyl Dipeptide
MFI	Mean Fluorescence Intensity
Mg	milligrams
MHC	Major Histocompatibility Complex
Mins	Minutes
MIP-2	Macrophage Inflammatory Protein 2
ml	Millilitres
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response gene 88
NET	Neutrophil Extracellular Trap
NFκB	Nuclear factor kappa B
NK	Natural Killer
ng	nanogram
nm	nanometer
nM	nanoMolar
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2
nTreg	Natural regulatory T cell
OD	Optical Density

PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PBST	PBS-Tween
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEC	Peritoneal Exudate Cells
PFA	Paraformaldehyde
PGN	Peptidoglycan
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear Neutrophil
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic Acid
Roryt	Retinoid-related orphan receptor γ t
ROS	Reactive Oxygen Species
RPM	Rotation Per Minute
RPMI	Roswell Park Memorial Institute
RT	Real Time
SIGIRR	Single immunoglobulin interleukin 1 receptor related molecule
SNP	Single nucleotide polymorphism
SPF	Specific Pathogen Free
SSc	Side Scatter
STAT	Signal transducer and Activator of Transcription
T-bet	T-box Transcription Factor

TCD	Trinity College Dublin
TCR	T Cell Receptor
TGF β	Transforming Growth Factor beta
Th1	T helper cell 1
Th17	T helper cell 17
Th2	T helper cell 2
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like Receptor
TMB	Tetramethylbenzidine
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
WT	Wild Type
$\alpha\beta$	Alpha Beta
$\gamma\delta$	Gamma Delta
μg	microgram
μl	microliter

Chapter 1: Introduction.

1. Introduction

1.1 Introduction to the Gastro-Intestinal tract.

The Gastro-Intestinal tract is essentially a long muscular tube running from the mouth to the anus. It contains specialized compartments that aid in the digestion of food and allow for nutrient absorption. The primary role of the GI tract is to break food down with the release of energy and to convert waste material into products that can be easily excreted.

In terms of structure, the GI tract is a muscular tube lined with epithelial cells. Its wall is divided into 4 main layers; the Mucosa, Submucosa, Muscularis Externa and Serosa/Mesentery (Fig 1A). Each Layer of the GI tract plays a distinctive role in gastro-intestinal physiology. The mucosa is the innermost layer and is in direct contact with the luminal contents. It is composed of specialized epithelial cells that are supported by a layer of underlying connective tissue called the lamina propria (LP). The LP contains a large blood supply network, nerves, lymphoid tissue and glands. Directly underlying the LP is the muscularis mucosa. This smooth muscle allows for the lumen to change shape. The second layer of the GI wall, the Submucosa, surrounds the Muscularis Mucosa. It is composed of fat, loose connective tissue and a rich supply of nerves and vessels that support the outer mucosa layer. The third layer consists of the Muscularis Externa. This smooth muscle layer is composed of an inner circular and outer longitudinal layer of muscle fibers. Its primary role is to facilitate peristalsis and movement of food through the GI tract. The Auerbach Plexus is a part of the enteric nervous system. It lies between the inner circular and outer longitudinal layers of muscle and functions autonomously as the major nerve supply that controls gut motility. The outermost layer of the GI tract is the Serosa/Mesentery. This is a layer

composed of fat and specialized epithelial cells called mesothelium that encloses the cavity of the GI tract. The mesothelium produces a lubricating fluid that allows low-friction intracoelomic movement.

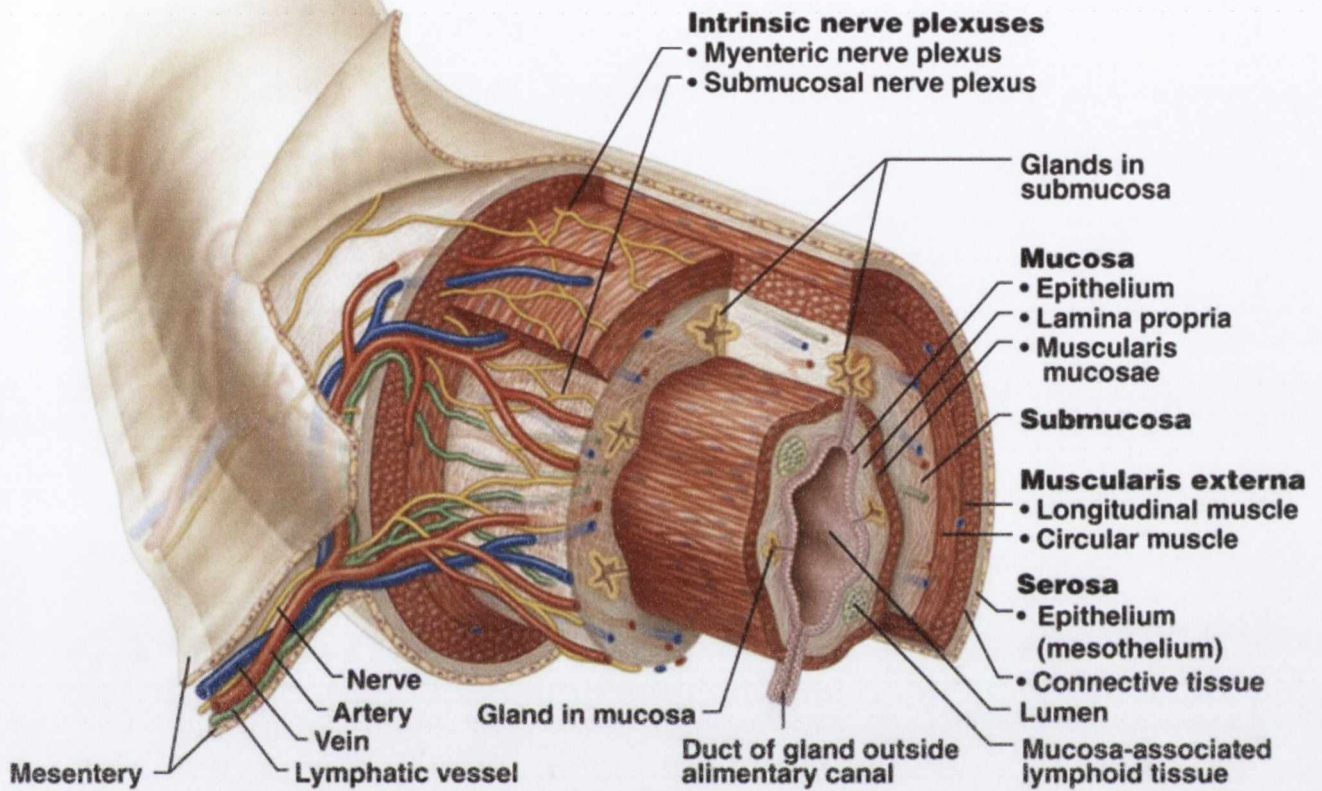


Figure 1.1. Gastro-Intestinal Tract structure.

The GI tract is composed of 4 layers. The innermost mucosa is in contact with the lumen. The submucosa is the second layer and is composed of connective tissue. The muscularis externa is outside of the submucosa and is responsible for movement of the gut wall. The serosa is the outermost layer of the gut and attaches the the GI tract to body cavity walls. (Marieb, Mallatt, Wilhelm, *Human Anatomy, Vol7*)

1.2 The Gastro-Intestinal Tract as a Lymphoid Organ

As the GI tract is in constant proximity with a range of potentially harmful pathogens, the ability to mount an immune response that can efficiently eliminate infection is paramount whilst also being able to remain “non-responsive” or tolerant to food antigens and commensals. The GI-tract contains a complex mucosal immune system that is organized into lymphoid tissue structures called the Gut-Associated Lymphoid Tissues (GALT). The GALT includes organized structures such as the Peyer’s patches, lymphoid follicles of the intestine, tonsils and adenoids and the mesenteric lymph nodes. In addition to the GALT, large numbers of immune cells are resident throughout the mucosal surfaces. Up to 70% of the body’s immunocytes exist in the GI tract. The mucosal immune system occurs in 2 distinct compartments, the epithelium and the Lamina Propria. The epithelium is the first line of defense in the GI tract and it provides a tight physical barrier that is in direct contact with vast quantities of antigen. The epithelial cells that line the tract are relatively inert in terms of immune activation. Accessing pathogens and antigen is done mostly through M-cells and dendritic cells (DCs) (Fig 1B). M-cells are epithelial cells that are specialized antigen-sampling cells[1]. These cells present antigen to immune cells found in underlying lymphoid follicles. M-cells are found in relatively low numbers in the intestine. Increased numbers of M-cells is associated with intestinal inflammation[2]. DCs play a role in antigen capture in the intestine. They reside in the lamina propria and extend processes across the epithelial layer to capture antigen from the lumen of the gut. DCs can then process antigens and present them via MHC molecules to CD4 T helper or CD8+ cytotoxic T cells.

B cells play a key role in mucosal immunity in the gut with IgA the dominant class of antibody found at this site. IgA exists in two isotype forms, IgA1 and IgA2, with IgA2 being more resistant to proteolytic degradation. IgA is transported through epithelium and released into the lumen of the gut, where it can act to prevent adherence of microorganisms and to neutralize bacterial toxins. Unlike other antibody isotypes, IgA fails to activate the complement pathway. The main function of IgA is to limit the translocation of bacteria across the epithelium, restricting micro biota to the lumen, while keeping inflammation to minimum. The role of IgA is integral to the homeostatic balance between effector and tolerogenic immunity observed in the gut.

The gastrointestinal mucosal immune system contains unusual populations of T cells. In the intestine, T cells are found in 2 compartments, the lamina propria and the epithelium. CD4+ and CD8+ T cells are most commonly found in the lamina propria at a ratio of about 3:1 respectively. These cells have a distinctly “memory” type phenotype. Intraepithelial Lymphocytes (IELs), as the name suggest, are found in the epithelium. The IELs make up one of the largest populations of lymphocytes in the body and are comprised primarily of CD8+ T cells and $\gamma\delta$ T cells.

Large numbers of lymphocytes circulate in the gut mucosa. These cells contain specific adhesion molecules and chemokines that make them tissue specific. Once naïve T and B cells encounter antigen in the GALT, they display an activated phenotype and lose their expression of CCR7 and L-selectin, therefore losing preference for homing to peripheral lymphoid organs. Lymphocytes primed in the gut upregulate the expression of gut homing molecules that renders them tissue specific. Activation of lymphocytes in the GALT induces expression of $\alpha_4\beta_7$

integrin. This binds to MadCAM-1, which is expressed selectively on endothelial cells within blood vessels in mucosal tissues. Lymphocytes that have been activated in the small intestine express CCR9, which homes these cells towards small intestinal epithelium where they bind to CCL25[3]. Lymphocytes that have been activated in the colon express CCR10 instead of CCR9. CCR10 can then bind to CCL28, which is found on colonic epithelium.

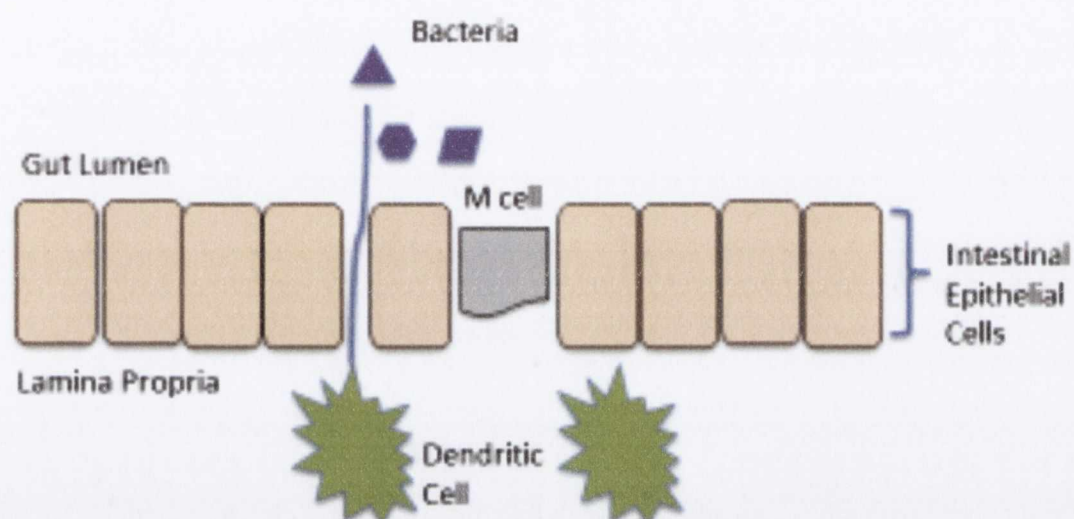


Figure 1.2 The Role of M cells and intestinal Dendritic Cells. Intestinal epithelial cells, M cells and Dendritic Cells regulate immune function in the gut. Epithelial cells are in contact with luminal contents and express pattern recognition receptors that can respond to commensal bacteria. Dendritic cells can extend their dendrites between epithelial cells to sample antigen in the lumen. M cells are specialized antigen-sampling epithelial cells. These cells act together to carry out immune surveillance in the gastrointestinal tract.

1.3 The role of the Microbiome

The interaction between the host and the microbiome is crucial in terms of digestion and nutrient uptake, development of the immune system and also competition with pathogens. The normal gut microflora are predominantly anaerobic. In a healthy human gut there are approximately 10^{14} resident bacteria from 500-1000 species that interestingly belong to only a few known bacterial phyla. The most prevalent phyla seen in the GI tract are Bacteroidetes and Firmicutes[4]. In very general terms these bacteria can be classified into 2 groups; allochthonous bacteria and autochthonous bacteria. Allochthonous bacteria are only present transiently while autochthonous bacteria are indigenous and form a biofilm.

The microbiome also plays a critical role in digestion and metabolism. It can process otherwise indigestible components of our diet such as plant polysaccharides. The microflora have also been well established as having a vital role in the synthesis of vitamins such as vitamin B and vitamin K. More recently, the microflora have been shown to be an environmental factor that directly regulates fat storage in mice.

The importance of the microflora in gut physiology and homeostasis is evident from the phenotype of germ-free mice. Germ-free mice have an increased susceptibility to infection[5]. They exhibit a reduction in vascularity, decreased function of digestive enzymes and reduction in muscle wall thickness. The mucosal immune system also appears in a more "naïve" state in germ-free mice. Germ-free mice also appear to have an altered immune phenotype. They produce less cytokines and have reduced immunoglobulin levels. Moreover, the

reconstitution of intestinal microflora into germ-free mice restores a functional mucosal immune system[6].

The gut microflora is key in immune regulation in the GI tract. It forms a biofilm on the epithelium whereby its physical presence prevents colonization by pathogens[7]. Its persistent presence also provides a continuous basal state of immune activation in the gastrointestinal environment. Resident lymphocytes in the gut display an “active” or “mature” phenotype. The prolonged stimulation of various innate receptors such as TLRs and NLRs can lead to a desensitization whereby a state of “tolerance” is established. The importance of microbiota-induced maturation of the GI tract is illustrated by the phenotypic difference of germ-free mice when compared to conventionally raised animals. Germ-free mice have a thinner mucus layer and altered mucus properties. Their lymphoid follicles, Peyer’s patches and mesenteric lymph nodes exist in an immature state. Furthermore GF mice have lower levels of serum IgA and antimicrobial peptides than their non-GF counterparts[8].

Alterations in the microbiome are associated with many disease states. The concept that gut microbial diversity is linked with obesity is beginning to be established [9, 10]. An aberrant immune response to commensal microflora is also a key factor attributed to the establishment of Inflammatory Bowel Disease (IBD)[5].

1.4 Factors affecting IBD pathogenesis

IBD is a chronic inflammatory disorder of the GI tract that is characterized by an imbalance between effector and regulatory arms of intestinal immunity. The major types of IBD are Crohn’s Disease (CD) and Ulcerative Colitis (UC). IBD

patients may present with a broad range of symptoms including abdominal pain, vomiting, diarrhea, rectal bleeding, severe cramps and weight loss. Although CD and UC have many similarities in terms of symptoms, they are immunologically distinct. CD can affect the entire GI tract from the mouth to the anus, with inflammation occurring in a patchy and segmented fashion. It is characterized by a Th1 cytokine profile and the presence of macrophage clusters known as granulomas. In contrast, UC is predominantly localized in the colon and appears to be mediated by a Th17 and Th2 type immune response. Ulceration in the large intestine is also a hallmark of disease in UC patients.

IBD is primarily a disease of the developed world. Although the highest incidence of disease is observed in western countries (5:1000 in western Europe)[11], there is an increasing incidence of disease in Asia, South America and southern and eastern Europe. The incidence of paediatric IBD is also on the rise and several studies have reported a particular increase in CD among paediatric cohorts. A study of an Irish paediatric cohort has shown that both UC and CD are on the rise in Irish children although the reasons for this have not yet been determined[12].

There is a well-established link between IBD and increased risk of colo-rectal cancer (CRC)[13]. Although the immune system is paramount to mounting an anti-tumor response, chronic inflammation facilitates the growth of many human tumors. There is evidence to suggest that the risk of IBD-associated CRC is directly linked to the durations and extent of inflammation. Various cytokines have been implicated as having tumor-promoting or tumor-suppressive properties. TNF- α , which is mainly produced by macrophages and T cells, is a pro-inflammatory cytokine that is implicated in IBD pathogenesis. At high

concentrations, TNF- α can stimulate reactive oxygen species (ROS), which leads to killing of transformed cells. However, prolonged production of low levels of TNF- α has been implicated in sustaining tumor growth and facilitating metastasis[14]. Furthermore, blockade of TNF- α reduces colorectal cancer associated with chronic colitis in a mouse model of disease[15]. The role of IL-6 in tumor development has also been investigated. Expression of IL-6 is elevated in patients that have active UC and those who had progressed to CRC, when compared with patients with inactive disease or control patients. IL-6 signaling activates STAT3, which induces anti-apoptotic and proliferative genes associated with tumorigenesis[16].

The underlying causes of IBD are multifactorial with genetic polymorphisms, environmental influences and dysregulation of immune responses all playing a role. A genetic component to IBD has been well established and is underscored by the fact that first-degree relatives of affected individuals have a relative risk of fivefold or greater of also having IBD. The genetic component of disease susceptibility appears stronger in CD than UC. A Genome Wide Association Study (GWAS) identified NOD2 as the greatest susceptibility loci in CD,[17, 18] with other genes such as IBD5, IL23R and ATG16L1 also conferring disease susceptibility[19].

The rapid increase in incidence of IBD highlights the fact that environmental factors are contributing to disease pathogenesis. Diet, Cigarette smoking, stress, infections, childhood hygiene and the use of antibiotics have all been implicated in the development of IBD. Many of the studies investigating the role of environmental factors have been somewhat inconsistent. However, the relapsing-remitting nature of the disease in combination with the importance of

a very fine balance between host and microbiome interactions, suggests that environmental factors may be pivotal in the establishment of IBD. The identification of NOD2 as a susceptibility allele links the pathogenesis of IBD to the dysregulation of the GI immune system and the host microbiome. An aberrant immune response, resulting from inappropriate activation of innate immune pathways may potentially lead to an overproduction of inflammatory cytokines and trafficking of effector leukocytes into GI tract. Fig 1.3 highlights the complex nature whereby environmental factors can affect a genetically pre-disposed individual, leading to alterations in barrier function in the gastrointestinal tract. These alterations can lead to translocation of luminal commensal bacteria and rapid innate immune activation in the gut wall. This schematic represents the potential manifestation of IBD.

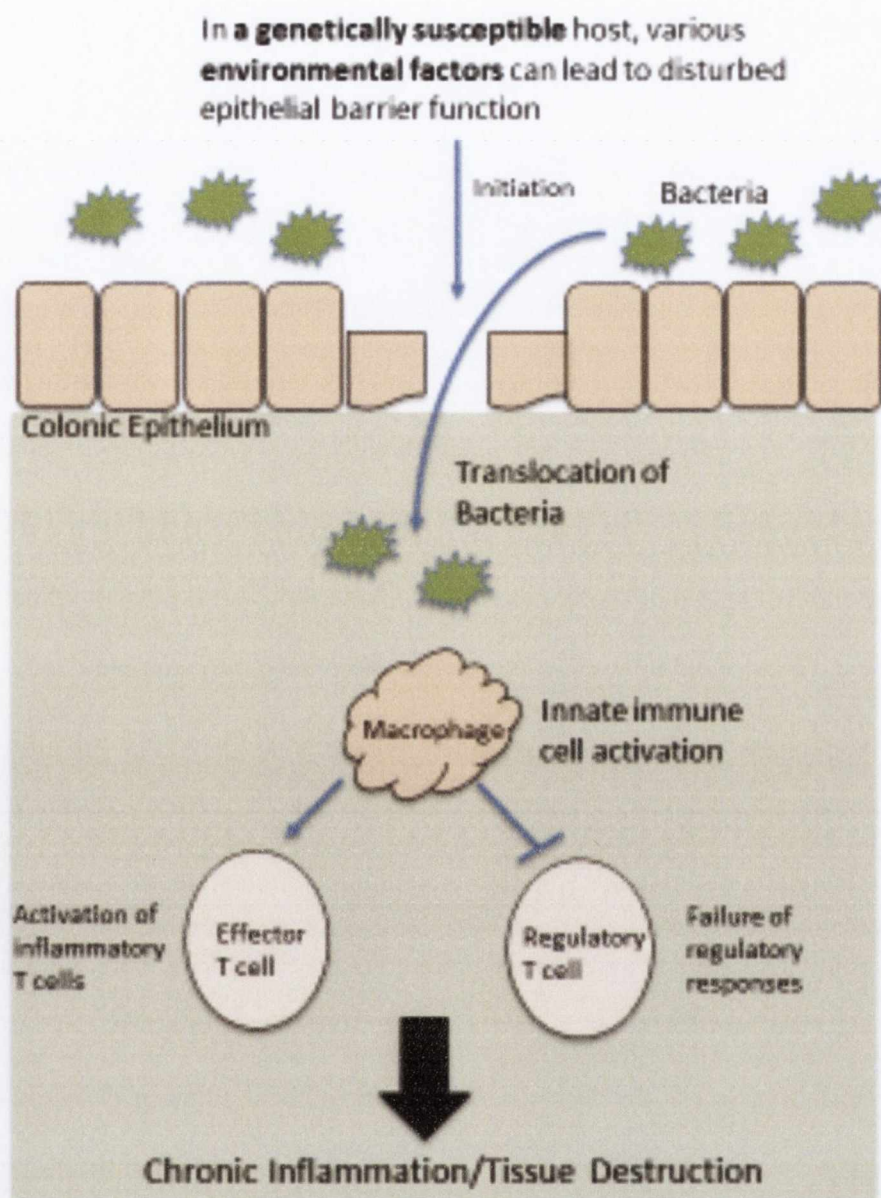


Figure 1.3 Conceptual Framework for IBD manifestation and pathogenesis.

This figure shows that environmental triggers can affect a genetically pre-disposed host and may trigger infections and alterations in barrier function which can subsequently lead to translocation of commensal bacteria from the lumen into the gut wall. This bacterial translocation can trigger an inflammatory response resulting in reduction of regulatory mechanisms leading to chronic inflammation.

The role of the Immune System in IBD

1.5 Innate Immune Component

1.5.1 The role of epithelial integrity in gut homeostasis

The epithelial barrier in the GI tract acts as a physical barrier and provides the first line of defense to the host from antigens found in the GI lumen. An alteration in permeability of the intestinal epithelium, observed in pathophysiological conditions, sees a disruption of the epithelial barrier and allows contact with noxious luminal antigens that may result in intestinal inflammation. Several proteins have been identified as key players in maintaining tight junction formation and epithelial integrity. These include the Claudin family of proteins, occludins, junction adhesion molecules and tricellulin. Various immune modulators have been found to affect the expression of such proteins. TNF- α has been shown to upregulate the expression of Claudin-1 while down regulating the expression of occludin in an intestinal epithelial cell line (IEC-18). Although TNF- α could induce the expression of Claudin-1, its increased expression was only observed in the cytoplasm and stimulation with TNF- α alone was not sufficient to direct expression to the cell membrane. The overall effect of TNF- α was to increase permeability of intestinal epithelial cells, which can be attributed to its ability to suppress occludin expression[20]. Claudin-2 has been shown to be consistently upregulated in IBD patients. It acts as a cation-permeable paracellular pore. The observed increased expression of claudin-2 in IBD leads to increased intestinal ion permeability which contributes to flux diarrhea, a common symptom of IBD. IL-6 has been shown to induce claudin-2 expression and can directly increase permeability to ionic solutes without affecting permeability to macromolecules[21].

A member of the Nod-like-receptors (NLRs) Nlrp3 forms a protein complex called the “inflammasome” which facilitates the caspase-dependent activation of cytokines. The Nlrp3 inflammasome has also been shown to play a protective role in maintaining intestinal epithelial integrity in murine models of colitis[22]. It is clear that many innate immune modulators directly affect epithelial integrity, which is key in the pathogenesis of IBD.

1.5.2 Anti-microbial peptides

One of the most common observations in IBD is the loss of tolerance to commensals. The intestinal epithelia act as more than a physical barrier to infection. Specialized epithelial cells, known as paneth cells, secrete anti-microbial peptides, particularly cryptdins and α -defensins that play a key role in restricting bacterial growth. Antimicrobial peptides are cationic peptides that can damage the bacterial cell wall leading to bacterial death. Many anti-microbial peptides exist as pro-peptides that require cleavage by trypsin or matrix metalloproteases for their activity. Paneth cells express α -defensins continuously and their expression confers protection of epithelial integrity[23]. CD patients have increased bacterial load in the proximal bowel than healthy control patients. Interestingly, they also show a reduced expression of the α -defensins HD5 and HD6[24]. The intracellular receptor NOD2 is expressed in intestinal epithelial cells at an even higher level in paneth cells. Stimulation of NOD2 can induce the expression of α -defensins. The loss of function mutation of the Nod2 gene associated with CD has been associated with reduced HD5 and HD6 expression[25]. Overall, antimicrobial peptides contribute to the maintenance of a homeostatic environment in the gut by preventing bacterial overgrowth and

invasion. Alterations in this dynamic could shift the balance towards the inflammatory phenotype observed in IBD.

1.5.3 The role of TLRs in the gut

The GI immune system has co-evolved with the microbiome. Commensal bacteria provide the intestinal epithelial barrier with continuous stimulation from Pattern Associated Molecular Patterns (PAMPs). In healthy individuals this innate immune stimulation facilitates the maintenance of an intact epithelial barrier and a state of homeostasis persists. However, alterations in Pattern recognition receptor (PRR) signaling pathways, as observed in IBD patients, results in a state of immune dysregulation and chronic inflammation. Toll like receptors (TLRs) are a family of membrane bound PRRs that recognize highly conserved microbial motifs. They are highly conserved receptors that contain a leucine-rich repeat extracellular domain, a trans membrane domain and a highly conserved intracellular TIR-domain. A protective role for various TLRs has been demonstrated *in vivo*. TLR2^{-/-}, TLR4^{-/-} and MyD88^{-/-} mice all show enhanced susceptibility to DSS-induced colitis[26]. An importance for TLR signaling maintaining a healthy epithelial barrier has been underscored by the observation that TLR4^{-/-} and MyD88^{-/-} mice show decreased epithelial proliferation[26, 27]. The location and expression levels of TLRs are fundamental to their role in maintaining a tolerogenic environment in the gut (Fig 1C). Colonic epithelial cells express relatively low levels of TLR4 and it's accessory receptor MD-2, and are poor responders to LPS. They have also been shown to respond poorly to TLR-2 stimulation. TLR5 is only expressed on the basolateral surface of intestinal epithelium[28]. Therefore TLR5 is only

accessible to bacteria that have breached the epithelial barrier and are now a potential risk to the host. TLR5 stimulation results in upregulation of IL-8 and MIP3 α , leading to the recruitment of immune cells to the site of damage. In most immune cells TLR9 expression is restricted to intracellular compartments called endosomes. However, intestinal epithelial cells express TLR9 on both their apical and basolateral surface. Stimulation of TLR9 with its ligand, CpG DNA, has a different outcome depending on which surface of the epithelium is stimulated. Stimulation of apical TLR9 results in reduced NF κ B activation while stimulation of basolateral TLR9 leads to increased NF κ B activity, increased JNK1 and JNK2 activity as well as upregulation of IL-8[29, 30]. The overall trend of TLR signaling in the gut shows that stimulation of epithelium from bacteria on the apical side of the epithelial barrier results in suppression of inflammation and a state of tolerance. However, once the epithelium has been breached and bacteria can access the basolateral surface of the epithelium, their presence stimulates a largely inflammatory profile.

1.5.4 The role of NLRs in the gut

Nod-like receptors (NLRs) are a family of intracellular, cytosolic PRRs. NLRs recognizes PAMPs and also danger associated molecular patterns (DAMPs). In terms of structure, Nod1 and Nod2 share a C-terminal leucine-rich repeat domain, a central NACHT/NOD domain and a N-terminal domain that can consist of a caspase recruitment domain (CARD). The NLRP subfamily of NLRs, contain a pyrin domain instead of a card domain in their N-terminus. The identification of a loss of function mutation in Nod2 as a susceptibility allele for CD, gave strong evidence for a protective role for Nod2 in intestinal homeostasis. The ligand for

Nod2 is a constituent of peptidoglycan, called Muramyl dipeptide (MDP). Nod2^{-/-} mice display an enhanced susceptibility to intestinal inflammation in a murine model of antigen-specific colitis[31], further highlighting its protective role in GI immunology. In healthy subjects, Nod2 stimulation induces expression of α -defensins from paneth cells [32, 33] and also causes the upregulation of negative regulators of TLR signaling such as IRF4 and IRAK-M from localized antigen presenting cells[34-36]. CD patients that have the Nod2 susceptibility allele, express lower levels of α -defensins and negative regulators of TLR pathways. These findings demonstrate that Nod2 plays a key role in maintaining a state of immune tolerance in the gut by upregulating anti-microbial peptides preventing overgrowth of bacteria, and also by inducing proteins that can dampen pro-inflammatory TLR pathways stimulated by the presence of commensal flora. Nod1 is highly expressed in the intestinal epithelium and plays a role in epithelial proliferation and maintaining epithelial barrier integrity. Similar to Nod2, peptidoglycan fragments stimulate Nod1. Although Nod1^{-/-} mice are also more susceptible to DSS induced colitis, Nod1 mutations have not been linked with human IBD susceptibility.

NOD2 mutations remain the most prevalent single susceptibility allele identified to date in the development of CD with approximately 35% of CD patients carrying at least one mutant allele, compared with approximately 14% of UC patients and 15% in control patients[37]. On a molecular level, Nod2 is shown to play a protective role in preventing excessive gut inflammation via its ability to dampen TLR responses in the gut. A “Two-hit theory” has been used to describe this tolerogenic effect of Nod2, whereby chronic Nod2 stimulation results in diminished subsequent TLR signaling[38, 39]. It has been shown that defective

Nod2 signaling results in increased TLR2 responses. Furthermore, Nod2^{-/-} mice show increased susceptibility to *E.coli* expressing ovalbumin (ECOVA) induced colitis, a T cell-driven form of colitis[31]. In a mouse model of DSS colitis, animals that had been pre-treated with MDP, a Nod2 agonist, were protected from disease. MDP-induced protection was lost in IRF4^{-/-} mice. This identifies Nod2 as a key factor in the induction of tolerance in the gut, resulting in diminished TLR responses that are regulated through IRF4 signaling[36].

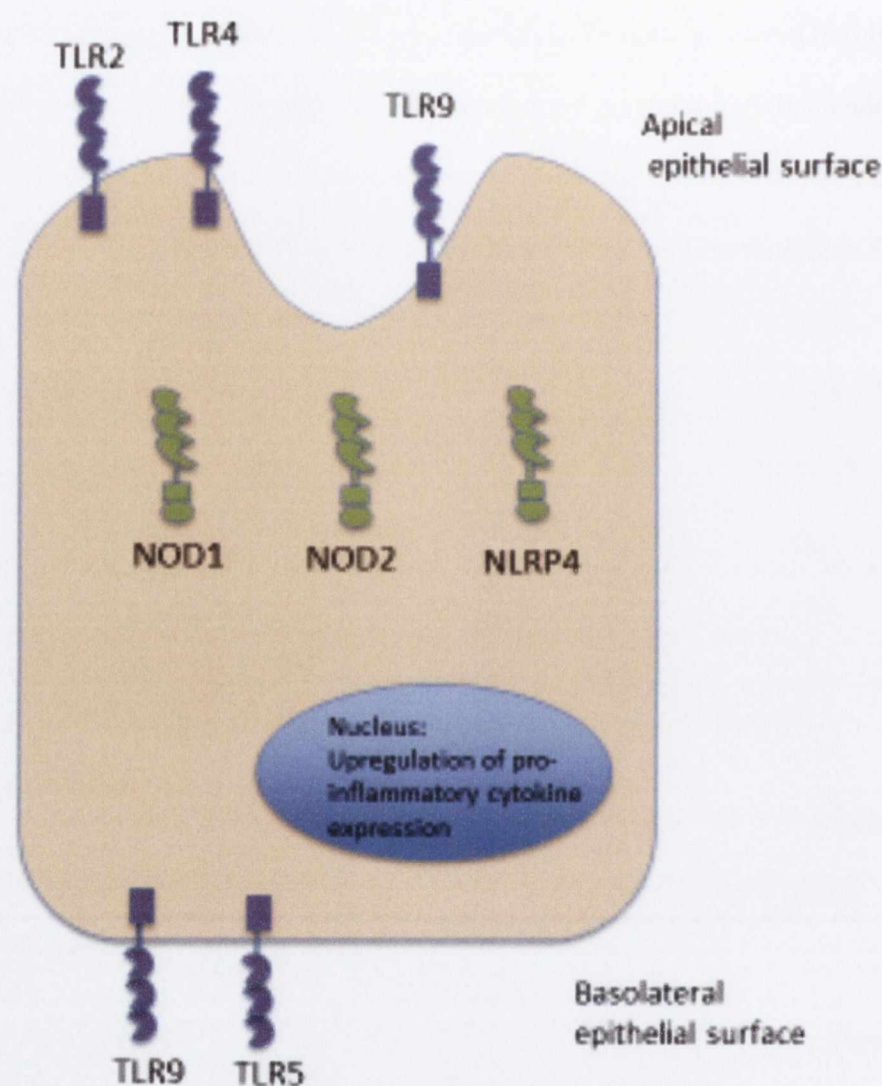


Fig 1.4. Location of TLRs and NLRs in gut epithelial cells.

The location of TLR expression on intestinal epithelium plays an important role in maintaining homeostasis. TLR5 is exclusively expressed on the basolateral surface of epithelium. TLR9 is expressed on both the apical and basolateral surface but only stimulation of basolateral TLR9 results in cell signaling. Epithelial cells express relatively low levels of TLR2 and TLR4. NLRs are located in the cytoplasm of epithelial cells.

1.5.5 The role of monocytes and macrophages in the gut

The intestine contains the largest pool of macrophages in the body, which are essential for maintaining mucosal homeostasis. They provide many key housekeeping roles in the gut, such as clearing of apoptotic or senescent cells. This is facilitated by their expression of the scavenger receptors that can associate with apoptotic cells resulting in their ingestion[40]. Furthermore, they produce PGE₂, which can facilitate the proliferation of epithelial progenitors in intestinal crypts, highlighting the role of gut resident macrophages in the maintenance of epithelial barrier function[41]. The main role of macrophages in the gut is to act as effector cells of the innate immune system. Their close proximity to the epithelium allows them to detect and capture any material that has invaded the epithelial barrier. However, the main difference between gut resident macrophages and the macrophages resident in other tissues, is that exposure to bacteria and various TLR ligands fails to elicit a pro-inflammatory response, showing no enhancement in the production of TNF- α , IL-6 or other pro-inflammatory cytokines or chemokines[42]. Although these cells appear largely anergic, they are highly phagocytic and are not completely inert. Gut resident macrophages have been shown to have high levels of MHCII expression and do express TNF- α . One of the main roles of gut resident macrophages is their ability to facilitate the expansion of FoxP3⁺ Tregs, that have been primed in the mesenteric lymph nodes (MLNs), through the production of IL-10[43, 44]. It has been shown that gut resident macrophages cannot directly activate naïve CD4⁺ T cells and that they do not migrate to the MLNs[45], but they can play a key role in the maintenance of gastrointestinal homeostasis through the expansion of Tregs. It has been well characterized that the composition of gut resident macrophages

is altered in the human intestine during IBD. There is an accumulation of pro-inflammatory macrophages in the mucosa of both UC and CD patients. These cells can produce high levels of IL-1, IL-6, TNF- α and reactive oxygen species, making them more akin to classical macrophages. Gut resident macrophages and the pro-inflammatory macrophages found in the mucosa of IBD patients can be differentiated by the expression levels of the surface receptor for LPS, CD14. Gut resident macrophages express low levels of CD14. In contrast, the pro-inflammatory macrophages found in the mucosa of IBD patients are characterized by high expression of CD14[46, 47]. Interestingly it is observed that the recruited macrophages in IBD mucosa behave differently in the CD or UC disease subgroups. For instance, the macrophages in the CD mucosa produce high levels of IL-23, which is associated with the Th1/Th17 phenotype in this condition[48]. In contrast, pro-inflammatory macrophages in the UC mucosa, express high levels of chemokines, particularly CCL11, promoting eosinophil infiltration into the mucosa[49].

1.5.6 The role of neutrophils in the gut

Polymorphonuclear leukocytes or neutrophils are the most abundant leukocyte in the blood. They play a key role in the innate immune response and the limitation of infection. Neutrophils exhibit several mechanisms of anti-microbial activity. They are avid phagocytes and also contain intracellular granules containing anti-microbial peptides, myeloperoxidase, hydrolytic enzymes as well as proteases which can be released upon interaction with microbes and can directly kill invading pathogens[50]. In recent years it has also been shown that neutrophils can also exhibit antimicrobial activity through neutrophil

extracellular traps (NETs), which are composed of DNA and chromatin and molecules from the neutrophil granules. These NET complexes can effectively kill invading pathogens[51]. Neutrophils play a key role in clearing bacteria that transverse across the gut epithelium. A study showing that depletion of neutrophils leads to increased translocation of bacteria in a murine model of colitis highlights their importance in limiting bacterial load[52]. Neutrophils have been shown to exhibit both beneficial and detrimental roles during intestinal inflammation. Animal models have led to conflicting reports as to the precise role of neutrophils in the maintenance of gut homeostasis. For examples, a mouse study of 2,4,6-trinitrobenzenesulfonic acid (TNBS)/dinitrobenzene sulfonic acid (DNBS)-induced colitis has shown that depletion of neutrophils exacerbates disease[52], suggesting that neutrophils play a protective role in colitis. In direct contrast, a rat model of DSS-induced colitis has shown that depleting neutrophils is protective against disease[53]. Neutrophils have a plethora of functions in the human gut. Their primary role is killing bacteria that penetrate across the epithelial barrier. They also contribute to cellular recruitment and the activation of immune cells in the gut. Neutrophils secrete significant levels of IL-8, IL-17 and IL-10 during intestinal inflammation [54-56]. Healing and repair has shown to be key part of the resolution phase of inflammation. Neutrophils have been shown to facilitate mucosal wound healing through the biosynthesis of growth factors and pro-resolution lipid mediators including lipoxins and resolvins[57]. Furthermore, defective neutrophil function is associated with pathological intestinal inflammation[58].

1.6 Adaptive Immune Component

1.6.1 Th1 cells

Th1 cells are CD4⁺ T cells characterized by the production of IFN- γ and expression of the transcription factor T-bet. Th1 cells promote cell-mediated immunity without being directly involved in killing themselves. Differentiation of a CD4⁺ T cell to the Th1 lineage requires the presence of IL-12, which is primarily produced by macrophages and DCs. IL-12 directs differentiation towards Th1 by promoting expression of IFN- γ and by having a suppressive effect on the expression of the Th2 defining cytokine, IL-4. Expression of T-bet, a transcription factor of the T-box family, is potently induced by the presence of IL-12. This transcription factor is responsible for the Th1-specific expression of IFN- γ as it is a strong inducer of the IFN- γ gene. It does this via the STAT4 signal transduction pathway. The importance of T-bet in determining Th1 phenotype can be seen when T-bet is transduced in a retroviral manner to other T helper subsets resulting in the induction of IFN- γ expression. T-bet is considered a master regulator of Th1 differentiation. Once T helper cells have been differentiated and committed to the Th1 lineage their main function in the immune system is activating macrophages and inducing proliferation of CD8⁺ T cells, therefore clearing intracellular pathogens. The activation of macrophages results in an increase in IL-12, thereby creating a positive feedback loop to enhance Th1 responses. Th1 cells have been implicated in IBD, particularly CD. CD is characterized by elevated IFN- γ in affected tissues[59, 60]. It has been observed that mucosal T cells from CD patients produce more IFN- γ than T cells from UC patients[61]. IFN- γ released by Th1 cells induces apoptosis of epithelial cells and triggers the release of TNF- α by activated mucosal macrophages. These

increased levels of TNF- α in turn induce the differentiation of stromal cells and promotes production of tissue-degrading enzymes called matrix metalloproteinases (MMPs)[62].

1.6.2 Th2 cells

Th2 cells are generally associated with priming the humoral response in response to extracellular pathogens, particularly parasitic infections. However, these cells also play a large role in mucosal immunology. Th2 cells produce a large range of cytokines including IL-4, IL-5, IL-9, IL-10 and IL-13. IL-4 is involved in both the differentiation of T helper cells into Th2 cells and also in the direct effector function of these cells. IL-4 drives differentiation of Th2 cells by inducing expression of GATA binding protein 3, a transcription factor and master regulator in Th2 differentiation. Once Th2 cells have differentiated and matured, they then become IL-4 secreting cells, creating a positive feedback loop. UC has been described as being mediated by an atypical Th2 response with elevated levels of IL-5 and IL-13 being expressed in UC patients, but not IL-4, compared to CD patients. IL-13 has been shown to increase intestinal permeability and induce epithelial cell differentiation and apoptosis[63, 64].

1.6.3 Th9 cells

In 2008 a newly identified subset of CD4⁺ T cells that specialized in IL-9 secretion was identified[65, 66]. They develop from naïve T cells in the presence of TGF- β and IL-4. Transcription of *IL9* is regulated by the transcription factor PU.1 which is induced in T cells upon stimulation with TGF- β [67]. *Gerlach et al.* have recently shown that *IL9* levels are elevated in UC patients. They have also

shown that PU.1 expression is elevated in cells from the lamina propria and IL-9R expression on epithelial cells is upregulated in UC patients. Furthermore, it has been shown that IL-9 and Th9 cells play a pathogenic role in colitis. IL-9 – knockout mice are protected from oxazalone-colitis and in this model IL-9 regulates Th2 cytokines and alters epithelial barrier function[68].

1.6.4 Th17 cells

Th17 cells are a relatively newly described subset of CD4⁺ T cells that are highly inflammatory and strongly associated with autoimmune diseases, such as Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), Psoriasis and Inflammatory Bowel Disease (IBD). IL-17, IL-22 and IL-21 are signature Th17 cytokines. IL-17 is thought to promote inflammation by causing an accumulation and activation of neutrophils in the intestine. Naive murine T cells differentiate into Th17 cells when stimulated with IL-6 in combination with TGF- β . IL-6 and TGF- β , together, can stimulate the induction of two orphan nuclear receptors, ROR γ t and ROR α , which are the characteristic transcription factors of Th17 cells. Differentiation of human Th17 cells requires the presence of IL-6, IL-1 β , IL-23 and TGF- β . STAT3 signaling induces upregulation of ROR γ t and ROR α , while STAT1 signaling inhibits this pathway. IL-6 is capable of inducing both STAT1 and STAT3 pathways, however it has been shown that STAT3 remains active, while STAT1 remains suppressed, in Th17 cells. IFN- γ inhibits Th17 development through activation of the STAT1 pathway. IL-2 has also been found to negatively regulate Th17 cell development; however, it does so via the STAT5 pathway. High transcript levels of IL-17A have been detected in both UC and CD patients compared to healthy controls. It has also been observed that IL-17 levels are

particularly high in the Lamina Propria of IBD patients[69, 70]. The precise role of IL-17 in the gut remains controversial. Although IL-17A induces pro-inflammatory cytokine production from macrophages, it also induces expression of anti-microbial peptides from epithelial cells and plays a protective role in tissue-remodelling in the gut. Recently, a subset of Th17 cells has been identified in CD patients that produce IFN- γ as well as IL-17A. In addition to this, a further subset of Th17 cell that express FoxP3 in the Lamina Propria of CD patients has also been identified[71]. To further highlight the complex nature of IL-17 in the gut, a recent study involving the blockade of IL-17A was ineffective in CD patients and actually appeared to exacerbate disease in a subset of patients[72]. This study highlights that the development of different subsets of Th17 cells and their relative pathogenic or protective contributions in IBD remains to be fully elucidated.

1.6.5 Regulatory T cells

A state of homeostasis occurs in the intestinal mucosa in which equilibrium exists between an effector immune response and tolerance to food antigens, self-antigens and commensals. A subset of T cells, known as Regulatory T cells (Tregs), play a key role in the maintenance of tolerance in the gut by reducing effector functions of other T cell subsets. In contrast to other T cells, naturally occurring Tregs are committed to a regulatory fate during their development and undergo positive selection in the thymus after encountering self antigen. They are CD4⁺ cells that constitutively express the α -chain of the IL-2 receptor (CD25) and Foxp3, the signature transcription factor expressed by Tregs. The presence of Foxp3 prevents activation of transcription of IL-2 within the Treg

itself. Inducible or adaptive Tregs also exist, and develop in the periphery from naïve CD4⁺ T cells. Adaptive Tregs are found in the mucosal immune system and a deficiency of these cells is linked to IBD[73]. Furthermore, the administration of oral antigen can lead to the expansion of adaptive Tregs. The importance of Tregs in peripheral immune tolerance is highlighted upon their depletion where huge levels of inflammation and autoimmunity is observed. Tregs can exert their suppressive effects through cell-cell contact dependent mechanisms involving CTLA-4 and Granzyme B. They can also secrete suppressive soluble factors such as IL-10 and TGF- β , which can directly affect neighboring cells. Tregs can also act as competition with effector T cells for the available cytokine/growth factor milieu.

1.7 The Role of IL-1 family members in IBD:

Cytokines play a crucial role in the pathogenesis of IBD. In particular an imbalance between pro-inflammatory and anti-inflammatory cytokines facilitates the development of IBD and subsequent tissue destruction. In recent years, it has become evident that IL-1 family members play an important role in gut homeostasis. The IL-1 family includes seven ligands with agonist activity (IL-1 α and IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ), three receptor antagonists (IL-1Ra, IL-36Ra, IL-38), and an anti-inflammatory cytokine (IL-37). The IL-1 receptor family consists of four functional receptor complexes, 2 decoy receptors and 2 negative regulators of IL-1R family signaling. The IL-1 agonists signal through their respective heterodimeric receptor complex (Fig1.7). A tight regulation via receptor antagonists, decoy receptors, and signaling inhibitors ensures a balance between positive innate immune signaling and uncontrolled inflammation. IL-1 family members play a key role in the differentiation and function of polarized innate and adaptive lymphoid cells. Although IL-1 family members have been classically described as mediators of inflammation, a dichotomous role for this cytokine family is now being established, particularly in IBD. Further studies are needed to fully understand the potential dual function of IL-1 family members in gut homeostasis.

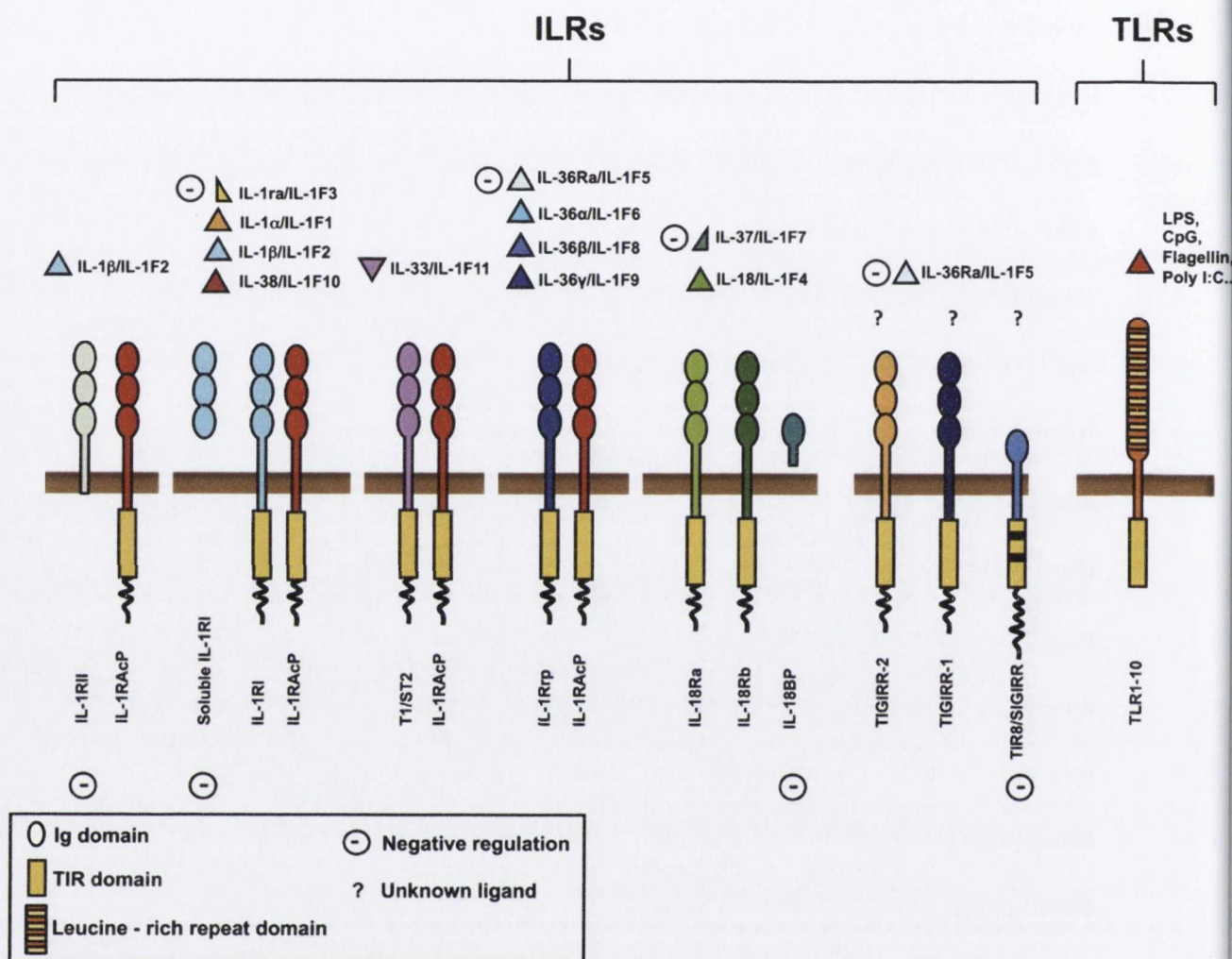


Fig 1.6. The IL-1 receptor family

The various IL-1 family members, IL-1 family of Receptors and their possible interactions are indicated in the above schematic. (Riva, F. *Front Immunol.*, vol 3, art 322, 2012.)

1.7.1 IL-1

IL-1 α and IL-1 β arise from two separate genes. However they display many similar effects, an observation that is underscored by the fact that they signal through the same IL-1 receptor. Upon ligand binding, the IL-1R1 forms a complex with its accessory protein IL-1RAcP. This complex facilitates the intracellular recruitment of the adaptor molecule MyD88, which activates a signaling pathway resulting in activation of NF κ B. IL-1 α is located in the cytosol and in association with the cellular membrane where it regulates the intracellular environment[74]. IL-1 α is expressed at high levels in epithelial cells where it is quickly released during necrosis and acts as an 'alarmin' mediating early phases of sterile inflammation[75]. It is also secreted from the cell where it can mediate its effects on neighboring cells. In contrast IL-1 β exists as a pro-form in the cytosol. Maturation of IL-1 β by caspase1 results in secretion of IL-1 β into extracellular space or translocation to the nucleus where it is believed to regulate gene expression[76]. Regulation of IL-1 signaling is mediated by IL-1R antagonist, which binds to IL-1R1 and prevents recruitment of IL-1RAcP[77]. IL-1RII also acts as a negative regulator of IL-1 signaling by acting as a decoy receptor that is capable of binding IL-1 but lacks an intracellular signaling domain[74]. Conflicting roles for IL-1 have been reported in animal models of IBD. In a rabbit model of disease, neutralization of IL-1 by the administration of IL-1RA appears to be protective in terms of disease severity[56]. However, the administration of IL-1 β also appears to have a beneficial role suggesting a potential role for IL-1 in gut homeostasis[78]. The dichotomous nature of IL-1 is

underscored by the fact that Phase I clinical trials of IL-1 neutralization in IBD have failed.

1.7.2 IL-18

IL-18 is also a member of the IL-1 family and is known as a potent inducer of IFN- γ , therefore it is regarded as strong inducer of inflammation. IL-18 signals through a heterodimeric receptor, consisting of the IL-18R α chain that is responsible for ligand binding, and the IL-18R β chain that is responsible for intracellular signaling. The IL-18 receptor is expressed on plethora of cells including T and B-lymphocytes, neutrophils, macrophages, Natural Killer cells, endothelial cells and smooth muscle cells[79, 80]. IL-18R is also upregulated on CD4+ T cells in response to IL-12. IL-18 signaling is controlled by the presence of soluble IL-18 binding protein (IL-18BP), which has a very high affinity for IL-18. IL-18BP sequesters IL-18 and renders it unavailable for signaling via the membrane bound IL-18R. IL-18 has been shown to be elevated in IBD patients, particularly patients with CD. Interestingly, less affected areas of the gut appear to show high levels of IL-18 in the epithelium, but as disease severity increases the primary location of IL-18 appears to shift to the Lamina Propria, with the most severe cases showing no IL-18 expression in the epithelium at all. This trend of differences in IL-18 expression in specific areas in the gut is specific to CD and is not observed in UC patients [81]. Elevation of IL-18 expression has also been observed in mouse models of DSS colitis[82]. Conflicting roles for IL-18 have been demonstrated in vivo in mouse models of disease. Neutralization of IL-18 has been shown to ameliorate disease[83]. However IL-18-/- mice are more susceptible to DSS-induced colitis[84]. These findings indicate that a pathogenic

or protective role for IL-18 in IBD may depend on the phase of disease as well as the cellular source of IL-18. The importance of the cellular source of IL-18 in IBD is highlighted by the observation that epithelial derived IL-18 plays a key role in NLR-dependent protection from DSS, shown using *Nlrp3*^{-/-} mice[22].

1.7.3 IL-33

Of the newer IL-1 family members, IL-33 is relatively well characterized in terms of its function and biological effects. IL-33 has been shown to be a ligand for the former orphan receptor ST2[85]. ST2 forms a complex with IL-1RAcP resulting in a functional IL-33R complex. ST2 exists in two splice variants, a transmembrane receptor (ST2L) and a soluble molecule that acts a decoy receptor sST2[86]. Two isoforms of IL-33 also exist, a full-length 30kDa protein and also a 25kDa protein generated by alternative splicing[87]. The shorter 25kDa variant of the IL-33 displays similar levels of bioactivity to the longer 30kDa version. However, it lacks a caspase cleavage site that is found in the longer protein. As well as two splice variants of IL-33 existing naturally, IL-33 protein can also be modified by a variety of enzymatic processes. It is well established that IL-1 β exists in a pro-form that is subsequently cleaved by caspase-1 resulting in enhanced bioactivity. Interestingly, IL-33 is fully active in its pro-form and processing by pro-apoptotic caspases 3 and 7 result in a less active form of IL-33[88]. However, full length IL-33 can also act as a substrate for neutrophil elastase (NE) and Cathepsin G[89]. Processing by these neutrophil serine proteases results in enhanced bioactivity of IL-33. IL-33 expression has been observed in a wide plethora of cell types including cells of hematopoietic origin such as macrophages and dendritic cells[85]. Non-hematopoietic cells

such as fibroblasts, adipocytes, smooth muscle cells, endothelial cells and bronchial and intestinal epithelial cells have also been shown to express IL-33[90, 91]. ST2 expression is observed on fibroblasts, mast cells and Th2 cells. IL-33 has been shown to activate mast cells and enhance Th2 responses[92]. It has been well established that IL-33 levels are elevated in associated with IBD, particularly UC[93, 94]. It has also been shown that ST2 levels are elevated in IBD compared with healthy controls. However, an investigation into the mechanistic role of IL-33 using various animal models has yielded mixed results. It has been shown that mice deficient for IL-33 exhibit less severe disease symptoms in the acute DSS model of colitis[95]. Furthermore, administration of exogenous IL-33 can also aggravate disease, in this model[96]. These data suggest a pathogenic role for IL-33 in acute intestinal inflammation. However, during a chronic model of DSS cycling, administration of recombinant IL-33 can reduce disease severity[97], underscoring the potential for a dual role for IL-33 in this context. SAMP1/YitFc (SAMP) mice are a particular strain of mice that are used as a model of Crohn's Disease. They develop spontaneous ileitis with a Th1/Th2 phenotype. It has been shown that over time IL-33 expression is elevated in SAMP mice and this elevation correlates with ileal inflammation[93]. Neutralization of IL-33 in these mice results in a reduction in eosinophil influx and reduces inflammation observed in the ileum, highlighting a pathogenic role of IL-33 in this model.

1.7.4 IL-36

The IL-36 family is a sub- family of the IL-1 family of cytokines. Five ligands of the IL-36R complex have been identified; IL-36 α , IL-36 β , IL-36 γ , IL-36RA and IL-

38. IL-36 α , IL-36 β and IL-36 γ have been shown to bind to the IL-36R leading to the recruitment of IL-1AcP, which results in downstream signaling similar to that of IL-1. Similar to the role of IL-1RA, IL-36RA can bind to the extracellular portion of the IL-36R but fails to recruit the IL-1AcP and thus the formation of a functional signaling receptor complex leading to inhibition of IL-36R signaling pathways[98]. Interestingly, it has been shown that the novel IL-1 family member, IL-38, shares 43% sequence homology with IL-36Ra. It too can bind to the IL-36R and act in an antagonistic fashion[99]. Post-transcriptional and post-translational regulation of IL-1 family members has been an emerging theme in recent years. Interestingly it has also been demonstrated that truncation of IL-36 α , IL-36 β , IL-36 γ and IL-36RA enhances their activity indicating that post-translational modification of IL-36 family members is required for their full activity[98]. However, unlike IL-1 β and IL-18 the mechanism whereby IL-36 members become processed and activated appears to be Caspase-1 independent. The enzymes responsible for the cleavage of IL-36 members are currently unknown.

It has been demonstrated that IL-36 cytokines are important in the maturation of dendritic cells and that they also play a role in T cell polarization both *in vitro* and *in vivo*. IL-36 cytokines were shown to act directly on DCs resulting in upregulation of co-stimulatory molecules and increased expression of pro-inflammatory cytokines such as IL-6 and IL-12. IL-36 can also act to induce IFN- γ , IL-4 and IL-17 from CD4 $^{+}$ T cells '*in vitro*' as well as acting as a potent Th1 inducing adjuvant '*in vivo*'[100].

A role for IL-36 in response to fungal infection was demonstrated in 2003, when it was shown that infection with the opportunistic fungus, *Aspergillus fumigatus*,

can drive the expression of IL-36 from human PBMCs and that the ability of *Aspergillus fumigatus* to induce a Th1 and Th17 phenotype in PBMCs is dependent on the IL-36R pathway[101]. In contrast, a study using an 'in vivo' model of mycobacterial infection showed that the IL-36R pathway played a dispensable role in the control of infection whereby IL-36r^{-/-} mice displayed a similar phenotype as wildtype mice in response to infection. In contrast TNF α KO mice completely succumbed to infection whereas IL-1r^{-/-} mice displayed an intermediate phenotype[102].

IL-36 family members appear to play a key role in the interplay between nonhematopoietic cells in tissue, such as keratinocytes in the skin, and hematopoietic cells such as T cells and DCs. Several reports have highlighted the prominent role for IL-36R pathway in the pathogenesis of inflammatory skin disorders, particularly in psoriasis. It has been shown that both IL-36 α and IL-36 γ are upregulated in the skin of psoriatic patients and that increased levels of these cytokines correlated with enhanced levels of IL-17, IL-23, IFN- γ and IL-23[103]. Murine models of psoriasis have also been employed to investigate the role of IL-36 in the context of skin inflammation. The imiquimod model is a model whereby mice are treated with the TLR7 agonist imiquimod on their skin leading to epidermal thickening, IL-17 production, and an influx of immune cells. Through the use of IL-36R^{-/-} and IL-36RA^{-/-} knockout mice, it has been shown that this model is almost completely dependent of the IL-36R pathway. IL-36R^{-/-} mice are protected from imiquimod-induced skin inflammation and in contrast, IL-36RA^{-/-} mice show a more severe disease phenotype [104]. To further highlight the role of IL-36 in psoriasis, it has been demonstrated that there is a

strong correlation between mutations in *IL36RN* and the occurrence of generalized pustular psoriasis, a severe, life-threatening form of psoriasis[105]. To date, most of the studies regarding IL-36 are related to its role in the pathology of skin inflammatory disorders. There are no known associations between IL-36 and intestinal inflammation

1.7.5 IL-37

IL-37 was discovered in 2000 and is a unique member of the IL-1 family as it is described as a “fundamental inhibitor of innate immunity”. Interestingly, no known mouse homolog of IL-37 has been identified to date. As with most IL-1 members, the gene for human IL-37 is located on chromosome 2. The IL-37 gene is subject to alternative splicing and there are 5 known splice variants (IL-37a-e) (Fig 1E). The complete IL-37 gene contains 6 exons. Exon 1 contains a caspase-1 cleavage site. Exon 2 contains a second cleavage site. The third exon contains a putative elastase cleavage site and a nuclear localization sequence. Exons 4-6 contain coding regions for the typical IL-1 family associated β -strands. IL-37b is the largest and best-studied splice variant, containing exons 1 and 2, forming its pro-domain, and also exons 4-6. Certain splice variants of IL-37 have been noted as being tissue-specific. For example IL-37a is the only isoform detected in the brain and IL-37b is the only isoform detected in the kidney. However other tissues express a variety of IL-37 isoforms. In 2010, IL-37 was characterized as having a distinctive immune-suppressive function. siRNA knockdown of IL-37 in peripheral blood mononuclear cells (PBMCs) resulted in elevated IL-1 β and TNF α expression in response to TLR stimulation[106]. Overexpression of IL-37 in THP-1 cells confirmed an immune-suppressive role for IL-37, by causing

reduced pro-inflammatory cytokine expression in response to LPS stimulation. An important role for the transcription factor, Smad3, has been identified in terms of IL-37 function. IL-37 was shown to co-localize with phosph-Smad3. Furthermore the use of Smad3 inhibitors caused a dose-dependent reversal of the inhibition of IL-6 and IL-1 β observed in LPS-stimulated RAW cells transfected to overexpress IL-37.[106]. In this transfection model, LPS induced upregulation and nuclear translocation of IL-37. Furthermore, nuclear translocation of IL-37 is completely blocked in cells treated with a caspase-1 inhibitor[107]. This indicates that, like other IL-1 family members, maturation of IL-37 is a caspase-dependent process.

In addition to the intracellular mechanisms of action of IL-37, an extracellular role for IL-37 has recently been described. It had previously been suggested that IL-37 could bind to IL-18R α , with little agonistic or antagonistic effect on IL-18-induced IFN- γ production[108]. More recently however, it has been demonstrated that IL-37 could act to inhibit innate immune signaling in an extracellular fashion, but this required the presence of the orphan receptor SIGIRR (TIR8)[109].

Although no mouse homolog of IL-37 has been identified, a humanized-transgenic mouse that over-expresses hIL-37 has been engineered. Interestingly IL-37tg mice are protected from DSS-induced colitis compared to their wild type counterparts. They exhibit a less severe disease score, a lower tissue injury and less leukocyte infiltration in colonic tissue[110]. The protective role of over-expression of IL-37 in these mice was independent of IL-10, as administration of anti-IL10 receptor-blocking antibody did not reduce the protective phenotype

observed in IL-37tg mice. Although this study highlights the potential protective role of IL-37 in the gut, the role of IL-37 in human IBD remains to be defined.

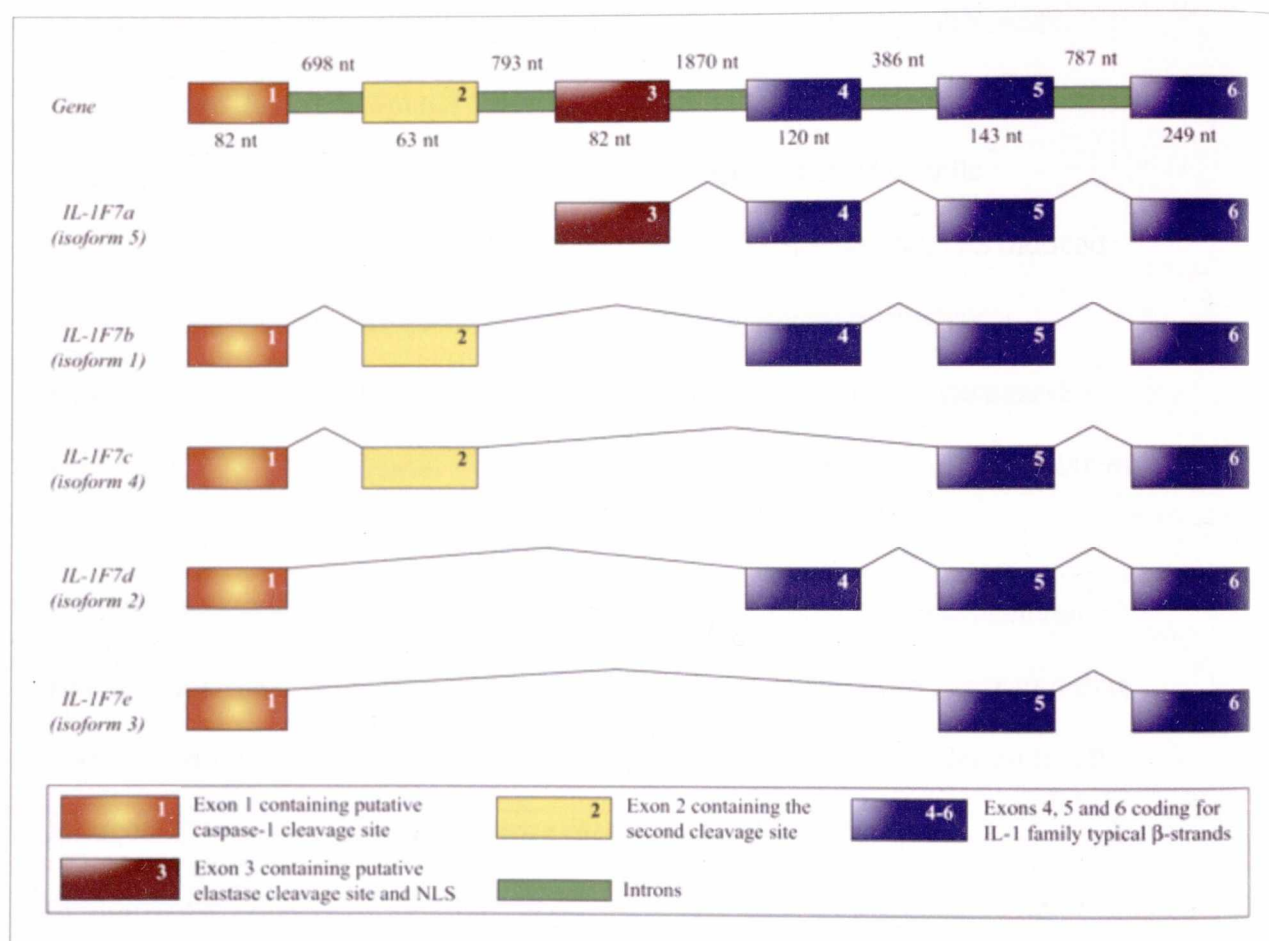


Figure 1.7. Five splice variants of IL-37

The IL-37 gene contains 6 exons that are alternatively spliced to form 5 separate isoforms. IL-37b is the largest isoform containing 5 of the 6 exons. (Boraschi D., *Eur. Cytokine Netw.*, vol 3, 127-47, 2011)

1.8 Negative regulators of IL-1 family

IL-1 family members play a key role in mediating innate immunity and inflammation. A tight regulation of these pathways is essential to maintain a balance between amplification of innate immunity and uncontrolled inflammation. Many pathways of negative regulation have evolved, including receptor antagonists, decoy receptors, soluble receptors and miRNA that negatively regulate these signaling pathways.

1.8.1 Receptor Antagonists

One important arm in the regulation of IL-1 signaling pathways is the existence of 3 naturally occurring receptor antagonists, IL-1RA, IL-36RA and IL-38. IL-1RA has been shown to bind to IL-1RI but unlike IL-1 β , it fails to recruit IL-1RAcP, therefore preventing signal transduction. Mice deficient for IL-1RA have been shown to develop spontaneous arthritis, psoriatic-like lesions and an enhanced susceptibility to LPS [111, 112]. Similarly, it has been described that patients with a deleterious mutation of *IL1RN* suffer with symptoms including pustular rash, osteopenia, lytic bone lesions, respiratory insufficiency and thrombosis. Interestingly, treatment with recombinant IL-1RA (Anakinra) could completely resolve the symptoms[113]. It has been previously suggested that an imbalance between the ratios of IL-1 levels to IL-1RA levels may play a role in the pathogenesis of IBD[114]. A naturally occurring IL-36RA also occurs. It can bind to the IL-36R complex but fails to elicit downstream signaling. The importance of IL-36RA in the regulation of inflammatory responses is highlighted by the fact that mice deficient for *il36rn* show significantly higher susceptibility to

imiquimod-induced skin inflammation[104], and in humans there is a high association between mutations in *IL36RN* and pustular psoriasis[105].

Interestingly it has also been shown that IL-38 shares 43% sequence homology with IL-36RA and can act in a similar fashion *in vitro* [99].

1.8.2 Soluble Receptors

Various soluble receptors for IL-1 family ligands exist which can essentially act as a 'sink' for these cytokines, by binding to their specific cytokine and reducing the bioavailability of these cytokines to the membrane bound receptor that could potentially propagate downstream signaling. IL-18BP is encoded from a separate gene to the IL-18R and has been shown to have a very high affinity for IL-18. It is present at 20molar excess compared to IL-18, in the serum of healthy patients[115]. IL-18 is a potent inducer of IFN- γ and it has been shown that IFN- γ itself can induce the expression of IL-18BP indicating a negative feedback mechanism for IFN- γ signaling[116]. Two splice variants of the IL-33 receptor ST2 exist, a membrane bound receptor and a soluble receptor (sST2). The soluble form of ST2 acts in a similar fashion to a decoy receptor by binding to IL-33 and decreasing its availability to the IL-33 signaling receptor[86]. Mast cells and fibroblasts have been shown to be a source of sST2[117].

1.8.3 Decoy Receptors:

IL-1RII is an IL-1 receptor decoy. Both IL-1RI (part of the functional IL-1R complex) and IL-1RII contain three extracellular Ig-like domains. However, unlike IL-1RI, IL-1RII lacks an intracellular signaling TIR-domain. A soluble version of IL-1RII has also been identified and can be generated by cleavage of

the membrane bound receptor by a metalloproteinase, or it can be expressed through alternative splicing[118, 119]. Although IL-1RI is expressed on a plethora of cell types, IL-1RII expression is more restricted and is found primarily on monocytes, neutrophils, B cell and T cells. IL-1RII expression is greatly enhanced in type-2 macrophages suggesting it may play a potential role in macrophage differentiation[120, 121]. Similarly Tregs express high levels of membrane-bound and soluble IL-1RII compared to effector T cells[122]. Expression of IL-1RII is influenced by surrounding inflammatory stimuli. Anti-inflammatory conditions, such as the presence of glucocorticoid hormones or Th2 cytokines, increases expression of IL-1RII, while pro-inflammatory stimuli such as LPS or IFN- γ suppresses IL-1RII expression[121, 123, 124].

1.8.4 Inhibitory receptors

Another member of the ILR TLR superfamily is Single Ig IL-1 Receptor Related molecule (SIGIRR). SIGIRR is an orphan receptor of the ILR family and has been shown to act as a negative regulator of IL-1 signaling[125]. Unlike the conventional three extracellular Ig domains observed in IL-1I and IL-1RII, SIGIRR is unique in having only a single Ig extracellular domain. It has been hypothesized that the shortened extracellular domain renders SIGIRR incapable of ligand binding and until recently, no known potential ligand had been identified. Like other ILR and TLR family members, SIGIRR contains an intracellular TIR domain. However, two amino acids considered essential for conventional for ILR signaling are substituted in the TIR domain of SIGIRR. Ser 447 and Tyr 536 are replaced by Cys 222 and Leu 305 in the SIGIRR TIR domain. The exact functional relevance of the amino acid substitutions remains unknown.

However, it has been shown that only the TIR domain of SIGIRR is required to inhibit TLR4 signaling. SIGIRR is constitutively expressed in certain tissues such as the kidney, gut, liver, lung and lymphoid organs[126]. In the GI tract, expression of SIGIRR is highest in intestinal epithelium, NKs, DCs, T and B cells. It has been observed that SIGIRR expression is reduced in human UC as well as murine models of colitis indicating a potential role for SIGIRR in intestinal inflammation[127]. SIGIRR has been shown to downregulate TLR and ILR-induced activation of NF κ B and JNK, without affecting TNF- α -dependent NF κ B activation[125]. It has been hypothesized that SIGIRR exerts a suppressive role by two mechanisms. The first being that it interferes with the dimerization of functional IL-1 receptor complexes, and secondly, that it uses its TIR domain to sequester adaptor proteins, such as MyD88, and prevents them interacting with TLRs and ILRs, thus preventing down-stream signaling (Fig 1F). SIGIRR has been identified as a key factor in controlling Th17 cell development and proliferation by modulating IL-1 induced phosphorylation of JNK and mTor kinase in Th17 cells[128]. Until recently there was no known ligand for SIGIRR. However, *Nold-Petry et al.* have shown that silencing of the SIGIRR gene *TIR8* or IL-18R α in PBMCs impairs the anti-inflammatory effect of IL-37, suggesting that SIGIRR and IL-18R α may form a receptor complex for IL-37[129]. A protective role for SIGIRR in the gut has also been demonstrated using the *Sigirr*^{-/-} mouse. *Sigirr*^{-/-} mice are more susceptible to DSS-induced colitis and also colitis-associated cancer. SIGIRR has been shown to regulate colonic epithelial homeostasis, inflammation and tumorigenesis. Hyperactivation of NF κ B and STAT3 is observed in the *Sigirr*^{-/-} mice and correlates with increased carcinogenesis in these mice. Furthermore, *Sigirr*^{-/-} mice have upregulated KC, MIP-2 and MIP-1 in

colonic tissue in response to DSS, suggesting a role for SIGIRR in cell trafficking in the colon[130].

Recently, new members of the ILRs have been identified such as TIGIRR-1, TIGIRR-1 and DIGIRR. TIGIRR-1 is expressed in the skin, placenta and fetal brain, whereas expression of TIGIRR-2 has been observed in tonsils, fetal liver, prostate, testis, small intestine, placenta and the colon. A role for TIGIRR as a negative regulator of ILR signaling has yet to be established. However *in vitro* studies using chimeric models have shown that cytoplasmic domains of TIGIRR-1 and TIGIRR-2 when fused to extracellular domains of IL-1RI and IL-1RAcP can inhibit NF κ B activation[131]. DIGIRR shares high homology with SIGIRR and also contains amino acid substitutes in its TIR domain. The DIGIRR protein contains two extracellular IG-like domains and siRNA studies in teleost fish have shown that knockdown of DIGIRR results in increased pro-inflammatory IL-1 β expression in the liver, kidney and leukocytes[132].

The existence of IL-1Rs acting as negative regulators for IL-1 family signaling emphasizes the importance of tight control of the IL-1 system. Dysregulated IL-1 family pathway signaling disrupts homeostasis on the gut resulting with uncontrolled inflammation, which contributes to the pathogenesis of IBD.

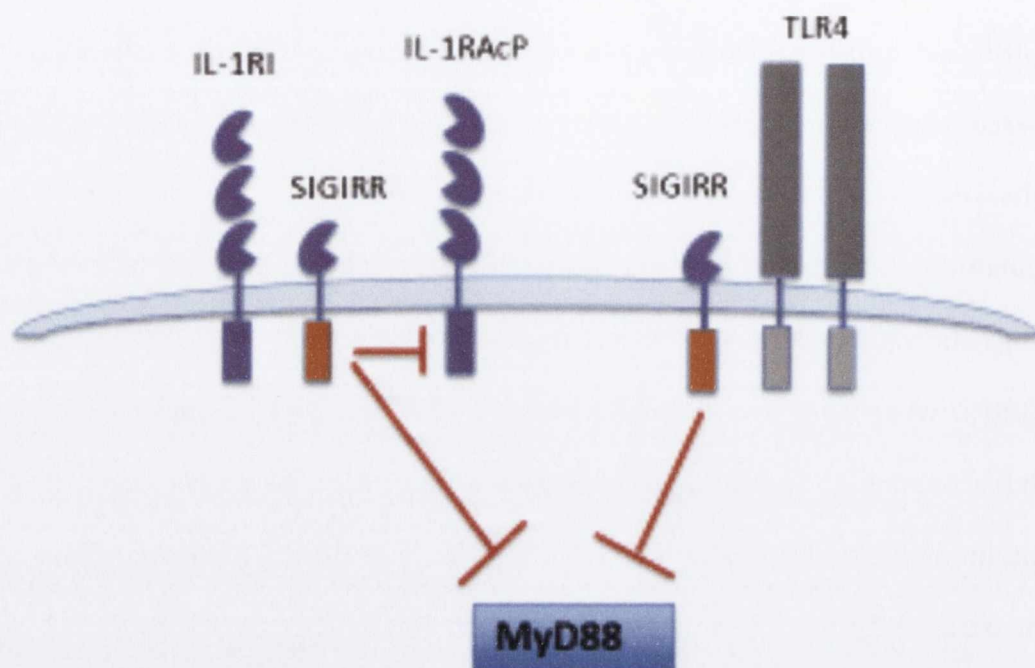


Fig 1.8. Hypothesized mechanism of action for SIGIRR
 SIGIRR has been shown to act as a negative regulator of IL-1R signaling and TLR signaling. It is proposed that SIGIRR acts by interfering with receptor heterodimerization and also by blocking the recruitment of adaptor proteins required for IL-1R/TLR signaling.

1.9 Aims of study:

It is clear that IL-1 family members play a key role in the maintenance of gastrointestinal homeostasis. However to date, targeting IL-1 as a potential therapeutic has had mixed results and clinical trials have failed. Based on this, it is key to identify novel mediators that may play a role in the pathology of IBD, which could lead to the development of new therapeutics. We have chosen to characterize expression levels of novel IL-1 family members in IBD patients, which, to date, are uncharacterized. We aim to identify novel targets and characterize their role on a molecular and cellular level, in the context of IBD.

Specifically the aims of this project are:

- To use a Paediatric IBD cohort to identify novel expression patterns of IL-1 family members
- To characterize cellular and molecular mechanisms of action of novel IL-1 family members in human cellular assays
- To use murine models of IBD to investigate the role of novel IL-1 family members in the pathology of intestinal inflammation
- To identify new potential therapeutic targets in IBD

Chapter 2:

Materials and methods.

2. Methods

2.1 RNA analysis

- RNA isolation from Human Biopsies/murine colon tissue/Cells

All RNA isolations were carried out using Bioline ISOLATE II RNA mini kit as per manufacturers instructions. RNA concentration was determined spectrophotometrically using a Nanodrop (Thermo Scientific) according to the manufacturer’s instructions.

- Reverse transcription and quantitative Real-Time PCR

0.5-1µg of each RNA sample was reverse-transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems), according to the manufacturer’s protocol, and the cDNA reverse transcriptase product was diluted 1:10 with nuclease free water. Transcripts were quantified by real time quantitative PCR on an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays and reagents according to the manufacturer’s instructions. For each sample, mRNA abundance was normalised to the amount of 18S ribosomal RNA (rRNA) and is expressed as fold difference compared to experimental control conditions.

Table 2.1.1 Materials for RNA analysis:

<u>Product</u>	<u>Use</u>	<u>Supplier</u>
ISOLATE II RNA mini kit	RNA extraction from tissue and cells	Bioline
High capacity cDNA	RT-PCR	Applied Biosystems

reverse transcriptase kit		
Sensifast Probe Hi Rox Kit	Master mix for quantitative real time PCR compatible with Taqman assays	Bioline
Sensifast SYBR Hi Rox kit	Master mix for quantitative real time PCR compatible with SYBR primer assays	Bioline

- Details of Human Taqman Primers for quantitative Real time PCR found in Appendix I
- Details of Murine Taqman Primers for quantitative Real time PCR found in Appendix I
- Details of Murine SYBR Primers for quantitative Real time PCR found in Appendix I

2.2 Cell culture

- Peripheral Blood Mononuclear Cell (PBMC) isolation from whole blood
Buffy coats were obtained from the Irish Blood transfusion Centre at St. James's Hospital. Blood was diluted 1:1 with sterile PBS. 25mls of the blood:PBS solution was carefully layered onto 25ml histopaque in a 50ml tube. This was spun at 400g with no brake for 25 minutes. PBMCs accumulate at the interphase in a white ring and were harvested with a

Pasteur pipette. PBMCs were washed twice in PBS and counted using Trypan blue.

- Purification of human CD14+ monocytes and CD4+ T cells from PBMCs, by Magnetic Activated Cell Sorting (MACS)

Human CD14+ and naïve CD4+ T cells were purified from total PBMCs using Magnetic Activated Cell Sorting kits from Miltenyi as per manufacturers instructions. Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >90%. Cells were seeded at $0.2-1 \times 10^6$ cells/ml (depending on the experiment) and stimulated as required.

- Purification of murine CD4+ and CD8+ T cells by Magnetic Activated Cell sorting (MACS)

Murine CD4+ and CD8+ T cells were purified from splenocytes using Magnetic Activated Cell Sorting kits from Miltenyi as per manufacturers instructions. Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >90%. Cells were seeded at $0.2-1 \times 10^6$ cells/ml (depending on the experiment) and stimulated as required.

- Generation of bone marrow derived dendritic cells (BMDCs)

Bone marrow derived DCs were generated from C57Bl/6 mice. Naive mice were sacrificed by cervical dislocation and their femurs and tibiae were removed and cleaned from the surrounding muscle and tissue. The bone marrow was then flushed from the bones using a 25-gauge needle

attached to a 20 ml syringe containing complete RPMI (cRPMI). Cell clusters were disrupted by aspirating the cell suspension through 19-gauge needle. The single cell suspension was centrifuged at 300 g for 5 min and cells were resuspended in 1 ml of warm 0.87% ammonium chloride for 2 min, to lyse contaminating red blood cells. Cells were washed in cRPMI and pelleted by centrifugation at 300 g for 5 min. The washed mononuclear cells were then resuspended in 10 ml cRPMI, cell viability and a total cell count was performed using trypan blue. The immature DCs were cultured at 0.5×10^6 cells/ml in complete RPMI with no more than 30 ml of cell suspension in a T175 tissue culture flask (Greiner). 40ng/ml of granulocyte macrophage-colony stimulating factor (GM-CSF) in the form of supernatant obtained from the J558 cell line was added. After culture for 3 days at 37°C and 5% CO₂, 25 ml of fresh medium containing 40 ng/ml of GM-CSF was added to each T175 flask. On day 6, the supernatant was carefully removed so as not to dislodge the loosely adherent immature DCs. This step removed contaminating cells, such as granulocytes from the culture. Removed supernatant was immediately replaced with 25 ml of warm fresh cRPMI containing 40ng/ml of GM-CSF. After a further 2 days at 37°C, (day 8) 20 ml of fresh medium containing GM-CSF (40ng/ml) was added to each of the flasks. On day 9 the loosely adherent cells were harvested by gentle pipetting. The viability of DCs was assessed and the cells were cultured at the required concentration in tissue culture plates. The immature DCs were allowed to rest for at least 2 h at 37°C before use.

- Conditions for skewing murine T helper cell subsets

Purified CD4+ T cells were counted in trypan blue and seeded at 2x10⁵ cells/well of a 96-well plate. Conditions for T cell skewing were as follows:

T helper subset	Activation condition	Recombinant cytokines/ cytokine neutralization antibodies
Th0	αCD3 (1μg/ml) αCD28 (2μg/ml)	None
Th1	αCD3 (1μg/ml) αCD28 (2μg/ml)	IL-12 (20ng/ml), anti-IL-4 (1μg/ml)
Th17	αCD3 (1μg/ml) αCD28 (2μg/ml)	TGF-β (2ng/ml), IL-6 (20ng/nl), anti-IL-4 (10μg/ml), anti-IFN-γ (10μg/ml)
iTreg	αCD3 (1μg/ml) αCD28 (2μg/ml)	TGF-β (5ng/ml)

- Conditions for skewing human T helper cell subsets

Purified naïve CD4+ T cells or total PBMCs were counted in trypan blue and seeded at 2x10⁵ cells/well of a 96-well plate. Conditions for T cell skewing were as follows:

T helper subset	Activation condition	Recombinant cytokines
------------------------	-----------------------------	------------------------------

Th0	αCD3 (5μg/ml) αCD28 (5μg/ml)	None
Th1	αCD3 (5μg/ml) αCD28 (5μg/ml)	IL-12 (50ng/ml)

- Culturing conditions for the murine macrophage cell line, RAW 267.4 cells/HT-29 cells/ HCT116 cells

Cells were cultured in DMEM supplemented with 10% FCS, 1% Pen/Strep and 1% L-Glutamine. Cells were grown in T75 flasks until they reached approx. 80% confluence. They were then washed with sterile PBS and treated with Trypsin-EDTA for approx. 2-3 minutes until cells loosened from the flask. They were then resuspended in complete DMEM and diluted 1:5 into new flasks.

- Cell counts

Cell counts were performed by diluting cells (usually 1/10 dilution) with trypan blue (Sigma). A 10μl volume of the cell suspension was then loaded onto a disposable hemocytometer (Hycor Biomedical, UK). The number of viable cells (appearing as white in contrast to dead cells, which stain blue) was counted using a light microscope. The number of cells per ml was calculated using the following formula: number of cells per ml = cell number x 10⁴ x dilution factor.

Table 2.2.1 Reagents used for cell culture:

<u>Reagent</u>	<u>Supplier</u>
RPMI	Sigma
DMEM	Sigma
Histopaque	Sigma
PBS	Biosciences
Fetal Calf Serum	Biosciences
Pen/Strep	Sigma
L-Glutamine	Sigma
Trypsin-EDTA	Sigma
Trypan Blue	Sigma

- Details of MACS antibodies in Appendix I
- Details of Human and Mouse T cell activating antibodies available in Appendix I
- Details of Recombinant murine cytokines/neutralization antibodies (used in cell culture) available in Appendix I
- Details of Recombinant human cytokines available in Appendix I
- Details of TLR/NLR ligands available in Appendix I

2.3 Transfection of RAW 267.4 cells with IL-37 plasmid

RAW 267.4 cells were plated in 6-well plates and transfected when cells were approximately 70% confluent. Targeting Systems ‘Targefect-Raw’ was used as per manufacturer’s instructions whereby transfection complexes containing transfection reagent and plasmid of interest were allowed to form in serum-free

Optimem. RAW 267.4 cells were then treated with transfection complexes and transfection efficiency was measured after 24 hours. The presence of Thy1.1 surface protein, as assessed by flow cytometry, was used to indicate transfection efficiency. Once positive transfection was confirmed, cells were counted and plated at equal numbers on a 96-well plate and subsequently treated with various TLR/NLR ligands.

2.4 NF- κ B alkaline phosphatase assay

RAW cells containing a NF- κ B-linked alkaline phosphatase-reporter (from Invivogen), were used to measure NF- κ B activation in stimulated RAW cells. Upon Activation of NF- κ B, an alkaline phosphatase is secreted into the supernatants. The amount of alkaline phosphatase secreted directly correlates with the extent of NF- κ B activation. To quantify the amount of alkaline phosphatase in the supernatant, supernatants were treated with the detection media Quanti-Blue, an alkaline-phosphatase substrate that turns from pink to blue in the presence of alkaline phosphatase. The blue colour, which reflects NF κ B activation, can then be quantified using a spectrophotometer at 655nm..

2.5 Measuring cytokine concentrations by ELISA

Cytokine concentrations were determined using commercially available ELISA kits according to the manufacturer guidelines. Antibody dilutions and incubation times were different between manufacturers. High binding microtitre plates (Greiner bio-one) were coated overnight at 4°C with 50 μ l per well of capture antibody diluted in PBS. Plates were washed 5 times in ELISA wash buffer (PBS / 0.05% Tween 20) and non-specific binding sites were blocked by incubation

with 150µl of blocking buffer for 2 h at room temperature. Plates were again washed 5 times before the addition of 50µl per well of sample supernatant or an eight point two-fold serial dilution from the top working standard recombinant protein for each cytokine diluted in reagent diluent. Plates were incubated at room temperature for 2 hours. After washing, 50µl of specific biotinylated detection antibody diluted in reagent diluent was added per well and incubated at room temperature. Plates were again washed 5 times and incubated with 50 µl/well horseradish peroxidase (HRP)-conjugated streptavidin for 20 min at room temperature in the dark. Plates were washed 7 times and 50µl of TMB substrate was added to each well. ELISA plates were developed and the enzyme reaction was stopped by the addition of 25µl/well of 1 M H₂SO₄. The OD values were determined by measuring absorbance at 492 nm using a BioTek SynergyMx plate reader and cytokine concentrations for supernatant samples were calculated using the standard curve.

- Details of Human ELISA kits available in Appendix I
- Details of Mouse ELISA kits available in Appendix I

2.6 Flow cytometric analysis

- Cell re-stimulation prior to the detection of cytokines by flow cytometry

Cell suspensions were added to a sterile FACS tube at 1-2 x 10⁶ cells in 500 µl per tube. Cells were activated with PMA (10ng/ml) and Ionomycin (1µg/ml) in the presence of Brefeldin A (BFA) (5 µg/ml) in cRPMI supplemented with 2-ME (50µM) and incubated at 37°C and 5% CO₂ for 6 h. After incubation, cells were washed twice with 3 ml of FACS buffer, pelleted by centrifugation at 300 g for 5 min and stained as required.

- Surface staining

Single cell suspensions, prepared from organs isolated from *in vivo* studies or obtained from *in vitro* cultures were added to a FACS tube at 1×10^6 cells in 100 μ l per tube. The cells were then washed in FACS buffer and centrifuged at 300g for 5 min. Cells were then incubated with 100 μ l of the appropriate FACS antibody directed against a surface antigen for 15 min at room temperature in the dark. In parallel, single stained compensation controls were prepared using FACS comp beads (BD). Excess staining antibody was removed by washing each tube twice in FACS Buffer, 1 ml per tube, with centrifugation at 300g for 5 min between each wash. Cells were resuspended in 0.5ml FACS buffer for the analysis.

- Intracellular staining

In order to detect intracellular epitopes such, as cytokines or transcription factors, cells were first stained with surface antibodies, as described above. Before surface staining, the cells were washed in PBS and stained with LIVE/DEAD Fixable Aqua Dead Cell stain (Aqua-Live/dead, Molecular probes, Life Technologies). The Aqua live/dead stain was diluted 1/600 in PBS for 20 min at room temperature in the dark. Aqua-Live/dead is an amine-reactive fluorescent dye that stains dead cells and can be detected by FACS using a filter band for V500/AmCyan. After the last wash the FACS buffer was completely removed and cells were resuspended in Fix/Perm buffer (eBioscience) and incubated for 30 min at 4°C in the dark. Cells were then washed twice with

Permeabilization Buffer (eBioscience) and stained with 100µl of appropriate fluorochrome-conjugated antibody (eBioscience) diluted in Permabilization Buffer. After 30 min cells were washed twice with Permabilization Buffer. After 30 min cells were washed twice with Permabilization buffer followed by centrifugation at 300 g for 5 min, resuspended in 0.5 ml FACS buffer and acquire as described above. Stained cell suspensions were acquired using BD LSR Fortessa flow cytometer according to the manufactures instructions. The flow cytometer was calibrated using single-stained suspensions of compensation beads (BD). FACS data analysis was performed using FlowJo (Treestar) software.

- Details of Human Flow Cytometry antibodies avialable in Appendix I
- Details of Mouse Flow Cytometry Antobodies available in Appendix I

Table 2.6.3 Other reagents used in Flow Cytometry analysis

<u>Reagent</u>	<u>Supplier</u>
PMA	Sigma
Ionomycin	Sigma
Brefaldin A	Sigma
Foxp3 Fix/perm Kit	Ebioscience
Compensation Beads	BD biosciences
Live dead aqua stain	Bioscience

2.7 Murine colonic explant stimulation

For colonic explant cultures, mice were sacrificed by cervical dislocation. Colons were excised and washed out with sterile PBS. A section of colon approx. 1cm long (closest to the caecum) was cut out and opened longitudinally. The tissue was cultured in cRPMI with appropriate cytokine treatment for 18-24 hours. After the stimulation the supernatant was harvest. The tissue was subsequently homogenized in 0.5ml sterile PBS containing protease inhibitors (Sigma). Once the samples were homogenized they were centrifuged at 400g for 10 minutes and the supernatents were harvested. ELISA readings from both the culture supernatents and the homogenates were made relative to total protein content as determined by BCA assay (Sigma).

2.8 I.P administration of recombinant IL-36 and cell isolation from peritoneal cavity

Mice were injected with rIL-36 α into the intraperitoneal space and sacrificed at either 1,4 or 6 hours post injection. The peritoneal cavity was lavaged with 1.5ml of sterile PBS in order to collect the peritoneal exudates cell (PECs). Lavage samples were centrifuged at 300xG for 10 minutes. The supernatents were harvested and chemokine levels were measured by ELISA. Cell pellets were re-suspended in PBS and stained with appropriate antibodies for Flow Cytometric analysis.

2.9 DSS-induction of colitis and relevant scoring system

Colitis was induced by adding Dextran Sodium Sulfate (MP Bio) to drinking water at either 1.5% (w/v) or 2% (w/v). Mice were monitored daily and weight loss, stool consistency and rectal bleeding were recorded and used to calculate

Disease Activity Index (DAI). For experiments that involved comparing 2 different genetic strains of mice, mice were co-housed for at least 7 days prior to DSS induction. Ths scoring system to measure disease severity was as follows:

DSS Scoring System. DAI (Disease Activity Index) is measured by adding the score from each of the 3 parameters. Minimum score of 0. Maximum score of 12		
Parameter	Observation	Score given
1. Weight Loss	0% weight loss	0
	1-5% weight loss	1
	5-10% weight loss	2
	10-20% weight loss	3
	20%+ weight loss	4
2. Stool consistency	Normal	0
	Loose	2
	Diarrhea	4
3. Rectal bleeding	none	0
	hemocult	2
	Gross bleeding	4

2.10 Cell isolation from murine colons

Murine colons and caecums were harvested and washed out with PBS. Tissue was opened longitudinally and cut into small pieces. Tissue pieces were then washed in

RPMI containing 5% FCS and 5mM EDTA for 40 minutes. Intra-epithelial cells were collected at this point from the washings. Tissue pieces were subsequently incubated in RPMI containing 5% FCS, collagenase VIII (1mg/ml) and DNase I (200U/ml) for 60 minutes. Digested colon tissue was subsequently passed through a cell strainer. Cells were then resuspended in 40% (v/v) percoll and layered onto 70% (v/v) percoll and centrifuged at 400xG with no brake. Lamina propria cells were collected at the interphase of the 2 layers and counted using Trypan blue. Cells were then stained for analysis by flow cytometry.

Table 2.10.1 Reagents used for tissue digestion

<u>Reagent</u>	<u>Supplier</u>
EDTA	Sigma
Collagenase VIII	Sigma
DNase I	Sigma
Percoll	Sigma

2.11 Histological analysis of human and murine colon tissue

Immunohistochemical staining was performed in 4µm thick formalin-fixed, paraffin-embedded whole-section slides obtained from the archives of Cork University Hospital. Sections were deparaffinized in xylene and rehydrated prior to analysis. Antigen retrieval was performed by microwave irradiation in 0.01 M citrate buffer, pH 6.0. Slides were washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.001% saponin. Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 min.

Nonspecific binding was blocked using 5% normal serum in wash buffer for 1 hr. Sections were incubated overnight at 4°C with primary antibody. Primary antibody binding was localized using a biotinylated secondary antibody, and visualised using avidin-conjugated HRP and DAB substrate, contained within the Vectastain ABC detection kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with haematoxylin and mounted. Parallel negative controls were performed, using rabbit IgG instead of the primary antibody. The IL36 Receptor antibody was obtained from Abcam (ab180894) and used at a dilution of 1:2000. Controls were treated as above omitting the primary antibodies and also by using an IgG control in place of primary antibody.

2.12 Mice

Female C57BL6/J mice (Harlan) between 6-12 weeks were utilized in all experiments. IL-36R^{-/-} mice and sigirr^{-/-} mice were bred in house. Mice were housed under SPF conditions at the Institute for Molecular Medicine, St. James Hospital Dublin. All animal experiments were performed in compliance with the Health Products Regulatory Authority (HPRA) regulations and approved by the Trinity College Dublin's Bioresources ethical review board

Chapter 3: Characterizing the role of novel IL-1 family members in Paediatric IBD

Chapter 3: The role of novel IL-1 family members in paediatric IBD

Introduction:

There has been a rapid rise in incidence in paediatric IBD in Ireland in the last 15 years[12]. As a result, a clinical IBD research project has been established at Our Lady's Children's Hospital, Crumlin (OLCHC). The DOCHAS study (Determinants and Outcomes in Children and Adolescents with IBD) is a prospective study following Irish children who have been newly diagnosed with IBD since 2012. The overall aims of the study are to identify the causes of paediatric IBD, improve diagnosis and attempt to better predict treatment and long-term outcomes. Importantly, the samples taken from the recruited patients are taken upon first presentation with disease and are therefore treatment naïve. Through a collaborative study with the gastro-intestinal unit in OLCHC, we were able to use this cohort of patients (table 3.1) to characterize gene expression levels of novel IL-1 family members that have not been previously characterized in the context of IBD.

Although various IL-1 family members have been studied in the context of IBD, there have been conflicting reports as to their role in disease pathogenesis. The dichotomous nature of IL-1 is underscored by the fact that Phase I clinical trials of IL-1 neutralization in IBD have failed. This highlights the necessity to unravel novel pathways involved in the IBD pathology and identify new potential therapeutic targets. As such, we have chosen to focus on characterizing the role of IL-36 and IL-37 as potential novel mediators of intestinal inflammation.

To date, it has been shown that IL-37 is an immuno-suppressive cytokine of the IL-1 family that acts as a fundamental inhibitor of inflammation[106]. As there is no known mouse homolog of IL-37, a previous study has employed an over-expression system whereby IL-37 transgenic mice over express the human IL-37 protein. These mice are protected from septic shock and murine colitis[110]. However the role of IL-37 in human IBD still remains largely unknown.

The IL-36 family of cytokines is best characterized by their pathogenic role in inflammatory skin conditions such as psoriasis. Various mouse models and clinical studies have demonstrated that IL-36 expression is elevated in psoriatic patients and in mouse models of skin inflammation and that it acts directly on keratinocyte and fibroblasts to promote disease symptoms[103]. Furthermore, it has been shown that the over expressing IL-36 α exacerbates skin lesions to a greater extent in Il36rn^{-/-} mice compared to WT mice[104], highlighting the role of IL-36 ligands as a driver of skin inflammation and the importance of IL-36RA in negatively regulating this pathway. Expression of IL-36 ligands appears to be very tissue specific. Although their expression has been characterized in skin, the lung and the brain, little has been shown in terms of the expression and role, if any, of IL-36 ligands in the gut.

Patient information		Numbers
Disease groups		22
	Ulcerative Colitis	15
	Crohn's Disease	18
Sex	Male	27
	FemaleControls	28
Age	0-5 years	4
	5-10 years	16
	10-15 years	24
	15+ years	11
UC phenotype	E1	2
	E2	0
	E3	0
	E4	13
CD phenotype	L1 any combination	1
	L2 any combination	3
	L3 any combination	10
	L4a	1
	L4b	1
	PA disease only	2
	Oral Disease only	0
UC disease severity	Mild	7
	Moderate	5
	Moderate-severe	0
	Severe	3
CD disease severity	Mild	4
	Moderate	0
	Moderate-severe	12
	Severe	2

Table 3.1. Paediatric IBD cohort information table

UC classification legend:

E1: proctitis only
E2: left sided disease
E3 extensive disease
E4: pancolitis

CD classification legend:

L1, L2 and L3 all classify colonic disease and are differentiated as follows:

L1: limited terminal ileal disease
L2: isolated colonic disease
L3: ileal-colonic disease

L4a and L4b both refer to upper GI tract disease and are differentiated as follows:

L4a: anything proximal to the ligament of treitz
L4b: anything distal to the ligament of treitz

PA: perianal disease

3.1 Increased expression of inflammatory cytokines and T cell lineage transcription factors in paediatric IBD

To investigate patterns of gene expression in a paediatric IBD cohort, rectal biopsies from healthy and diseased patients were homogenized and RNA was isolated for analysis. RNA was reverse transcribed into cDNA, which was then analyzed by qPCR. Results were analyzed and made relative to expression of the 18S RNA housekeeping gene. To firstly demonstrate that our cohort follows the same expression profiles as is well documented in the literature[70, 133-135], we have measured expression levels of cytokines and transcription factors that are hallmarks of Th1, Th2 and Th17 CD4+ T cell subsets. These CD4+subsets have been well documented as playing a role in the pathogenesis of IBD. As described previously, the signature Th1 cytokine, *IFNG*, is significantly elevated in IBD patients (fig3.1A). The signature Th17 cytokine, *IL17A*, is significantly elevated in IBD patients, particularly UC patients and to a lesser extent in CD patients (fig3.1B). Th2 associated *IL13*, is specifically upregulated in UC (fig3.1C). *IL22* expression is significantly elevated in IBD whereas levels of the naturally occurring IL-22 binding protein (*IL22RA2*) remain unchanged (fig3.1D&E). Levels of the neutrophil recruiting chemokine *IL8* are significantly elevated in the mucosa of IBD patients (fig3.1F). Furthermore the transcription factors that regulate T cell differentiation also appear to be altered in the gut mucosa of IBD patients. The Th1 and Th17 associated transcription factors *TBX1* and *RORC* respectively, are upregulated in CD patients (fig3.1G&H). These data demonstrate that this paediatric IBD cohort shows similar alterations in the

expression of pro-inflammatory mediators as has been published previously (reviewed in [136]).

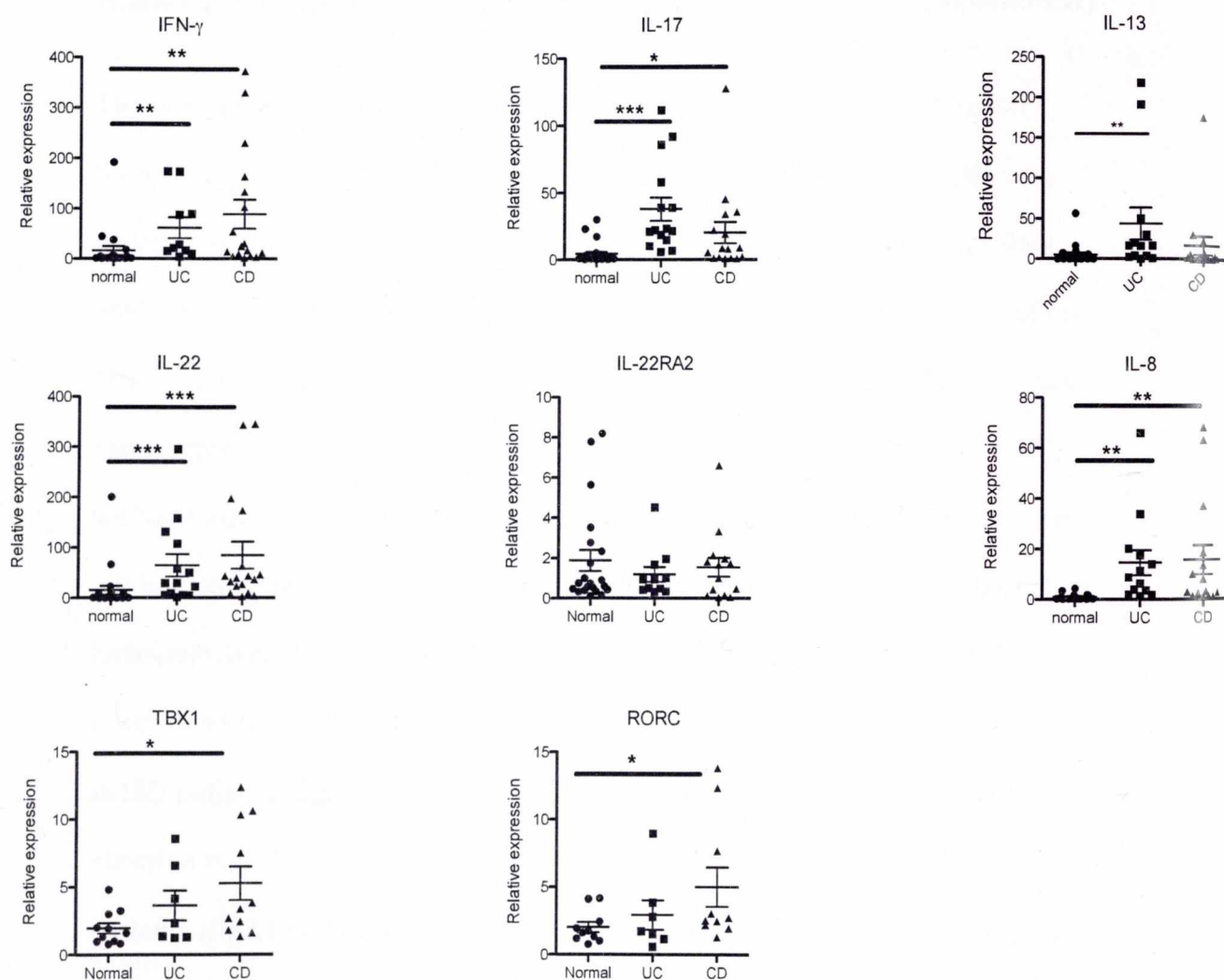


Figure 3.1. Increased expression of inflammatory cytokines and T cell lineage transcription factors in paediatric IBD. Rectal biopsies from a paediatric cohort were classified as normal, Ulcerative Colitis (UC) or Crohn's Disease (CD). RNA was extracted from biopsies and gene expression was measured by qPCR for *IFNG* (A), *IL17* (B), *IL13* (C), *IL22* (D), *IL22RA2* (E), *IL8* (F), *TBX1* (G) and *RORC* (H). All genes were normalized to 18S RNA expression. Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Mann-Whitney test. P values of less than 0.05 were considered significant. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$.

3.2 Expression of the orphan receptor SIGIRR and its putative ligand IL-37 in a paediatric IBD cohort.

Having shown that the paediatric IBD cohort demonstrates elevated levels of the pro-inflammatory cytokines *IFNG*, *IL17A*, *IL13*, *IL22* and *IL8*, we next decided to focus on a group of IL-1 related genes. Firstly, we chose to examine expression levels of the orphan receptor SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling[125]. A previous study has shown that SIGIRR expression is reduced in an adult cohort of IBD patients[127]. Furthermore, murine models have demonstrated that SIGIRR plays an important role in gastrointestinal homeostasis as *sigirr*^{-/-} mice show enhanced sensitivity to gut inflammation[130]. To investigate whether reduced levels of *SIGIRR* expression are also evident in paediatric IBD patients, *SIGIRR* expression in rectal biopsies was measured by qPCR. No significant difference in *SIGIRR* gene expression levels between normal, UC and CD patients were observed (fig3.2A). Although no difference in SIGIRR expression was observed, we next sought to investigate expression levels of the novel IL-1 family member IL-37, which has recently been identified as a putative ligand for SIGIRR and has been shown to suppress innate inflammation[109]. Total *IL37* expression is significantly reduced in paediatric CD patients compared to normal patients (fig3.2B). Furthermore *IL37B*, the most commonly studied immunosuppressive isoform of IL-37 is significantly reduced in paediatric UC patients and CD patients (fig 3.2C). These data demonstrate that although levels of SIGIRR appear unaltered between control and diseased groups, expression levels of *IL37* are significantly reduced in the mucosa of paediatric IBD patients.

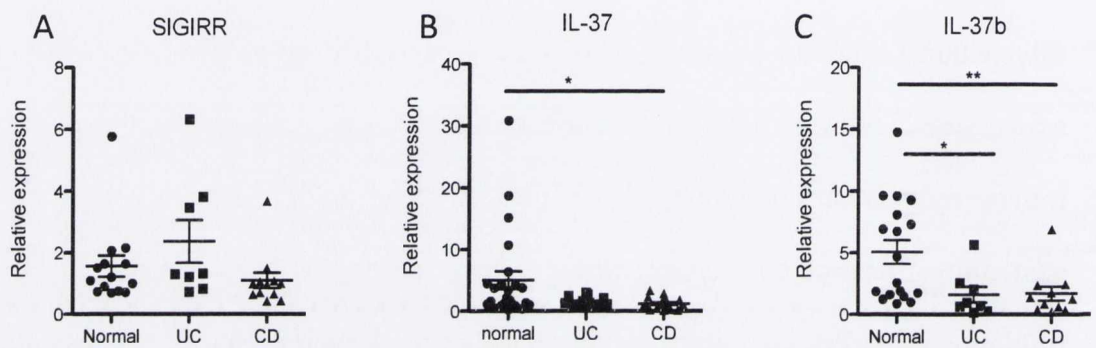


Figure 3.2 Expression of the orphan receptor SIGIRR and its putative ligand IL-37 in a paediatric IBD cohort. Rectal biopsies from a paediatric cohort were classified as normal, Ulcerative Colitis (UC) or Crohn's Disease (CD). RNA was extracted from biopsies and gene expression was measured by qPCR for *SIGIRR* (A), *IL37* (B) and *IL37B* (C). All genes were normalized to 18S RNA expression. Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Mann-Whitney test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

3.3 Stimulation of PRRs leads to upregulation of IL-37 expression in various cell types

As IL-37 has been demonstrated to suppress innate immune responses upon intracellular over expression[109], we next examined which factors might alter IL-37 expression within certain cell types. PBMCs were stimulated with a TLR-2 ligand Pam3CSK4 (500ng/ml) or a TLR-4 ligand LPS (100ng/ml) and harvested at 24 and 48 hours post-stimulation. RNA was extracted from cells and reverse transcribed into cDNA. *IL37B* gene expression was detected by qPCR. TLR-2 and TLR-4 stimulation significantly induces *IL37B* expression in PBMCs (fig 3.3A). Similarly, stimulation of CD14+ primary monocytes with a NOD2 agonist MDP (10µg/ml), Pam3CSK4 (500ng/ml) or LPS (100ng/ml) leads to significant upregulation of *IL37B* 24 hours post stimulation (fig 3.3B). Human colonic epithelial cells play a key role in gut homeostasis. We therefore examined HT-29 colonic epithelial cell line for *IL37B* expression. Nod2 stimulation with MDP (10µg/ml) induces significant upregulation of *IL37B* in these cells. These data demonstrate that PRR stimulation can induce expression of *IL37B* in cells that are found in the gut. This highlights the potential importance of the presence of PAMPs derived from gut microflora in regulating IL-37 levels in the colon.

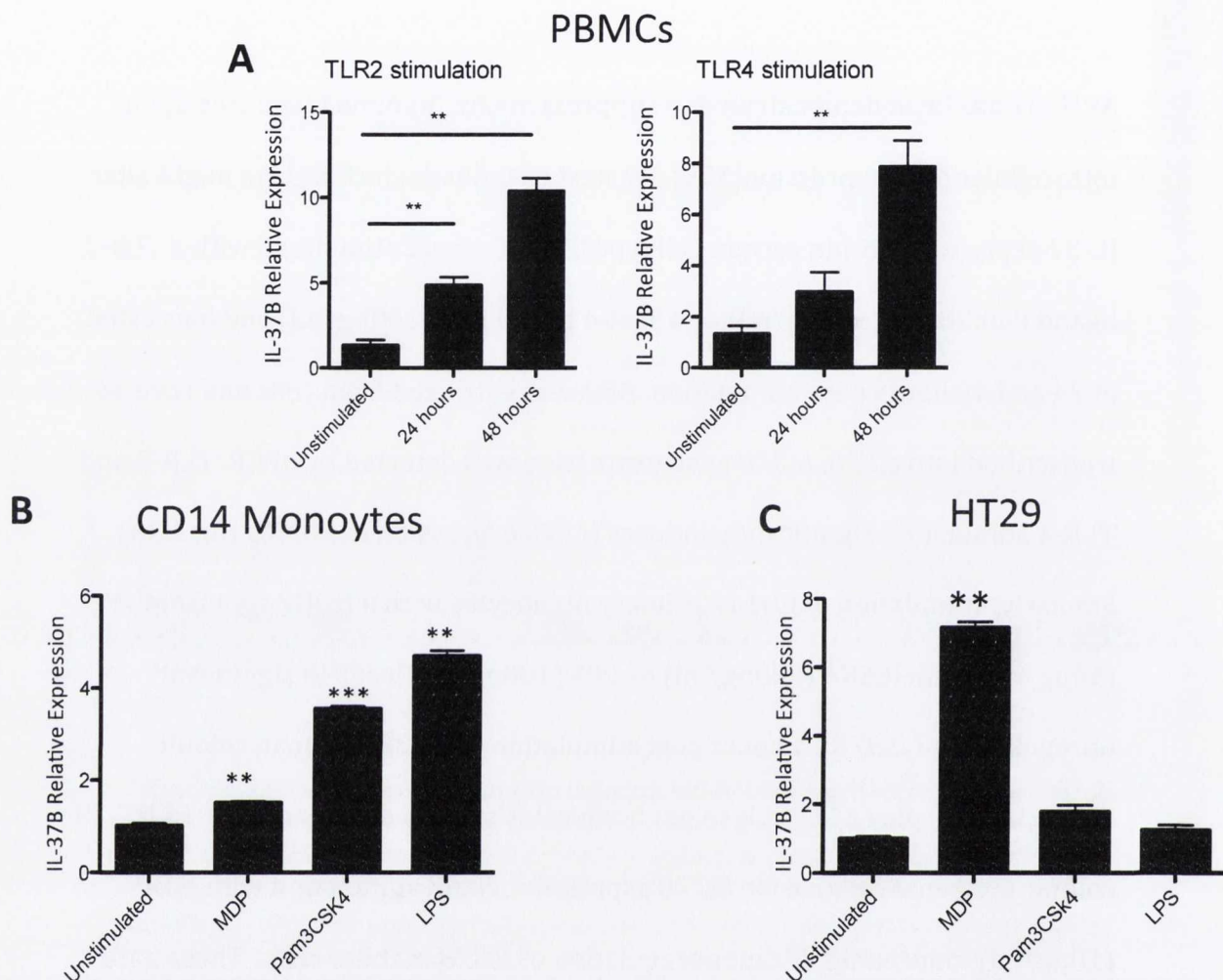


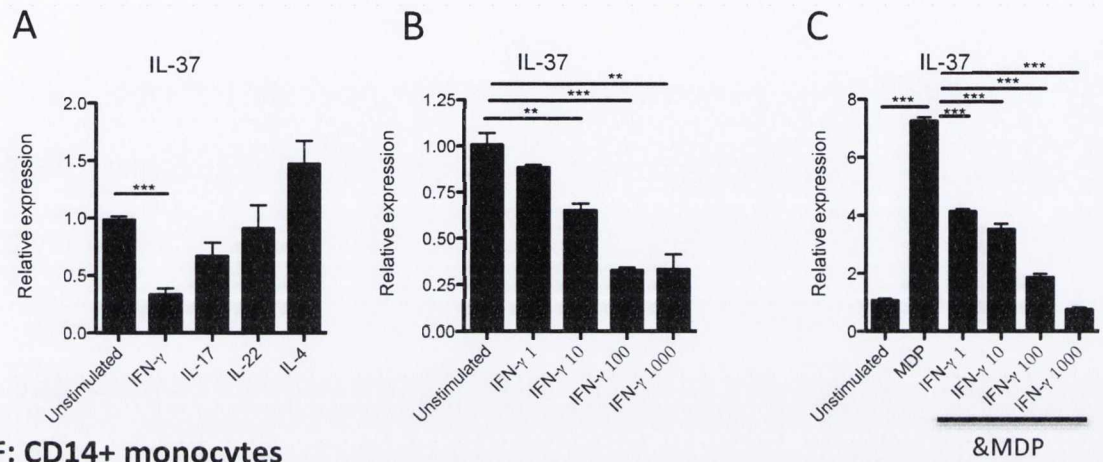
Figure 3.3. Stimulation of Pattern Recognition receptors leads to upregulation of IL-37b expression in various cell types. PBMCs (A), CD14+ monocytes (B) or HT29 colonic epithelial cells (C) were stimulated with MDP (10 μ g/ml), Pam3CSK4 (500ng/ml) or LPS (100ng/ml) as stated. PBMCs were stimulated for 24 and 48 hours, while CD14 monocytes and HT29 cells were stimulated for 24 hours. RNA was extracted from cells and IL-37 expression was measured by qPCR. Gene expression was made relative to 18S RNA. Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

3.4 The pro-inflammatory cytokine IFN- γ inhibits IL-37 expression

We have demonstrated that stimulation of various TLR and NLR stimulation can up-regulate IL-37b expression in monocytes and colonic epithelial cells. As well as defective innate immune pathways being pathogenic in IBD, various CD4+ T helper subsets have also been shown to play an important role in chronic inflammation in this disease. Despite this, the specific effect of the cytokine environment produced by the various T helper subsets in IBD is currently ill defined. UC has been characterized as having a Th17 or Th2 phenotype[134, 137] and CD has been shown to have a prominent Th1 cytokine profile[138]. To investigate the effect of the T cell environment in IBD on IL-37 expression, HT-29 cells were treated with Th1, Th17 and Th2 associated cytokines and IL-37b expression was measured. HT-29 cells were treated with IFN- γ , IL-17, IL-22 and IL-4 (all 100ng/ml) for 24 hours. IL-37 was measured by qPCR. IFN- γ significantly down-regulates IL-37b in HT-29 cells while IL-17, IL-22 and IL-4 appear to have no effect (fig 3.4A). This data demonstrates that inflammatory mediators, that we have shown to be elevated in our paediatric IBD cohort, can negatively regulate IL-37b expression. Thereby suggesting that IL-37 expression may be being suppressed in sites of inflammation. To further demonstrate the inhibitory effect of IFN- γ on IL-37b expression in colonic epithelium, HT-29 cells were treated with various concentrations of IFN- γ (1-1000ng/ml) for 24 hours. RNA was isolated and *IL37B* expression was quantified by qPCR. Endogenous *IL37B* expression is inhibited in a dose-dependent manner in response to IFN- γ . We have already shown that the NOD2 agonist MDP, which is thought to be

protective in Crohn's Disease, up-regulates *IL37B* in HT-29 cells. HT-29 cells were stimulated with MDP (10µg/ml) in combination with a range of IFN-γ concentrations (1-1000ng/ml) for 24 hours and then gene expression was analyzed by qPCR. IFN-γ also inhibits MDP-induced *IL37B* in a dose-dependent manner (fig 3.4A). To investigate whether the inhibitory effect of IFN-γ is specific to colonic epithelium, human primary monocytes were also tested. We have previously shown that Pam3CSK4 and LPS are potent inducers of *IL37B* expression in primary monocytes. We therefore tested the effect of IFN-γ (1-1000ng/ml) on endogenous *IL37B* expression as well as Pam3CSK4-induced and LPS-induced *IL37B*. IFN-γ reduced endogenous, Pam3CSK4-induced and LPS-induced total *IL37B* in primary monocytes in a dose-dependent manner (fig 3.4B). This highlights a potential mechanism of reducing *IL37B* expression in an inflamed environment associated with IBD.

A-C: HT-29 Cells



D-F: CD14+ monocytes

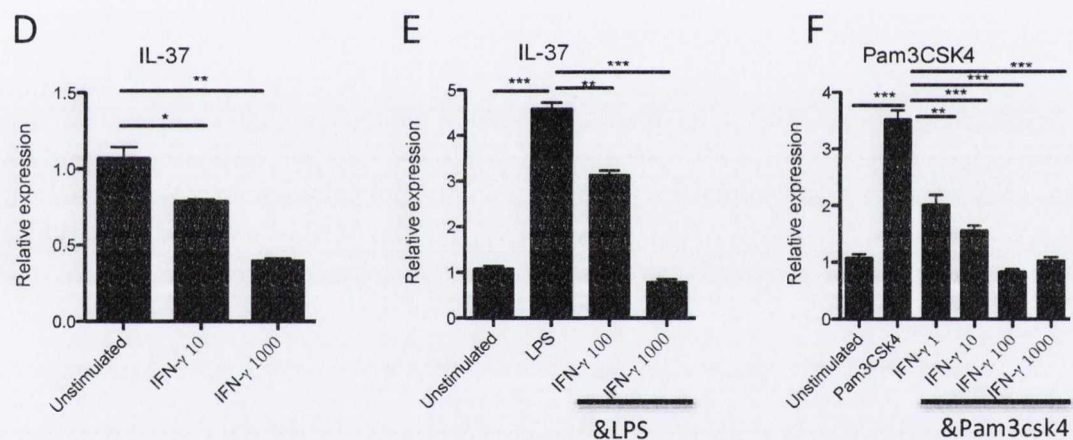


Figure 3.4. The pro-inflammatory cytokine IFN- γ inhibits IL-37 expression. HT-29 cells were treated with IFN- γ (100ng/ml), IL-17 (100ng/ml), IL-22 (100ng/ml) or IL-4 (100ng/ml) (A) for 24 hours and then IL-37 levels determined by qPCR. HT29 cells were subsequently treated with a range of concentrations of IFN- γ (1-1000ng/ml) (B) or stimulated with both MDP (10 μ g/ml) and a range of concentrations of IFN- γ (1-1000ng/ml) (C). After 24 hours of stimulation RNA was extracted and IL-37 expression was analysed by qPCR and made relative to 18S RNA expression. Similarly human CD14+ monocytes were treated directly with IFN- γ (10-1000ng/ml) or stimulated with LPS (100ng/ml) or Pam3csk4 (500ng/ml) and a range of concentrations of IFN- γ (1-1000ng/ml) (E&F) Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

3.5 Extracellular IL-37 does not appear to alter basic immune cellular responses '*in vitro*'

Recently it has been shown that extracellular IL-37 can inhibit innate inflammatory responses in human M1 macrophages[109]. Furthermore, IL-37 has been shown to be secreted after caspase-1 mediated processing in a similar manner to IL-1 β and IL-18[139]. Therefore, we sought to examine whether exogenous recombinant IL-37b may act to suppress both innate and adaptive immune responses. To address this, Peripheral Blood Mononuclear Cells (PBMCs) and CD4⁺ T cells were stimulated and treated with or without recombinant IL-37b. PBMCs were isolated from buffy coats using a histopaque gradient. PBMCs were pre-treated with IL-37b (2.5 μ g/ml) and subsequently stimulated with Pam3Csk4 (500ng/ml) +/- IL-37b for 24 hours. TNF- α and IL-6 levels were detected by ELISA. Pre-treatment with IL-37b did not affect Pam3CSK4 induced TNF- α or IL-6 from PBMCs (fig 3.5A). CD4⁺ T cells were purified from PBMCs using Magnetic Activated Cell Sorting (MACS). Purified CD4⁺ T cells were activated with anti-CD3 (5 μ g/ml) and anti-CD28 (5 μ g/ml) T cell activation antibodies and subsequently polarized to Th0 (cell media), Th1 (IL-12 50ng/ml) or Th17 (IL-23 50ng/ml, IL-1 β 50ng/ml, IL-6 30ng/ml) conditions. Th0, Th1 and Th17 cells were cultured for 4 days with and without recombinant IL-37b (2.5 μ g/ml). Supernatants were harvested and cytokine levels were measured by ELISA. Supernatants from Th1 cells had increased IFN- γ levels relative to Th0 cells. However, the presence of exogenous IL-37b did not affect IFN- γ levels in Th0 or Th1 cells. IL-17 levels were elevated in Th17 cells

supernatants compared to Th0 supernatants. The presence of exogenous IL-37b did not alter IL-17 expression in Th17 cells (fig 3.5B). Although these data demonstrated no effect of extracellular IL-37b on the cell types and responses examined that we have examined, the possibility remains that IL-37b is capable of modulating immune responses in an extracellular fashion in a different cell type or under different experimental conditions from the ones tested here.

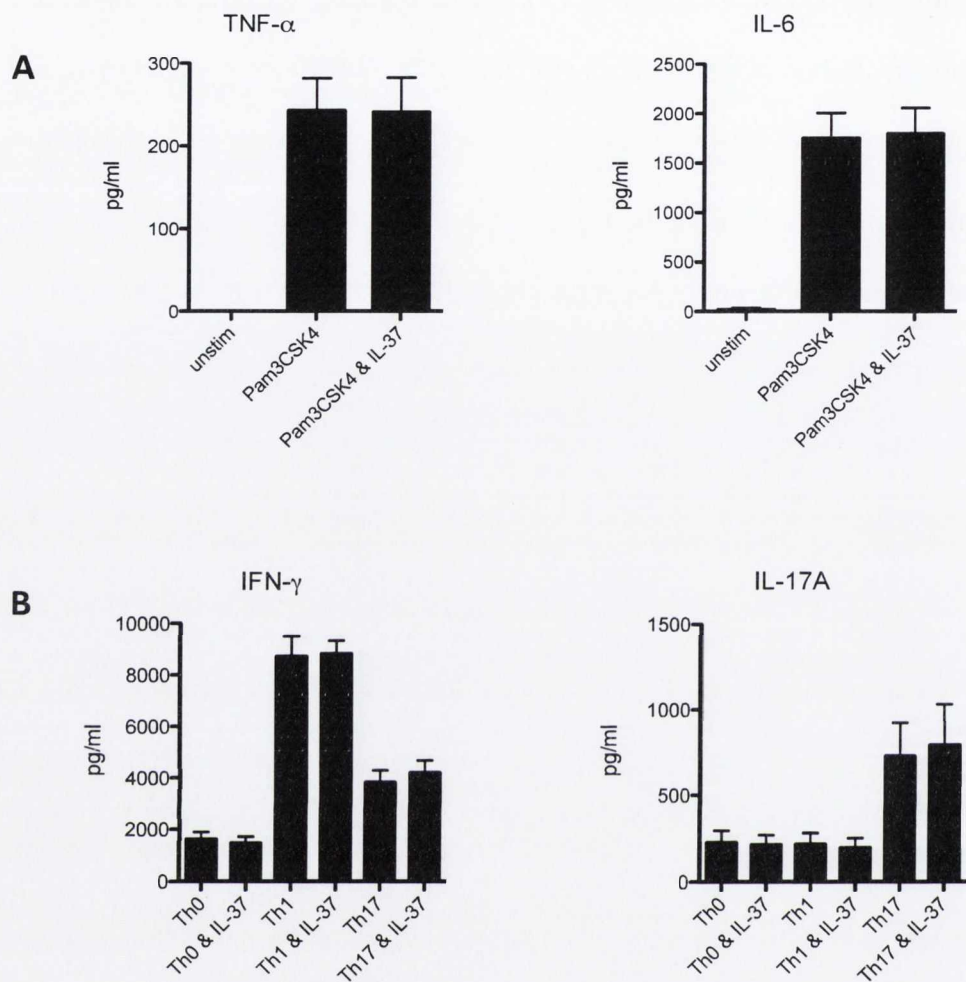


Figure 3.5. Extracellular IL-37 does not appear to alter basic immune cellular responses 'in vitro'. Peripheral Blood Mononuclear Cells were isolated from buffy coats and stimulated with Pam3CSK4 (500ng/ml) +/- IL-37 (2.5 μ g/ml). Cultured supernatants were collected at 24 hours and analysed for cytokine production by ELISA (A) CD4⁺ T cells were purified from PBMCs by MACS separation and polarized as Th0, Th1 or Th17 phenotype T cells +/- IL-37 (2.5 μ g/ml). Cultured supernatants were collected at 96 hours and analysed for cytokine production by ELISA (B). Data is represented as mean values, with error bars representing standard error of the mean.

3.6 Over expression of IL-37 in RAW 264.7 cells suppresses subsequent innate immune stimulation

To begin to further investigate a mechanism of action of IL-37 on a molecular level, an over-expression system was developed where RAW 267.4 cells were transfected with a MSCV plasmid containing the IL-37 gene. A gene encoding the surface protein Thy1.1 occurs downstream, and separated by an internal ribosomal entry site, of the IL-37 gene within the plasmid. The presence of Thy1.1 is used to identify positively transfected cells. RAW 267.4 cells were seeded on 6-well plates. This murine macrophage cell line was chosen as these cells are responsive to a wide variety of TLR and NLR ligands, do not express endogenous human *IL37* and are easily transfected. When the cells were 70-80% confluent they were treated with a RAW 264.7 cell-specific transfection agent containing the IL-37 plasmid or an 'empty' control plasmid. The cells were incubated with the transfection complexes for 24 hours at 37°C at 5% CO₂. Transfected cells were then washed and surface stained with a fluorescently labelled Thy1.1 antibody. Transfection efficiency was then measured using flow cytometry. Transfection efficiency of the control plasmid was >50% where it was >30% for IL-37 transfected cells (fig 3.6A). Non-transfected RAW 267.4 cells were used as a negative staining control. RNA was also isolated from IL-37-transfected cells and control-transfected cells and reverse transcribed into cDNA. *IL37* gene expression was quantified using qPCR to confirm positive transfection. Up-regulation of *IL37* was seen in IL-37 transfected cells but not in control transfected cells (fig 3.6A). Once ectopic *IL37* expression was confirmed, cells were washed and re-plated in equal numbers on a 96-well plate. Control and IL-

37-transfected cells were stimulated with LPS (100ng/ml), Pam3CSK4 (500ng/ml) or MDP (10µg/ml) for 24 hours. Supernatants were harvested and cytokine levels were determined by ELISA. Cells over-expressing IL-37 secrete significantly less TNFα and IL-6 in response to TLR and NLR stimulation than control-transfected cells (fig 3.6B). This system has allowed us to confirm previous reports showing that over-expression of IL-37 leads to a reduction in pro-inflammatory cytokines in response to innate stimulation.

In an effort to determine which signaling pathways IL-37 influences to exert its immunosuppressive effects we have employed the over-expression of IL-37 in RAW 267.4 cells described above. NF-κB is a master-regulator transcription factor. Its activation leads to the up-regulation of pro-inflammatory cytokines. To investigate whether IL-37 exerts its immunosuppressive function by modulating NF-κB activity, RAW 267.4 cells that contained a secreted embryonic alkaline phosphatase reporter construct that is induced by NF-κB activation were used. Upon NF-κB activation an alkaline phosphatase is secreted into the supernatant. This can be quantified using a colourimetric assay whereby a substrate for the alkaline phosphatase is added to supernatants. In the presence of alkaline phosphatase the substrate turns from pink to blue and can be quantified using a spectrophotometer at 650nm. RAW cells were transfected with IL-37 plasmid or control plasmid. Cells were stimulated with LPS (100ng/ml), Pam3CSK4 (500ng/ml) or MDP (50µg/ml) for 6 hours and then supernatants were harvested for analysis. Over-expression of IL-37 reduces NFκB activity in response to LPS, Pam3CSk4 and MDP (fig 3.6C). These data demonstrate that IL-37 exerts its immunosuppressive effect through modulating NFκB activity.

Further investigation into what stage of the signaling pathway involved in NFκB activation that is targeted by IL-37 is required.

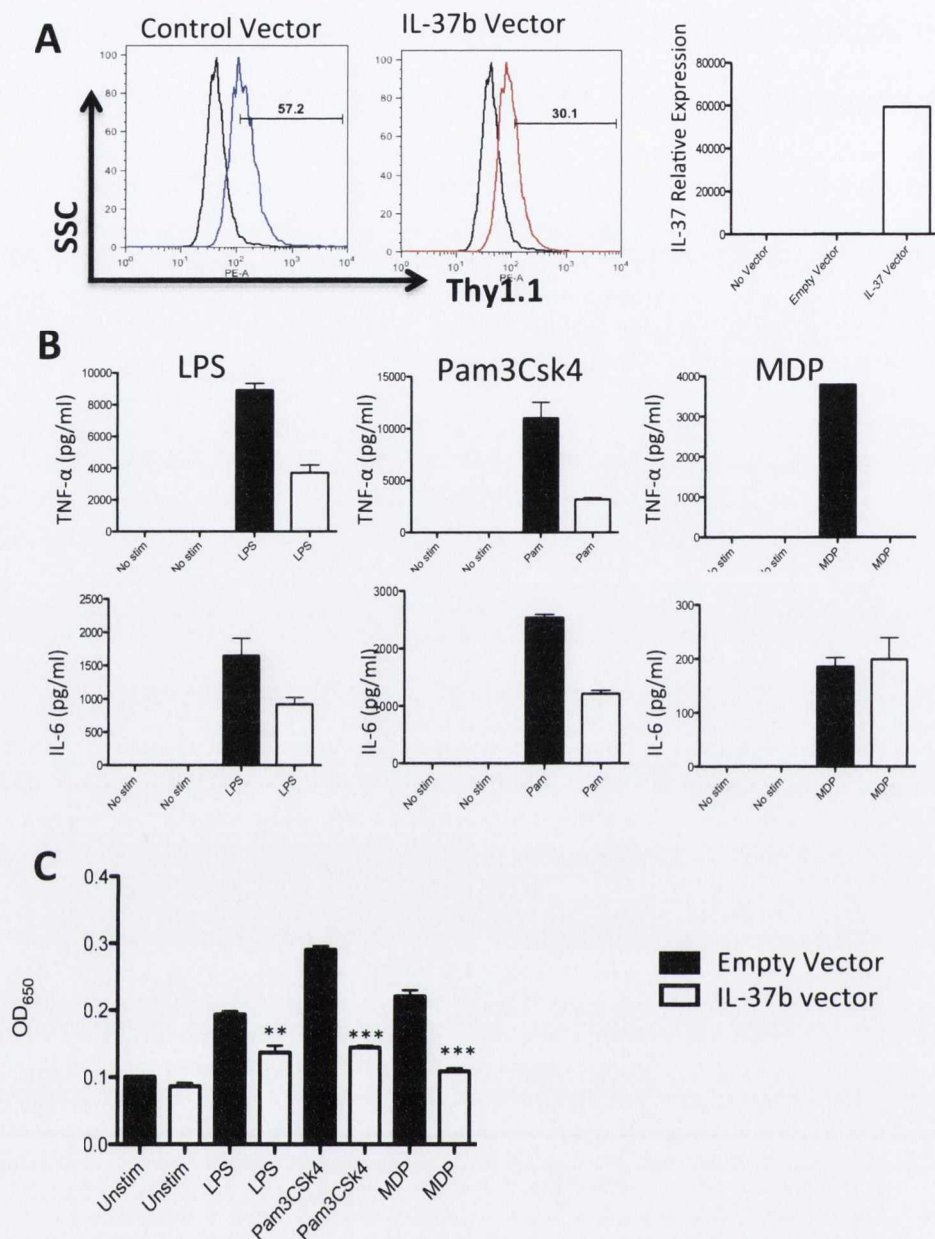


Figure 3.6 Over expression of IL-37 in RAW cells suppresses subsequent innate immune stimulation. RAW cells were transfected with IL-37-containing plasmid and a control plasmid. Presence of Thy1.1 indicates positive transfection. Transfection efficiency was measured by flow cytometry and by measuring IL-37 expression by qPCR (A). Transfected cells were stimulated with LPS (100ng/ml), Pam3CSK4 (500ng/ml) or MDP (10μg/ml). Cultured supernatants were collected at 24 hours and analyzed for cytokine production by ELISA (B) RAW cells contained a secreted embryonic alkaline phosphatase reporter construct that is induced by NFκB activation. Transfected cells were stimulated with LPS (100ng/ml), Pam3CSK4 (500ng/ml) or MDP (10μg/ml) for 6 hours. NFκB activity was quantified using a calorimetric assay whereby a substrate for the alkaline phosphatase is added to supernatants. Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

3.7 Expression of the novel IL-1 family member IL-36 α is significantly elevated in paediatric UC

We next sought to characterize the role of IL-36 in the context of IBD. It has been previously shown that IL-36 cytokines play a highly pathogenic role in various skin inflammatory conditions[104]. It is currently unknown whether IL-36 family members are expressed in the gastrointestinal tract and if so, whether they play a role in intestinal homeostasis. In an effort to characterize expression patterns of IL-36 members in IBD, rectal biopsies from control, UC and CD patients were analyzed by qPCR. Interestingly *IL36A* is significantly upregulated in the mucosa of UC patients compared to healthy controls. There is no significant difference in *IL36A* expression between CD and control patients (fig 3.7A). *IL36B* expression is completely undetected in the mucosa of healthy, UC and CD patients. Although *IL36G* is expressed in the gut mucosa, there is no significant difference between healthy and diseased groups (fig 3.7B). It has previously been shown that single point mutations in the naturally occurring IL-36RA lead to enhanced susceptibility to psoriasis indicating that it plays an important regulatory role in skin inflammation[105]. Interestingly, we have observed a reduced expression of *IL36RN* in the mucosa of UC patients compared to healthy controls (fig3.7C). For the first time we have demonstrated that IL-36 family members are expressed in the GI tract. Furthermore we have shown that *IL36A* is significantly elevated in UC and *IL36RA* levels are trending towards being lower. This suggests that an imbalance between IL-36 α /IL-36RA levels may play a role in gut homeostasis.

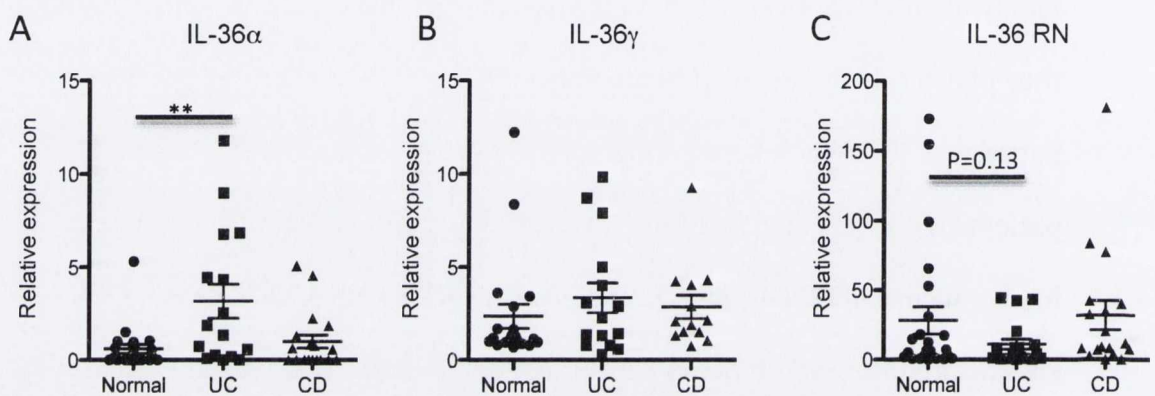


Figure 3.7. Expression of the novel IL-1 family member IL-36 α is significantly elevated in paediatric IBD. Rectal biopsies from a paediatric cohort were classified as normal, Ulcerative Colitis (UC) or Crohn's Disease (CD). RNA was extracted from biopsies and gene expression was measured by qPCR for *IL36A* (A), *IL36G* (B) and *IL36RN* (C). All genes were normalized to 18S RNA expression. Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Mann-Whitney test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

3.8 Expression patterns of IL-36 family members in various cell types

Having shown that IL-36 α expression is elevated in the mucosa of UC patients, we next sought to identify whether different cell types differentially express IL-36 α , IL-36 β and IL-36 γ , to potentially address which cell types might be responsible for the increased IL-36 α expression levels. Firstly total PBMCs were harvested from whole blood and stimulated with various TLR and NLR ligands. Interestingly we observed that TLR-4 stimulation and TLR-2 stimulation leads to induction of *IL36B* and *IL36G* whereas Nod2-stimulation leads to a modest induction of *IL36G* but no significant induction of *IL36B*. Furthermore, although *IL36A* expression was detected in PBMCs, TLR/NLR stimulation failed to upregulate its expression. In fact, it appeared to down regulate *IL36A* expression in PBMCs (fig 3.8A). We next sought to examine MACS purified CD14⁺ primary monocytes. Similar to total PBMCs, TLR2 and TLR4 and Nod2 stimulation leads to a strong induction of both *IL36B* and *IL36G*. However, no significant upregulation of these genes is observed in response to TLR3 or TLR9 stimulation. We have also shown that *IL36A* expression is not detected in human primary monocytes (fig 3.8B). It has been well described that T cells play a key role in the maintenance of intestinal homeostasis and that aberrant T cell responses are strongly implicated in IBD[140]. Interestingly, we have shown that naive CD4⁺ T cells express *IL36A* and that this expression is further enhanced upon T cell activation. *IL36B* expression is not detected in human CD4⁺ T cells. Although *IL36G* expression is detected in these cells, T cell stimulation does not enhance expression levels (fig 3.8C). We also tested the colonic epithelial cell line HCT116 for IL-36 expression. These cells are highly responsive to MDP stimulation (Nod2

agonist), in terms of IL-8 secretion. Neither *IL36A* nor *IL36B* expression could be detected in non-stimulated or MDP treated HCT116 cells. Although HCT116 cells do express *IL36G*, MDP treatment of these cells does not affect expression levels (fig 3.8D). Here we have shown that PBMCs and in particular CD14⁺ monocytes are a source of IL-36 β and IL-36 γ . Of the cell types that we have tested, only CD4⁺ T cells express IL-36 α , which is further upregulated upon T cell activation.

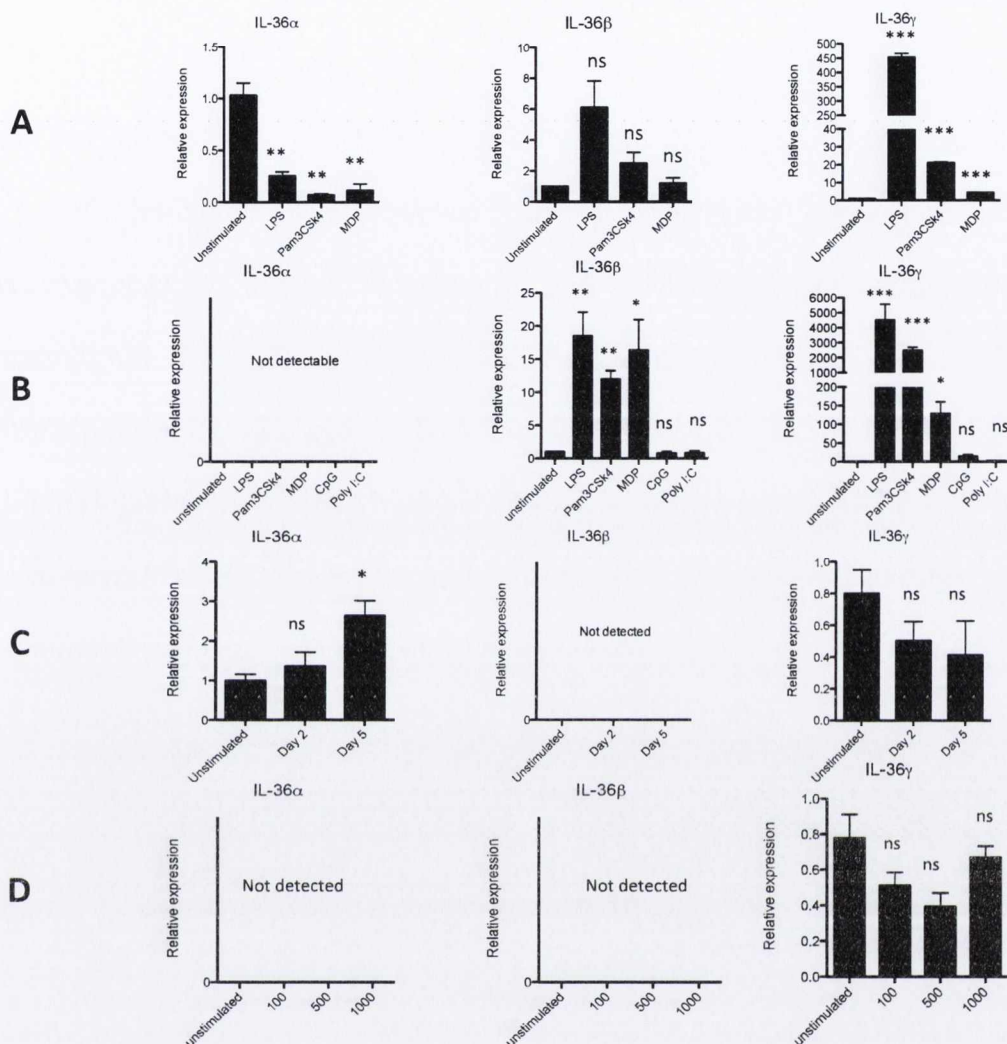
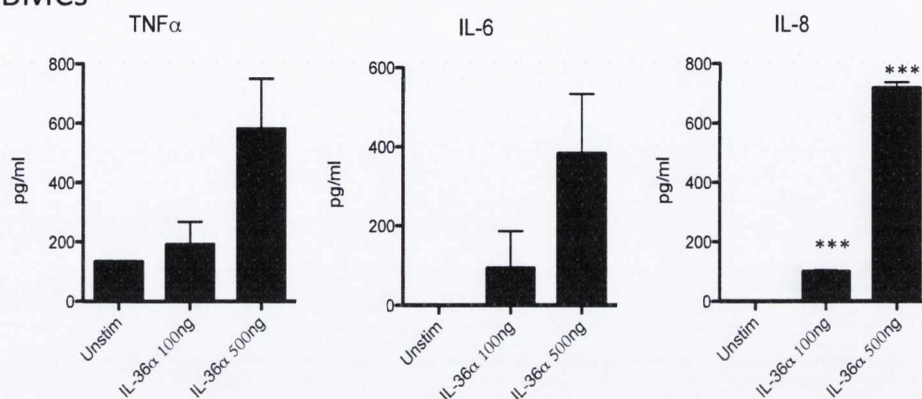


Figure 3.8. Expression patterns of IL-36 family members in various cell types. PBMCs were stimulated with LPS (100ng/ml), Pam3CSK4 (500ng/ml or MDP (1 μ g/ml) for 24 hours (A). CD14 $^{+}$ monocytes were stimulated with LPS (100ng/ml), Pam3CSK4 (500ng/ml), MDP (1 μ g/ml), CpG (5 μ g/ml) or Poly I:C (1 μ g/ml) for 24 hours (B). CD4 $^{+}$ T cells were activated with anti-CD3 (1 μ g/ml) and anti-CD28 (1 μ g/ml) for 48 and 120 hours (C) and HCT116 colonic epithelial cells were stimulated with various concentrations of MDP (100-1000ng/ml) for 24 hours. Once stimulation times were reached, cells were harvested and RNA was extracted. *IL36A*, *IL36B* and *IL36G* was measured using qPCR and made relative to 18S RNA expression. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

3.9 IL-36 stimulates innate inflammatory responses in PBMCs and human primary monocytes

In an effort to characterize how elevated IL-36 α levels in the gut mucosa may affect the inflammatory profile; various cell types were stimulated with recombinant IL-36 α '*in vitro*'. PBMCs were treated with IL-36 α (100ng/ml and 500ng/ml) for 18 hours and supernatants were harvested for ELISA. Treatment with IL-36 α induces significant amounts of IL-8 from both PBMCs and purified CD14 $^{+}$ monocytes in a dose-dependent manner. Although the result is not significant, TNF- α and IL-6 also appear to be induced in these cell types in response to IL-36. These data show that IL-36 can act as an innate immune stimulus enhancing inflammatory mediators from innate cellular sources.

A PBMCs



B CD14+ Monocytes

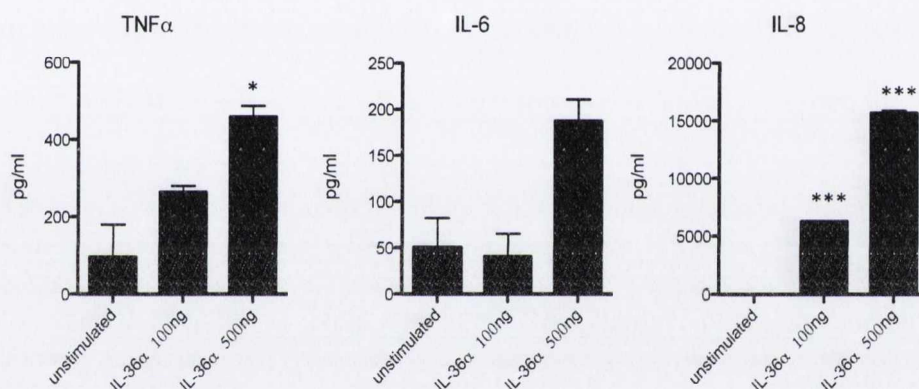


Figure 3.9. IL-36 stimulates innate inflammatory responses in PBMCs and human primary monocytes. PBMCs (A) and purified CD14+ monocytes were stimulated with rIL-36 α (100 or 500ng/ml) for 24 hours. Supernatants were harvested and cytokine release was determined by ELISA. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

3.10 IL-36 indirectly promotes Th1 responses '*in vitro*'

As well as acting as an innate immune stimulus, we also sought to investigate whether IL-36 can act directly on T cells to alter their function. It has been shown that IL-36 can act as a strong driver of a 'Th1' phenotype in murine cells[100]. However, its role in human T cell polarization remains ill defined. Naïve CD4⁺ T cells were purified from total PBMCs. CD4⁺ T cells were activated with anti-CD3 and anti-CD28 T cell activation antibodies (both 5µg/ml) and cultured in Th0 and Th1 (IL-12 50ng/ml) conditions in the presence and absence of rIL-36α (500ng/ml). Addition of IL-36α does not enhance IFN-γ levels from Th0 or Th1 conditions from purified CD4⁺ T cells (fig 3.10A). In contrast, when total PBMCs are stimulated with anti-CD3 and anti-CD28 activating antibodies in TH0 and Th1 conditions, addition of rIL-36α into these cultures enhances IFN-γ levels from both Th0 and Th1 cells (fig 3.10B). These data demonstrate that IL-36 can act to drive a Th1 cellular phenotype in an indirect fashion. Although CD4⁺ T cells do not respond directly to IL-36, the mixed population of PBMCs can respond to IL-36 and promote IFN-γ expression from CD4⁺ T cells.

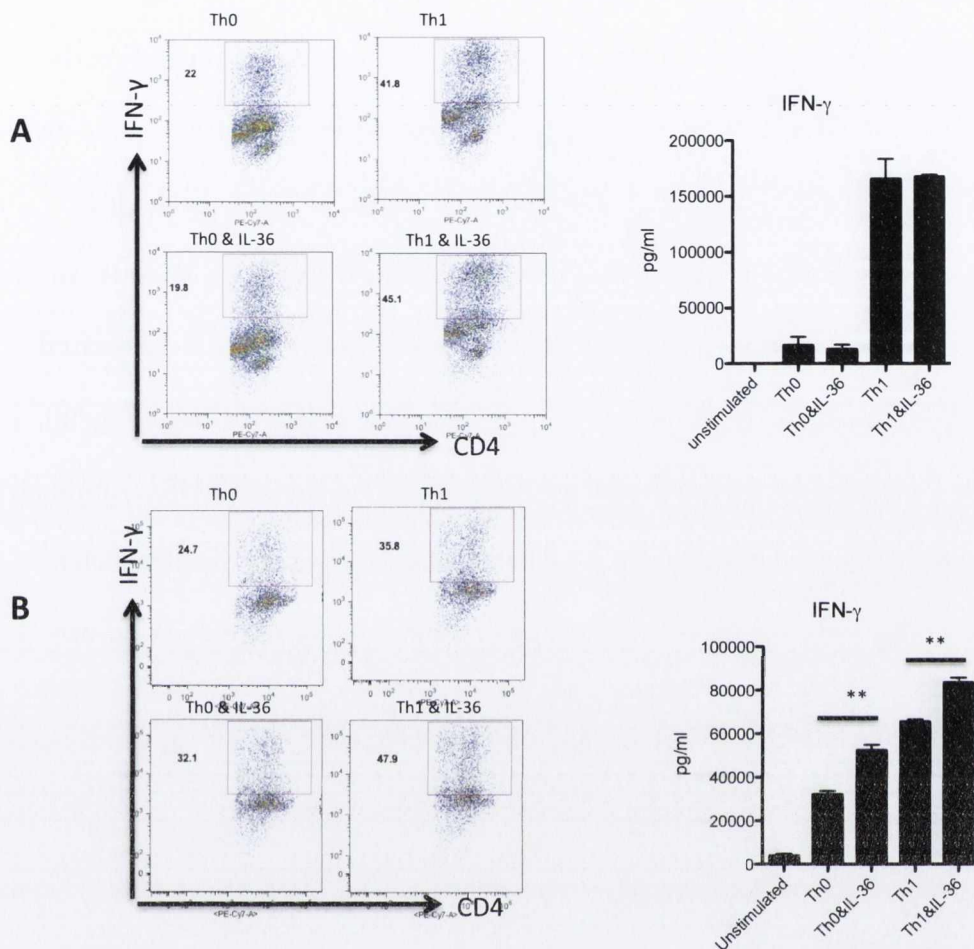


Figure 3.10. IL-36 indirectly promotes Th1 responses *'in vitro'*. Total PBMCs (A) or purified CD4⁺ T cells (B) were activated with anti-CD3 (5 μ g/ml) and anti-CD28 (5 μ g/ml) in Th0 or Th1 (IL-12 50ng/ml) promoting conditions in the presence and absence of recombinant IL-36 α (500ng/ml). After 120 hours of culture, supernatants were harvested for ELISA and cells were restimulated with PMA, Ionomycin and BrefeldinA and subsequently surface and intracellularly stained for flow cytometric analysis.

3.11 IL-36 does not appear to stimulate HCT116 colonic epithelial cells

Having shown that IL-36 can induce innate immune responses and indirectly enhance adaptive responses, we sought to investigate whether IL-36 could also act on non-hematopoietic cells. It is well documented that IL-36R is highly expressed on keratinocytes and that these cells are a target of elevated IL-36 levels in psoriatic patients[104]. HCT116 colonic epithelial cells were treated with rIL-36 α (100ng/ml, 500ng/ml, 1 μ g/ml) and MDP (100ng/ml, 500ng/ml, 1 μ g/ml) for 18 hours and then supernatants were harvested for ELISA. Although MDP induces secretion of IL-8 from HCT116 cells, IL-36 α fails to elicit such a response (fig 3.11). Further studies using primary colonic epithelial cells are required to fully elucidate whether IL-36 α can stimulate these cells.

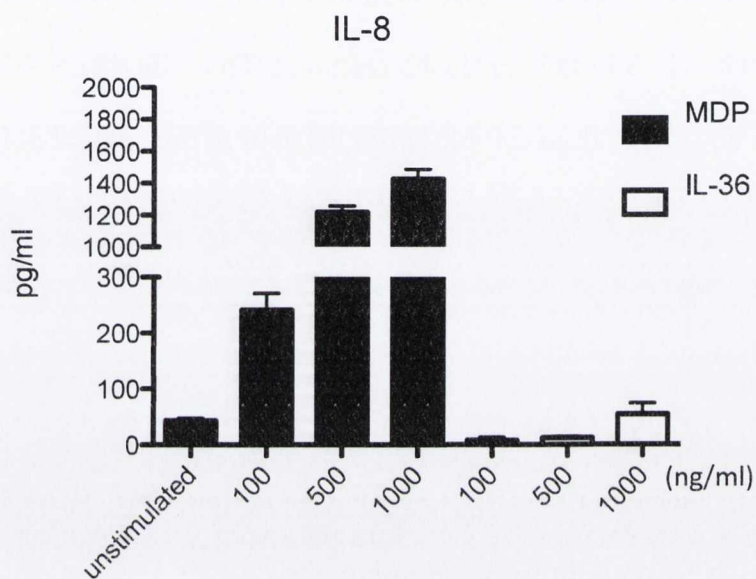
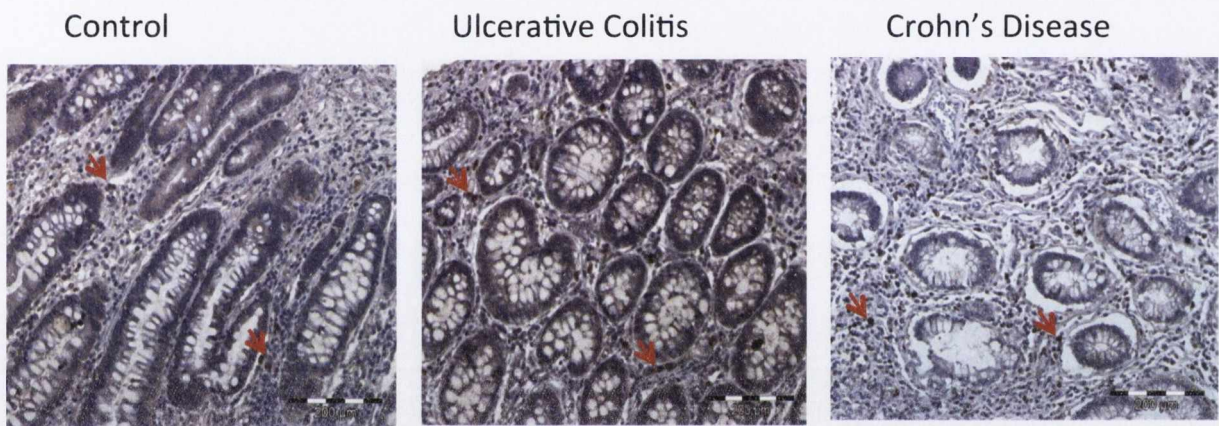


Figure 3.11. IL-36 does not appear to stimulate HCT116 colonic epithelial cells. HCT116 cells were treated with MDP (100-1000ng/ml) or rIL-36 α . After 24 hours supernatants were harvested and IL-8 levels were determined by ELISA.

3.12 IL-36R is expressed on infiltrating cells in the colonic mucosa

We have demonstrated using a variety of 'in vitro' assays that recombinant IL-36 α can directly stimulate PBMCs and specifically primary CD14⁺ monocytes. We have also shown that T cell polarization can be affected by the presence of IL-36 α in an indirect manner. However, stimulation of the colonic epithelial cell line HCT116 with rIL-36 α fails to elicit a response. To verify that IL-36 acts on immune cells rather than colonic epithelial cells in the context of IBD, we have taken biopsy samples from healthy, UC and CD patients and stained them for IL-36R. This analysis was carried out in collaboration with Dr. Beth Brint at the Dept. of Pathology in UCC. Interestingly, IL-36R staining was evident on infiltrating cells exclusively (fig 3.12). No positive staining was observed on the epithelium. This data is consistent with our previous data, highlighting IL-36 acting primarily on infiltrating immune cells.



(Beth Brint, UCC)

Figure 3.12. IL-36R is expressed on infiltrating cells in the colonic mucosa. Human colonic whole-section slides were obtained from the archives of Cork University Hospital. Sections were stained with an antibody against IL-36R and counterstained with haematoxylin. IL-36R is expressed on infiltrating cells rather than on the colonic epithelium. Magnification of 100X.

3.13 IL-36RA inhibits the effect of IL-36 *in vitro*

From our paediatric IBD cohort we have shown that expression levels of *IL36RN* are trending towards being lower in UC patients. To demonstrate how IL-36RA acts *in vitro*, CD14+ monocytes were pre-treated were treated with IL-36RA for 45 minutes and subsequently stimulated with IL-36 α for 18 hours. Pre-treatment with IL-36RA significantly reduces the ability of IL-36 α to induce an IL-8 from primary monocytes. Although the result is not significant, pre-treatment of CD14+ monocytes with IL-36RA reduces IL-36-induced IL-6 and TNF- α . These data suggest that the imbalance of IL-36 α and IL-36RA levels observed in the mucosa of UC patients may be resulting in an over-active IL-36R signaling pathway.

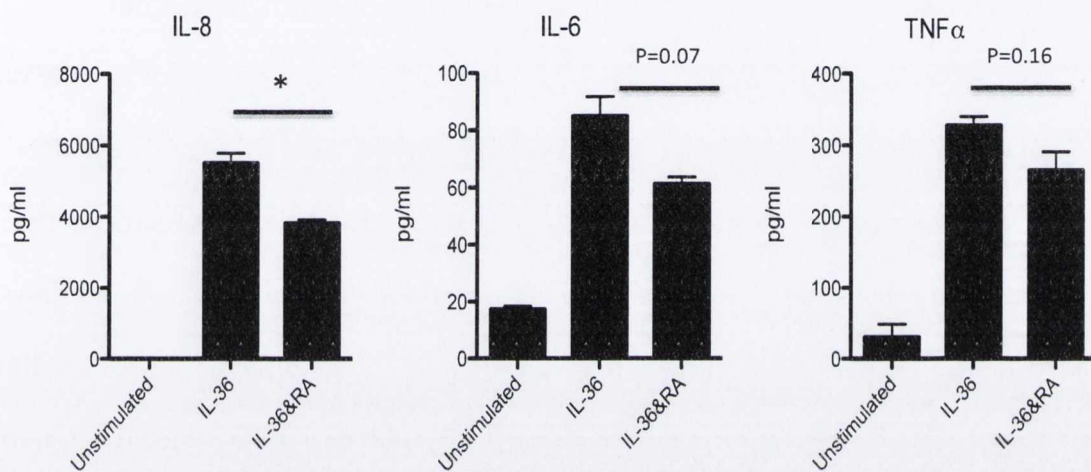


Figure 3.13. IL-36RA inhibits the effect of IL-36 'in vitro'. Purified CD14+ human monocytes were pre-treated with IL-36RA (500ng/ml) for 1 hour and subsequently stimulated with IL-36α (100ng/ml) for 24 hours. Levels of IL-8, IL-6 and TNF-α were determined by ELISA. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001

3.14 Activated CD4 T cells can act as a source of IL-36 that can subsequently activate CD14+ monocytes

We have previously demonstrated that activated T cells express IL-36 α . We have also shown that rIL-36 α strongly induces IL-8 from CD14 primary monocytes. To try and replicate an *in vitro* system to demonstrate the potential interplay between these cells we set up a system whereby the supernatants from unstimulated and activated T cells were used as a potential source of IL-36 α . We then used these supernatants to treat CD14+ purified cells that have been pre-treated with rIL-36RA (100 and 500 μ g/ml). Firstly, we have demonstrated that activated T cell supernatants induce significantly higher levels of IL-8 and IL-6 from CD14+ monocytes than inactive T cells (Fig 3.14). Importantly we can show that the induction of these pro-inflammatory cytokines can be reduced when the monocytes are pre-treated with IL-36RA. This suggests that IL-36 exists in the T cell supernatants and it directly plays a role in inducing IL-8 and IL-6 from primary monocytes.

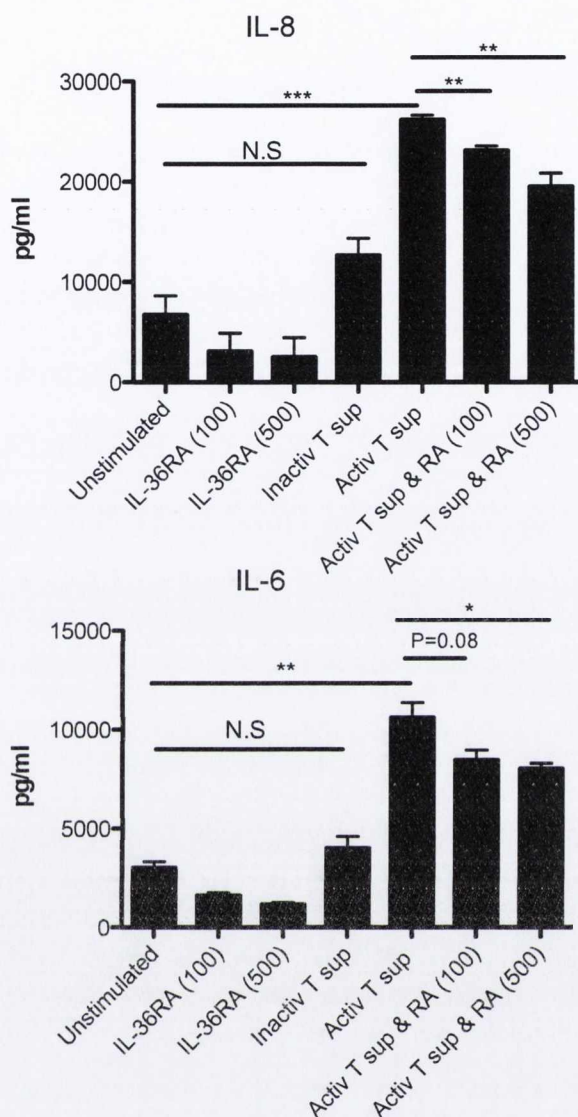


Figure 3.14. Activated CD4 T cells can act as a cellular source of IL-36 that can subsequently activate CD14+ monocytes. CD14+ monocytes were pre-treated with or without IL-36RA(100 or 500µg/ml) for 1 hour and then subsequently treated with inactive or activated CD4+ T cell supernatants. CD14+ monocytes were stimulated for 18 hours and then supernatants were harvested and analyzed by ELISA. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001, N.S non significant.

3.15 Chapter Discussion:

It is well established that classic IL-1 family cytokines, such as IL-1 and IL-18, are involved in the maintenance of normal gut homeostasis. Genetic knockout studies and neutralization studies have demonstrated dichotomous functions of these cytokines depending on the phase of disease. Although there have been many studies focusing on the role of these cytokines in IBD, to date, the roles of newer members of the IL-1 family, such as IL-33, IL-36 and IL-37, remain ill-defined. Recently, however, it has been demonstrated that IL-33 can play a protective role in terms of GI inflammation where it has been shown to play a key role in epithelial regeneration. Our study aimed to investigate the potential role of IL-36 and IL-37 in gastrointestinal inflammation and homeostasis.

We have characterized expression levels of novel IL-1 family member cytokines in the rectal mucosa of paediatric patients upon first diagnosis with disease. Through this analysis we have shown that *IL37* expression is reduced in the mucosa of IBD patients, particularly in CD patients. We have also investigated expression patterns of IL-36 family members, in the GI tract, for the first time. This study has identified *IL36A* gene expression as being significantly elevated in UC patients when compared to controls. Although *IL36G* expression is detected, no difference in expression levels between patients groups occurs. In contrast, *IL36B* gene expression was not detected in any of our samples, indicating that unlike the skin, this IL-36 family member is not prominently expressed in the colon. We have also attempted to do stain for IL-36 α protein on patient histology slides. However, antibody detection reagents for IL-36 family members are quite new and the results we obtained showed a lot of non-specific binding

(data not shown). With the development of improved protein detection for IL-36 family members, we could add to our gene expression data and further characterize the role of IL-36 in the context of IBD.

Further to identifying expression patterns of these cytokines in a human cohort of IBD, we sought to identify cellular functions of these novel mediators in human cellular assays. It has been well established that IL-37 acts as an inhibitor of innate immune signaling. However, treatment of PBMCs or purified CD4⁺ T cells with recombinant IL-37 prior to stimulation does not affect pro-inflammatory cytokine expression from these cells. We have however, been able to replicate previously published data, whereby over-expression of IL-37 in RAW cells leads to subsequent diminished responses to TLR and NLR stimulation[106]. Taken together, these data highlight that IL-37 can act as an immuno-suppressive cytokine, but it mainly functions intracellularly. However, more studies using recombinant IL-37 in different cellular assays are required to definitively rule out an extra-cellular role. Furthermore, we have highlighted that IL-37 expression is particularly reduced in the mucosa of CD patients. It is also well characterized that CD is a Th1 mediated disease[61]. Interestingly we have demonstrated that IL-37 expression is suppressed by the Th1 signature cytokine IFN- γ in both human epithelial cells and human primary monocytes. This highlights a potential explanation for the lower expression levels of IL-37 observed in these patients.

As we had identified that *IL36A* gene expression specifically was upregulated in UC patients, we sought to identify a potential cellular source of IL-36 α and also

investigate human cellular targets of IL-36 α . Interestingly we observed that although human primary monocytes express high levels of *IL36B* and *IL36G*, they do not express any *IL36A*. We have demonstrated that CD4+ T cells express *IL36A* and that its expression is induced further upon T cell activation. We have also shown that recombinant IL-36 can induce a response from PBMCs and purified monocytes, which can indirectly alter T cell immune responses. We failed to see any response in human colonic epithelial cells in response to stimulation with IL-36 α . This observation is backed up by our histological analysis in UC patients where we observe that IL-36R is highly expressed on infiltrating cells as opposed to the colonic epithelium. This is in contrast to the high levels of IL-36R expression observed on keratinocytes in the skin lesions of psoriatic patients.

Our analysis of a paediatric IBD cohort has revealed two major alterations in expression levels of novel IL-1 family members in the context of IBD, namely reduced levels of *IL37* in CD and elevated levels of *IL36A* in UC. Due to the fact that a mouse homolog of IL-36 exists and the availability of both murine and human reagents, we have chosen to pursue a study investigating the role of IL-36 in a murine model of colitis, which will be the focus for the remainder of the project.

Chapter 4:
**Using the DSS model of colitis to investigate the
role of IL-36 in gastrointestinal homeostasis**

Chapter 4: Using the DSS model of colitis to investigate the role of IL-36 in gastrointestinal homeostasis

Introduction:

In the previous chapter we have identified IL-36 as a potential new mediator of inflammation in IBD. We have shown that expression level of *IL36A* are elevated in paediatric UC and that *IL36A* can be expressed by T cells, which can act directly on primary monocytes to induce a plethora of innate inflammatory cytokines. Given the complex role of classic IL-1 family members in gastrointestinal homeostasis, we sought to utilize a murine model of colitis whereby we could study the specific role of the IL-36R pathway in the pathology of disease. There are a wide variety of commonly used murine models of colitis. We have chosen to use the Dextran Sodium Sulfate (DSS) model, as it is primarily an innate model of colitis.

Although IL-1 family members have been classically described as inflammatory mediators of inflammation, a dichotomous role for this cytokine family is now becoming apparent. Conflicting roles for IL-1 have been reported in animal models of IBD. In a rabbit model of disease, neutralization of IL-1 by the administration of IL-1RA appears to be protective in terms of disease severity[56]. However, the administration of IL-1 β also appears to have a beneficial role suggesting a potential role for IL-1 in gut homeostasis[78]. Conflicting roles for IL-18 have been demonstrated *in vivo* in mouse models of disease. Neutralization of IL-18 has been shown to ameliorate disease[83]. However IL-18-/- mice are more susceptible to DSS-induced colitis[84]. These

findings indicate that a pathogenic or protective role for IL-18 in IBD may depend on the phase of disease as well as the cellular source of IL-18. Although there are consistent findings to show that IL-36R pathway plays a highly pathogenic role in skin inflammatory disorders, its role in the context of IBD, if any, remains uncharacterized.

As IL-36 appears to act in a pro-inflammatory capacity, we also wish to investigate mechanisms by which IL-36R signaling may be regulated. Similar to IL-1, a naturally occurring receptor antagonist specific for the IL-36R exists. As we have observed lower expression of IL-36RA in human UC population, we sought to investigate the role of IL-36RA in the pathology of colitis. Another potential mechanism of regulation of the IL-36R signaling pathway is through the orphan receptor SIGIRR. It is well characterized that SIGIRR negatively regulates IL-1R and TLR signaling pathways[125]. It has also been shown that SIGIRR plays a role in the prevention of gastrointestinal inflammation[130]. However its role in the regulation of the IL-36R pathway is unknown. Therefore, we sought to investigate whether SIGIRR can in fact act as a negative regulator of IL-36R signaling.

4.1 Elevation of IL-36 cytokines during DSS-induced Colitis

Having shown that IL-36 is elevated in human UC and that it acts primarily as an innate immune stimulus in human *in vitro* assays, we decided to use the Dextran Sodium Sulfate (DSS) model of murine colitis to further investigate the role of IL-36 in gastrointestinal homeostasis. DSS acts by directly compromising epithelial integrity allowing subsequent stimulation of innate immune cells. We employed this model, as it is an innate model of colitis. To induce disease, mice are given 2% DSS in their drinking water. Daily recordings of weight loss, rectal bleeding and stool consistency are recorded to allow Disease Activity Index (DAI) to be calculated. To demonstrate how disease progresses in wildtype mice, a time course was run over a 6-day period, whereby 3 mice were sacrificed every day over the time course. Here we can demonstrate that mice lose weight over time and a significant shortening in colon length over time parallels this weight loss. This is characteristic of DSS induced colitis disease course. Levels of IL-6, CXCL1 and CXCL2 were measured from homogenized colon tissue by ELISA and made relative to total protein content, as determined by BCA assay. It is observed that as disease progresses, levels of these pro-inflammatory cytokines and chemokines increase (fig 4.1A). Importantly, we investigated whether IL-36 family members were elevated during DSS-induced colitis as is seen in human UC. Interestingly *il36a* and *il36g* are significantly elevated at day 6 during DSS colitis. In contrast, the levels of *il36b*, *il36rn* and *il36r* do not show any changes (fig 4.1B). These data demonstrate that as disease course progresses during DSS colitis, IL-36 cytokine expression is increased suggesting that IL-36 may play some role in disease pathogenesis. This also reflects elevated levels of *IL36A*

expression observed in UC patients and therefore makes this model a relevant model in which to study the role of the IL-36 family in modulating gastrointestinal inflammation.

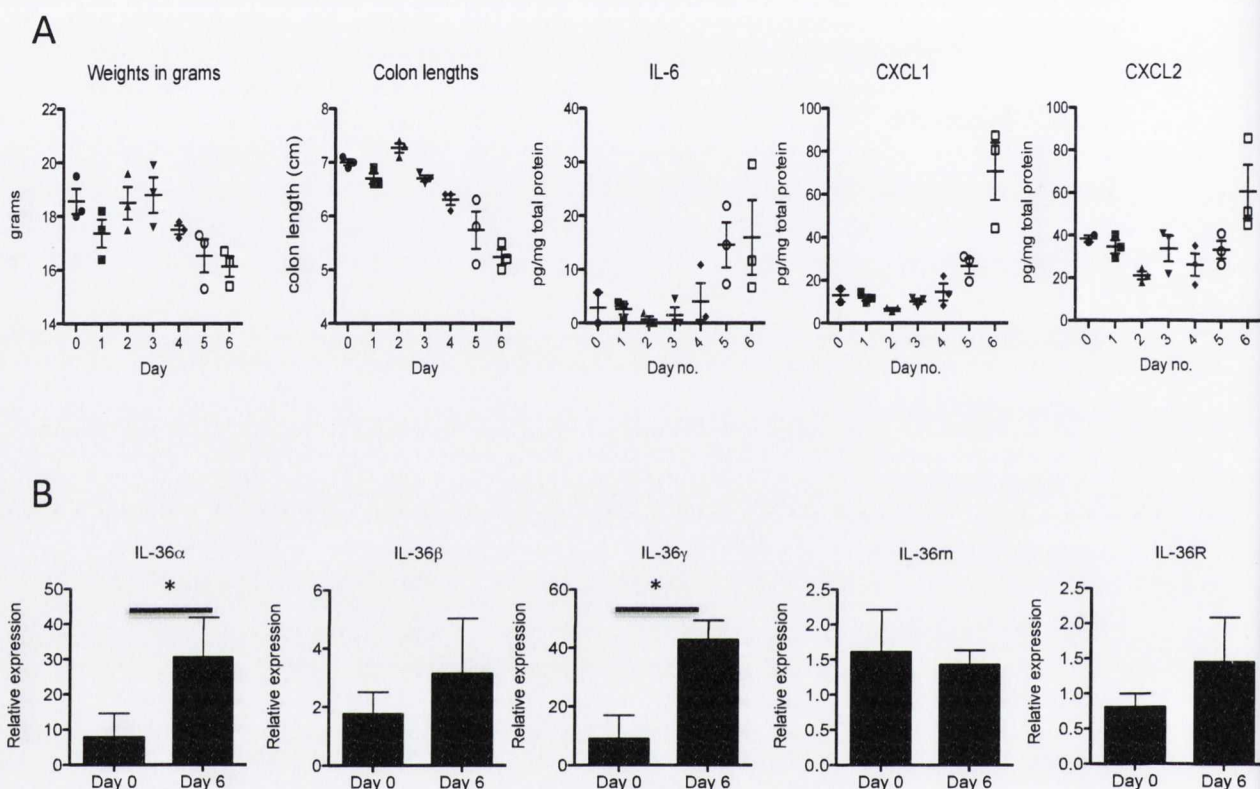


Figure 4.1 Elevation of IL-36 cytokines during DSS-induced colitis. C57Bl6 mice were subject to 2% DSS in their drinking water over a 6 day period. 3 mice were every day over the disease course. Weight and colon length was recorded and levels of IL-6, CXCL1 and CXCL2 from colon tissue homogenates were determined by ELISA and made relative to total protein content (A). RNA was extracted from colon tissue and expression levels of IL-36 family members was determined by qPCR (B). Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

4.2 IL-36 induces pro-inflammatory cytokines from murine BMDCs

To characterize murine cellular responses to IL-36 'in vitro', we firstly grew wild-type Bone Marrow-derived Dendritic Cells (BMDCs) and treated them with various concentrations of recombinant IL-36 α . Cells were cultured for 24 hours and then supernatants were analyzed by ELISA. IL-36 acts as an innate stimulus on BMDCs by driving elevation of TNF- α , IL-6 and also the chemokines CXCL1 and CXCL2 in a dose-dependent manner (fig 4.2).

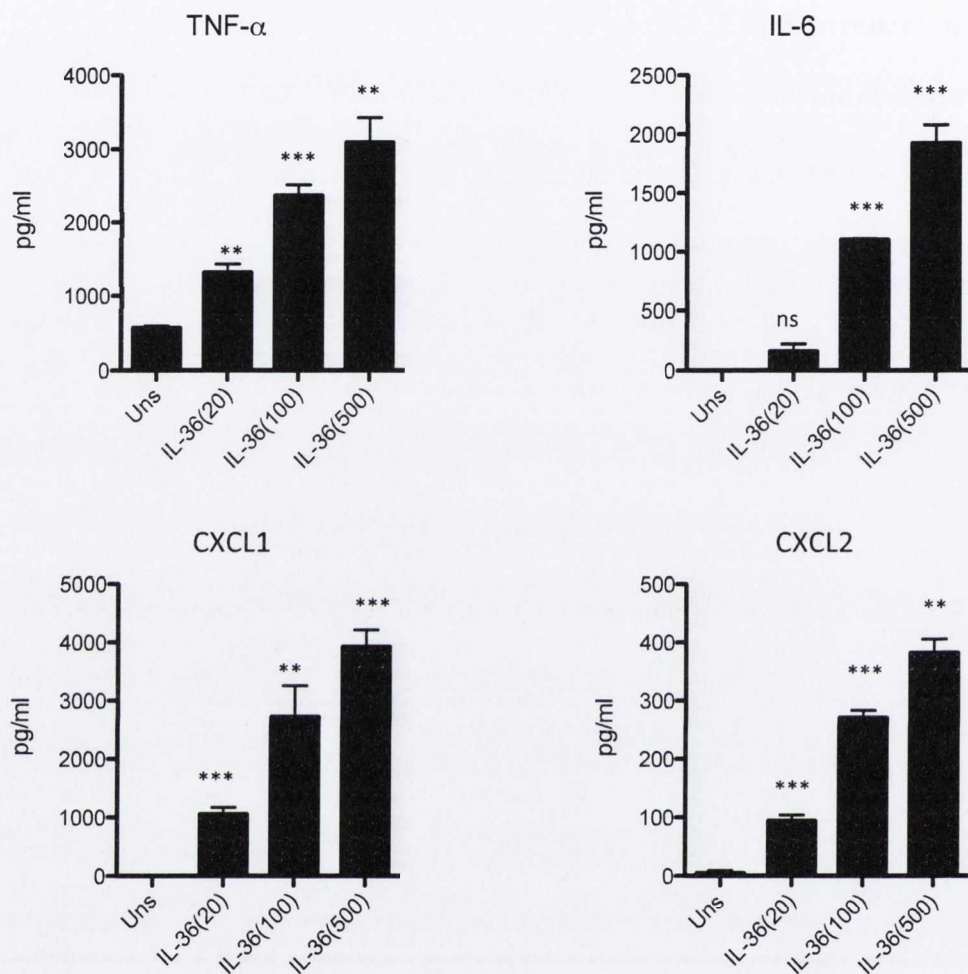


Figure 4.2 IL-36 induces pro-inflammatory cytokines from murine BMDCs. Murine BMDCs were treated with recombinant IL-36 (20, 100, 500ng/ml) for 24 hours. TNF- α , IL-6, CXCL1 and CXCL2 levels in the culture supernatants were then determined by ELISA. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

4.3 IL-36 can directly stimulate murine T cells

Although IL-36 does not appear to act directly on human T cells, it has been published that murine T cells do respond to IL-36 directly and that IL-36 can act as a Th1 polarizing cytokine and as a Th1 adjuvant *in vivo* in mice[100].

Therefore we differentiated MACS purified murine CD4⁺ T cells under Th0, Th1, Th17 and Treg murine cultures in the presence and absence of IL-36 α to address whether IL-36 plays a role in murine T cell polarization. By ELISA, we have demonstrated that IL-36 α enhances IL-2 responses from Th1 and TH17 conditions *in vitro* after 24 hours indicating that IL-36 can act as an early co-stimulus in the activation of mouse CD4⁺ T cells. Furthermore IL-36 enhanced IFN- γ from Th1 cultures and IL-17 from Th7 cultures after 72 hours.

Interestingly IL-36 also enhanced IL-22 from both Th1 and Th17 cultures (fig 4.3A). Expression of Foxp3 from inducible Tregs was inhibited by IL-36 in a dose dependent fashion (fig 4.3B). We also sought to address whether IL-36 can alter CD8⁺ cytotoxic T cell responses *in vitro* as well as CD4⁺ T cells. Interestingly, IL-36 enhanced IFN- γ expression from activated CD8⁺ T cells (fig 4.3C).

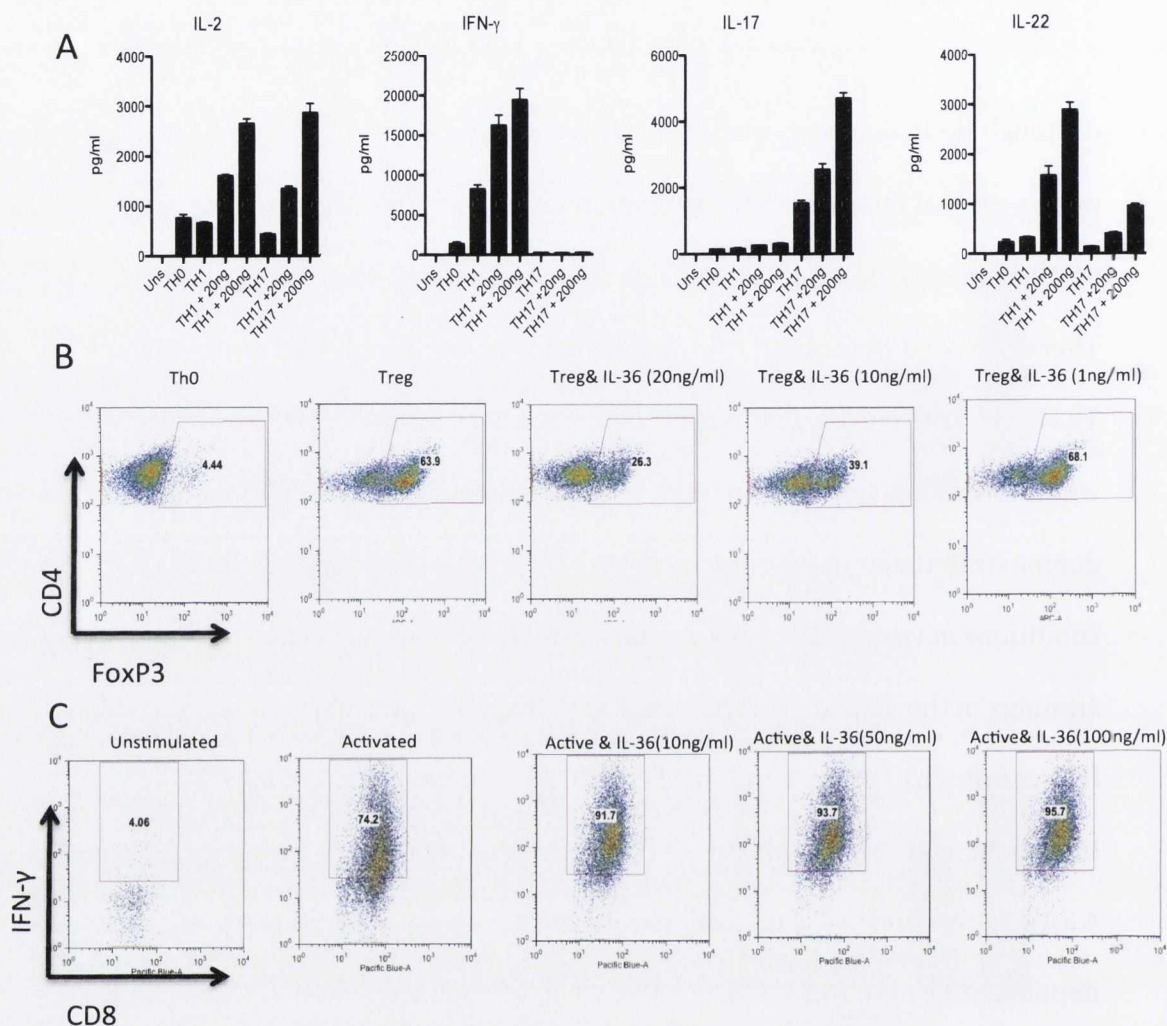


Figure 4.3 IL-36 can directly stimulate murine T cells. CD4⁺ T cells were MACS purified from splenocytes and activated with anti-CD3 (1 μ g/ml) and anti-CD28 (2 μ g/ml) T cell activation antibodies and cultured in the presence of the relevant Th0, Th1 or Th17 polarizing cytokines. Recombinant IL-36 (20, 200ng/ml) was added into the cultures and supernatants were harvested at 24 hours for IL-2 ELISA and 72 hours for IFN- γ , IL-17 and IL-22 ELISA (A). MACS purified CD4⁺ T cells were also activated and polarized towards a Treg phenotype in the presence of IL-36 (20, 10, 1ng/ml) for 72 hours. Cells were then fixed and permeabilized and Foxp3 levels were measured by flow cytometry (B). CD8⁺ T cells were MACS purified and activated with anti-CD3 (1 μ g/ml) and anti-CD28 (2.5 μ g/ml) T cell activation antibodies and cultured in the presence of rIL-36 (10, 50 and 100ng/ml) for 72 hours. Cells were then restimulated with PMA, Ionomycin and Brefeldin A for 6 hours before being fixed and permeabilized and stained for IFN- γ , subsequently measured by flow cytometry

4.4 IL-36 induces the neutrophilic chemokine CXCL1 from murine colon tissue 'ex vivo'

Having shown that IL-36 can act on purified primary murine dendritic cells and T cells *in vitro* to upregulate several pro-inflammatory cytokines, we next sought to investigate whether IL-36 could act directly on colon tissue 'ex vivo'. Colonic tissue was cultured in the presence and absence of recombinant IL-36 (100ng/ml) for 18 hours. Homogenized tissue samples and culture supernatants were both analyzed by ELISA and the results were made relative to total protein content in the tissue sample, as determined by BCA assay. CXCL1 (murine IL-8) levels were detected in unstimulated colon homogenates as well as the supernatants from these cultures. Stimulation with IL-36 α significantly enhanced CXCL1 expression detected in both supernatants and colonic tissue. Although TNF- α levels were detected in untreated colon samples, culture with IL-36 does not induce any significant increase in TNF- α levels from murine colonic tissue. IL-6 and IFN- γ levels were undetected from the colonic explant cultures with or without IL-36 α treatment (data not shown).

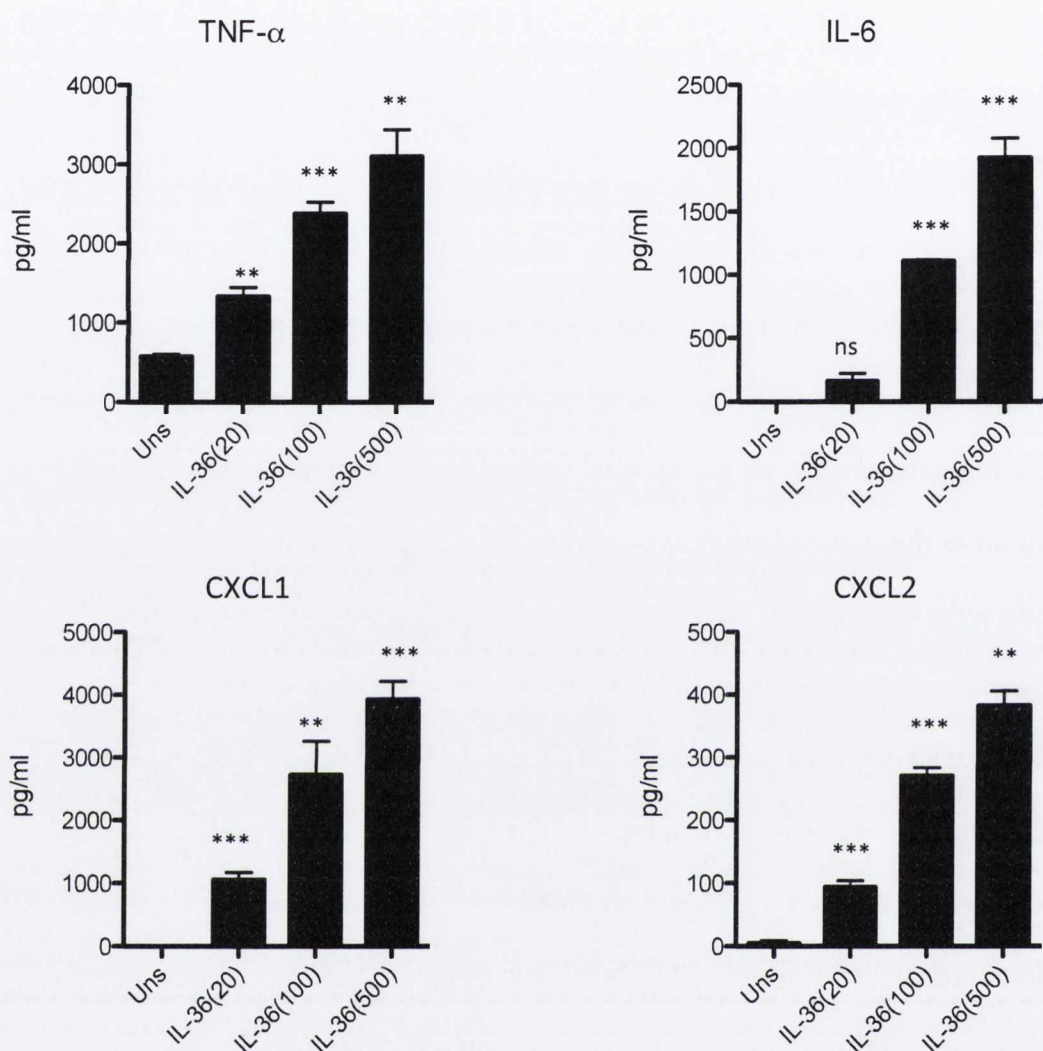


Figure 4.2 IL-36 induces pro-inflammatory cytokines from murine BMDCs. Murine BMDCs were treated with recombinant IL-36 (20, 100, 500ng/ml) for 24 hours. TNF- α , IL-6, CXCL1 and CXCL2 levels in the culture supernatants were then determined by ELISA. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001, ns (non-significant).

4.5 Intraperitoneal administration of rIL-36 leads to neutrophil influx

We have demonstrated that IL-36 α can act as a potent proinflammatory stimulus on mouse cells *in vitro*. In an effort to examine whether this is mirrored *in vivo* and potentially define a cellular mechanism through which IL-36 α promotes intestinal inflammation, we next examined whether IL-36 α could promote innate responses '*in vivo*' in the mouse. Intraperitoneal administration of recombinant IL-36 α (100ng) led to a significant infiltration of neutrophils to the peritoneal cavity. Neutrophils were characterized as CD45+CD11b+F4/80-Ly6G+ expressing cells. This increase was first apparent 1-hour post injection and by 6 hours there was a strong neutrophil infiltration. Levels of macrophages (CD45+CD11b+F480+) as well as T (CD45+CD3+) and B cells (CD45+B220+) were not altered (fig 4.5A). Importantly, this response was preceded by the induction of the chemokines CXCL1 and CXCL2 within 1 hour after injection (fig 4.5B). As we have found that IL-36 α induces CXCL1 expression in mouse colonic explant tissue, these data indicate that IL-36 α may also regulate neutrophil infiltration in the inflamed colons of mice.

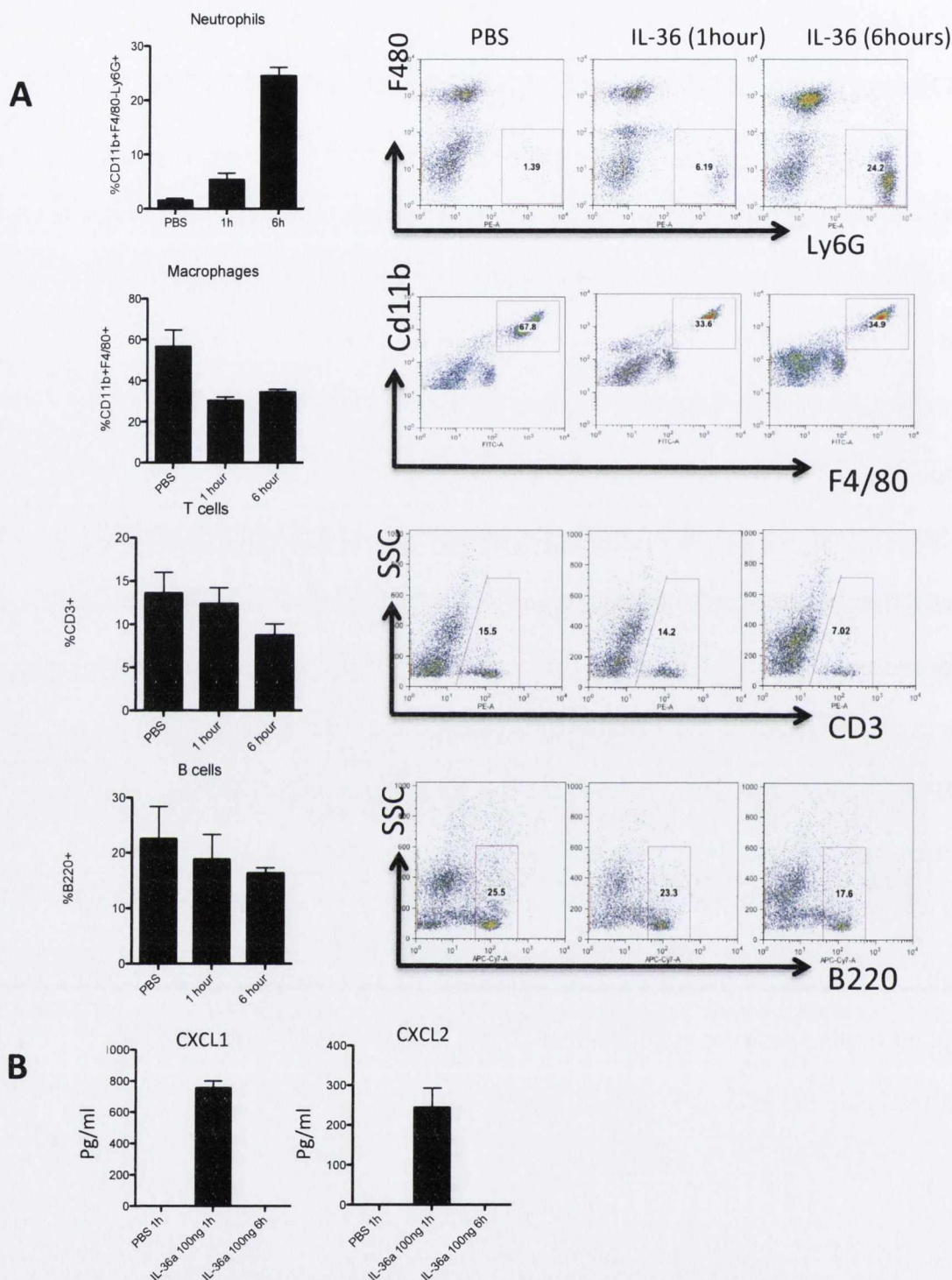


Figure 4.5 I.P administration of rIL-36 leads to neutrophil influx. Wildtype mice were injected I.P with 100ng rIL-36. After 1 hour and 6 hour timepoints, cells were harvested from the peritoneal lavage and spun down for flow cytometric analysis. Cells were surface stained for neutrophil, macrophage, T cell and B cells surface markers and percentage of cellular populations was determined (A). Levels of chemokines in the supernatants were analyzed by ELISA (B).

4.6 Neutrophils are recruited into the Lamina Propria during DSS colitis

During intestinal inflammation, resident monocytes can facilitate the recruitment of neutrophils through secretion of neutrophil attracting chemokines. The chemokine gradient that is created can attract neutrophils from the blood into the Lamina Propria. As our data above demonstrate that IL-36 α can promote neutrophil recruitment *in vivo* we sought to investigate whether neutrophil infiltration into the Lamina Propria is a feature of DSS-induced colitis. As described above, mice were treated with 2% DSS in their drinking water. On day 6 of disease the mice were sacrificed and the colons were put through various digestion steps to allow leukocyte isolation from the Lamina Propria for examination by flow cytometry. We have demonstrated, a significant recruitment of neutrophils into the colon tissue occurs during the DSS disease course at the same timepoint at which *il36a* expression is elevated (fig 4.6).

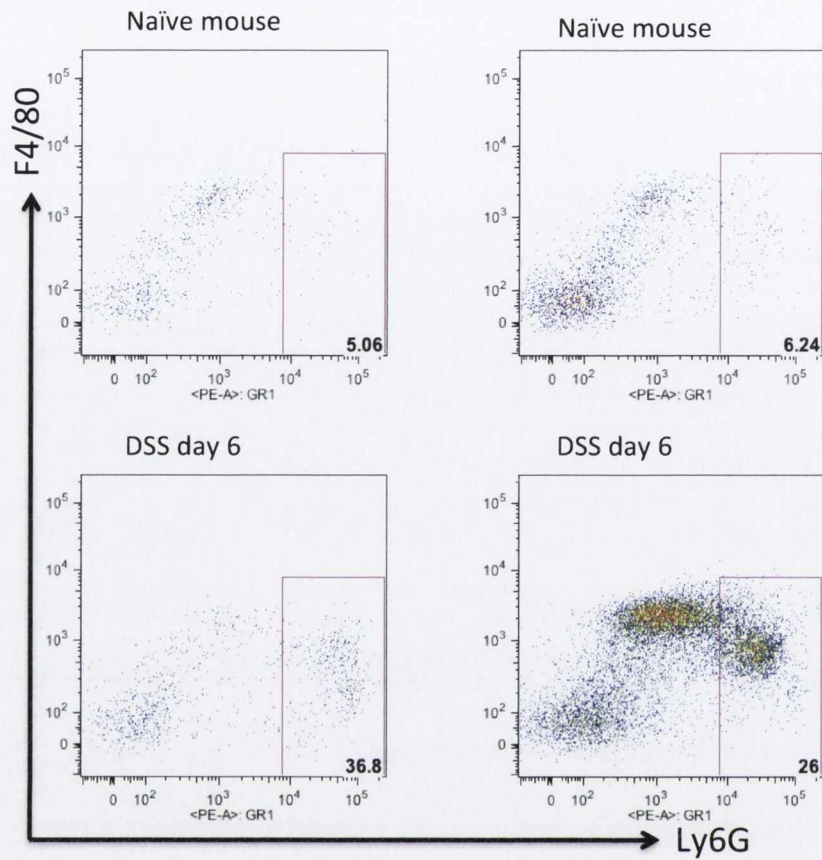
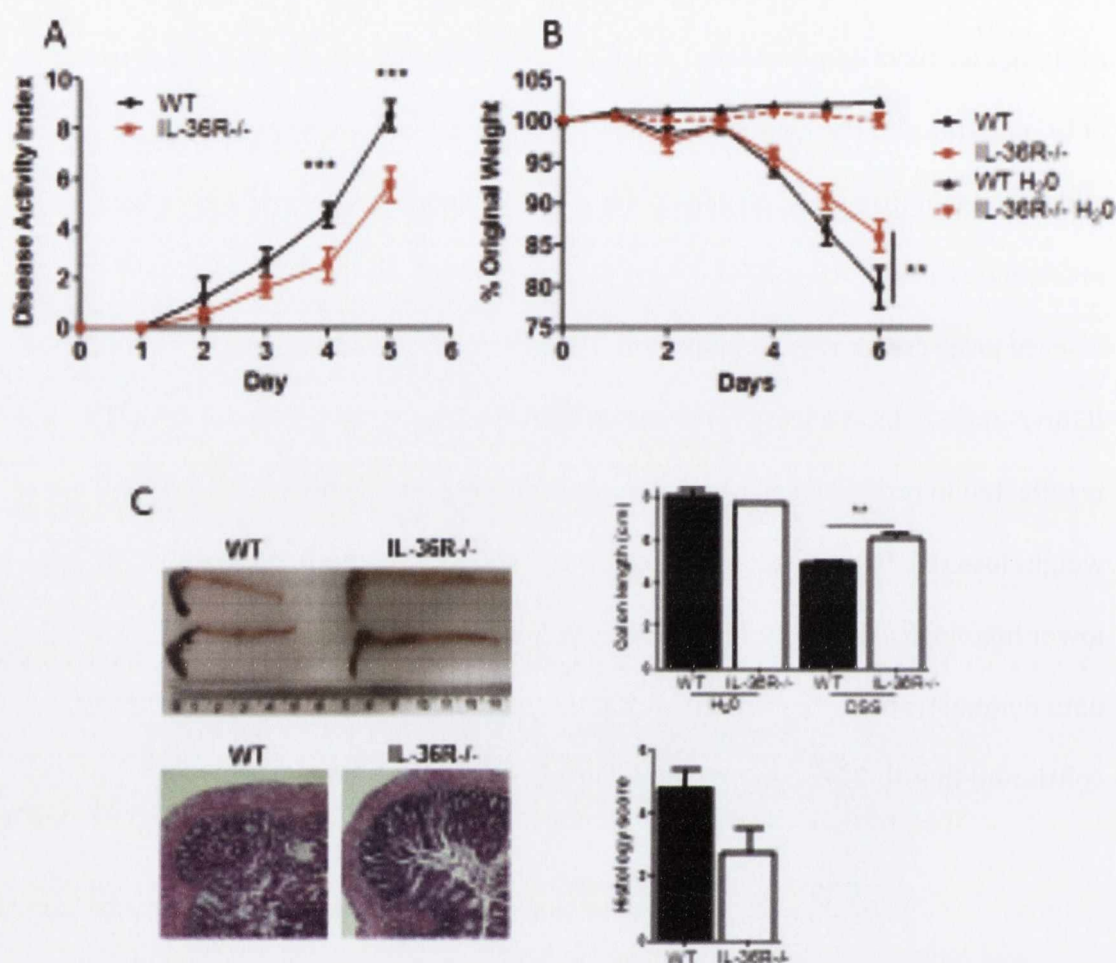


Figure 4.6 Neutrophils are recruited into the Lamina Propria during DSS colitis. Wild-type mice were subject to 2% DSS in their drinking water. On day 6 the mice were sacrificed and colons were harvested. Tissue was subject to a digestion process which allowed isolation of Lamina Propria cells. Cells were subsequently stained for neutrophil markers CD45, CD11b, F4/80 and Ly6G and analyzed by Flow cytometry.

4.7 *Il36r*^{-/-} mice show reduced susceptibility to DSS induced colitis

Although we have demonstrated that IL-36 expression is elevated in the mucosa of UC patients and the colonic tissue of DSS treated mice, we have not yet addressed whether IL-36 directly plays a role in the pathology of disease. To investigate this question, *Il36r*^{-/-} mice were treated with 2% DSS and their disease progression was compared to WT controls. We have demonstrated that *Il36r*^{-/-} mice exhibit a less severe disease course compared to control mice. This is reflected in terms of a significantly lower DAI (A) and a lower susceptibility to weight loss (B). *Il36r*^{-/-} mice show less shortening in their colon length and a lower histological score compared with WT controls (C). Taken together, these data demonstrate for the first time that IL-36 cytokines are elevated during DSS colitis and that IL-36R signaling plays a pathogenic role in disease.

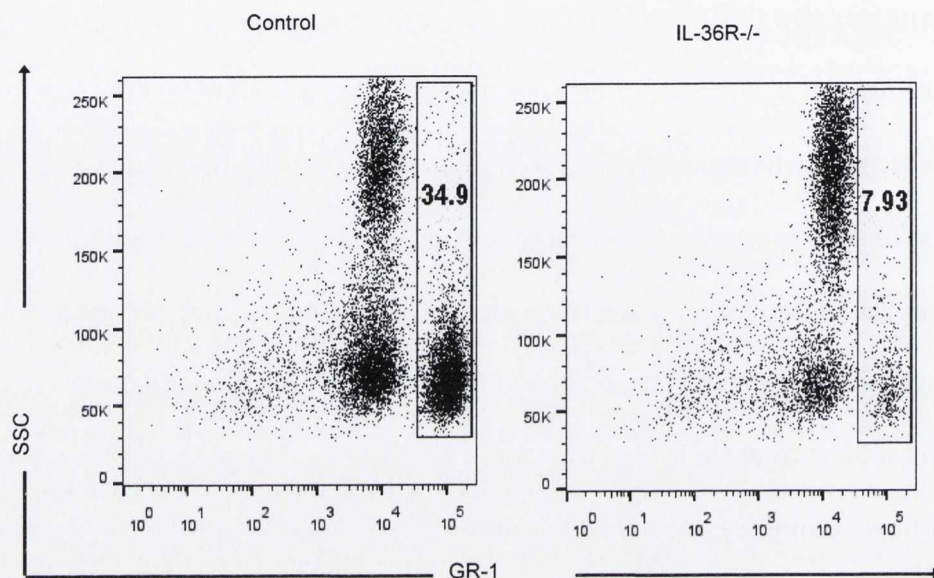


(Exp carried out with Shane Russell)

4.7 IL-36R^{-/-} show reduced susceptibility to DSS colitis. WT and IL-36R^{-/-} were subject to 2% DSS in their drinking water and disease course was monitored over time (n=6 mice per group). Disease activity index was measured taking into account percentage weight loss, rectal bleeding and stool consistency (A). Weight loss was measured daily (B) and colon length and histological analysis was measured on the final day of the experiment (C). Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Student's unpaired t-test and one-way ANOVA. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

4.8 Reduced neutrophil influx in the Lamina Propria of *il36r*^{-/-} mice during DSS colitis

We have previously demonstrated that there is a large neutrophil influx into the Lamina Propria in WT mice during DSS colitis. Separately, we have also demonstrated that I.P administration of rIL-36 α could lead to neutrophil recruitment *in vivo*. Based on these two separate experiments we wanted to investigate whether increased IL-36R signaling during DSS colitis was responsible for increased numbers of neutrophils observed in the Lamina Propria. WT and *il36r*^{-/-} were treated with 2% DSS in their drinking water and 6 days later mice were sacrificed and colon tissue was harvested. The colon tissue was subject to various digestion steps to isolate leukocytes. Cells were analyzed by flow cytometry. Fig 4.8 demonstrates that in the absence of IL-36R, there is less recruitment of neutrophils into the Lamina Propria during DSS. These data, carried out in collaboration with Dr. Shane Russell, highlight a novel role of IL-36 as a pathogenic cytokine involved in neutrophil recruitment in the gut.



(Shane Russell)

Figure 4.8 Reduced neutrophil influx in the Lamina Propria of IL-36R^{-/-} mice during DSS colitis. Wild-type and IL-36R^{-/-} mice were subject to 2% DSS in their drinking water. On day 6 the mice were sacrificed and colons were harvested. Tissue was subject to a digestion process which allowed isolation of Lamina Propria cells. Cells were subsequently stained for neutrophil markers CD45, CD11b, F4/80 and Ly6G and analyzed by Flow cytometry.

4.9 *Sigirr*^{-/-} mice show enhanced susceptibility to DSS induced colitis

SIGIRR has been demonstrated to act as a negative regulator of TLR/IL-1R signalling[125]. To establish the role of SIGIRR in a murine model of colitis, DSS (1.5% wt/vol) was administered in drinking water *ad libitum* for 7 days, before being returned to normal water for the remainder of the disease course. *Sigirr*^{-/-} mice display a more severe disease phenotype than wild type mice in response to DSS-induced colitis. This can be seen in terms of percentage weight loss where *sigirr*^{-/-} mice lose significantly more weight than WT mice. Healthy wildtype and *sigirr*^{-/-} mice that are not subject to DSS treatment show no differences in colon length. However, during DSS-treatment *sigirr*^{-/-} mice show a more severe shortening of the colon than WT mice. *Sigirr*^{-/-} mice display a higher DAI score than WT mice during DSS-induced colitis (fig 4.9). These data confirm previous results demonstrating the important protective role that SIGIRR activity plays in regulating disease severity in murine models of Inflammatory Bowel Disease[130].

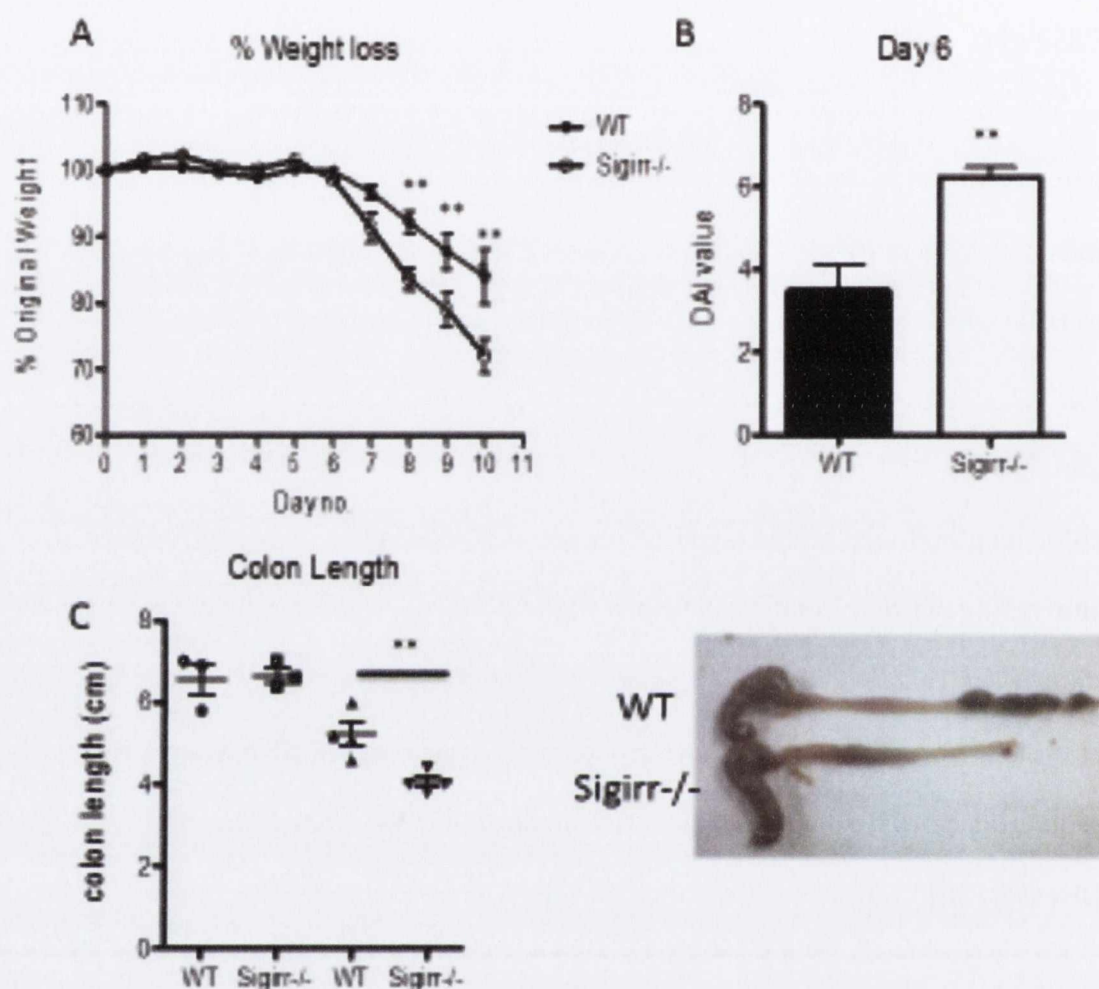


Figure 4.9 Sigirr^{-/-} mice show enhanced susceptibility to DSS induced colitis. Mice were treated with 1.5% DSS for 7 days then returned to normal drinking water. Percentage weight lost was monitored daily (A). Disease activity index was measured on day 6 of disease taking into account percentage weight loss, rectal bleeding and stool consistency (B). Mice were sacrificed on day 6 of disease and colon length was measured (C). Data is represented as mean values, with error bars representing standard error of the mean (n=3 mice per group). Statistics were calculated using the Student's unpaired t-test and one-way ANOVA. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

4.10 Elevated T cell cytokine expression observed in the colons of *sigirr*^{-/-} mice during colitis

SIGIRR has been shown to act as a negative regulator of IL-1 signaling[125]. As IL-1 has been shown to play a role in T cell polarization, we investigated whether SIGIRR modulates T cell associated cytokines in the context of DSS-induced colitis. DSS (1.5% wt/vol) was administered in drinking water ad libitum and mice were sacrificed after 6 days of treatment with DSS. Colonic tissue was taken from *sigirr*^{-/-} and WT mice for cytokine analysis. Cytokine levels were detected by ELISA and normalized per mg of total protein. IL-22, IL-17 and IFN- γ were found to be elevated in the colons of *sigirr*^{-/-} mice suggesting that these proinflammatory cytokines may play a role in enhanced disease severity (fig 4.10A). RNA was also extracted from colonic tissue and cytokine gene expression levels were detected by qPCR. Similar to the protein data described above *il22* gene expression levels are also elevated in *sigirr*^{-/-} mice. However, there was no difference in the gene expression levels of *ifng* between WT and *sigirr*^{-/-} mice and *il17a* gene expression was not detected. IL-1 β and IL-23 are innate cytokines that have been shown to play important roles in IL-17A production. *Il1b* and *il23* gene expression are the same in WT and *sigirr*^{-/-} colon tissue on day 6 of disease after administration of DSS suggesting that the differences observed in IL-17A protein may not occur as a consequence of elevated IL-1 β and IL-23 in the absence of SIGIRR expression.

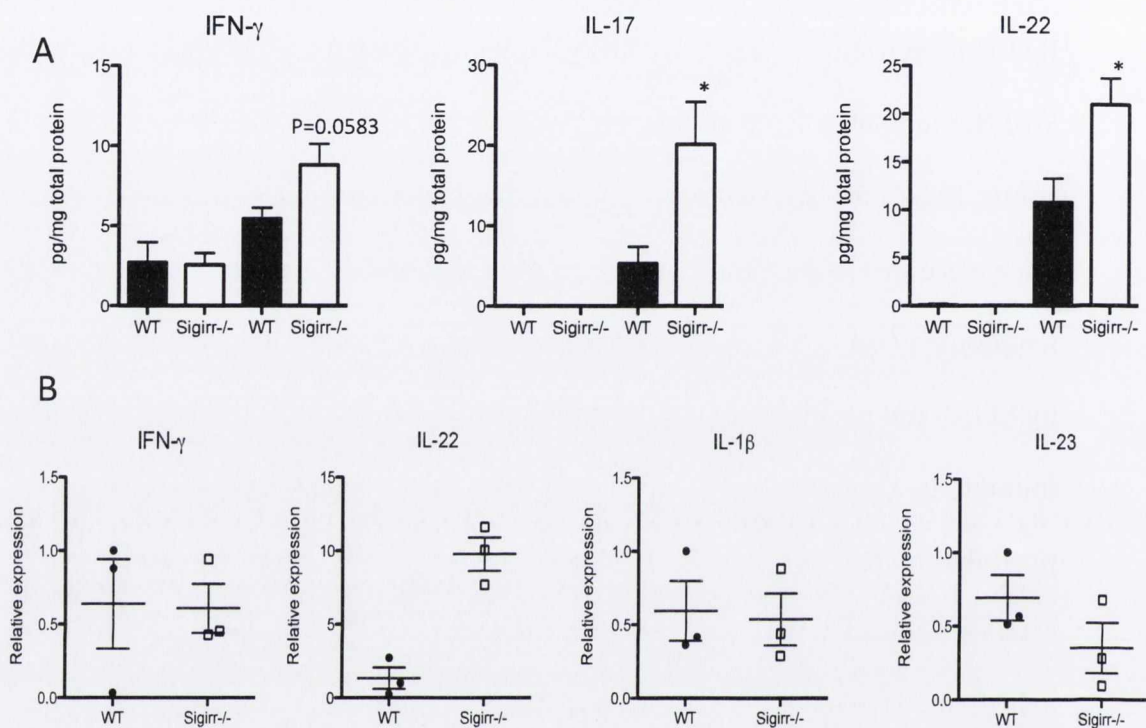


Figure 4.10 Elevated T cell cytokine expression observed in the colons of sigirr^{-/-} mice during colitis. Colon tissue was isolated from SIGIRR^{-/-} and WT mice on day 6 of disease. Protein homogenates were made and IL-22, IL-17A and IFN- γ levels were detected by ELISA and made relative to total protein content (A). RNA analysis was also carried out on colon tissue and expression levels of *ifng*, *il22*, *il1b* and *il23* was measured by qPCR and normalized to 18S RNA expression. Data is represented as mean values, with error bars representing standard error of the mean. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

4.11 *Sigirr*^{-/-} BMDCs and splenocytes secrete higher levels of pro-inflammatory cytokines in response to IL-36

Although it has been demonstrated that SIGIRR can act as a negative regulator of IL-1/TLR signaling, it has not been investigated whether SIGIRR also regulated the IL-36 signaling pathway. To address this question WT and *sigirr*^{-/-} BMDCs were treated with rIL-36 and LPS as a positive control. *Sigirr*^{-/-} BMDCs secreted significantly enhanced levels of IL-6, CXCL1 and CXCL2 in response to IL-36 than WT cells (fig 4.11A). Similarly, WT and *sigirr*^{-/-} splenocytes were cultured with rIL-36 and LPS as a control. Increased levels of CXCL1 and CXCL2 are induced from *sigirr*^{-/-} splenocytes than WT splenocytes (fig 4.11B). These data demonstrate that the presence of SIGIRR acts to limit IL-36R signaling *in vitro* and in the absence of SIGIRR, IL-36R signaling is enhanced.

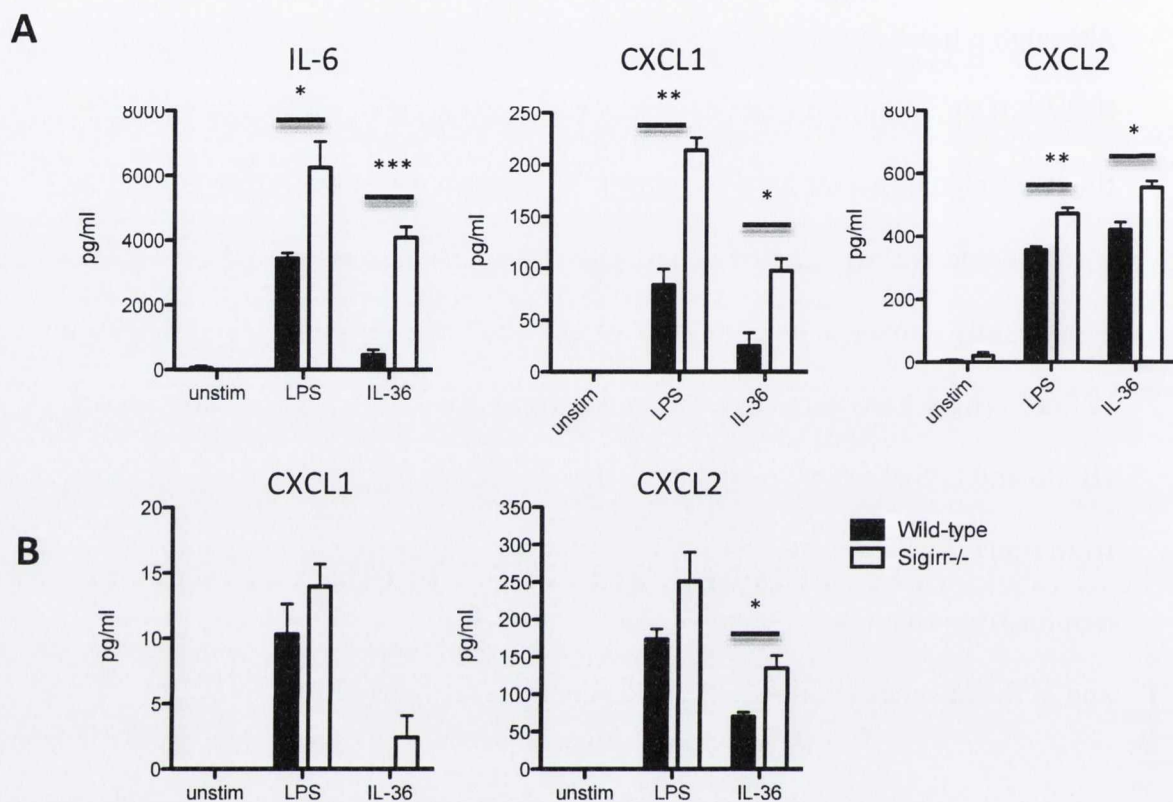


Figure 4.11 Sigirr^{-/-} BMDCs and splenocytes secrete higher levels of pro-inflammatory cytokines in response to IL-36. WT and sigirr^{-/-} BMDCs (A) and splenocytes (B) were plated at equal numbers and treated with LPS (100ng/ml) or rIL-36 α for 18 hours. Supernatants were harvested and IL-6, CXCL1 and CXCL2 levels were determined by ELISA. Statistics were calculated using the Student's unpaired t-test. P values of less than 0.05 were considered significant. *P<0.01, **P<0.01, ***P<0.001.

4.12 Intraperitoneal administration of rIL-36 leads to enhanced neutrophil influx in *sigirr*^{-/-} mice compared to wildtype controls

To investigate whether our observation that SIGIRR regulates IL-36 signaling *in vitro* holds true in an *in vivo* system, we used the I.P injection model described above whereby WT and *sigirr*^{-/-} mice are administered rIL-36 α (100ng/ml) into the intraperitoneal cavity. As seen previously, IL-36 drives an influx of neutrophils into the peritoneal cavity. However, we can now show that the recruitment of neutrophils is greatly enhanced in *sigirr*^{-/-} mice (fig 4.12). This demonstrates that IL-36 signaling *in vivo* is regulated by the orphan receptor SIGIRR and may suggest that enhanced responsiveness to IL-36 signaling may play a role in the increased susceptibility towards disease in *sigirr*^{-/-} mice observed in Fig4.9.

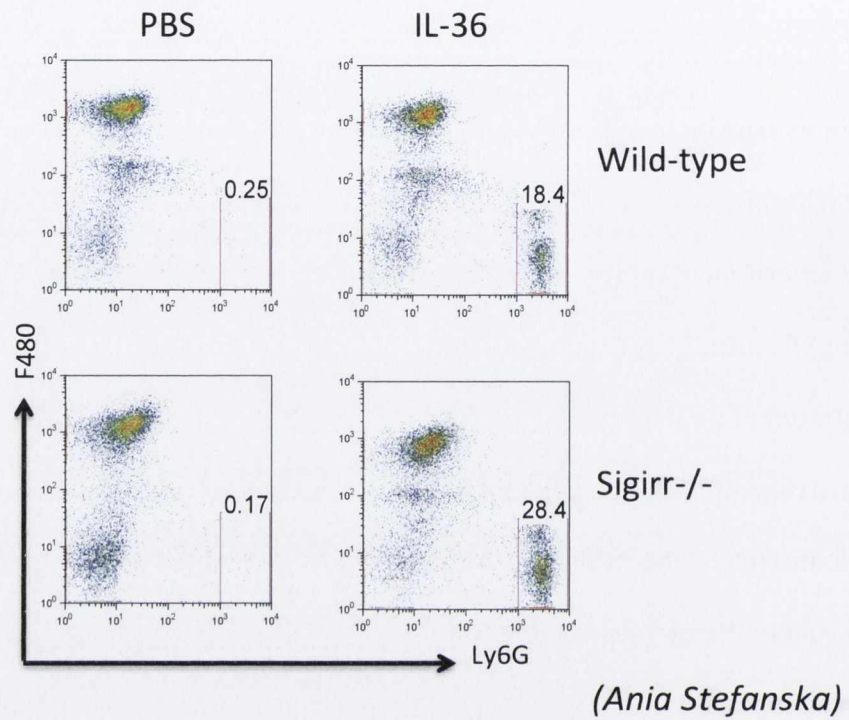


Figure 4.12 I.P administration of rIL-36 leads to enhanced neutrophil influx in sigirr-/- mice compared to wildtype controls. WT and Sigirr-/- mice were injected I.P with 100ng of rIL-36. 4 hours later cells were harvested by peritoneal lavage. Cells were washed and then surface stained with CD45, CD11b, F4/80 and Ly6G. Neutrophils were gated as CD45+CD11b+F4/80-Ly6G+.

4.13 Chapter Discussion:

IBD is a chronic, relapsing inflammatory disorder of the GI tract. In recent years it has become clear that a combination of genetic susceptibility factors and certain environmental triggers are involved in disease progression. A combination of these factors can ultimately lead to disturbance of the epithelial barrier and subsequent translocation of bacteria into the gut lumen. This in turn can lead to aberrant innate immune signaling resulting in excessive inflammatory cytokine responses. Cytokines involved in disease pathogenesis represent popular therapeutic targets for IBD. This is highlighted by the fact that TNF- α neutralization therapy is currently used in the clinic. However, there are many side effects of TNF therapy and newer, relevant targets are always being sought. In this chapter, we have used a murine model of colitis to demonstrate that IL-36 plays a pathogenic role in terms of gastrointestinal inflammation and could potentially be a novel target in terms of IBD therapy.

We have used the DSS model of colitis to investigate the role of IL-36 in murine colitis and to unravel a mechanism of action to complement our findings in our human study whereby we demonstrate that *IL36A* expression is elevated in human UC. Firstly, we have shown that expression of IL-36 members are increased in the colons of DSS treated mice over time. Crucially we have shown, using the *il36r*^{-/-} mouse, that IL-36 plays a pathogenic role in disease as *il36r*^{-/-} display a less severe phenotype than WT mice. Subsequent to these observations we have shown that IL-36 could directly induce the neutrophil recruiting chemokine CXCL1, from murine colon tissue. Furthermore, I.P administration of recombinant IL-36, leads to a rapid elevation of CXCL1 and subsequent influx of

neutrophils into the peritoneal cavity of these mice. This demonstrates the capacity for IL-36 to act as a driver of neutrophil infiltration 'in vivo'. To tie these results together we have also demonstrated that during the course of DSS colitis, *il36r*^{-/-} mice have lower numbers of neutrophils in the Lamina Propria than WT mice, indicating a potential mechanism of action of IL-36 in the context of murine colitis. Our data suggests that as a result of the tissue damage and destruction caused by DSS in the gastrointestinal tract, IL-36 expression levels are elevated which drives a large influx of neutrophils into the colonic tissue. This results in increased Disease activity index and percentage weight loss than is observed in *il36r*^{-/-} mice highlighting that the IL-36R signaling pathway plays a pathogenic role in DSS colitis. To relate this to our human cohort, as well as seeing elevated *IL36A* in the mucosa of UC patients we have also shown significantly increased *IL8* expression in these patients. Our *in vitro* data also shows that recombinant IL-36 can induce high levels of IL-8 from human PBMCs and primary monocytes. As IL-8 is the primary human chemokine responsible for neutrophil recruitment, it is plausible that elevated IL-36 in the gut mucosa in UC patients may play a role in enhancing neutrophil recruitment in this setting.

Based on the fact that our data shows a pathogenic role for IL-36 in colitis, we determined that it was also important to investigate potential mechanisms of negative regulation of this pathway. We have examined the role of the orphan receptor SIGIRR in this context. It is well characterized that SIGIRR can negatively regulate IL-1R and TLR signaling. As IL-36R utilizes the same IL-1RAcP as The IL-1R complex, we wanted to investigate whether SIGIRR could directly inhibit IL-36R. It is well documented that the naturally occurring IL-

36RA can bind to IL-36R and prevent IL-36R signaling. Firstly we, and others, have shown that *sigirr*^{-/-} mice are more susceptible to DSS induced colitis, indicating that it plays a protective role in the gastrointestinal tract, which is the opposite phenotype of IL-36R mice. We have shown utilizing *sigirr*^{-/-} cells, that SIGIRR can negatively regulate IL-36R signaling *in vitro*. Furthermore, I.P administration of IL-36 in WT and *sigirr*^{-/-} mice also reveals that IL-36 signaling is largely enhanced in the absence of SIGIRR. These data indicate that SIGIRR can in fact regulate IL-36R signaling. However, it does not directly address whether enhanced IL-36R signaling may be responsible for the more severe disease phenotype observed in *sigirr*^{-/-} mice. We are currently generating double knockout (*sigirr*^{-/-}*il36r*^{-/-}) mice to definitively address that question.

Chapter 5: **General Discussion and Future Work**

5. General Discussion

Intestinal homeostasis is dependent on various interactions between microbiota, the epithelium that line the intestinal tract, and the complex immune system in the gut. Genetic susceptibility alleles, alterations in the composition of the microbiome and impaired epithelial barrier function are factors that can disturb intestinal homeostasis and can tip the balance towards intestinal inflammation. During this study we have characterized expression patterns of novel IL-1 family members in a paediatric IBD cohort that have not been studied in this context previously. Our study has revealed two major findings. We have shown for the first time that expression levels of the immunosuppressive cytokine IL-37 is significantly reduced in the mucosa of paediatric Crohn's Disease patients compared to control patients. We have also demonstrated significantly increased levels of the pro-inflammatory mediator IL-36 α specifically in the mucosa of Ulcerative Colitis patients, compared to controls. To date it has been well established that IL-37 can act as a fundamental inhibitor of innate immunity. We, and others, have shown that over- expression of IL-37 can dampen innate immune stimulatory responses[106]. Although there is no known mouse homolog of IL-37 a transgenic mouse that overexpresses human IL-37 has been shown to be protected from DSS-induced colitis[110], indicating a role for IL-37 in the maintenance of a tolerogenic environment in the gut. Based on the fact that IL-37 can reduce TLR/NLR responses, it is reasonable to assume that the reduced IL-37 levels we have observed in the mucosa of a CD patients could result in exacerbated TLR/NLR signaling and an enhanced inflammatory cytokine profile. Furthermore we have shown that the pro-inflammatory

environment that occurs in CD can negatively affect the expression of IL-37 and that stimulation of colonic epithelial cells with a Nod2 agonist leads to induction of IL37 expression. It is well noted that Nod2 plays a protective role in IBD and that mutations in the *NOD2* gene are one of the biggest genetic susceptibility factors for CD[39]. Further investigation into whether CD patients with mutations in *NOD2* specifically, have altered *IL37* will provide greater insight into the regulation of *IL37* expression in this disease setting. It is well established that CD is associated with a Th1 response, characterized by elevated IFN- γ levels[60]. We have shown that IFN- γ can suppress IL-37 expression in both epithelial cells and monocytes. Significantly higher levels of *IFNG* expression in the mucosa of CD patients may in part contribute towards the lower levels of *IL37* expression observed in these patients. Although it has been shown previously that IL-37 levels are enhanced in the mucosa of an IBD cohort[141, 142], we have found that expression of IL-37 is decreased in paediatric IBD, particularly in CD patients. It is important to note that this paediatric cohort was treatment naïve, and this may play some role in explaining differences between our cohort and other cohorts studied. We have also used several antibodies in an attempt to characterize protein expression of IL-37 in IBD, however several problems with non-specific binding were encountered.

As well as screening the paediatric biopsies for *IL37* expression, we also investigated expression patterns of IL-36 family members. The skin is a prominent site where IL-36 cytokines have been shown to be expressed and there have been several reports of IL-36 cytokines playing a pathogenic role in skin inflammatory disorders[104, 115, 143, 144]. However, it has not yet been

reported whether IL-36 cytokines are expressed in the gut and if so, whether they played a role in intestinal inflammation. We have shown for the first time that *IL36A*, *IL36G* and *IL36RN* are expressed in the gut and that *IL36A* is significantly and specifically upregulated in the mucosa of UC patients. We have also shown that *IL36RN* expression appears to be reduced in UC patients, although this observation is not significant. In the context of skin inflammation, it has been shown that keratinocytes act as a prominent source of IL-36 cytokines[104]. We sought to identify a potential cellular source of IL-36 α specifically as this is the IL-36 family member that is elevated during UC. Although we could not detect *IL36A* expression in human primary monocytes or colonic epithelial cells, we have identified CD4+ T cells as expressing *IL36A* and expression levels can be further induced upon T cell activation. We have shown that IL-36 α can act as a powerful innate immune stimulus driving the production of IL-8, TNF α and IL-6 from PBMCs and primary monocytes. Interestingly we have shown that although IL-36 α does not appear to act directly on CD4+ T cells, it can enhance Th1 responses indirectly, whereby addition of rIL-36 α into PBMCs that have been treated with T cell activation antibodies, leads to enhanced IFN- γ expression from CD4+ T cells. Taken together, these data suggest that elevated levels of *IL36A* in the mucosa may come from infiltrating CD4+ T cells and may subsequently promote an inflammatory environment with elevated levels of pro-inflammatory chemokines and cytokines.

Due to the lack of a murine model of IL-37 and limited reagents, we chose to pursue a study investigating the role of IL-36 in a murine model of colitis. We have shown that expression of *il36a* and *il36g* are upregulated in the colon

during the course of DSS-induced colitis. Furthermore, mice deficient for the IL-36R^{-/-} are less susceptible to DSS colitis, indicating that not only is IL-36 expression elevated during disease, IL-36R signaling plays a pathogenic role in the gut during inflammation. We have shown that IL-36 can act as a powerful innate and adaptive immune stimulant *in vitro*. IL-36 can directly induce TNF- α , IL-6, CXCL1 and CXCL2 from BMDCs. Interestingly, unlike our observations in human CD4⁺ T cells; IL-36 can directly enhance CD4⁺ T cell subset responses as well as acting as a powerful co-stimulating factor inducing IL-2 expression from CD4⁺ T cells. This observation highlights that there are differences in the role of IL-36 cytokines between human and mouse.

DSS-induced colitis is an innate model of colitis. We have shown that during the course of DSS, IL-36 cytokine expression is enhanced. At the same time point, levels of the neutrophil recruiting chemokine CXCL1 are significantly enhanced. Furthermore we have shown that recombinant IL-36 can directly induce CXCL1 expression in colon tissue *ex vivo*. To further characterize the role of IL-36 in neutrophil recruitment, we have used an *in vivo* model of I.P administration of IL-36 showing that IL-36 can lead to a rapid increase in CXCL1 levels and subsequent neutrophil infiltration into the peritoneal cavity. This demonstrates that IL-36 can act to enhance neutrophil recruitment *in vivo*. A key feature of DSS-induced colitis that we have demonstrated is a massive influx of neutrophils into the lamina propria as disease activity progresses. A key finding is that *il36r*^{-/-} mice show a significantly lower number of neutrophils in the lamina propria during DSS-induced colitis, than the wild type counterparts. Although it has been shown that neutrophils can play a protective role in the gut in terms of limiting

bacterial burden, it is key that this neutrophil response is diminished to allow resolution of inflammation[145]. Taken together these data suggest that IL-36 is induced during intestinal inflammation and plays a pathogenic role through the recruitment of neutrophils.

Given the fact that IL-36 is a powerful inflammatory stimulant and acts to promote intestinal inflammation it is key to investigate mechanisms of negative regulation of this pathway. SIGIRR is a membrane bound receptor that has been shown to act as a negative regulator of IL-1 and TLR signaling[125]. It is widely expressed in intestinal epithelial cells and hematopoietic cells[126]. *Sigirr* deficiency leads to exacerbated inflammation and it has been shown that SIGIRR plays a key role in the maintenance of intestinal homeostasis. We and others have shown that *sigirr*^{-/-} mice are more susceptible to DSS-induced colitis[130]. In fact, the profile is the reverse of the protective effect of *il36r* deficiency. This led us to question whether SIGIRR could regulate IL-36R signaling and if so, was enhanced IL-36R signaling responsible for the increased susceptibility to DSS-induced colitis seen in *sigirr*^{-/-} mice. Our *in vitro* data demonstrates that SIGIRR can regulate IL-36R signaling. Furthermore I.P administration of rIL-36 leads to enhanced neutrophil recruitment in *sigirr*^{-/-} mice than in wild type mice, indicating that SIGIRR regulates IL-36 signalling *in vivo*. To address the role of SIGIRR regulating IL-36R signaling in colitis, we are currently generating double knockout mice (*il36r*^{-/-}*-sigirr*^{-/-}) to investigate whether the increased susceptibility to colitis seen in *sigirr*^{-/-} mice is reduced once the IL-36R pathway has been abolished in this setting.

Future Work:

Although we have identified alterations in expression of *IL37* and *IL36A* in IBD patients, it is well documented that regulation of IL-1 family members can occur at various post-translational levels. Limited reagents, particularly antibodies, have curtailed our investigation into the regulation of these cytokines. It has been shown that a truncated form of IL-36 displays enhanced bioactivity than the pro-form. However, unlike IL-1, the processing of IL-36 appears to be caspase-independent, as the amino acids surrounding the truncation site do not resemble a caspase-cleavage site[98]. The proteases involved in IL-36 cleavage are currently unknown. Interestingly, the IL-1 family member IL-33 has been shown to be processed by neutrophil derived proteases, which can lead to enhanced activity of IL-33[89]. In contrast, caspase-mediated cleavage of IL-33 can reduce its functional activity. Future work to address how IL-37 and IL-36 can be processed and what factors can control their processing is required to increase our understanding of the role of these cytokines in intestinal inflammation. The development of improved antibodies against IL-37 and IL-36 can also lead to a more in-depth characterization of their expression profiles in patients. Histological analysis of control, UC and CD patients would complement our gene expression studies and reveal exact cellular sources and targets of these cytokines in IBD.

During this project we have chosen to use the DSS-induced colitis model as our murine model of disease. We chose this model as we had observed a predominantly innate role for IL-36 in human cellular assays and DSS has been shown to represent an innate model of murine colitis. Although we have been

able to make many valuable observations from this model, in terms of the role of IL-36 in intestinal inflammation, it is important to note that IL-36 can also play a direct role in murine adaptive immunity. Therefore investigation into the role of IL-36 in an adaptive model of murine colitis warrants investigation. We have begun to address this question by subjecting wild-type and *il36r*^{-/-} mice to the *Citrobacter Rodentium* model of infection colitis. We have preliminary data to suggest that *il36r*^{-/-} succumb to a higher bacterial load during this model of disease than wild-type mice. Further studies to investigate the effect of the absence of IL-36R pathway in T cell responses in this model are required.

Another area that we plan to address in future experiments is to further investigate potential negative regulators of the IL-36R pathway in the context of IBD. As mentioned earlier, we have shown that SIGIRR can act as a negative regulator of IL-36R signaling. We plan to use a double knockout mouse to address whether enhanced IL-36R^{-/-} signaling is the reason for increased disease severity in SIGIRR^{-/-} mice. As well as SIGIRR, IL-36RA is a naturally occurring IL-36 receptor antagonist that has the potential to play a key role in intestinal homeostasis. Our patient data suggests that IL36RN expression is slightly lower in UC patients than in control patients, further highlighting that a balance of IL-36 and IL-36RA levels could be a key factor in the maintenance of intestinal homeostasis. We are currently breeding *il1f5*^{-/-} (IL-36RA) mice. We plan to investigate whether they show enhanced susceptibility to DSS colitis, and if so, we will investigate the use of rIL-36RA as a potential therapeutic target in the context of DSS-colitis. As we have already shown that IL-36 plays a

pathogenic role in colitis, administration of IL-36RA could lead to protection of mice from DSS-induced colitis.

In conclusion, in recent years much has been learned about the biology of IL-36 and IL-37. Although IL-37 has been characterized reasonably well on a molecular level, its role in inflammatory diseases has remained largely uncharacterized. For the first time we have shown that IL-37 levels are suppressed in IBD. This study compliments a previous study showing the over-expression of human IL-37 protects mice from DSS colitis. These observations taken together provide rational to further explore the immuno-modulatory capacity of IL-37 in intestinal homeostasis. Similarly, much progress has been made in characterizing the role of IL-36 in skin inflammation. However, we have now identified IL-36 cytokines as major players in intestinal inflammation. Combining a human patient study with a murine model of disease, we have identified IL-36 as a potential therapeutic target for ulcerative colitis. Currently, anti-TNF- α therapy is the most common anti-cytokine therapy used for IBD patients. However although this treatment has been shown to be highly effective, not all patients respond to anti-TNF therapy and many patients that are initially responsive, lose their responsiveness over time[146]. Therefore, new additional therapeutic strategies are in demand. Further studies to explore the role of IL-36 in different mouse models of colitis as well as other inflammatory disorders could open up the development of specific anti-cytokine therapies.

Chapter 6:
Appendix I

Human Taqman Primers for quantitative Real time PCR:

<u>Gene</u>	<u>Protein</u>	<u>Product code</u>	<u>Supplier</u>
<i>18S rRNA</i>	18S rRNA	4319413E	Biosciences
<i>IFNG</i>	IFN- γ	Hs00989291_m1	ABI
<i>IL17A</i>	IL-17a	Hs00174383_m1	ABI
<i>IL13</i>	IL-13	Hs00174379_m1	ABI
<i>IL22</i>	IL-22	Hs01574154_m1	ABI
<i>IL22RA2</i>	IL-22BP	Hs00364814_m1	ABI
<i>IL8</i>	IL-8	Hs00174103_m1	ABI
<i>TBX21</i>	T-bet	Hs00203436_m1	ABI
<i>RORC</i>	Ror- γ	Hs01076122_m1	ABI
<i>SIGIRR</i>	Sigirr	Hs00222347_m1	ABI
<i>IL1F7</i>	IL-37	Hs00367201_m1	ABI
<i>IL1F7B</i>	IL-37b	Hs00367199_m1	ABI
<i>IL36A</i>	IL-36 α	Hs00205367_g1	ABI
<i>IL36B</i>	IL-36 β	Hs00758166_g1	ABI
<i>IL36G</i>	IL-36 γ	Hs00219742_g1	ABI
<i>IL36RN</i>	IL-36ra	Hs01104220_g1	ABI

Murine Taqman Primers for quantitative Real time PCR:

<u>Gene</u>	<u>Protein</u>	<u>Product code</u>	<u>Supplier</u>
<i>18S rRNA</i>	18S rRNA	4319413E	Biosciences
<i>ifng</i>	IFN- γ	Mm01168134_m1	ABI
<i>Il22</i>	IL-22	Mm01226722_g1	ABI

<i>Il1b</i>	IL-1 β	Mm00434228_m1	ABI
<i>Il23</i>	IL-23	Mm01160011_g1	ABI

Murine SYBR Primers for quantitative Real time PCR:

<u>Gene</u>	<u>Protein</u>	<u>Product code</u>	<u>Supplier</u>
<i>18S rRNA</i>	18S rRNA	Ppm72041A	Qiagen
<i>Il1f5</i>	IL-36RA	Ppm03758C	Qiagen
<i>Il1f6</i>	IL-36 α	Ppm04200G	Qiagen
<i>Il1f8</i>	IL-36 β	Ppm29241D	Qiagen
<i>Il1f9</i>	IL-36 γ	Ppm35005C	Qiagen
<i>Il1rl2</i>	IL-36R	Ppm03542A	Qiagen

MACS antibodies:

<u>MACS cell isolation Kit</u>	<u>Product Code</u>	<u>Supplier</u>
Naïve CD4+ T cell Isolation kit II, human	130-094-131	Miltenyi Biotec
CD14 microbeads, human	130-050-201	Miltenyi Biotec
CD4 (L3T4) Microbeads, mouse	130-049-201	Miltenyi Biotec
CD8a (Ly-2) Microbeads, mouse	130-049-401	Miltenyi Biotec

Human and Mouse T cell activating antibodies

<u>Antibody</u>	<u>Product Code</u>	<u>Supplier</u>

Anti-mouse CD3e functional grade purified (clone 145-2C11)	16-0031	ebioscience
Anti-mouse CD28 functional grade purified (clone 37.51)	16-0281	ebioscience
Anti-Human CD3 functional grade purified (clone OKT3)	16-0037	ebioscience
Anti-Human CD28 functional grade purified (clone CD28.2)	16-0289	ebioscience

Recombinant murine cytokines/neutralization antibodies (used in cell culture)

<u>Protein/Neut antibody</u>	<u>Product Code</u>	<u>Supplier</u>
IL-36 α	7059-ML-010	R&D
IL-36RA	2714-ML-005	R&D
IL-12	419-ML-010	R&D
IL-4	404-ML-010	R&D
TGF- β	130-095-067	Miltenyi Biotec
IL-6	575702	Medical Supply
Anti-IL-4	16-7041-85	ebioscience
Anti-IFN- γ	BE0055	Bioxcell

Recombinant human cytokines

<u>Protein</u>	<u>Product Code</u>	<u>Supplier</u>
IL-36 α	6995-IL-010	R&D
IL-36RA	1275-IL-025	R&D
IL-12	Cyt-362	MSC
IFN- γ	Cyt-206	MSC

TLR/NLR ligands

<u>Ligand</u>	<u>Specific for</u>	<u>Product Code</u>	<u>Supplier</u>
Ultra-pure LPS	TLR4	tlrl-peklps	Invivogen
Pam3CSK4	TLR2	Tlrl-pms	Invivogen
CpG	TLR9	Tlrl-2216	Invivogen
Poly I:C	TLR3	Tlrl-plc	Invivogen
Muramyl Dipeptide	NOD2	tlrl-lmdp	Invivogen

Human ELISA kits

<u>ELISA KIT</u>	<u>Product Code</u>	<u>Supplier</u>
IL-8	88-8086-88	Ebioscience
IL-6	88-7066-88	Ebioscience
TNF- α	88-7324-88	Ebioscience
IFN- γ	88-7316-88	Ebioscience
IL-17	88-7876-88	Ebioscience

Mouse ELISA kits

<u>ELISA KIT</u>	<u>Product Code</u>	<u>Supplier</u>
IFN- γ	88-7314-88	Ebioscience
IL-17A	88-7371-77	Ebioscience
IL-22	88-7922-88	Ebioscience
IL-2	88-7024-88	Ebioscience
TNF- α	88-7324-88	Ebioscience
IL-6	88-7064-88	Ebioscience
KC	DY453-05	R&D
MIP-2	DY452-05	R&D

Human Flow Cytometry antibodies

<u>Antibody</u>	<u>Clone</u>	<u>Fluorochrome</u>	<u>Product code</u>	<u>Supplier</u>
CD4	SK3	PE-Cy7	25-0047-42	ebioscience
CD14	61D3	APC	17-0149-42	ebioscience
IFN- γ	4S.B3	FITC	11-7319-41	ebioscience

Mouse Flow Cytometry Antibodies

<u>Antibody</u>	<u>Clone</u>	<u>Fluorochrome</u>	<u>Product code</u>	<u>Supplier</u>
CD45	30-F11	E450	48-0451-82	ebioscience
CD3	145-2C11	FITC	11-0031-82	ebioscience
CD3	145-2C11	APC	17-0031-82	ebioscience
CD4	GK1.5	PE-Cy7	25-0041-82	ebioscience
CD4	GK1.5	PE	12-0041-82	ebioscience
CD8	53-6.7	APC	17-0081-82	ebioscience

CD11b	M1/70	PE-Cy7	25-0112-81	ebioscience
CD11b	M1/70	eFluor 450	48-0112-82	ebioscience
F4/80	BM8	FITC	11-4801-82	ebioscience
Gr1	RB6-8C5	PE	12-5931-82	ebioscience
Foxp3	FJK-16s	APC	17-5773-82	ebioscience
Thy1.1		PE	551401	BD Pharmingen
IFN- γ	XMG1.2	PE	12-7311-82	ebioscience
IFN- γ	XMG1.2	PE-Cy7	25-7311-41	ebioscience

Chapter 7:
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