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Understanding the Role of IL-36 Family Cytokines in Paediatric IBD

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By

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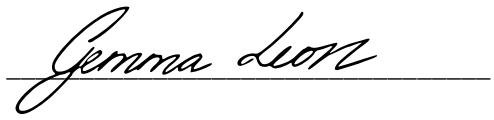

Gemma Leon

SUMMARY

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastro-intestinal (GI) tract. Incidence of the disease is on the rise globally, and approximately 25% of these cases arise in childhood or adolescence. Over the past few years, our understanding of the disease has advanced tremendously, and we now know that an inappropriate $CD4^+$ T_H cell response is critical in driving the inflammation and pathology we see in IBD. Cytokines are key innate mediators of the adaptive response, and emerging evidence suggests that IL-1 family cytokines in particular play a prominent role in the regulation of $CD4^+$ T_H cells in the intestines. Recently, IL-36, a newly identified member of the IL-1 superfamily, has been associated with IBD and regulation of the adaptive response.

Using quantitative RT-PCR, we revealed significantly elevated levels of *IL36A* in the colonic tissue of UC patients, when compared to healthy controls or CD patients. This elevated expression translated into enhanced IL-36α protein in the serum of these UC patients when measured by ELISA. Furthermore, we also reported increased serum levels of IL-36α in CD patients when compared to controls. Serum analysis by ELISA also revealed a negative correlation between IL-36α and IL-36Ra, indicating in IBD there is an environment permissive to IL-36α signalling. Importantly, using immunofluorescence, we identified the presence of the IL-36R on $CD3^+$ T cells in the colonic tissue of paediatric IBD patients, as well expression of IL-36α in this tissue, indicating that the elevated levels of IL-36α present have the potential to act on T cells.

Mechanistic studies using $CD4^+$ T_H cells isolated from *wt* mice in culture revealed a prominent role for IL-36 in the activation, and polarisation of $CD4^+$ T_H cells. Interestingly, we demonstrated that IL-36 can synergise with CD3, the TCR, to activate $CD4^+$ T_H cells, circumventing the necessity for CD28 co-stimulation. Furthermore, IL-36 enhances the polarisation of pro-inflammatory T_H1, T_H2 and T_H9 T_H lineages, whilst inhibiting the generation of T_H17 cells and tolerogenic iTreg cells. As T_H1 cells are associated with CD, and T_H2 and T_H9 cells are associated with UC, and Tregs are the predominant regulators of their response in the intestines, this indicates that IL-36 enhances the generation of colitogenic T_H cells and facilitates their evasion from homeostatic suppression. Accordingly, using FACs sorted T effector cells and nTregs from *wt* and *Il36r*$^{-/-}$ mice, we demonstrated that IL-36 does indeed allow T effector cells to evade suppression by nTregs in an *ex-vivo* tissue culture assay.

To determine the effect of IL-36R signalling in the development of T_H cell responses in IBD, we used the T cell transfer model of colitis. In this system, T effector cells were

FACs sorted from *wt* and *Il36r*$^{-/-}$ mice and transferred via *i.p.* injection into adaptive immune deficient *Rag1*$^{-/-}$ hosts. Importantly, while *wt* recipient mice develop colitis over the course of disease, as is evident by significant weightloss, enhanced intestinal damage, determined by H&E staining, and enhanced generation of pro-inflammatory T_H1 cell population in the periphery, and their subsequent infiltration into the intestinal tissue as determined by FACs analysis, *Il36r*$^{-/-}$ recipient mice exhibit none of these features. In contrast, *Il36r*$^{-/-}$ recipient mice appear protected from disease and display a weightloss pattern similar to control mice, furthermore, they contain a reduced T_H1 population, and enhanced Treg population in the periphery as measured by FACs, and significantly less of these cells migrate to the intestines, than they do in *wt* recipient mice, thereby, resulting in little intestinal damage by H&E staining compared to *wt* recipients. Remarkably though, *Il36r*$^{-/-}$ mice do display splenomegaly and lymphadenopathy. And later mechanistic studies using *in-vivo* T cell transfer, and *in vitro* $CD4^+T_H$ cell culture techniques, revealed this may be due a potential role for IL-36 in $CD4^+$ T_H cell gut homing. IL-36 signalling can promote expression of $\alpha4\beta7$ and CCR9 on T_H cells, even in the presence of tolerogenic retinoic acid (RA), promoting the generation of proinflammatory T_H1 cells with a gut homing phenotype.

Collectively these findings suggest that IL-36 can utilize an alternate pathway in T_H cell activation, allowing the T_H cells to activate and expand in conditions where conventional co-stimulatory signalling is low, and synergistically evading natural regulation of this classical activation. What's more, this unregulated activation promotes the generation of pathogenic $CD4^+$ T_H cell lineages and inhibits tolerogenic Treg polarisation, allowing T effector cells to evade another level of natural suppression. These proinflammatory T_H1 cells can then expand in the periphery, and migrate unrestricted to the intestines, whereby the presence of pro-homeostatic RA, further promotes the generation of gut homing phenotype, but fails to regulate the pathogenicity of the cell population. In conclusion, IL-36 promotes the generation of colitogenic $CD4^+$ T_H cells and facilitates their evasion of multiple levels of homeostatic suppression, thereby inducing the inflammation and pathology we see in IBD.

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ABBREVIATIONS AND ACRONYMS

α	Alpha
α4β1	Alpha 4 beta 1
α4β7	alpha 4 beta 7
β	Beta
γ	Gamma
γδ	gamma delta
γδ T cells	gamma delta T cell
δ	Delta
ϰ	Kappa
μg	microgram
μl	microliter
μM	micromole
°C	degrees Celsius
AD	Atopic dermatitis
AE	Atopic eczema
AhR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptides
APC	Antigen presenting cell
ATRA	all-trans Retinoic acid
B cell	Bone marrow derived lymphocyte
Batf	Basic Leucine Zipper ATF-Like Transcription Factor
BCA	BICINCHONIC ACID PROTEIN
Breg	Regulatory B cell
C-Maf	Musculoaponeurotic fibrosarcoma oncogene homolog
CCL27	C-C Motif Chemokine Ligand 27
CCR1	C-C Motif Chemokine Receptor 1
CCR10	C-C Motif Chemokine Receptor 10
CCR3	C-C Motif Chemokine Receptor 3
CCR4	C-C Motif Chemokine Receptor 4
CCR5	C-C Motif Chemokine Receptor 5
CCR6	C-C Motif Chemokine Receptor 6
CCR8	C-C Motif Chemokine Receptor 8
CCR9	C-C Motif Chemokine Receptor 9

CD	Crohns disease
CD103	Cluster of differentiation 103
CD11b	Cluster of differentiation 11 b
CD123	Cluster of differentiation co-receptor 123
CD137	Cluster of differentiation 137/4-1BB
CD138	Cluster of differentiation 138
CD14	Cluster of differentiation 14
CD161	Cluster of differentiation co-receptor 161
CD25	Cluster of differentiation co-receptor 25
CD27	Cluster of differentiation co-receptor 27
CD28	Cluster of differentiation co-receptor 28
CD3	Cluster of differentiation co-receptor 3
CD4	Cluster of differentiation co-receptor 4
CD45Rβ	Cluster of differentiation co-receptor 45β
CD49b	Cluster of differentiation 49 b
CD62L	Cluster of differentiation 62 L
CD69	Cluster of differentiation 69
CD8	Cluster of differentiation co-receptor 8
CD8α	Cluster of differentiation co-receptor 8 alpha
CD80	Cluster of differentiation co-receptor 80
CD86	Cluster of differentiation co-receptor 86
CFSE	Carboxyfluorescein succinimidyl ester
cmLN	Colon draining mesenteric lymph node
CNS	Central nervous system
CRC	Colorectal cancer
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4 (CD152)
CTLA-4-Ig	Cytotoxic T-lymphocyte-associated protein 4 immunoglobulin
CTV	Cell trace violet
CXCR3	C-X-C Motif Chemokine Receptor 3
CXCR4	C-X-C Motif Chemokine Receptor 4
DAI	Disease activity index
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay

FAC	Follicle associated epithelium
FACs	Flow cytometry
FAE	Follicle-associated epithelium
FDC	Follicular dendritic cell
FMO	Fluorescence minus one
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
GATA3	GATA Binding Protein 3
GC	Germinal Center
GC	glucocorticoid steroids
GF	Germ free
GI	Gastro-intestinal
GWAS	Genome wide association studies
H&E	Haematoxylin and eosin
H_20	Water
H_2O_2	Hydrogen peroxide
HEV	High endothelial venules
IBD	Inflammatory bowel disease
ICAM-1	intercellular adhesion molecule 1
ICOS	inducible T cell co-stimulator
IEC	Intestinal epithelial cell
IFNγ	Interferon gamma
$IFN\gamma^{-/-}$	Interferon gamma knockout
IFNγR1	Interferon gamma receptor 1
Ifnγr1$^{-/-}$	Interferon gamma receptor 1 knockout
IFNγR2	Interferon gamma receptor 2
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL-10	Interleukin 10
Il-10$^{-/-}$	*Il-10* knockout
IL-12	Interleukin 12
IL-12p70	Interleukin 12 p70 subunit
IL-12Rβ	Interleukin 12 receptor beta
IL-13	Interleukin 13
IL-17A	Interleukin 17 A
IL-17E	Interleukin 17 E

IL-17F	Interleukin 17F
IL-18	Interleukin 18
IL-18bp	Interleukin 18 binding protein
Il-18bp$^{-/-}$	Interleukin 18 binding protein knockout
IL-18Rα	Interleukin 18 receptor alpha
IL-18Rβ	Interleukin 18 receptor beta
IL-1R1	Interleukin 1 receptor 1
IL-1R10	Interleukin 1 receptor 10
IL-1R2	Interleukin 1 receptor 2
IL-1R8	Interleukin 1 receptor 8 (SIGIRR)
IL-1R9	Interleukin 1 receptor 9
IL-1Ra	Interleukin 1 receptor antagonist
IL-1RAcP	Interleukin 1 receptor accessory protein
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
Il-2$^{-/-}$	*Il-2* knockout
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-22bp	Interleukin 22 binding protein
IL-22R	Interleukin 22 receptor
IL-23	Interleukin 23
IL-23R	Interleukin 23 receptor
IL-25	Interleukin 25
IL-27	Interleukin 27
IL-27Rα	Interleukin 27 receptor alpha
IL-2R	Interleukin 2 receptor
IL-33	Interleukin 33
Il-33$^{-/-}$	Interleukin 33 knockout
IL-36R	Interleukin 36 receptor
Il-36r$^{-/-}$	*Il-36 receptor* knockout
IL-36Ra	Interleukin 36 receptor antagonist
IL-36α	Interleukin 36 alpha
IL-36β	Interleukin 36 beta
IL-36γ	Interleukin 36 gamma
IL-37	Interleukin 37

Il-38	Interleukin 38
IL-4	Interleukin 4
IL-4R	Interleukin 4 receptor
IL-4Rα	Interleukin 4 receptor alpha
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
ILC3	Type 3 innate lymphoid cells
IRF1	Interferon Regulatory Factor 1
IRF4	Interferon response factor 4
iTreg	induced regulatory T helper cell
LAG-3	Lymphocyte-activation gene 3
LAM	Leukocyte adhesion molecule
LCN2	Lipocalin 2
LD	Live/Dead
LN	Lymph node
LP	Lamina propria
LPMC	Lamina propria mononuclear cells
LPS	Lipopolysaccharide
M	Molar
M cells	Microfold cells
mAb	Monoclonal antibody
Mac-1	macrophage-1 antigen 1
MAdCAM-1	mucosal vascular addressin cell adhesion molecule 1
MAPK	mitogen-activated protein kinase
MBP	Myelin basic protein
MCP-3	monocyte-chemotactic protein
Mg	milligram
MHCI	Major histocompatibility complex 1
MHCII	Major histocompatibility complex 2
MIP-1	macrophage inflammatory protein 1
mL	millilitre
mLN	Mesenteric lymph node
mRNA	Messenger RNA
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response 88

NFAT	Nuclear factor of activated T cells
NFκβ	nuclear factor kappa beta
Ng	nanogram
NK	Natural killer cells
NLRP3	NLR family pyrin domain containing 3
NLRP6	NLR family pyrin domain containing 6
nM	nanomole
NMOSD	neuro-myelitis optica spectrum disorders
NOD mouse	Non-obese diabetic mouse
npT$_H$17	Non-pathogenic T helper cell 17
nTreg	naturally occurring regulatory T helper cell
OA	Osteoarthritis
OX-40	Tumour necrosis factor receptor superfamily, member 4
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PDGFR	platelet-derived growth factor receptor
PECAM-1	Platelet/endothelial cell adhesion molecule 1
Pg	picogram
PP	Peyer's patches
PRR	Pathogen recognition receptors
PSA	Polysaccharide A
PSGL-1	Selectin P ligand
pTreg	peripherally derived regulatory T helper cell
PU.1	PU-box 1
RA	Retinoic acid
RA	Rheumatoid Arthritis
Rag$^{-/-}$	Recombination-activating gene knockout
Rag1$^{-/-}$	Recombination-activating gene-1 knockout
RARα	Retinoic acid receptor alpha
RNA	Ribonucleic acid
RORγt	Retinoic acid receptor-related orphan receptor gamma-T
RPM	Revolutions Per Minute
RT	Room temperature
$_{RT}$PCR	Real-time PCR
Runx1	Runt-related transcription factor 1
Runx3	Runt-related transcription factor 3

$S1P_1$	Sphingosine-1-phosphate receptor 1
s-Le^x	Sialyl-Lewis x antigen
SAMP mice	Senescence accelerated mice
SED	Sub-epithelial dome
SFB	Segmented filamentous bacteria
SI	Small intestine
SIGIRR	Single Ig And TIR Domain Containing
Sigirr$^{-/-}$	Single Ig And TIR Domain Containing knockout
SLE	Systemic lupus erythematosus
Smad 2	Mothers against decapentaplegic homolog 2
Smad 3	Mothers against decapentaplegic homolog 3
smLN	Small intestine draining mesenteric lymph node
SNP	Single nucleotide polymorphisms
SPF	Specific pathogen free
ST2	Interleukin 33 receptor
St2$^{-/-}$	Interleukin 33 receptor knockout
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
STAT4	Signal transducer and activator of transcription 4
STAT5	Signal transducer and activator of transcription 5
STAT6	Signal transducer and activator of transcription 6
T cell	Thymus derived lymphocyte
T1D	Type 1 Diabetes
Tbet	T-box transcription factor
Tbet$^{-/-}$	T-box transcription factor knockout
TCR	T cell receptor
TCR α/β	T cell receptor alpha beta
TGFβ	Transforming growth factor beta
TGFβRII	Transforming growth factor beta receptor type 2
T_H	T helper cell
T_H-POK	T helper inducing POZ-Kruppel like factor
T_H1	T helper cell 1
T_H17	T helper cell 17
T_H2	T helper cell 2
T_H22	T helper cell 22
T_H3	T helper cell 3

T_h9	T helper cell 9
TJ	Tight junctions
TLR	Toll like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
TNFα	Tumour necrosis factor alpha
TNFβ	Tumour necrosis factor beta
Tr1	Type 1 regulatory T cell
Treg	Regulatory T helper cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule 1
VDJ	Variable (V), diversity (D), and joining (J) gene segments of the TCR
VLA-4	Very late antigen 4
Wt	C57BL/6 mice

CHAPTER 1.

INTRODUCTION

Chapter 1

Introduction

1.1 The Gastrointestinal System

The human gastrointestinal system, also known as the GI tract, is a series of hollow twisting tube like organs connecting from mouth to anus. The GI tract is composed of the mouth, oesophagus, stomach, small intestine (SI), large intestine (colon), rectum, and anus (Fig.1.1.1), and their primary function is to absorb ingested nutrients, and to excrete the waste products of digestion[1]. Many nutrients are ingested in a form that is either too complex for absorption, or insoluble, and it is the principle responsibility of the stomach and SI to aid in their digestion and absorption. These organs incorporate both physical (retropulsion in the stomach) and chemical (bile acids and enzymes in the SI) mechanisms to solubilize these nutrients and further degrade them enzymatically into simple molecules, that are sufficient in size and form to permit absorption across the mucosal epithelium. Once nutrients and the majority of H_20 is absorbed, the desiccation and compaction of waste occurs in the colon, and the waste product is then stored sigmoid colon and rectum prior to elimination[1].

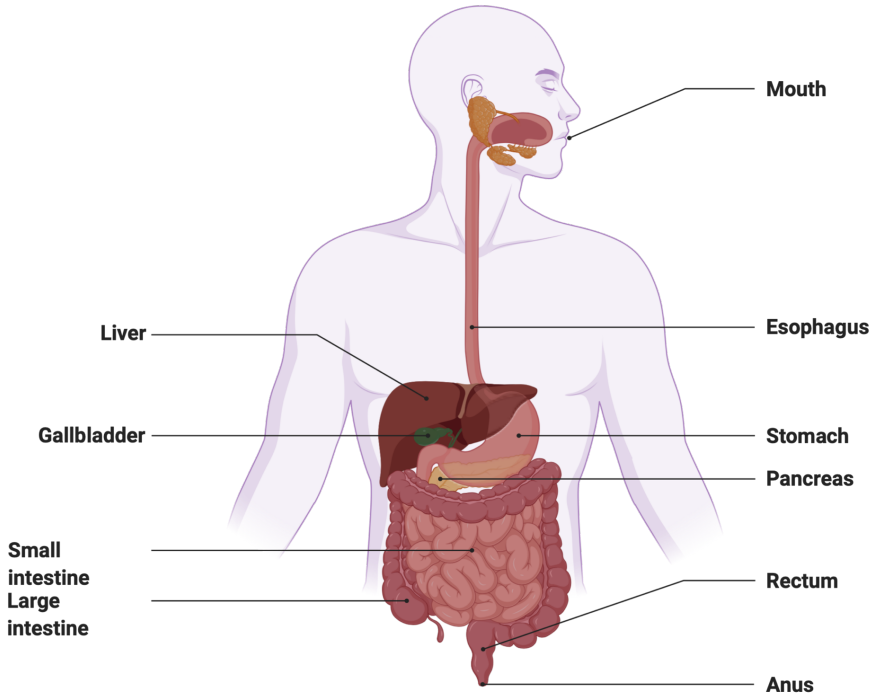

Figure 1.1.1.: The Organs of the Digestive System and GI Tract. The digestive system stretches from mouth anus and encompasses all the organs necessary to digest food and process waste. Food is taken in through the mouth

and transported to the stomach via the oesophagus. Here it is mechanically degraded, so nutrients can be chemically degraded and absorbed and in the small intestine. H_20 is then absorbed in the large intestine, and the waste is expelled as faeces through the anus. *Figure created with biorender.com*.

Morphologically the wall of GI tract is separated into 4 layers: the mucosa, the innermost layer; the submucosa, a layer of connective tissue that supports the mucosa; the muscularis externa, muscular wall surrounding the submucosa; and the adventitia or serosa, the outmost layer[2] (Fig.1.1.2).

The mucosa is composed of a layer of epithelial cells on the luminal side. Directly beneath the epithelium there lies a layer of supporting loose connective tissue, the lamina propia (LP), joined to a thin layer of smooth muscle cells, the muscularis mucosae. These cells form the boundary between the mucosa and the submucosa. The main function of the mucosa is nutrient absorption and mucus secretion, although as the epithelium is the interface between the luminal contents and the GI tract it also serves a protective role[2].

The submucosa is an irregular layer of fibrous connective tissues containing blood vessels, lymphatics, and nerves that separates the mucosa from the muscularis externa. The muscularis externa is responsible for the segmental contractions and peristaltic movements in the GI tract. The serosa lies outside of the muscularis externa, and is a smooth membrane that consists of a thin layer of connective tissue and a layer of epithelial cells. This provides a partition between the internal organs and the abdominal cavity[2].

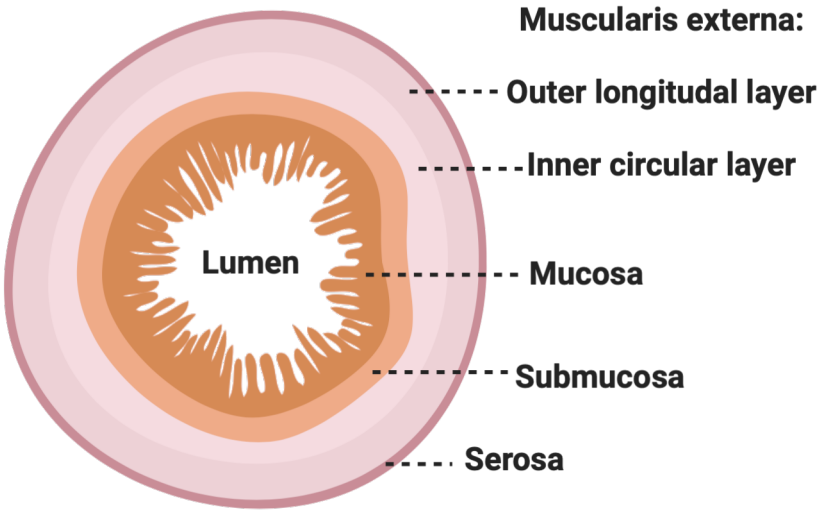

Figure 1.1.2.: The Intestinal Wall. The GI wall is composed of four main layers, the mucosa, submucosa, muscularis externa, and serosa. The mucosa is responsible for mucus secretion, nutrients absorption, and also forms a protective barrier from luminal antigens penetrating the intestinal tissue. The submucosa contains blood and lymphatic vessels. The muscularis externa is responsible for the segmental contractions and peristaltic movements in the GI tract. The serosa provides a partition between the internal organs and the abdominal cavity. *Figure created with biorender.com*.

1.2 IMMUNOREGULATION OF THE GI TRACT

Aside from its key role in digestion and absorption of nutrients, the GI system represents the largest and most sophisticated immune organ of the entire body. It is the main source of contact with the external environment, and is continuously overloaded with external stimuli, such as ingested food, pathogens and commensal microflora. This commensal flora, also known as the gut microbiome, has co-evolved with its host, and has proven beneficial in many ways; such as aiding in digestion and production of nutrients, detoxification, and protection against pathogens, such as virus's, pathogenic bacteria and bacteriophages[3,4]. As many of these pathogens enter the body though the intestinal mucosa, it is critical that the intestinal immune system, the gut-associated lymphoid tissues (GALT), provides effective and efficient responses when necessary. However, these responses need to be intricately regulated as inappropriate responses against innocuous antigens, from food and commensal microflora, lead to inflammatory disorders such as inflammatory bowel disease (IBD) and coeliac disease[3,4]. Therefore, dynamic layers of mucosal immunoregulation are vital in maintaining the delicate balance between vigilance and tolerance that is central to GI homeostasis.

1.2.1 GUT-ASSOCIATED LYMPHOID TISSUE

The intestinal immune system is comprised of the gut-associated lymphoid tissues (GALT), which is organised into immune inductive sites, such as the Peyer's patches (PP)[5] and the mesenteric lymph nodes (mLNs), and immune effector sites, such as the intestinal epithelium and underlying lamina propria (LP) (Fig.1.2.1.). In these regions, many different immune cells reside, such as activated T cells, mast cells, dendritic cells (DCs) and macrophages. The fact there are so many effector cells present in these regions and it does not result in tissue pathology reflects the tight regulatory mechanisms that are taking place.

1.2.1.1 PEYER'S PATCHES

Peyer's patches are composed of aggregated lymphoid follicles which are surrounded by the follicle-associated epithelium (FAE) in the intestinal mucasa[5]. The FAE forms an interface between the GALT and the luminal microenvironment, and contains specialized microfold (M) cells that facilitate the transport of antigens and bacteria in the lumen to the underlying immune cells in the PP. These immune cells then either activate or inhibit the immune response, thus leading to tolerance or the elicitation of an inflammatory response[5].

Morphologically, PPs are split into three domains; the follicular area, the interfollicular area, and the follicle-associated epithelium[5]. The germinal center (GC) is located within the follicular and interfollicular areas; and contains follicular dendritic cells

(FDCs), macrophages, and proliferating B cells. The follicle containing the GC is surrounded by the sub-epithelial dome (SED), which itself contains a variety of immune cells such as T cells, B cells, macrophages and DCs[5]. Endothelial venules connect the PPs to the body, and it is through these venules that naïve lymphocytes migrate into the PPs. In the intestine, these venules express the mucosal addressin MAdCAM-1[6,7]. MAdCAM-1 is recognized by lymphocytes expressing the $\alpha_4\beta_7$ integrin; the mucosal gut homing molecule[8]. These lymphocytes then exit the PPs via efferent lymphatic vessels which connect the PPs to the mLNs[6,7].

The FAE, which surrounds the PPs, is quite different from the mucosal epithelium. For instance, the basal lamina is more porous compared with the regular epithelium, it lacks the sub-epithelial my fibroblast sheath and, exhibits weaker mucus production[5,9]. The FAE is also infiltrated by many immune cells, but its main feature is the presence of micro fold cells (M cells). M cells are specialised enterocytes, that facilitate the transcytosis of intact luminal material like bacteria, viruses, soluble proteins, and antigens[10]. Interestingly, these cells can exploit a variety of mechanisms such as endocytosis, phagocytosis, pinocytosis, and micropinocytosis to ingest the extracellular material[10]. The presentation of these ingested antigens induces the activation of B and T lymphocytes in the follicular area of the PPs[11].

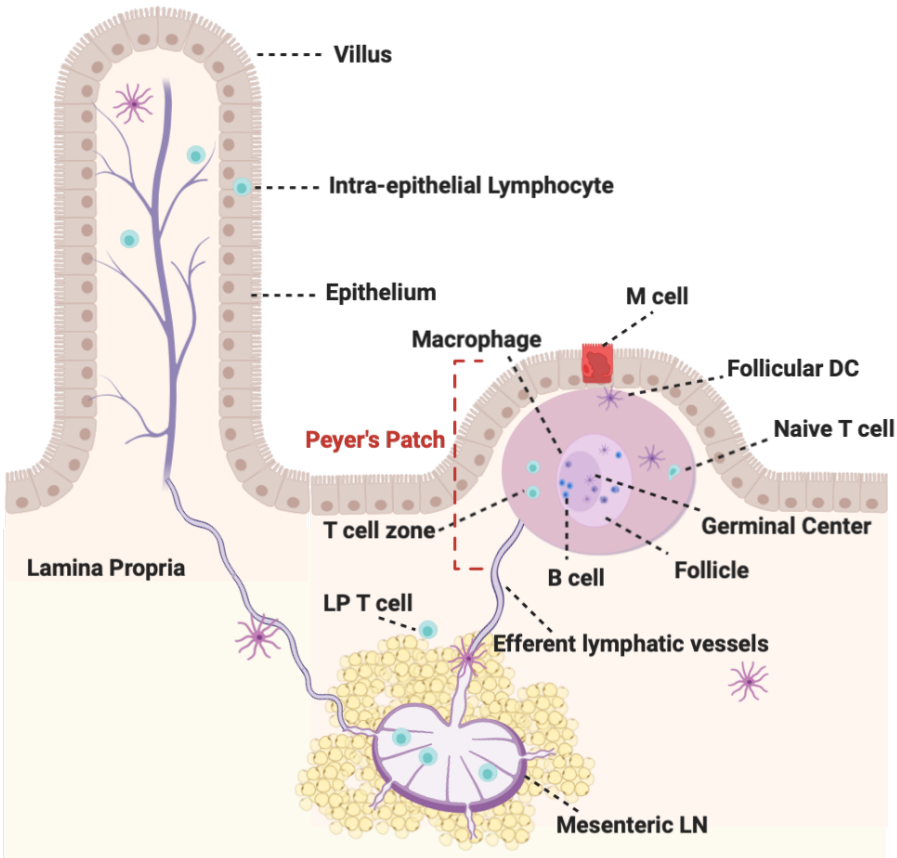

Figure 1.2.1.: The gut-associated lymphoid tissue (GALT). A mono layer of epithelial cells separates the intestinal lamina propria from the luminal gut flora. The intestinal immune system can be functionally divided into inductive sites, which include the mesenteric lymph nodes and Peyer's patches, and immune effector sites, which include the epithelium and the LP. The M cells present in the epithelium, transport lumenal antigens to Peyer's patches for sampling by DCs. These DCs that can also sample antigens from apoptotic epithelial cells, and either present to B cells in the PPs or migrate to mesenteric lymph nodes through the lymph to present antigens to T cells and induce an inflammatory response. The LP contains large numbers of immune cells such as T cells, IgA-producing plasma cells, DCs and macrophages, which all perform immune surveillance and promote either the induction of tolerance or an inflammatory response. *Figure created with biorender.com*.

1.2.1.2 MESENTERIC LYMPH NODES (MLNS)

The mesenteric lymph nodes (mLNs) are the first organ that nutrients and microbial substances must pass through when entering the lymph fluid in the intestinal LP. As such, they serve as a key site for induction of tolerance to innocuous antigens, but synergistically act as a firewall to prevent systemic spread of pathogens (Fig.1.2.1). Preclinical studies have demonstrated that mice lacking in mLNs, fail to induce oral tolerance to fed antigens, but mice lacking in PPs are unaffected[12,13], thus indicating that LNs are the primary sit where tolerance to food antigens occur. Furthermore, observations of DCs constitutively trafficking endocytosed intestinal epithelial cell remnants from the epithelium and Peyer's patches to the mLNs[14], indicate a potential mechanism whereby antigen can be picked up at the intestinal epithelial surface and transported to the mLNs, where T cell tolerization occurs.

Food antigens are certainly not the only immunogenic substance in the GI tract. The intestines are colonised by high loads of commensal, and some pathogenic bacteria, the antigens of which are extremely immunogenic. DCs that have sampled commensal intestinal bacteria induce IgA^+ B cells, that recirculate through the vascular and lymphatic systems and populate the LP with IgA-secreting plasma cells[15]. These B cells are mostly induced in the PPs, and although commensal laden DCs do traffic to the mLNs, they are stopped here from entering the systemic circulation, thereby preventing constant systemic penetration and triggering of the inflammatory response[15].

Interestingly, a recent study by Housten *et al*. reported that the mLNs draining from the SI and colon are anatomically distinct[16]. This study demonstrated that SI draining mLNs (smLNs), are presented with fed antigen specifically by $CD103^+CD11b^+$ DCs, in keeping with the SI's physiological role in nutrient absorption. However, the colonic draining mLNs (cmLNs) contain a unique population of $CD103^+CD11b^-$ DCs, which efficiently cross-present self-antigen[16,17]. Furthermore, the DCs in the smLN produce more retinoic acid (RA), a

vitamin A metabolite important in immune homeostasis, than DCs from cmLNs, and also induce more CCR9^{+}α4β7^{+} expressing CD4^{+} T cells[16]. These functional differences in the DC subsets found in the cmLNs and smLNs possibly reflect the necessary anatomical compartmentalization of immune responses between the different intestinal sites. Where the SI requires an immune response tailored to dietary factors, and the colon needs a response that can handle high bacterial burden.

1.2.2 THE MICROBIOME

The human GI tract is home to an enormous and complex community of commensal bacteria. While approximately 100 trillion (10^{14}) microbes reside in the intestines, the vast majority of them inhabit the colon, with densities reaching 10^{11}–10^{12} cells/ml[18]. As we are born germ free (GF), these microbes that populate our intestines must come from the external environment. However, the intestines are remarkable in the exclusivity of bacterial phyla which they will allow to colonise. In human adults, the microbiome is dominated by Bacteroidetes and Firmicutes, as well as just one member of Archaea[19,20].This microbiome has co-evolved symbiotically with humans over millennia, and is extremely valuable to our health, aiding in processes such digestion, detoxification, and regulation of our immune system[19,21].

Due to the many benefits the GI microbiome provides for its host, it is not surprising that the intestinal immune system has developed mechanisms to tolerate these foreign symbiotes (Fig.1.2.2.).For instance, intestinal epithelial cells (IECs), typically hypo-responsive to microbial stimuli[2], use spatio-temporal organization and compartmentalisation of pathogen recognition receptors (PRRs) to avoid constant interaction with luminal antigens[22,23], and innate and adaptive immune cells resident in the gut adopt an anti-inflammatory regulatory phenotype[24-32].

Given the intimate interplay between the intestinal immune system and the microbiome, it is not surprising that some species of the gut microbiota have been associated with autoimmune diseases, in particular GI autoimmunity. Unsurprisingly, microbial dysbiosis is strongly associated with the pathogenesis of IBD, which will be discussed in detail in section 1.3. However, the effects of the microbiome are far reaching and defects in its functioning have also been shown to impact immunity systemically. For instance, microbial dysbiosis has been associated with Rheumatoid Arthritis (RA)[33], experimental autoimmune encephalomyelitis (EAE), a preclinical model of MS[34,35,36,35,37,38,39], and Type 1 Diabetes (T1D)[40-42], highlighting the importance of the microbiome not only in GI homeostasis but systemic immune equilibrium.

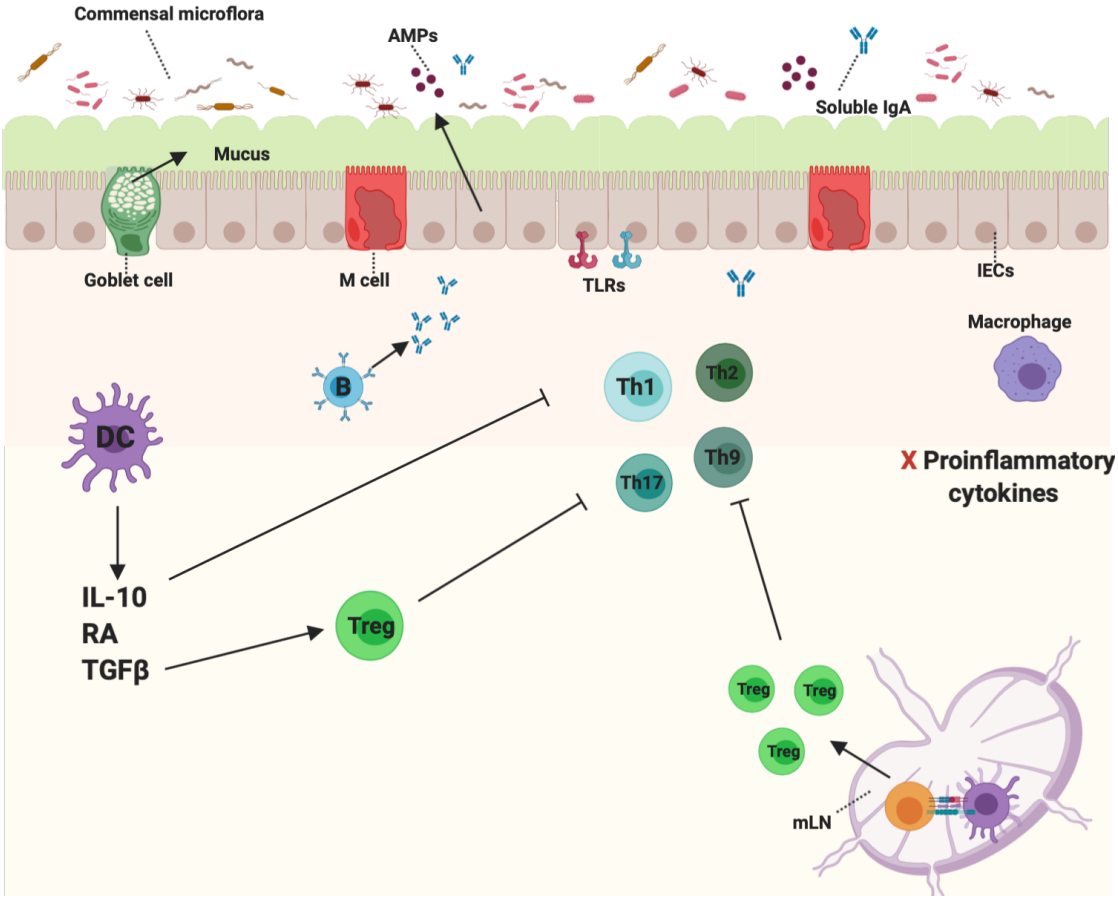

Figure 1.2.2.: Tolerance in the GI tract. The GI immune system has developed many mechanisms of tolerance to facilitate the close proximity of commensal bacteria and food antigens with the GALT. Goblet cells produce mucus to form a protective layer on top of the epithelial barrier, both functioning to keep luminal antigens from entering the underlying tissues. IECs also produce antimicrobial peptides (AMPs), and restrict expression of the PRRs to the basolateral surface of their membrane to reduce the induction of inflammation. LP macrophages, LP DCs also secrete anti-inflammatory cytokines and RA to promote the generation of tolerogenic Treg cells, which in turn inhibit the generation and expansion of proinflammatory T cell lineages. The lack of macrophage secreted proinflammatory cytokines also inhibits the induction of proinflammatory responses. *Figure created with biorender.com.*

1.3 INFLAMMATORY BOWEL DISEASE

Inflammatory Bowel Disease (IBD), typically subcategorised as either Crohns disease (CD) or ulcerative colitis (UC), is a chronic inflammatory disorder of the GI tract whose incidence is on the rise globally[43]. Its aetiology is unknown, but there is accumulating evidence demonstrating that interactions between environmental, genetic and immunological factors promote the development and pathogenesis of these chronic inflammatory disorders. Prevalence of IBD is highest in developed western countries, with approximately 1.6 million people in United States and up to 2 million people in Europe affected[43, 44]. More recent epidemiological studies have also shown an increase of disease incidence in developing countries, such as in Eastern Europe, South America, Africa, and Asia[43, 44]. Although the precise causes of IBD remain relatively unknown, such differences in epidemiological patterns highlight the likely importance of environmental and genetic factors in the development of the disease.

Interestingly, approximately 20-25% of reported IBD cases present in patients under 20 years old[43, 45, 46], with a reported male predominance in incidence. Yet, diagnostic delay is common, with the median age of diagnosis being 12-14 years, although a substantial proportion of patients exhibit symptoms before their tenth birthdays[47-49]. For those who are diagnosed before 10 years of age, they present a much more severe disease, that impacts not only their health, but psychological well-being and schooling[50-52]. Interestingly, in patients under 6 years of age, UC is most commonly diagnosed, whereas CD is more often reported in patients over 6 years[48, 49, 53]. Incidence of IBD in paediatric populations is also on the rise[46, 47, 54]. Benchimol *et al*. recently demonstrated an increase of incidence in a Canadian paediatric population from of 7.9/100,000 in 1999 to 10.6/100,000 in 2008[46], and Ghione *et al*. reporting an overall incidence of IBD increasing in a French paediatric population from 6.0 to 13.8 per 100,000 from 1988-2011[54]. As these trends occurred over a relatively short period of time it indicates that the increase in incidence observed is likely is not genetically driven, but may be predominantly driven by environmental influences[55]; and the increased risk of developing IBD among immigrants moving to a high prevalence region from a low prevalence area supports this hypothesis[56, 57]. Environmental and familial influences such as duration of breast feeding, time of gluten introduction, adherence to a Mediterranean diet, sibling bed sharing and pet ownership have all been closely linked with early IBD onset[55], as has the association between antibiotic exposure in childhood and increased risk of developing the disease[58-60]. Another consideration is whether the rising incidence of IBD observed in childhood and

adolescence reflects an improved ability to diagnose CD and UC among children, and also, to make this diagnosis earlier in the disease course[61].

For adult and paediatric onset alike, while both CD and UC share the common characteristic of GI inflammation, their disease presentation, histological, and immunological profiles are quite distinct (Fig.1.3.1). CD can affect anywhere along the GI tract from mouth to anus, most frequently the ileum and colon, and commonly causes inflammation in a discontinuous transmural pattern, resulting in a thickened submucosa, granulomas, fissuring ulceration and the development of fistulas and strictures in the affected areas[62, 63]. In contrast, in UC, inflammation is continuous, presenting from the rectum to colon, and only involving the mucosal and sub-mucosal layers[62]. In both disorders, disease course in highly variable, some patients experience chronic abdominal pain, rectal bleeding, fever and diarrhoea, whilst others experience long periods of remission from active disease[62, 63]. This heterogeneity in disease presentation and severity highlights the complexity of differential immunological factors and inflammatory pathways that are involved in the pathogenesis of IBD, and will be discussed further in section 1.3.2.

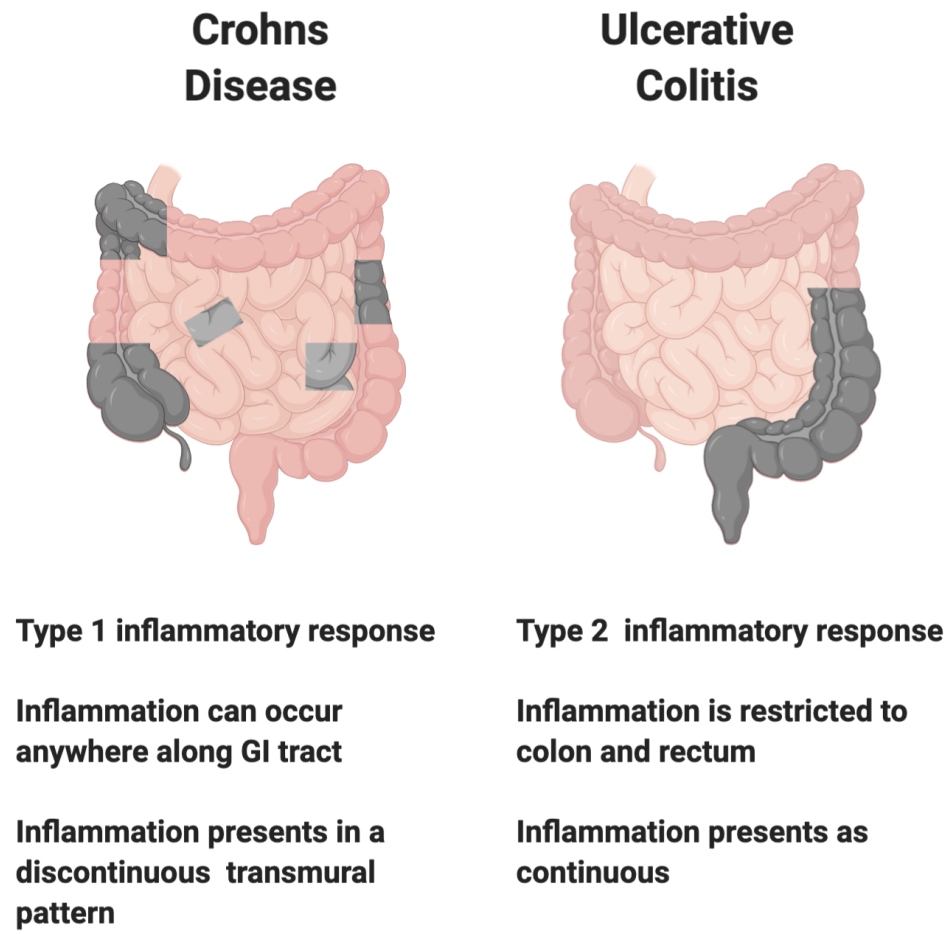

Figure 1.3.1.: Distinguishing Characteristics of CD and UC. *Figure created with Biorender.Com.*

1.3.1 TREATMENT OF IBD

Traditional treatment of IBD involves a multistep "step up" therapeutic approach using pharmaceutical compounds, such as glucocorticoid (GCs) steroids, immunomodulators, antibiotics and mesalamine compounds[64], with the ultimate goal of inducing and maintaining remission in patients (Fig.1.3.2). Failure on these pharmaceuticals typically leads to surgical intervention, but in recent years the advent of novel biologics has served as a key step in preventing surgery. There have already been some notable successes using this approach, for example, with the use of anti-TNFα biologics, infliximab, adalimumab, and certolizumab[65,66], as well as Ustekinumab, a monoclonal antibody which targets the common p40 subunit of IL-12 and IL-23, thereby inhibiting the generation of T_H1/T_H17 cells[67]. Both of these strategies have been shown to induce and maintain clinical response and remission in patients with IBD[68-71]

The success of these biologics have created much debate on where biologics should be positioned within the current treatment strategy for IBD, to maximise efficacy and minimise risk. Many gastroenterologists subscribe to the "step up" approach, consisting of treatment progression from melasamine compounds and antibiotics, to steroids, through immunomodulators, followed by biologics, and culminating with surgery. However, gastroenterologists endorsing a "step-down" strategy argue that traditional approaches focus on symptomatic relief, and do not consider the possibility of modifying the natural history of the disease. They believe employing biologics as an initial therapy may avoid disease progression and be more beneficial to patients in the long run[72].

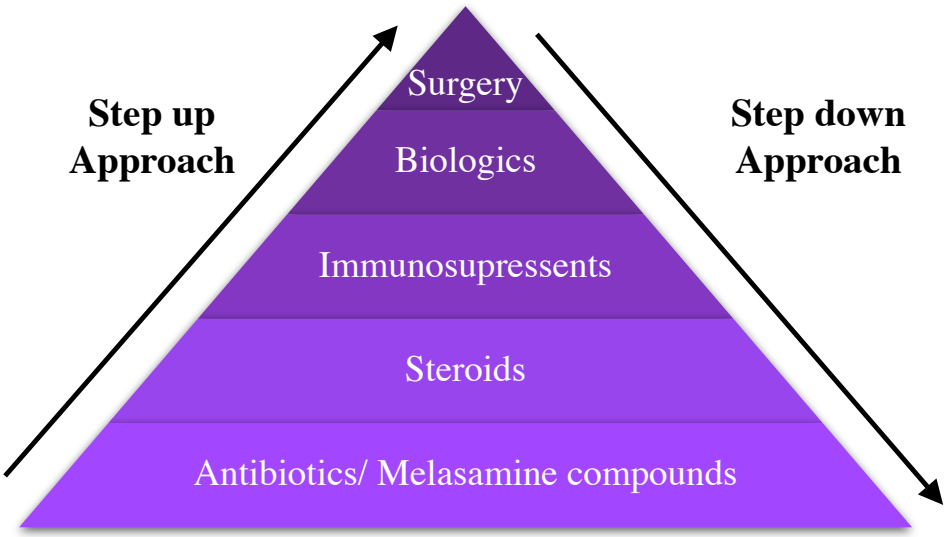

Figure 1.3.2: Treatment strategies for IBD. Traditional IBD therapy involves a "step up" approach from milder therapies to more aggressive treatments. The new "step down" approach does the opposite.

Similar to adult onset IBD, paediatric IBD is managed by a multi-step treatment approach of GC steroids, immunomodulators, antibiotics and mesalamine compounds in a "step up" approach[64, 73-75]. As earlier described, in this method, the milder, less toxic compounds are employed first, such as antibiotics and steroids, and failure with these causes the patients to "step up" to the more aggressive anti-inflammatory agents, such as biologics. However, due to the limited studies carried out in children using these treatments, paediatric gastroenterologists are restricted in their ability to interpret whether this is the correct strategy, and are often forced to extrapolate information from adult cohorts, which may not be applicable to a child[76].

Following the success of Infliximab in inducing clinical remission and mucosal healing in adult IBD populations[66, 77-80], a multicentre trial was established evaluating the safety of this biologic in 112 children with CD[81, 82]. The REACH trial was the first in its kind, and while not powered for efficacy the results do support the use of infliximab in paediatric cohorts, with a response rate of 90% at week 10, and a remission rate of 58% at week 54. Longitudal follow up studies revealed that the children's responsiveness to infliximab correlated with reduced rates of hospitalization, surgery for stricture and fistulae formation, as well as exhibiting overall improved growth while maintaining an effective disease remission[81, 82].

The success of infliximab in these children have led gastroenterologists to question whether employing a "step down" approach in paediatric populations may be more beneficial to patients in the long run (Fig.1.3.2). Since then a number of other cytokine based therapies have been gaining popularity in the clinic for paediatric IBD, both on and off label, such as other anti-TNF treatments, Adalimumab[83] and certolizumab[84], as well Ustekinumab[85] which targets the p40 subunit of IL-12.

1.3.2 IMMUNE INVOLVEMENT IN THE PATHOGENESIS OF IBD.

Due to the 10^{14} microbes residing in our GI tract[18], it not surprising that many lines of evidence point to a direct involvement of the microbiome in the pathogenesis of IBD. For instance the gut phyla of healthy adults differs greatly to that of CD patients[86], with an overgrowth of proteobacteria, alongside a reduction in Firmicutes and Bacteroides species evident in the IBD patients. Accordingly, in both IBD patients, and preclinical models alike, treatment with antibiotics and probiotics is often beneficial in treating inflammtion[86-88].

The reduced severity and protection from disease observed in numerous GF colitis models, such as *Il-2*$^{-/-}$[89], *Il-10*$^{-/-}$[90] and *TCR* $\alpha/\beta^{-/-}$[91], also highlights the importance of the intestinal microflora in regulating the inflammation we see in IBD. Furthermore, mechanistic studies using *Tbet*$^{-/-}$*Rag*$^{-/-}$ TRUC mice, that develop a colitis similar to human UC, demonstrated that

Klebsiella pneumoniae and *Proteus mirabilis*, in concert with other members of the endogenous microbial community, are the driving force behind the induction of inflammation[92, 93].

Conversely, the presence of "beneficial" commensal bacteria are also critical in maintaining GI homeostasis. In *Helicobacter hepaticus* induced colitis colonisation of *B. fragilis* can reduce disease via PSA production, which downregulates IL-17 production and enhances IL-10 production from colonic $CD4^+$ T_H cells[94]. Furthermore, in dextran sulfate sodium (DSS) induced colitis the introduction of *Clostridium* resulted in an enhanced colonic Treg population and a reduction in disease severity[95]. The expansion of $CD4^+$ populations in the intestines are also reliant of signals from the microbiome. Feng *et al.* demonstrated using the *Rag*$^{-/-}$ adoptive transfer model, that transferred T cells fail to proliferate in GF conditions. In the intestines, $CD4^+$ T cells require microbiota mediated IL-6 production by DCs in order to homeostatically expand and initiate inflammation[96].

In the intestines, DCs are critical in the development of antigen specific responses, and their dysregulation has been implicated in the pathogenesis of IBD. In the inflamed intestines DCs are shown to have temporal effects, protecting from inflammation early in disease[97, 98], but exacerbating inflammation in the later stages of disease[99]. One of the key roles of DCs in mediating homeostasis is the production of anti-inflammatory RA by the $CD103^+$ subset, this in turn modulates adaptive immune responses, enhancing tolerogenic Treg populations and inhibiting proinflammatory effector lineages[31].

Neutrophils are key effector cells in the GI system and are important in our front-line defence against invading pathogens. Aside from destroying infiltrating bacteria themselves, they secrete numerous inflammatory cytokines to elicit an immune response. There are increased neutrophil numbers in the blood of UC patients[100], and this coincides with elevated levels of their enzyme neutrophil elastase in colonic tissues[101]. In DSS colitis, neutrophil recruitment to the colon occurs early in disease, prior to DC and macrophage infiltration[102]. Furthermore, blocking neutrophil recruitment to the intestine attenuates colitis[102-104], highlighting their pathogenic role in the induction of GI inflammation.

Intestinal barrier dysfunction also plays a key role in the pathogenesis of IBD[105]. The intestinal epithelium consists of a monolayer of specialised cells, that are functionally different, but all essential to GI homeostasis[106-108]. Dysregulation in these cells' differentiation, and consequent disruption to correct formation of the epithelium barrier, is associated with IBD[109], and accordingly, genes responsible for the differentiation of these cells are aberrantly expressed in during disease[110-112]. This epithelial monolayer forms the main compartment of the epithelial barrier, but its ability to act as a protective barrier, separating luminal contents from the underlying GALT, depends on strict regulation of a web of tight junctions (TJ),

connecting the IECs together[113]. Accordingly, altered expression and structural changes of the intestinal TJ proteins are strongly associated with IBD[114-116]. SAMP1/YitFc mice develop spontaneous colitis due to epithelial dysfunction and increased permeability[117]. Furthermore, pro-inflammatory cytokines, such as TNFα and IFNγ, have been shown to induce apoptosis of IECs and increase TJ permeability[118-120], thereby reducing epithelial barrier function. Abnormal GI permeability has been strongly associated with mucosal inflammation in both CD and UC patients[121], and interestingly anti-TNF therapy has been shown to restore epithelial permeability[122, 123].

As described, many facets of the innate immune response play important roles in the pathogenesis of IBD. However, arguably one of the most studied pathogenic immune characteristics of IBD is the aberrant infiltration of pro-inflammatory $CD4^+$ T_H cells into the intestinal mucosa.

1.4 THE T CELL

In 1961 the world of T cell biology came into being following Professor Jacques Millers seminal discovery of a group of thymocytes that are essential in the immune response. Just 7 years earlier, while studying for his PhD in London, Miller first identified the thymus gland as an essential immune organ. Following on from Ludwik Gross's research on viral induced leukaemia, which revealed that inoculating low-leukaemia strain (C3Hf/ Gs) mice with filtered leukemic extracts from high-leukaemia strain (Ak mice) induced leukaemia in the immediate neonatal period[124], Miller went on to determine that the development of this disease occurred in the thymus.

Using thymectomized adolescent C3Hf/Gs mice that were injected at birth with leukemic extracts, he discovered that absence of this organ protected the mice from the development of leukaemia. However, transplant of a healthy thymus to these mice, restored the potential for disease development, even when the thymus was transplanted in mice at 6 months of age[125, 126]. These results led miller to believe that the virus must have spread from the thymus and be lying latent in some other tissue, and led him to question what was the cellular source of the viral spread? Why the mice had to be infected neonatally? If the mice were thymectomized earlier than 4/5 weeks would this inhibit viral spread?

In answering these questions he soon discovered that the thymus was essential for life. Thymectomized neonatal mice soon wasted away and died after weaning, whether inoculated with leukaemia or not, and post mortem examination of these mice revealed low levels of lymphocytes in their blood, as well as lesions on the liver indicating hepatitis infection[127]. These results implied the thymectomized neonatal mice were immunodeficient, and failure of these mice to mount an immune response and reject skin grafts from allogenic mice and rats confirmed this theory[128]. This lead Miller to conclude that immunocompetent lymphocytes were produced in the thymus; these later became known T cells.

Further experiments elucidated that T cells could be activated specifically by antigens and that they were essential to help bone marrow derived lymphocytes (B cells) to generate antibodies in response to antigen[129, 130]. This was the first indication of lymphocyte collaboration, demonstrating that cellular communication underlies the immune response to infection. It has become a central tenant of the adaptive immune response, in which the immune cells of the body "learn" and "remember" antigens so they can elicit a rapid and heightened response to future exposure. In the decades following this seminal work, the field of T cell biology and adaptive immunity has exploded.

1.4.1 T CELL GENERATION IN THE THYMUS

In the years since Professor Millers ground-breaking discovery, our knowledge of T cell development and function has grown massively. In mammals there are two central lymphoid organs essential for lymphocyte development – the bone marrow for B cells and the thymus for T cells[131]. In early life, T cells are produced in the thymus, and migrate to populate the peripheral lymphoid tissues. However, as the person ages, this production of "new" T cells slows down, rather the remaining T cells are "maintained" by cell division of mature T cells outside of the periphery.

In early T cell development the precursor cell diverges into one of two distinct lineages based on their T cell receptor (TCR) phenotype, either α:β or γ:δ. This occurs through a process called VDJ recombination, which involves somatic recombination in the thymus of variable (V), diversity (D), and joining (J) gene segments of the TCR[131]. First the β-chain of the TCR undergoes D-J recombination, involving the joining of $D_{\beta}1$ to $J_{\beta}1.1$- $J_{\beta}1.6$ gene segments, or $D_{\beta}2$ to $J_{\beta}2.1$- $J_{\beta}2.6$. Once D_{β} J_{β}combination is complete, an upstream V_{β} segments joins the $D_{\beta}J_{\beta}$ complex, followed by the incorporation of the constant domain gene C_{β}. All gene segments between the newly formed V_{β}-D_{β}-J_{β}-C_{β} complex are deleted, and the primary transcript is polyadenylated, generating the mRNA product $V_{\beta}D_{\beta}J_{\beta}C_{\beta}$poly-a, which forms the β chain of the TCR. The rearrangement of the α chain involves V-J joining, followed by the addition of the C segment. This is then transcribed into a primary transcript, polyadenylated, and the intervening gene segments are spliced out, leaving a final product of $V_{\alpha}J_{\alpha}C_{\alpha}$poly-a. The α and β chains then assemble, forming the αβTCR (Fig.1.4.1), which is present on the majority of T cell [132]. The γδ TCR population of T cells undergo the same recombination process, in this case the δ chain forms a V-D-J-C complex similar to the β chain of αβ T cells, whilst the γ chain forms a V-J-C complex similar to the α chain[133]. This produces a highly diverse repertoire of antigen receptors with distinct specificities, providing the versatility that is fundamental in immune homeostasis. The diversity of antigen receptors expressed allows for T cells to recognise foreign pathogens, such as virus and bacteria, self "altered" cells, such as cancer, or detrimentally, host own cells and tissues, leading to autoimmunity[131, 132].

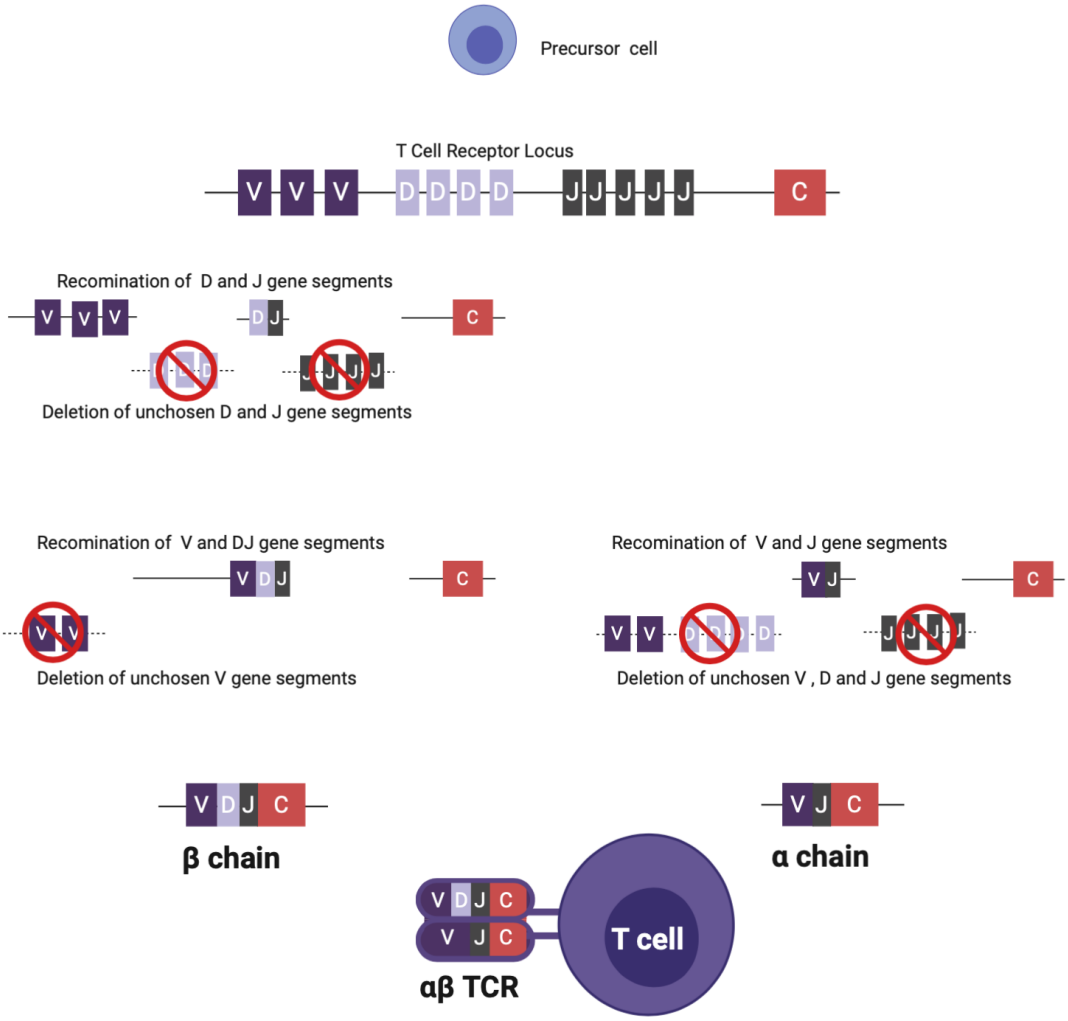

Figure 1.4.1.: VDJ Recombination. VDJ recombination is a process in which the variable (V), diversity (D), and joining (J) gene segments of the TCR are assembled. Three gene segments are joined to form the α chain, first recombination of V and J, followed by joining of C, to form VJC. β chain assembly occurs in three steps, first recombination of D and J segments, followed by joining of the V segment, and lastly assembly with the C section now attached. In γδ t cells, γ chain follows the same process as the α chain, and δ follows the same process as the β chain. *Figure created with biorender.com*.

1.4.2 T CELL SUBSETS

After TCR gene assembly and expression, immature α:β T cells undergo further bifurcation into two distinct effector lineages based on their expression of the cell surfaces receptors, cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8). This divergence allows the T cells to specifically recognise one of the two major histocompatibility complex (MHC) molecules, MHC class I and MHC class II, that are differentially expressed in the body. $CD8^+$ T cells recognise and interact with MHCI, and $CD4^+$ T cells associate with MHCII. This interaction is necessary in antigen recognition by the T cell, in which either the CD4 or CD8 molecules interact with the TCR and bind to the invariant sites on the MHC:peptide ligand to elicit an effective T cell response. This role renders CD4 and CD8 as TCR coreceptors[131,132].

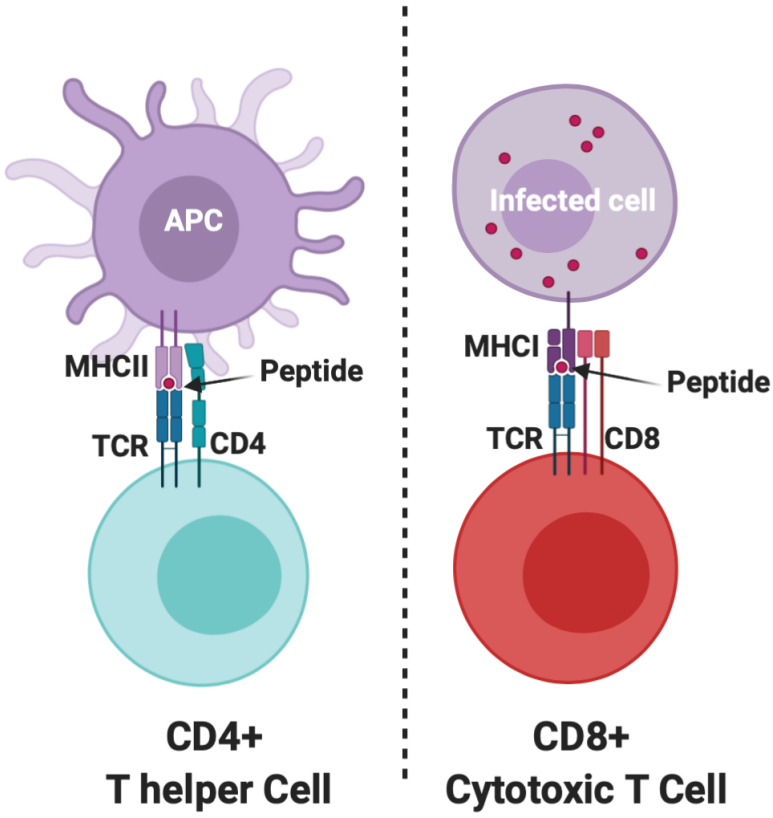

Figure 1.4.2.: Antigen Recognition and T cell Fate. Following TCR gene assembly in the thymus, the immature T cells undergo antigen recognition in order to mature into their specific lineage and mount an efficient effector response. If the thymocyte recognises a peptide presented by the MHCII molecule as well as receiving a moderate TCR signal this induces expression of T_H-POK, regulatory gene that promotes the commitment of the cell to a $CD4^+$ fate, and represses CD8 gene expression. T_H-POK, a gene-regulatory protein that in turn controls other genes (including repression of the CD8 gene), causing the cell to adopt a CD4 fate. However, if the thymocytes recognises MHC1 molecule, whilst also receiving a weak TCR this fails to induce T_H-POK expression, and facilitates commitment to the CD8 fate. *Figure created with biorender.com.*

MHC1 molecules present peptides from pathogens to $CD8^+$ cytotoxic T cells (Fig.1.4.2). These pathogens are commonly viral, and as such can infect almost any nucleated cell. Therefore, almost all such cells express MHC class 1 molecules, although constitutive expression differs among cell type, with immune cells abundantly expressing the molecules whereas red blood cell express little or no MHC molecules. $CD8^+$ T cells are specialised to kill any cell they specifically recognise, leading to the designation as cytotoxic T cells[131, 132].

In contrast, $CD4^+$ $T_н$ cells recognise peptides presented by MHC class II molecules (Fig.1.4.2), and rather than eliciting cell death, they activate and recruit other immune cells to initiate an effector response, such as B cell antibody production and macrophage phagocytosis of pathogenic cells. MHC class II is expressed by hematopoietic cells and by thymic stromal cells, as well as activated $CD4^+$ $T_н$ cells in humans. Specialised MHCII expressing antigen presenting cells (APCs) circulate the lymphatic system and present to and activate naïve $T_н$ cells in the lymphoid tissue[131, 132].

While this system is efficient in eliciting an immune response to pathogen, in certain cases it is the hosts own self peptides that are presented by MHC molecules, leading to the development of autoimmune and autoinflammatory conditions. The ability to distinguish between self and non-self is known as immunological tolerance, and is facilitated by the removal of autoreactive T cells during T cells ontogeny in the thymus. However, sometimes this mechanism is imperfect, and can result in T cell mediated autoimmune diseases such as Multiple Sclerosis (MS), in which $CD8^+$ MHCI and $CD4^+$ MHCII restricted T cells react against central nervous system antigens, such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein[134, 135], and in Type I Diabetes (T1D), in which $CD4^+$ MHCII restricted $T_н$ cells destroy insulin-secreting pancreatic β cells[136]. Furthermore, aberrant $CD4^+$ $T_н$ cell responses are strongly associated the inflammation and inflammation induced pathology evident in IBD. This will be discussed further in section 1.4.4.

1.4.3 CD4+ T HELPER CELLS: FUNCTION AND DIFFERENTIATION

CD4+ T helper cells are fundamental in mediating the immune response, and maintaining host homeostasis in the body. These cells carry out multiple roles, such as the activation of cells of the innate and adaptive branches of the immune system, B cells[137], dendritic cells (DCs)[138], and CD8+ T[137] cells, as well as non-immune cells such as IECs[139]. Following CD4+ $T_н$ cell development, the naïve $T_н$ cell's then undergo further specialisation into one of a number of CD4+ $T_н$ helper cell lineages, such as the classical $T_н1$ and $T_н2$ phenotypes, and the newer $T_н17$, $T_н9$, iTreg, $T_н13$ and $T_н22$ lineages. These lineages are classified by the prominent cytokine the newly differentiated cells produce, known as their signature cytokine (Table 1.1).

The initial step in this differentiation process is the antigenic stimulation of the TCR by a professional APC. This induces a downstream signalling complex which induces naïve $T_н$ cell proliferation and effector differentiation. Differentiation of the naïve T cell into one of the effector lineages occurs through a number of mechanisms, which depends on the cytokine milieu in the microenvironment, the type of APC and the strength of the antigen it presents to the T cells, in addition to input from the co-stimulatory molecules on the $T_н$ cell itself [140, 141].

For instance, DCs are potent APCs that possess a unique ability to stimulate naïve T cells and influence their lineage determination. DCs themselves are comprised of distinct subsets, and these differ in their surface molecule expression, biological function and $T_н$ cell lineage affiliation[142]. In the mouse CD8α+ lymphoid DCs are able to induce a $T_н1$ response, whereas CD8α - myeloid DCs induce a $T_н2$ response[143, 144], and immature human CD83- DCs induce a regulatory response via Tr1 cells[145].

Furthermore, research has shown that $T_н$ cell co-stimulatory signals, such as those from CD28 and members of the TNF superfamily, amplifies TCR signalling, further promoting differentiation and proliferation[146, 147]. In CD4+ $T_н$ cells, CD28 functions as the main co-stimulatory molecule and is expressed in all naïve $T_н$ cells. The ligand for this receptor are CD80 and CD86, and are expressed on DCs, as well as B cells and other APCs, and upregulated upon activation [146]. TNF receptor superfamily members, CD27, DR-3, 4-1BB, and OX-40, also act as less potent co-stimulatory molecules for T cells[147]. Other costimulatory molecules include the CD28 homologue, inducible co-stimulator (ICOS)[146].

Once the $T_н$ cell has been adequately activated, it is the cytokines present in its vicinity, usually being produced by nearby APCs and innate immune cells, which initiate and instruct the differentiation of the CD4+ $T_н$ cells into particular phenotypes, which will be detailed below.

1.4.3.1 T_H1

In T_H1 development, presence of Interleukin 12 (IL-12) and interferon γ (IFNγ) is critical in initiating downstream signalling in the T cell to develop a T_H1 effector phenotype[148]. Following APC activation via PRRs, the cells secrete large amounts of IL-12, this in turn activates nearby NK cells, which then produce IFNγ[148-150]. The newly activated $CD4^+$ T_H cell is then stimulated by the IL-12 and IFNγ to induce expression of T-box transcription factor (Tbet) and in turn to secrete IFNγ, their signature cytokine[151]. Tbet is the master regulator of T_H1 differentiation, as well as inducing expression and activation of critical genes and transcription factors in T_H1 lineage determination, it also induces a set of pathways to supress opposing lineage differentiation[151]. Tbet inhibits the important *IL4* gene and impairs the function of GATA3, the T_H2 master transcription factor, thereby supressing T_H2 cell development and differentiation[151-153]. Similarly, Tbet inhibits the activation of RORγt, the T_H17 master transcription factor, thus limiting T_H17 generation[154]. T_H1 cells play a critical role in the elimination of intracellular pathogens, and are implicated in numerous diseases such as psoriasis and IBD[155, 156].

1.4.3.2 T_H2

For T_H2 differentiation IL-4 and IL-2 are the key cytokines instructing their polarisation. In addition, STAT6 is necessary to activate GATA-3, the T_H2 master transcription factor[157-159]. Interestingly, expression of GATA-3 has been shown to suppress T_H1 generation via inhibition of STAT4[160]. T_H2 cells are strongly associated with type 2 immunity, they mount a humoral immune response against extracellular parasites, and are strongly implicated in the induction and persistence of allergic diseases, such as Atopic Dermatitis (AD)[156, 161], asthma, chronic rhinosinusitis, and eosinophilic gastrointestinal disorders[162].

1.4.3.3 T_H9

Originally characterised as an IL-9 producing sub-group of T_H2 cells, T_H9 cells have recently gained their own lineage classification. TGFβ and IL-4 are their differentiation inducing cytokines, and PU.1 is their master transcription factor; but they also require regulation by IRF4 (interferon response factor 4), and GATA-3[163-166].

Although T_H9 cells have been associated with numerous autoimmune diseases, such as colitis[163, 165], MS[167, 168], and are linked to the induction of allergic inflammation[166, 169], their production of immunosuppressive IL-9 and IL-10 cytokines indicates they have a role in immune tolerance[165, 170].

1.4.3.4 T_H17

TGFβ is the critical signalling cytokine in T_H17 differentiation, but IL-6, IL-21, and IL-23 are also necessary[171-173]. Retinoic acid receptor-related orphan receptor gamma-T (RORγt) is their master regulator, and IL-17a is the signature cytokine they produce[173, 174]. Unlike T_H1 and T_H2 cells which have their signature cytokines, IFN-γ and IL-4, working in a positive feedback loop to amplify their respective responses, IL-17a does not serve to amplify T_H17 differentiation. Instead, IL-21, which T_H17 cells secrete to a lesser extent, performs this task, and APC derived IL-23 stabilizes the population of T_H17 cells and allows their expansion[175, 176].

The main function of T_H17 cells is to mount an immune response against extracellular bacteria and fungi. They are also associated with a number of autoimmune diseases, such as MS, RA and IBD, but are perhaps most well known for their role in psoriasis[177]. Elevated numbers of T_H17 cells are found in patients with psoriasis[155, 156], alongside a dysregulated T_H17/Treg balance which correlates with disease activity [178]. Furthermore, targeting these cells in psoriatic patients with the use of monoclonal antibodies, such as Secukinumab and Ustekinumab, is already a success in the clinic, and are now undergoing trials in IBD[69, 70, 177, 179].

1.4.3.5 TREG

Tregs are regulatory members of the $CD4^+$ T_H helper cell group, and similar to T_H17 cells, TGFβ plays a significant role in their differentiation. Where TGFβ is present alone in high concentrations, the naïve T_H cell will skew towards iTreg generation, though the induction of master iTreg transcription factor forkhead box P3 (FOXP3)[180]. However, when TGFβ is present at a lower quantity with IL-6 also present, the naïve T cell will differentiate towards a T_H17 phenotype, via enhanced STAT3 induced RORyt expression [171, 172, 181-183].

Interestingly, deficiency in STAT3 results in enhanced Tbet and FOXP3 expression, thus promoting T_H1 and Treg differentiation[182]. iTreg differentiation also involves TGFβ induced Smad2 and Smad3 activation of FOXP3, which both enhances iTreg generation and expansion as well as inhibiting T_H17 generation by blocking RORyt[184-186]. IL-2 is also a critical cytokine necessary for iTreg generation. IL-2 induces STAT5, which in turn enhances FOXP3 and inhibits STAT3, synergistically promoting iTreg and blocking T_H17 generation[187, 188].

Tr1 cells are a member of the regulatory T_H cell lineage and are characterised by their expression of IL-10. IL-10 and IL-27 are key cytokines involved in their differentiation, along with C-Maf, their master transcription factor, IL-21, which acts as an autocrine growth factor, and the costimulatory receptor ICOS[189].

Tregs are critical in immune homeostasis. Following clearance of pathogens by other immune cells Tregs negatively regulate the immune response, protecting against the development of immunopathologies[190].

1.4.3.6 T_H22

T_H22 cells are a characterised by their production of signature cytokine IL-22, and small amounts of IL-13 and TNFα, without any IFNγ (T_H1 marker), IL-4 (T_H2 marker) and IL-17 (T_H17 marker) being expressed[191-193]. IL-6 and TNFα are key cytokines involved in their differentiation, and their master regulator of transcription aryl hydrocarbon receptor (AhR) is key in their expansion[191, 192, 194]. The addition of increasing concentrations of TGFβ inhibits their generation[191].

The main function of T_H22 cells is in epithelial barrier homeostasis, but they may also contribute to the pathogenesis of skin disease. One of the main characteristics of these cells is their expression of the chemokine receptors CCR4 and CCR10, whose specific cognate chemokines are strongly expressed in the skin[191]. Keratinocytes strongly produce the CCR10 ligand CCL27, and so induce T_H22 migration to the upper epidermis[193, 195]. It is therefore unsurprisingly that elevated levels of these cells have been found in patients of skin diseases such as psoriasis[155], AD and atopic eczema (AE)[156, 161]. T_H22 cells have also been implicated in the pathogenesis of chronic asthma[196], Systemic lupus erythematosus (SLE)[197, 198] and Rheumatoid arthritis (RA)[199, 200].

TABLE 1.1: CD 4+ T CELL LINEAGE DEFINING FACTORS

Lineage	Cytokines Secreted	Transcription Factors	Key cytokines in differentiation	Surface Markers	Inhibitory Transcription Factors
Th1	**IFNγ** IL-2 Lymphotoxin-α TNFα/β	**T-bet** STAT1 STAT4 Runx 3 Eomes Hlx	IFNγ IL-12 IL-27	CD4+ CXCR3+ IFNγR1+ IFNγR2+ IL-12Rβ+ IL-18Rα+ IL-27Rα+ CCR1+ CCR5+	GATA3
Th2	**IL-4** IL13 IL-5 IL-9 IL-17E IL-25	**GATA3** STAT6 STAT5 STAT3 Gfi-1 c-Maf IRF4	IL-4 IL-2 IL-7 TSLP	CD4+ IL-4Rα+ CXCR4+ ST2+ CCR3+, CCR4+, CCR8+	T-bet Runx3 Runx1
Th9	**IL-9** IL-10	**PU.1** IRF4 GATA3	TGFβ IL-4	CD4+ IL-4Rα+ TGFβRII+	STAT4 T-bet FOXP3
Th17	**IL-17** IL-22 IL-21 IL-23, TGFβ	**RORγt** STAT3 RORα Runx1 Batf IRF4 AhR	TGFβ IL-6 IL-21 IL-23	CD4+ CCR4+ CCR6+ CXCR3- CD161+ IL-23R+	Tbet Runx1 Smad3 Runx1 FOXP3 STAT5
iTreg	**IL-2** TGFβ	**FOXP3** Smad2 Smad3 STAT5 NFAT	TGFβ IL-2	CD4+ CD25+ FOXP3+	STAT3
Tr1	IL27 IL10	c-Maf AhR IRF1 Batf	IL-21 IL-27 ICOS	CD4+ CD49b+ LAG-3+	
Th22	**IL-22** IL-13 TNFα IL10 IL-21 MCP-3 MIP-1	AhR Batf STAT3	IL-6 TNFα	CD4+ CCR4+ CCR6+ CCR10+ PDGFR+	STAT1

*Text in bold represents **signature cytokines** or **master transcription factors**

1.4.4 CD4 T_H CELLS IN IBD

Inflammatory bowel disease is a chronic inflammatory disorder of the gastrointestinal (GI) tract. Its aetiology is unknown, but there are multiple factors contributing to its pathogenesis, including genetic, environmental, and immunological interactions. $CD4^+$ T_H cells are believed to be major initiators in the disease process, with enriched populations of these cells found in inflammatory lesions from IBD patients[201, 202]. Furthermore, as mentioned above, therapeutic blockade of these cells as well targeted blockade of $CD4^+$ differentiating cytokines can be an effective therapeutic approach[78, 201-204].

1.4.4.1 T_H1 CELLS IN IBD

IBD is commonly subcategorised into CD or UC, with each exhibiting a distinct $CD4^+$ lineage profile involved in their pathogenesis. T_H1 cells are commonly associated with CD, with accumulations of these cells, and elevated expression of their signature cytokine, IFNγ, found in the GI tract of CD patients.

The pathogenicity of T_H1 cells in IBD has been demonstrated with numerous functional studies demonstrating the necessity of IFNγ to initiate disease in multiple pre-clinical models of colitis, including $CD45R\beta^{hi}$ T cell transfer[205] and DSS[206] induced colitis. In these studies, IFNγ appears to be promoting inflammation via disruption of the mucosal epithelium, reduced epithelial cell proliferation, enhanced chemotactic signalling, and subsequent inflammatory cell infiltration of the intestine[120, 205, 206]. Accordingly, Prowrie *et al*., demonstrated that development of T cell induced colitis can be attenuated with the use of an anti-IFNγ mAb, and disease could be completely abrogated with in mice also treated systemically with the regulatory cytokine, IL-10[205].

However, in opposition to these findings, some studies have described a protective function for the cytokine at the mucosa. Muzaki *et al*. showed that $IFN\gamma^{-/-}$ mice were more susceptible to disease development in the DSS model[207], while Simpson *et al*., found that IFNγ was not necessary to induce disease in a T cell transfer model[208], and Camoglio *et al*. saw no difference in disease between *wt* and $Ifn\gamma r1^{-/-}$ mice in 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis[209]. These discrepancies in the functional role of IFNγ in IBD indicate that there are alternate factors involved that may compensate for the loss of IFNγ in some models, or may perhaps be attributed to the different strains of mice used and their diversity in gut microbiome across institutes.

Despite this reported dichotomy, human GWAS studies have implicated a clear role for IFNγ in the pathogenesis of IBD. Both Silverberg *et al*.[210] and Anderson *et al*.[211] report several SNPs in IBD-associated risk areas comprised of several regions up and downstream of the human *IFNG* gene (Table 1.2). Furthermore, the IBD associated rs1861494 SNP,

which is located in the *IFNG* gene, has been functionally associated with enhanced IFNγ expression in IBD patients. It has also been linked with a more aggressive disease and enhanced expression of serological markers such as ANCA autoantibodies, a biomarker associated with treatment resistance to anti-TNF therapy in IBD patients[212]. In addition, there has also been numerous SNPs reported in the *IL12B* gene[211, 213-218], which codes the IL-12 cytokine key in T_H1 differentiation, in the *STAT4* gene, an important T_H1 transcription factor[219-220], and in the *TNFA* gene, which codes the cytokine TNFα commonly produced by T_H1 cells[221-224].

Similar to IFNγ, the T_H1 associated cytokine, TNF, has been shown to have pleiotropic roles in murine models of disease. Various studies report protection from the onset of colitis when mice are deficient in TNF signalling or the pathway is blocked[225, 226]. On the other hand, there are also reports that absence of TNF signalling fails to protect from disease in oxazolone model of colitis[225], and actually exacerbates disease in DSS induced colitis[227, 228] Regardless of these contrasting findings, anti-TNF therapy is already used in the clinic for the treatment of patients with CD and UC, with proven significant benefits for many. However, it is notable that in patients that achieve complete clinical remission of IBD following anti-TNF treatment, only 24-50% exhibit mucosal wound healing[78, 79, 203, 229, 230] leaving the patients open to relapse after ceasing the anti-TNF therapy.

Interestingly, dysregulation of the T_H1 associated transcription factor STAT4, has also been associated with T_H1 driven IBD. In mice, overexpression of the *Stat4* gene leads to the development of an IBD like disease following administration of dinitrophenyl-conjugated keyhole limpet hemocyanin[231], whereas deficiency of *Stat4* inhibits onset of IBD in numerous models[208, 232, 233].

In spite of the reported dichotomy of T_H1 in the pathogenesis of IBD in preclinical models, the success of anti-TNF therapy and strong associations of T_H1 associated SNPs in IBD patients indicates a clear role for T_H1 cells in the pathogenesis of IBD, and inhibiting their polarisation, expansion and/or migration to the intestines represents a potential target for therapy.

1.4.4.2 T_H2 Cells in IBD

While T_H1 cells are classically associated with CD, T_H2 cells are associated with the pathogenesis of UC. Unlike IFNγ in CD, the T_H2 signature cytokine, IL-4, is not overly expressed in UC patients[234, 235], and SNPS identified in the *IL-4* gene locus associated with IBD are believed to be loss of function mutations[236], suggesting a protective role for the cytokine in the pathogenesis of the disease. Accordingly, functional studies have revealed that IL-4 synergizes with IL-10 to inhibit proinflammatory $TNF\alpha^+$ and $IL\text{-}1\beta^+$ cells in PBMCs isolated

from IBD pateints[237]. However, treatment of colitogenic mice with an anti-IL-4 antibody prevents disease[238, 239], thereby suggesting there may be pleiotropic roles for this cytokine in the intestines.

In contrast IL-13, another cytokine that is highly produced by T_H2 cells, appears to have a much clearer role in IBD. Several studies have shown that IL-13 is derived from numerous cellular sources in the mucosa, including $CD4^+$ T cells, NK cells, ILCs, and LP mononuclear cells (LPMC)[115, 240-243], and promotes the pathogenesis of colitis via disruption to the epithelial barrier[115], as well as induction of fibrosis in TNBS induced colitis[244, 245]. Despite such findings highlighting the pathogenic role of IL-13 in the intestines, IL-13-specific antibodies (anrukinzumab and tralokinumab) have already been trialled and did not achieve significant clinical response in UC patients[246, 247], although Tralokinumab did show some improvement in clinical remission rates and mucosal healing[247].

Furthermore, STAT6, which is activated by IL-4 and IL-13, was also found to be upregulated in UC patients[248, 249], and has been shown to be pathogenic in mouse models of colitis[250, 251]. In turn, STAT6 can activate GATA3, the T_H2 master regulator of transcription. Accordingly, GATA3 expression is also elevated in IBD, and is found to be expressed at higher levels in the colonic tissues of UC patients compared to CD patients[252], endorsing the dichotomy of T_H cell profiles between IBD sub groups. Fittingly, these patients also exhibited higher levels of $CD4^+GATA3^+$ cells, and expression of *GATA3* correlated with disease severity[252]. Overexpression of *GATA3* has been shown to exacerbate disease in DSS and oxazolone induced colitis[252, 253], and its deficiency prevented disease onset[252]. Furthermore, treatment with a GATA3 specific DNAzyme, hgd40 showed a significant decrease in disease severity DSS and oxazolone induced colitis[252], and has shown success in a phase IIa trial with UC patients exhibiting clinical and endoscopic improvement[254].

IL-5, another T_H2 associated cytokine is upregulated in UC patients when compared to CD patients or healthy controls[255]. Yet functional studies reveal no impact on disease severity when IL-5 is genetically deleted in DSS induced colitis. Stevceva *et al*., reported that while there may be a reduction in eosinophilia in *Il-5*$^-$ mice, this has no impact on disease progression, and indicates that IL-5s main role in colitis is to induce the infiltration of eosinophils[256]. However, Kobayashi *et al*. [257], indicate that this infiltration of eosinophils may in fact be beneficial in colitis, as IL-5 transgenic mice show a marked increase in eosinophil infiltration, less pronounced pathologic features of disease, and lower levels of pro-inflammatory IL-1β and TNFα. Therefore, IL-5 may have a pro-homeostatic role during colitis, and serve to naturally regulate T_H2 induced autoimmunity.

1.4.4.3 T_H9 CELLS IN IBD

As originally believed to be a subset of T_H2 cells, T_H9 cells were initially associated with the T_H2 like inflammation observed in UC colitis[258, 259]. Accordingly elevated levels of IL-9[258, 259] and IL-13 were reported in these patients[259], alongside lower levels of IL-4[234, 235], as just described above. However, in recent times elevated levels of IL-9 and its signature transcription factor PU.1 have been reported in both UC and CD cohorts[260, 261]. In agreement, both mouse models of UC, such as, DSS[260] and oxalazone induced colitis[262] and CD, such as $CD45R\beta^{hi}$ T cell transfer[165] and TNBS induced colitis[263], report a pathogenic role for the cytokine in the pathogenesis of disease, with disruption in the intestinal epithelium and enhanced pro-inflammatory T cell responses reported. Fittingly, deficiency in IL-9 signalling or treatment with a neutralizing antibody has been shown to alleviate disease[260-263]. Interestingly, in healthy individuals, circulating IL-9 was positively correlated with inflammatory cytokines, IL-1β and IL-6, and IL-13, the T_H2-associated cytokine, in univariate analysis. What's more, a correlation of IL-9 with IL-13 was observed exclusively in CD[259], indicating there may be some type 2 responses being induced in a classically type 1 disease. Furthermore, in both CD and UC patient's, higher levels of IL-9 cytokine correlates with a worse prognosis, increased incidence of cachexia and lower haemoglobin concentrations[259, 264].

1.4.4.4 T_H17 CELLS IN IBD

The discovery of T_H17 cells provided a challenge to the T_H1/T_H2 CD/UC paradigm[265], as elevated amounts of the T_H17 signature cytokine, IL-17a have been reported in both patient cohorts. Enhanced amounts of IL-17a, and IL-17a$^+$ T_H cells were found in the serum of CD patients[266], and intestinal biopsies from UC and CD patients with active disease exhibited increased numbers of IL-17a$^+$ cells, compared to controls or patients with inactive IBD[267]. However, whether these cells serve a pathogenic or regulatory purpose in the context of IBD is still under investigation.

While some studies found IL-17 signalling necessary to induce disease in pre-clinical models of colitis, such as TNBS[81], other authors report IL-17 signalling critical in maintaining intestinal homeostasis, in models such as DSS[83, 268] and T cell transfer[269]. In these studies IL-17 exhibited prominent roles in maintaining epithlial barrier functioning, regulating the microbiota[83], and inhibiting the development of Type-1 responses[269]. In keeping with these findings, while the IL-17 blocking monoclonal antibodies, Secukinumab and Brodalumab, were effective in treating arthritis and psoriasis, they exacerbated disease among some IBD patients, resulting the premature termination of two clinical trials[270, 271].

However, alternate therapies inhibiting T_H17 cells in IBD have been immensely successful. Targeting the shared p40 or p19 subunits of IL-23, has proven efficacious in both mouse[272-274] and human CD studies[69, 275, 276].

TABLE 1.2: SNPS ASSOCIATED WITH CD4+ DEFINING GENES AND IBD

Lineage	Gene	SNP	Disease
Th1	IFNγ	rs1861494	IBD
		rs7134599	UC
		rs11614178	IBD
	TNFα	rs1799964	CD
		rs1800629	IBD
		rs1799724	CD
	IL12B	rs6556412	IBD
		rs10045431	IBD
		rs6871626	IBD
		rs2288831	CD
		rs6887695	IBD
		rs3212227	IBD
		rs56167332	CD
	IL12RB2	rs11581607	IBD
	STAT4	rs925847	UC
		rs7574865	UC
Th17	STAT3	rs12948909	UC
		rs744166	IBD
		rs12942547	CD
	JAK2	rs10758669	IBD
		rs75900472	IBD
	CCR6	rs1819333	IBD
	IL-23R	rs11805303	IBD
		rs7517847	CD
		rs1004819	CD
		rs11209026	IBD
		rs11465804	CD UC
iTreg/Tr1	IL-2	rs7657746	IBD
	IL-2RA	rs12722515	IBD
	IL-10	rs3024505	IBD
	SMAD7	rs7240004	IBD
	SMAD3	rs17293632	IBD
	STAT5	rs12942547	IBD
Th2/Th9	STAT6	rs324015	CD
	IL-4	rs2243250	IBD
		rs2243248	IBD
Th22	AhR	rs1077773	IBD

Interestingly, in CD45RβHiT cell colitis, although protected from intestinal pathology, IL-23p19 deficient mice show little difference in the weight loss that is characteristic with murine models of colitis[272]. Similarly, mice deficient in IL-12p40 mirrored these effects, exhibiting minimal intestinal pathology but reduced weightloss in anti-CD40 induced colitis[277]. These studies suggest that in the setting of colitis weightloss and intestinal pathology can be independently regulated, and illustrate the complexities in targeting the disease therapeutically.

In addition, IL-23 has also been shown to have a protective role in IBD. Using DSS, TNBS and *C. rodentium* models of colitis, mice deficient in IL-23 or IL-23p19 signalling exhibit enhanced disease, and an increase in IFNγ expression[278, 279]. However, disease could be attenuated with the use of IL-12 or IFNγ neutralizing antibodies, suggesting a compensatory role for T_H1 responses in the absence of T_H17 cells.

Furthermore, the T_H17 associated TF *STAT3* is elevated in IBD patients, with its expression correlating with disease severity[280, 281]. Using the T cell transfer model of colitis Durant *et al*. demonstrated that *Rag*$^{-/-}$ mice reconstituted with STAT3-deficient T cells were protected from the development of colitis. These mice were unable to differentiate the transferred T cells into IL-17^{+} T_H17 cells, thus allowing the expansion of Tregs in the intestine, which in turn regulated

the IFNγ^{+} T_H1 population to inhibit inflammation and maintain intestinal homeostasis[282]. A *STAT3* SNP, rs744166, has also been found to be associated with enhanced susceptibility and severity to CD and UC[283, 284].

1.4.4.5 T_H22 CELLS IN IBD

In keeping with the dichotomy reported for many of the other T_H cell lineages, T_H22 cell possess both a protective and pathogenic role in IBD. Functional studies using CD35Rβ^{hi} T cell transfer colitis demonstrated the capacity of IL-22 to supress T cell mediated colitis via STAT3 signalling using a microinjection-based local gene-delivery system[285]. Accordingly, overexpression of IL-22bp exacerbated disease during the recovery phase of DSS induced colitis[285].

Similarly, other authors report in both DSS and T cell transfer colitis models, deficiency in IL-22 signalling enhanced disease, promoting weightloss and enhanced colonic inflammation[285-288]. Furthermore, these IL-22 deficient mice are more susceptible to *C. rodentium* induced colitis[289, 290], and this is further enhanced when *Stat3* is knocked out specifically in T cells[290]. Fittingly these mice can be rescued when IL-22 signalling is reintroduced[289, 290], and the intestinal inflammation observed in this model is reportedly due to IL-22 mediated epithelial disruption, AMP dysregulation and an increased bacterial burden in the intestines[291].

Therefore, it is perhaps unsurprising that IL-22 expression is enhanced in patients with IBD[288, 292], perhaps functioning to naturally counteract the inflammation in the intestines. Opposingly, expression of IL-22bp is also enhanced in both CD and UC patients compared to healthy controls, and CD4^{+} T_H cells derived from these patients produce elevated levels of the protein[288], indicating why the elevated levels of IL-22 found in these patients may not be eliciting mucosal homeostasis.

Additionally, expression of Aryl Hydrocarbon Receptor (AhR), a TF critical for T_H22 differentiation, is also enhanced in IBD patients[289, 292]. In murine models of colitis, AhR deficiency, like IL-22 deficiency, leads to a more severe disease[285-287, 292, 293], and colitis induced in *wt Ahr*$^{+}$ mice can be attenuated with AhR agonist 6-formylindolo(3, 2-b)carbazole (FICZ), a tryptophan derivative[292]. In the mucosa tryptophan derivatives have been shown to alter the microbiome, allowing the expansion of *Lactobacilli* species which activate AhR to promote IL-22 transcription[294]. Collectively, these studies propose a role for T_H22 cells in the pathogenesis of IBD, with dichotomous roles reported for the ligands, while IL-22 is predominantly associated with promoting homeostasis and IL-22bp promotes inflammation.

1.4.5 CD4+ T_H CELL TRAFFICKING

The inflammatory response is a double-edged sword, while necessary to fight pathogens, parasites, and heal injury, most pathology involves aberrations in the inflammatory process. This includes inflammation that is self-directed, can occur in the wrong place at the wrong time, and frequently persists too long, resulting in the development of chronicity. The physical manifestation and damage observed in IBD is due to an inappropriate T cell response at the mucosal surface. The processes involved in the trafficking of these cells to the gut provide an attractive target for therapeutics in T_H cell mediated disease.

1.4.5.1 LEUKOCYTE EXTRAVASATION

The migration of leukocytes to the site of injury or infection is crucial in maintaining tissue homeostasis across the entire body and occurs through an elaborate process called extravasation. This involves the intricately regulated movement of leukocytes out of the circulatory system through a series of adhesion events through the endothelium to a specific site of injury[295]. In the steady state, leukocytes travel passively along the bloodstream in the central stream of the laminar flow of blood. However, at sites of inflammation or injury, in the post capillary high endothelial venules (HEV) there is a change in the hemodynamics resulting in a reduced rate of blood flow. This increases the chance of the leukocytes contacting the endothelial cells that line the vessel. To migrate through the endothelium, the leukocytes must express leukocyte adhesion molecules (LAM), such as L-selectin and very late antigen 4 (VLA-4), and encounter their counteracting LAM receptors, s-Le^x (sialyl-Lewis x antigen) and vascular cell adhesion molecule 1 (VCAM-1) respectively, that have been induced on the surface of the endothelial cells at the site of injury. Once a loose binding has occurred, the leukocytes begins a process of "rolling" along the endothelial wall. This rolling is facilitated by P-selectin binding with its ligand, PSGL-1, and the interaction between E-selectin and ESL-1, which begins to slow down the rolling of the leukocyte by inducing partial expression of integrin's on its surface[296, 297]. The purpose of this is to allow the leukocyte time to encounter chemokines and other inflammatory molecules present on the endothelial cell surface. Once the chemokine binds with its associated chemokine receptor (CCR) on the leukocyte, this induces full expression of leukocyte integrins and facilitates adhesion to the endothelium[298-300]. Leukocytes express specific integrin family members, and lymphocytes express B1 integrins, VLA-4 and $\alpha 4\beta 1$ integrin, which binds to VCAM-1[301-303]. The now adherent leukocyte "crawls" to the endothelial border and begins transendothelial migration (TEM), a process known as diapedesis. During this process, the leukocyte squeezes through the borders of the endothelial cells in an amoeboid fashion[298, 304].

Transmigration of the leukocyte through sub endothelial basement membrane, pericytes, and interstitial tissue involves homophilic interactions of Platelet/endothelial cell adhesion molecule 1 (PECAM-1) and macrophage-1 antigen (Mac-1)[298, 305-307].

In the intestines, $CD4^+$ T_H cells are recruited from the blood via high endothelial venules which highly express mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1)[308, 309]. MAdCAM-1 is the corresponding receptor for the $\alpha_4\beta_7$ integrin, which is induced on T cells following antigen presentation by $CD103^+$ DCs in the mLNs[6, 310-312]. MAdCAM-1 also contains a mucin domain, comprised of functionally specialized ligands for L-selectin (CD62L)[313]. $\alpha_4\beta_7$ expression directs $CD4^+$ T_H cells specific for gut antigens back to the intestines and GALT. The small intestinal epithelium expresses the chemokine CCL25, which interacts with CCR9 on $CD4^+$ T_H cells. Co-expression of $\alpha_4\beta_{7\text{ and}}$ CCR9 recruits $CD4^+$ T_H cells into the small intestine epithelium[6, 7, 314] (Fig.1.4.3.).

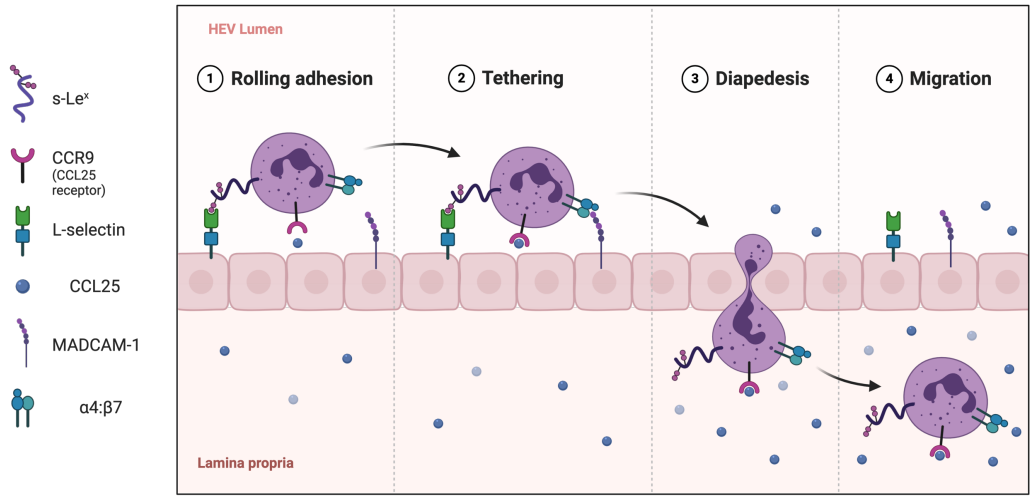

Figure 1.4.3.: CD4+ T cell Gut Homing. Localization of T cell to the intestines is mediated by expression of α4β7 and CCR9 on the T cell membrane. These gut homing molecules interact with their counterparts, MAdCAM-1, on HEV in the intestinal vasculature, and with CCL25 on the epithelial surface, and these interaction facilitate T cell gut homing. *Created with biorender.com.*

While the trafficking of lymphocytes to the intestines is of clear significance in in the pathology of IBD, how pro-inflammatory signals in the intestinal microenvironment influence these migratory processes is unclear. However, there is some evidence suggesting that innate cytokines can affect lymphocyte gut tropism, with reports that TNFα can induce expression of MAdCAM in the intestine, colon and MLN[315]. Furthermore, pro-inflammatory members of the IL-1 cytokine family have demonstrated roles in lymphocyte trafficking, with IL-1β inducing the mucosal addressin MAdCAM in the intestinal endothelial cells[315], IL-18 inducing the intercellular adhesion molecule ICAM-1 on monocytes[316], and IL-33 inducing

expression of ICAM-1 and vascular cell adhesion molecule, VCAM-1, on coronary artery and umbilical vein endothelial cells[317]. Collectively, these studies indicate that proinflammatory signals, in particular from IL-1 family cytokines in the microenvironment, may not just influence polarisation of the distinct $CD4^+$ T_H cell subsets described above, but also influence their trafficking to the site of inflammation as well.

1.5 THE IL-1 SUPERFAMILY OF CYTOKINES: INNATE MEDIATORS OF THE ADAPTIVE RESPONSE

Proinflammatory cytokines have an established role at the interface between innate and adaptive inflammation and are integral mediators of GI inflammation with a significant impact on the pathogenesis of IBD. The IL-1 cytokine family in particular has been reported to play key and often dichotomous roles in this setting. The IL-1 family contains 11 immunomodulatory cytokines, comprising of eight agonistic ligands (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, and IL-37) and three natural antagonists (IL-1Ra, IL-36Ra, and IL-38)[318]. In humans, the genes encoding these cytokines are located on chromosome two, with the exception of IL-18 and IL-33, which are mapped on chromosome 11 and 9 respectively[319].

These cytokines elicit their effects through four specific heterodimeric receptor complexes, with each receptor consisting of an intracellular TIR domain, a transmembrane helix and an extracellular ligand specific binding domain (IL-1R1, IL-33R (ST2), IL-18Rα and IL-36R). Recruitment of an accessory protein is necessary to initiate signal transduction. Upon appropriate ligand interaction with either the IL-1R1, the IL-33R or the IL-36R, the IL-1R accessory protein (IL-1RAcP) is recruited, whereas in the case of IL-18, the IL -18Rβ chain is recruited to form the IL-18R. Dimerization of these receptor complexes initiates nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pro-inflammatory signalling cascades[318] (Fig.1.5.1).

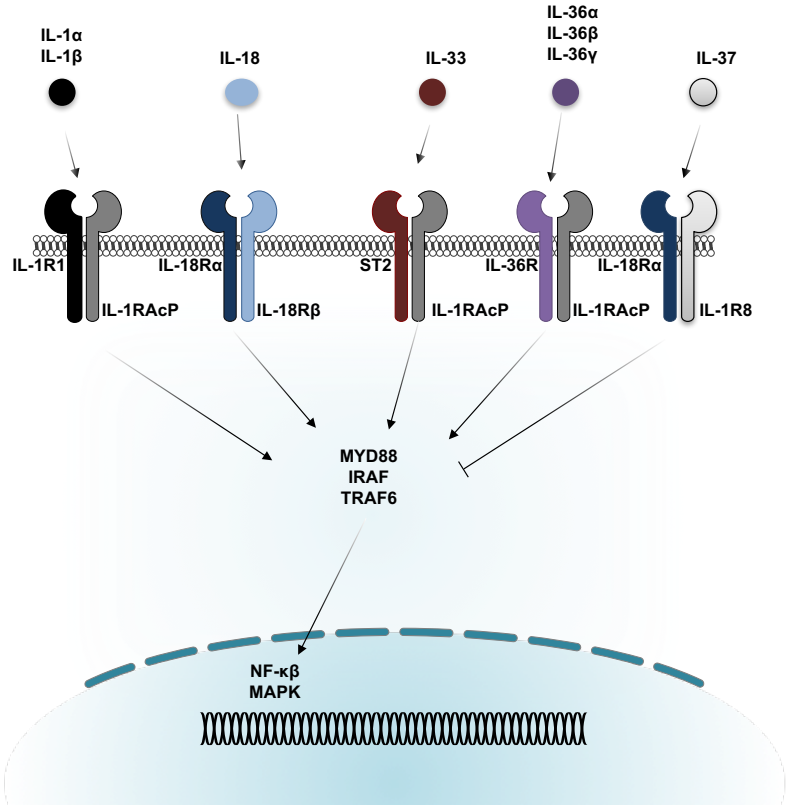

FIGURE 1.5.1: Members of the IL-1 family of cytokines and their cognate receptors. IL-1, IL-33 and IL-36 cytokines initiate signalling by binding to the IL-1R, ST2 and IL-36R respectively, which, upon ligand binding, form heterodimeric receptors with the IL-1RAcP. This results in the activation of MAPK and NFKB signalling, and subsequent pro-inflammatory gene expression, via MyD88, IRAF and TRAF6 dependent signalling mechanisms (as denoted by the arrows). Similarly, IL-18 activates these same pathways through the formation of an IL-18Rα and IL-18Rβ heterodimeric receptor. In contrast, IL-37 initiates an immunosuppressive signalling programme through interacting with IL-18Rα in association with IL-1R8 (SIGIRR). Figure taken from Hernandez-Santana, Y. E., Giannoudaki, E., Leon, G., Lucitt, M. B., & Walsh, P. T. (2019). Current perspectives on the interleukin-1 family as targets for inflammatory disease. *European journal of immunology*, 49(9), 1306-1320.

Due to the central role of IL-1 family member signalling in inflammation, a number of distinct mechanisms have evolved to regulate their activity. These include receptor antagonists, decoy receptors, and neutralizing binding proteins[318, 320] (Fig.1.5.2). The IL-1Ra and IL-36Ra, alongside IL-38, preferentially bind the IL-1R and IL-36R respectively and this action disrupts the recruitment of the IL-1RAcP, thereby preventing receptor dimerization and downstream signal transduction[320]. The decoy receptor, IL-1R2, lacks the intracellular TIR domain required for signal transduction, and so it acts as a molecular sink for both IL-1α and IL-1β cytokines. Interestingly, IL-1R2 also exists in a soluble form and can exert these neutralizing effects in a cell extrinsic manner, similar to the mechanisms through which soluble IL-33R (ST2) and IL-18 binding protein (IL-18bp) have been described to neutralize their respective cytokines signalling[318, 320, 321].

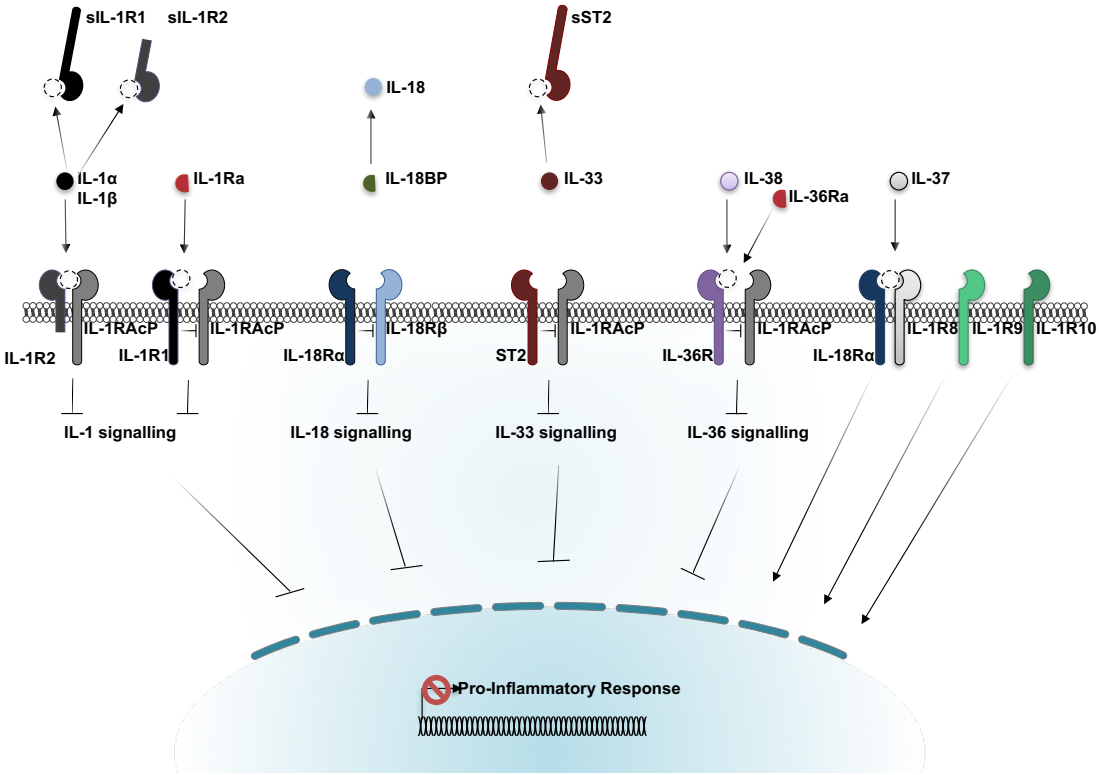

Figure 1.5.2.: Negative Regulation of IL-1 family signalling. (From left to right) Specific mechanisms have evolved to regulate proinflammatory signalling by IL-1 family cytokines and their receptors. These include inhibition of IL-1α/β through neutralisation with soluble forms of the IL-1R (sIL-1R1/2), specific inhibition of the IL-1R complex through association with cell surface expressed IL-1R2, or direct antagonism through excess IL-1Ra expression. IL-18 activity is regulated through activity of the IL-18 binding protein (IL-18BP) which preferentially binds free IL-18 restricting its ability to interact with the IL-18R. Similarly, IL-33 bioavailability and activity is limited by the expression of the soluble form of its receptor ST2 (sST2). Initiation of IL-36 dependent signalling is regulated through competitive binding to the IL-36R of the specific IL-36Ra and also through the activity of IL-38. Uniquely, IL-37 acts to restrict proinflammatory responses through engagement with the IL-18Rα chain, utilizing IL-1R8 as a co-receptor. IL-1R9 and IL-1R10 are more recently described IL-1 family receptors which are also thought to exert a negative regulatory/ inhibitory function. Figure taken from Hernandez-Santana, Y. E., Giannoudaki, E., Leon, G., Lucitt, M. B., & Walsh, P. T. (2019). Current perspectives on the interleukin-1 family as targets for inflammatory disease. *European journal of immunology*, 49(9), 1306-1320.

1.5.1 THE ROLE OF THE IL-1 FAMILY IN IBD

Given the potent ability of IL-1 family members to drive inflammation, it is not surprising that many members of this family have been implicated as being involved in gastrointestinal inflammation, and in particular with the pathogenesis of IBD. However, for many IL-1 family members a more complex picture has emerged with often conflicting mechanistic roles as both mediators of inflammation as well as homeostasis and resolution described.

The prototypical family members, IL-1α and IL-1β have been described to play a pathogenic role in the context of IBD[322, 323]. Accordingly, elevated levels of monocyte derived IL-1β have been reported in IBD patients[322,324,325,326] with disease severity correlating with the increased IL-1 levels found in inflamed tissue biopsies[327]. Furthermore, deletion of IL-1β was shown to confer protection in the TNBS model of colitis[328]. While such reports indicate a pathogenic role for IL-1 in the intestine a more complex picture is beginning to emerge which demonstrates that IL-1 can also play critical, context dependent, roles in GI homeostasis. For example, a recent study by Zhou *et al*. described the necessity for constitutive expression of IL-1β in the steady state to facilitate Treg generation via ILC3 mediated IL-2 production[329]. In addition, specific deletion of IL-1 signalling in ILC3 cells causes disruption in GI homeostasis, oral tolerance to dietary antigens, and Treg maintenance, while ILC3 mediated IL-2 production was found to be significantly reduced in Crohns disease patients[329]. These studies highlight the importance of a balanced and tightly regulated IL-1 signalling axis in the intestinal mucosa to facilitate gut homeostasis and allude to the intricacies involved in targeting cytokines as a potential therapy. Whilst inhibition of IL-1 signalling has proven effective in other inflammatory conditions[330], there has been little success therapeutically targeting IL-1 in IBD.

Similar to IL-1α, IL-18 is also expressed constitutively in the gut mucosa indicating a potential role in GI homeostasis[331]. IL-18 is generally regarded as a pro-inflammatory mediator, enhancing the production of IFNγ, stimulating the differentiation of T_H1 cells[332] and priming NK cell cytotoxicity[333]. Interestingly, IL-18 activity can be regulated through cleavage of pro-IL-18 mediated by the NLRP6 and NLRP3 inflammasomes, which are known regulators of colonic homeostasis[334, 335]. NLRP6 is predominantly expressed in intestinal epithelial cells (IECs) and promotes mucosal renewal, mucus secretion and proliferation[335], and IEC derived IL-18, mediated by the NLRP6-inflammasome, has been shown to prevent gut colonization by colitogenic microbiota[336]. Recent studies have shown that deficiency in NLRP3 exacerbated chemical induced colitis. This increased severity in disease occurred in association with insufficient amounts of IL-18 in the mucosa, and administration of recombinant IL-18 (rIL-18) induced protection in this model via enhanced barrier repair and epithelial regeneration[334]. In addition, IL-18 has also been reported to regulate the adaptive inflammatory response in the GI tract by orchestrating the balance between pro-

inflammatory T_H17 and anti-inflammatory Treg differentiation in the colon[337]. In this context, IEC derived IL-18 promotes the generation of Tregs, whilst synergistically inhibiting the generation of T_H17 cells, thus maintaining colonic homeostasis in the steady state[337].

On the other hand, increased levels of IL-18 have been observed in patients with CD[338,339], correlating with studies highlighting the role of IL-18 in the breakdown of the mucosal barrier and amplifying epithelial inflammation during disease. Specific deletion of IL-18 or its receptor, IL-18R in IECs, has conferred protection in DSS colitis[340], and this protection is further enhanced in a IL-18/IL-1β double knockout model[328]. Furthermore, *Il-18bp*$^{-/-}$ mice exhibit significantly exacerbated disease, alongside a reduction in mature goblet cells. This phenotype is reversed in the absence of IL-18R expression on IECs, suggesting a pathogenic role for IL-18R signalling in the colonic epithelium. Further mechanistic studies demonstrated a role for IL-18 in inhibiting goblet cell development and maturation, thereby promoting mucosal barrier dysfunction, which is a common characteristic of UC[340]. It is also noteworthy that genome-wide association studies (GWAS) have linked mutations in genes involved in the IL-18 signalling pathway with increased IBD susceptibility[217,341], enhancing the rationale for targeting the IL-18 pathway therapeutically.

IL-33 is generally regarded as a mucosal alarmin, typically involved in type 2 immune responses, which can play important roles in innate immunity in the intestine[342]. While IL-33 is constitutively expressed in the epithelium and endothelium, its receptor ST2 (IL-33R) is primarily expressed on immune cells, including T cell and ILC subsets. This pattern of expression is indicative of the key role IL-33 plays in the orchestration of immune responses at mucosal surfaces[321]. Elevated levels of IL-33 have been observed in the colon and serum of ulcerative colitis (UC) patients in which a T_H2 like inflammatory profile is commonly observed[343,344,345,346]. Furthermore, when mesenteric lymph node $CD4^+T_H$ cells from piroxicam-accelerated colitis (PAC) *Il-10*$^{-/-}$ mice were challenged with rIL-33, T_H2 associated cytokines were produced[344]. While these data indicate a proinflammatory role for IL-33 in the pathogenesis of UC, pre-clinical studies have also revealed a complex and dichotomous role for this cytokine often with conflicting results. On one hand, various studies have shown that *Il-33*$^{-/-}$ mice are highly susceptible to the development of TNBS and DSS induced colitis[347] and administration of rIL-33 attenuates disease[348,349,350]. In contrast, using the same model, other groups have observed opposing results[351,352,346]. Sedholm *et al*. observed elevated levels of IL-33 in both DSS and TNBS induced colitis, and in this case administration of rIL-33 increased disease severity whilst *St2*$^{-/-}$ mice were somewhat protected[346]. Furthermore, investigations using senescence accelerated (SAMP) mice, which develop an enteric UC like disease, revealed that IEC derived IL-33 correlates with disease severity, and that blockade of this pathway alleviates disease[350,353]. A recent study by Groβ *et al*. provided an interesting insight

into complexity of IL-33 signalling in IBD, while offering a potential explanation for conflicting data described above. In these studies an exacerbation of disease was evident when rIL-33 is administered during the acute phase of DSS induced colitis, whereas prolonged rIL-33 administration was found to lead to enhanced recovery from disease, and ameliorated intestinal inflammation in the chronic phase[354]. These results suggest distinct temporal roles for IL-33 signalling in disease. With such complex and dichotomous roles, the precise impact of IL-33 on the development and progression of IBD is still unclear and requires further study before it can begin to be assessed therapeutically.

IL-37 is a unique anti-inflammatory member of the IL-1 family. Expression of this cytokine has been found to be elevated in the sera of both UC and CD patients, and higher levels of IL-37 producing cells have also been found in inflamed colon tissue biopsies in CD [355, 356]. In paediatric patients, elevated levels of this cytokine correlate with disease severity[357], and humanized transgenic mouse studies have demonstrated that IL-37 can act to dampen mucosal inflammation and protect against DSS induced colitis[358].

IL-37 is thought to signal through a IL-18Rα/IL-1R8 (SIGIRR) complex, and may play a role in SIGIRR mediated anti-inflammatory effects[359] (Fig.1.5.2). SIGIRR itself is expressed in the IECs of the human colon, and its expression is highest in inactive vs active disease cohorts of IBD patients. Accordingly, SIGIRR expression in murine IECs decreases rapidly following the induction of colitis, which was revealed to be due to an inhibition of SP1 signalling[360]. Furthermore, *Sigirr*$^{-/-}$ mice develop exaggerated colitis following *C. rodentium* infection[361], highlighting the importance of negative regulation of IL-1 signaling to maintain GI homeostasis.

1.6 THE IL-36 FAMILY OF CYTOKINES

IL-36 is a relatively novel member of the IL-1 cytokine superfamily. It was initially discovered two decades ago when DNA database screens identified 4 new members of the IL-1 family based on their homology at both gene and protein levels[362, 363]. They were originally designated names based on their order of discovery, from IL-1F5 to IL-1F9, but were later reclassified as IL-36Ra (IL-1F5), IL-36α (IL-1F6), IL-36β (IL-1F8) and IL-36γ (IL-1F9) and are collectively known as the IL-36 subfamily of cytokines. As mentioned above, the IL-36 family is composed of 3 agonistic ligands (IL-36α, IL-36β and IL-36γ) and one specific receptor antagonist (IL-36Ra)[362, 363].

Like other IL-1 family members, such as IL-1β[364], IL-36 agonists are generated in a biologically inactive "pro" form, that require proteolytic processing to enable their pro-inflammatory activity[365]. Truncation of their n-termini results in over a 1,000 fold increase in their activity[365], and Henry *et al*. recently reported that this cleavage is due to neutrophil granule derived proteases. IL-36α, IL-36β, and IL-36γ are activated differentially by cathepsin G, elastase, and proteinase-3[366]. Elastase has also been shown to activate IL-36Ra, facilitating its anti-inflammatory effects[367]. IL-36 ligands signal by first binding to the IL-36R; this induces the IL-1RAcP to act as a co-receptor, and the dimerization of this IL-36R/IL-1RAcP complex facilitates downstream proinflammatory signal transduction. Regulation of IL-36 family signalling is mediated by both the IL-36Ra, and possibly IL-38, which preferentially bind the IL-36R and inhibit IL-1RAcP recruitment, thereby preventing activation of IL-36 mediated signalling cascades[365].

IL-36 ligands are expressed by epithelial cells in several tissues of the body, including the oesophagus, tonsils, skin, brain and gut, and also possess the potential to act on the epithelium in turn, as well as cells of both the innate and adaptive branches of the immune system[368-370].In the skin, IL-36α and IL-36γ are noticeably up-regulated in psoriatic lesions[368-370], in which they have been found to be produced predominantly by keratinocytes, but also by fibroblasts, monocytes, macrophages and dendritic cells, although to a lesser extent[370-372]. In these lesions, they can act on IL-36R expressing keratinocytes, endothelial cells, and/or Langerhans cells, and induce the release of pro-inflammatory chemokines and cytokines. This leads to the recruitment of immune cells, mainly neutrophils, that induce the pathology we associate with the disease [373-378]. Recently, Muller *et al*., found that IL-36 elicits these psoriatic effects via NF-ϰB and STAT3 activation of the transcriptional regulator, IϰBζ. Induction of IϰBζ by IL-36 was found to be necessary for the expression of a number of psoriasis-related genes, with roles in neutrophil chemotaxis, leukocyte activation and inflammatory signalling. Furthermore, expression of this transcription factor regulator was

required for the induction of IL-36 mediated dermatitis, with *IκBζ*$^{-/-}$ mice exhibiting reduced immune cell infiltration and downregulated expression of pro-inflammatory genes[379].

Similarly in the lung, IL-36γ is potently induced from bronchial epithelial cells and adjacent fibroblasts by a number of stimuli, including bacteria, rhinovirus infection, smoke and cytokines[380-383]. Furthermore, expression of this cytokine is thought to contribute to bronchial dysfunction via enhanced neutrophil infiltration of the airways[383-385], which may impact the pathogenesis of numerous airway pathologies such as asthma, COPD and bronchial infections [381, 384-386].

In patients with neuro-myelitis optica spectrum disorders (NMOSD) IL-36β and IL-36γ are significantly upregulated in patients serum compared to healthy controls. Furthermore, expression of these ligands correlates with disease activity and may serve as a an important biomarker in these neuro inflammatory disorders[387]. However, in EAE, despite the presence of elevated levels of neutrophil derived IL-36γ, which was found to activate microglial cells, IL-36 expression was found to have no role in the pathogenesis of the disease[388].

All IL-36 cytokines have been identified in both humans and mice. Interestingly, their general chromosomal location and gene organisation is quite similar, sharing a considerable sequence homology between the species of 91% for IL-36Ra, 62% for IL-36β, 56% for IL-36γ and 54% IL-36α. This genomic conservation serves to highlight the importance of these cytokines between species[389].

1.6.1 IL-36 EXPRESSION IN THE INTESTINES

In recent years IL-36 family members have gained significant attention due to their dysregulation in inflammatory conditions, such as psoriasis[390], rheumatoid arthritis (RA)[391], osteoarthritis (OA)[392], SLE[393], and IBD[394], and have been shown to regulate both parenchymal and immune cell responses[262, 377, 394]. In the GI tract IL-36 cytokines are derived from various sources, such as the intestinal epithelium and parenchyma cells, as well as immune cells[395]. In a cross-sectional comparative study of adult IBD patients and healthy controls Fonseca-Camarillo *et al*., used gene and immunohistochemical analysis to determine expression of IL-36 family members in the GI tract. Using gene expression analysis, the authors reported differential expression of the different IL-36 members between cohorts, with *IL36A*, *IL36B*, *IL36G* and *IL36RN* expression significantly increased in the mucosa of patients with active UC compared to those with non-active UC, CD, and healthy control patients[395].

The authors went on to determine the cellular sources of the increased levels of IL-36 in the mucosa by using immunohistochemistry. They determined epithelial cells as the cell type responsible for producing IL-36Ra in the mucosa, whereas in the sub mucosa it appears that IL-36Ra is derived from perivascular mononuclear cells[395]. While the majority of IL-36α was detected in nonimmune cells, in cells of the gut epithelium and parenchyma, in the muscularis and serosa there was presence of IL-36α producing CD14+ macrophages and CD123+ plasmacytoid dendritic cells detected. Similarly, IL-36β was found to be produced from CD14+ macrophages in the muscularis and serosa, in addition to lymphocytes in submucosa, muscularis, and serosa. Interestingly, while there was detection of CD8α+ IL-36γ+ cells in IBD patients, there was none of these double positive cells found in the healthy patient cohort, and similar to other IL-36 agonists, IL-36γ was also found to be produced by plasmacytoid DCs[395]. Expression levels of IL-36α and IL-36γ in particular have been shown to be elevated in the inflamed intestinal mucosa of both mice and IBD patients and such increases appear to be driven by tissue damage and the intestinal microbiota (Fig.1.6.1)[262, 394, 396, 397].

These data contrast with other tissue sites where IL-36 cytokines have also been reported to be mediators of inflammation. For example, in psoriatic skin, IL-36β is produced by, and can act on keratinocytes, which express the IL-36R[374]. In addition, elevated levels of expression of IL-36γ are found in the serum of psoriasis patients and may represent a biomarker for disease severity[390, 391]. IL-36 family members are also highly expressed in the synovial tissues of patients with RA, OA and psoriatic arthritis (PsA)[391, 392]. In these tissues, CD138+ plasma cells are thought to be the main cellular source of IL-36α.

1.6.2 IL-36 AND IBD

Historically, research on immune involvement in IBD was starkly divided into groups focusing either on the innate arm or the adaptive arm of the immune response, and crosstalk between the two was generally ignored[398]. However, recent GWAS confirmed the relevance of already characterized pathways, and also revealed strong evidence for an association between innate and adaptive immunity in mucosal homeostasis[217]. As IL-36 cytokines have been described to mediate both innate and adaptive inflammation, they may play critical roles in this crosstalk and the sensitive balance of mucosal immunity in the intestine. (Fig.1.6.1). This possibility will be explored in the coming sections.

1.6.2.1 IL-36 AS A MEDIATOR OF INNATE INFLAMMATION IN THE INTESTINE.

Similar to other IL-1 family members, IL-36 cytokines have been shown to have a dichotomous role in regulating the innate immune response in mouse models of colitis. Recently, Russell *et al*. observed a significant increase in expression of IL-36α mRNA in the colonic mucosa of UC patients. The authors confirmed this elevated expression at a protein level using immunohistochemistry, and determined intestinal epithelial cells and lamina propia mononuclear cells (LPMCs) to be the cellular source of the protein. Furthermore, they found these two cell types to also express the IL-36R, indicating that that the elevated levels of IL-36α present has the potential to act on both immune and nonimmune cells in the mucosa. The authors went on to report enhanced expression of both IL-36α and IL-36γ mRNA in the colons of mice undergoing active DSS induced colitis[394]. To determine the pathophysiological effect of the elevated levels of IL-36 observed in colitis, Russell *et al*., compared acute DSS onset and severity between *wt* mice and *Il-36r*$^{-/-}$ mice. This analysis demonstrated that in the absence of IL-36R signalling a less severe form of colitis was induced, alongside reduced inflammatory cell infiltrate into the colonic lamina propria, with decreased numbers of neutrophils and macrophages detected when compared to *wt* mice[394].

Similarly, Medina-Contreras *et al*. also observed elevated expression of IL-36γ mRNA in both IBD patients and in the colons of wt mice undergoing acute DSS colitis[397]. This study reported enhanced mRNA expression of IL-36γ by M1 macrophages and IECs in the colonic lamina propria (Fig.1.6.1), as well as a decreased disease activity index (DAI) in *Il-36r*$^{-/-}$ mice compared to their *wt* counterparts. Notably, they also uncovered a significant defect in the ability of *Il-36r*$^{-/-}$ mice to resolve DSS induced inflammation. Following 5 days of DSS exposure, both *wt* and *Il-36r*$^{-/-}$ mice were returned to normal drinking water, at which point *wt* mice began to spontaneously recover from colitis as expected. In contrast, *Il-36r*$^{-/-}$ mice continued to exhibit an elevated DAI, and the failure to appropriately resolve disease was observed upon study termination, where *Il-36r*$^{-/-}$ mice were found to exhibit a significantly higher grade of colonic inflammation[397]. Using flow cytometry, they determined

that the failure to recover from DSS induced damage was associated with a marked reduction in expression of IL-36γ induced IL-22 by colonic neutrophils[397]. In follow on studies the same group demonstrated that IL-36R signalling acts as an upstream driver of the IL-23/IL-22/AMP pathway during intestinal injury and barrier repair[399].

In agreement with Medina-Contreras *et al*., Scheibe *et al*. also found that *Il-36r*$^{-/-}$ mice exhibited increased DSS disease severity, along with diminished survival rates and an increased bacterial burden in the colonic wall[377]. Consistent with other studies, they also reported elevated levels of IL-36 cytokines in the colons of patients with IBD[377, 394, 397]. Using immunofluorescence of colonic biopsies, the authors determined that IL-36α was expressed by colonic inflammatory macrophages and DCs, whereas IL-36γ expression was found in IECs[377], and in agreement with other reports, expression of these cytokines was elevated in IBD patients when compared to healthy controls[377, 394, 397]. Scheibe *et al*. further reported that stimulation of murine mucosal IECs with IL-36R ligands resulted in marked proliferation, both *in vitro* and *in vivo*, via Myd88 pathway signalling[377], indicating this response is a direct mechanism of intestinal inflammation resolution[377](Fig.1.6.1). Furthermore, using RNA-seq analysis of primary colonic fibroblasts, the authors report significant enrichment for several biological processes, including inflammatory immune responses, defence responses, responses to wounding and positive regulation of cell proliferation in response to stimulation with IL-36γ [377].

Collectively these studies indicate that IL-36 plays a pathogenic role in the "damage phase" of innate colitis by amplifying the inflammatory response in the mucosa, however as the disease resolves, IL-36R signalling is necessary to modulate the innate responses of IECs and neutrophils to promote intestinal barrier repair and wound healing. While these findings bear striking similarities with related IL-1 family members, as described above, it remains to be determined whether IL-36 cytokines play a more prominent role in either intestinal inflammation or homeostasis and resolution in such settings. The development of tissue/cell specific knock out mice will play an important role in segregating the importance of these apparently opposing effects[373, 400].

1.6.2.2 THE REGULATION OF THE ADAPTIVE RESPONSE BY IL-36

As well as playing a significant role in modulating innate inflammatory responses and homeostasis in the intestine, there is significant evidence that IL-36 cytokines can also influence the activation of adaptive immune cell subsets and thereby play key overlapping instructive roles in immune crosstalk. T cells represent the key cell population from the adaptive immune response involved in the pathogenesis of IBD and in recent years numerous groups have reported a role for IL-36 in directly regulating T cell responses[262,394,401,402]. Using gene expression, immunofluorescence and flow cytometric techniques, Penha *et al.* detected expression of the IL-36R on both T and B lymphocytes in human blood, and the presence of IL-36R+ CD4+ T cells in the intestinal lamina propia[403], confirming that cells of the adaptive immune are directly receptive to IL-36 ligand signalling. IL-36β and IL-36γ have been shown to promote $CD8^+$ T cell activation[401], T_H1 polarization is enhanced by IL-36α[394] and IL-36β[402], while IL-36γ promotes T_H9 polarization[394], and inhibits iTreg differentiation via MyD88 and NFκBp50 dependent signalling pathways[262]. As dysregulated T cell responses in the gut mucosa are a defining characteristic of IBD, these studies highlight the potential impact IL-36 may have on disease development and severity.

Accordingly, Russell *et al.* reported altered colonic T cell responses in *Il-36r*$^{-/-}$ mice using the *C. rodentium* model of colitis, demonstrating reduced mucosal T_H1 responses in the absence of IL-36R signalling by gene expression and flow cytometric analysis[394]. Subsequently, Harusato *et al.* found elevated levels of IL-36γ mRNA expression in *wt* mice in both the *Helicobacter hepaticus* and T cell transfer models of colitis[262]. Using the oxazolone induced model of disease, which elicits a T_H2/T_H9 dependent colitis comparable to human UC, they further demonstrated that *Il-36r*$^{-/-}$ mice also exhibited a milder form of disease[262]. A significant increase in IL-36γ mRNA was also observed in the inflamed colonic tissue of *wt* mice during active disease, and *Il-36γ*$^{-/-}$ mice also displayed attenuated disease[262]. Significantly, a diminished population of IL-9 producing $CD4^+$ T cells, and increased numbers of $CD4^+$ Treg cells were found in the colonic tissue of *Il-36r*$^{-/-}$ mice following oxazolone treatment by flow cytometry[262]. Mechanistic *in vitro* studies using flow cytometry, gene expression and ELISA techniques further demonstrated that IL-36 dependent regulation of the T_H9/Treg balance occurs through modulation of IL-2/STAT5 and IL-4/STAT6 signalling. In addition, it was also demonstrated using the T cell transfer model of colitis, that recipients of *Il-36r*$^{-/-}$ effector $CD4^+$ T_H cells exhibited a significantly milder form of disease compared to recipients of *wt* $CD4^+$ T_H cells[262]. As the T cell transfer model is characterized by T_H1/T_H17 induced mucosal inflammation, the reduction in disease severity could be hypothesized to be due to a reduced ability to mount an effective T_H1 response in

the absence of IL-36 signalling. In agreement with these findings, Scheibe *et al*. have recently reported a pathogenic role for IL-36 in the T_H1 dependant TNBS model of colitis. *Il-36r*$^{-/-}$ mice exhibit a significantly less severe form of disease, and neutralization of IL-36 signalling using an anti-IL-36R antibody ameliorates disease in *wt* mice undergoing chronic TNBS[404] colitis. These data indicate that IL-36 cytokine dependent direct and indirect modulation of mucosal T cell responses may represent an important mechanism through which these cytokines influence inflammation in the GI tract (Fig. 1.6.1).

Furthermore, the potent stimulatory effects of IL-36 family members across a wide range of mucosal inflammatory cell responses, indicate a vital role for this family as mediators at the interface between innate and adaptive inflammation in the intestine. IL-36 cytokines are predominantly produced by cells of the innate immune, particularly the intestinal epithelium[394, 395, 397]. As these cells are widely recognised as the "first line of defence" in the gut[405], this indicates that IL-36 cytokines are crucial in the early innate driven response to damage and pathogens. Furthermore, the IL-36R is expressed by a number of innate effector cells, such as macrophages and DCS, and IL-36 agonist stimulation of these cells has been shown to elicit and amplify the innate immune response[374, 375, 397]. This induction of the innate response commonly elicits the activation of the adaptive immune system, which forms a more robust T and B cell driven response, and the generation of immunological memory[131]. The IL-36R is also expressed by these adaptive immune cells[403], indicating they are receptive to innate generated IL-36 stimulation. IL-36 has been shown to have a potent regulatory effect on the adaptive immune system, with key roles in modulating CD4+ T cell polarisation and pathogenicity[262, 394, 404], key mechanisms involved in the pathogenesis of IBD[240, 406, 407]. As recent GWAS studies have revealed strong evidence for an association between innate and adaptive immunity in mucosal homeostasis[217], this indicates that IL-36 may be a key player in the crosstalk of the innate and adaptive immune responses associated with GI inflammation and the onset of IBD.

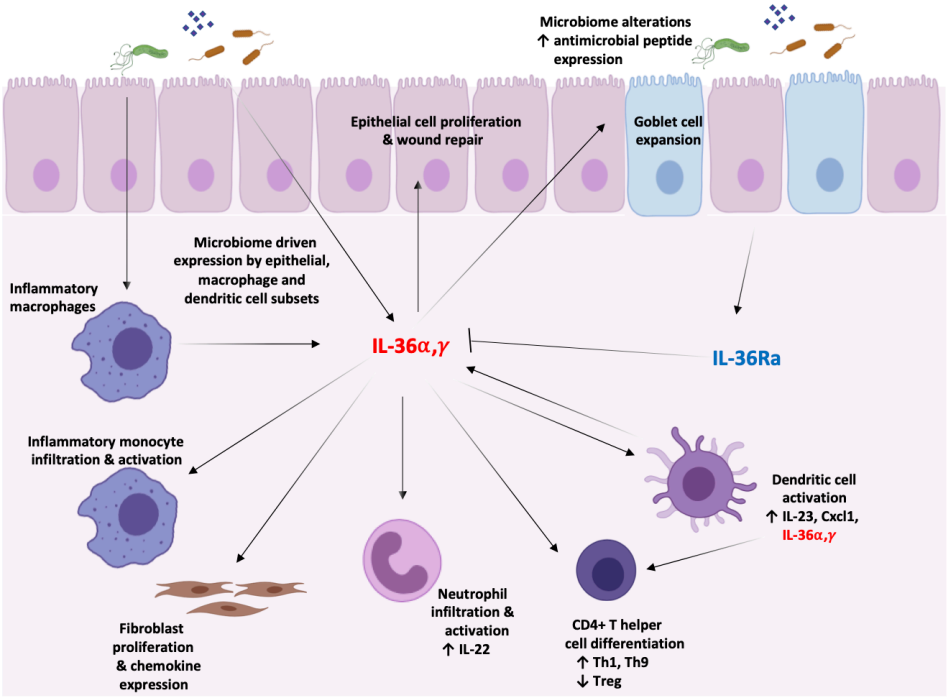

Figure 1.6.1: The effects of IL-36 family members at the intestinal mucosa. The IL-36 family of cytokines are mediators of inflammation and homeostasis at the intestinal mucosa where they can stimulate diverse responses from both immune and parenchymal cell subsets. Their effects can be dichotomous, simultaneously promoting mucosal homeostasis and barrier integrity while also having the potential to recruit and activate inflammatory cells to the mucosa, expand pathogenic cell populations associated with IBD, and induce fibrosis and microbiotic dysbiosis. *Figure created with biorender.com*. Figure taken from Leon, G., Hussey, S., & Walsh, P. T. (2020). The diverse roles of the Il-36 family in gastrointestinal inflammation and resolution. *Inflammatory bowel diseases*.

1.7 AIMS:

There is emerging evidence for a role for the IL-36 cytokine family in the pathogenesis of IBD. Numerous adult patient cohorts express elevated levels of the cytokines, and aberrant IL-36 signalling is reported in preclinical models of colitis. While reports in the literature are unclear as to whether this cytokine family is pathogenic or protective in the pathogenesis of the disease, there is clear evidence that it has a substantial influence of the adaptive response. Therefore, this study aims to:

1. Determine the expression of IL-36 family members in a paediatric IBD cohort
2. Characterize the effect of IL-36 cytokines on the generation of CD4$^+$ T_H helper cell responses
3. Evaluate the effect of IL-36 signalling in the generation of CD4$^+$ T_H cells *in vivo*
4. Assess the impact of IL-36R signalling on the development of T_H cell mediated colitis *in vivo*
5. Determine potential mechanisms of IL-36R signalling in the development of T_H cell mediated colitis *in vivo*

CHAPTER 2.

MATERIALS AND METHODS

Chapter 2

Material & Methods

2.1. STUDY SUBJECTS. All human samples were provided with consent/assent from paediatric IBD patients and control participants recruited in the Determinants and Outcomes of CHildren and AdolescentS with IBD study (DOCHAS) at the gastroenterology unit at Our Lady's Children's Hospital (Crumlin, Ireland). The analysis of IL-36 family gene and protein expression, and IFNγ protein expression in intestinal tissue specimens and serum samples from these participants is under approval from the institutional Research Ethics Committee (GEN/193/11), and included 97 participants (CD, n = 42; UC, n = 31; uncategorised IBD n = 1 Ctrl, n = 23). Post-hoc analysis revealed this sample size to have a power of 88.7%, with the probability of a type 1 error set at α=0.05. Information of the demographics of the patients enrolled in this study are detailed in Appendix 1.

2.2. MICE. Wild-type (*wt*) C57BL/6 mice (bred in house), IL-36 Receptor knockout mice (*Il36r*$^{-/-}$) (Amgen), *Rag1*$^{-/-}$ mice (provided by Professor Padraic Fallon, TCD) and *Sigirr*$^{-/-}$ mice (from Dr. C Garlanda and Professor A Mantovani[408]) were housed under specific pathogen-free (SPF) conditions in a temperature-controlled unit with a 12-h light/dark cycle at the Comparative Medicine Unit (CMU) in Trinity Translational Medicine Institute (TTMI), St. James Hospital (Dublin). Water and food were provided *ad libitum*. All mice used were aged between 6-10 weeks old and were performed under license from the Irish Health Products Regulatory Authority (Project No. AE19136/P036) and in compliance with Irish Department of Health regulations (license number B100/4272), with approval by the institutional ethical review boards.

2.3. DNA EXTRACTION FOR GENOTYPING. DNA extraction was performed by incubating mouse ear hole punches in Direct PCR Ear (Viagen)(90μl/ear punch) and Proteinase K (10ng/ml)(10μl/ear punch), at 55^{o}C for 3 hours in a 1.5ml Eppendorf. The ear punches were then vortexed gently to aid in the break-up of the ear tissue, followed by deactivation at 85^{o}C for 45 minutes. Eppendorf's were then centrifuged at 1300RPM for 5 minutes at room temperature. Lysates were then pipetted off the top of pellet of fur and debry that had accumulated at the bottom of the tube, and transferred to a fresh Eppendorf. These lysates were then either purified using the DNeasy Blood and Tissue Kit (Qiagen) in accordance with manufacturer's instruction, or used in their crude form for genotyping.

DNA samples were either used immediately for DNA quantification (2.4) and PCR (2.5), or stored at -20°C until needed.

2.4. DNA AND RNA QUANTIFICATION BY SPECTROPHOTOMETRY. Prior to PCR genotyping the concentration of the extracted DNA was measured in the ear punch lysates using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). This method was also used to measure the amount of RNA in retro-transcribed samples (2.17.2). The machine was first "blanked" using nuclease-free dH_2O. 2µL of undiluted DNA or RNA samples were then loaded onto the spectrophotometer to determine nucleic acid quantity. This was achieved by measuring the absorbance of the samples at 260/ 280 nanometres (nm) for DNA and, 260/230nm for RNA. ND -1000 software then utilizes a modified Beer-Lambert equation to output DNA concentration in ng/μl:

$$c=(A*e)/b$$

c = nucleic acid concentration in ng/µL

A = the absorbance in AU

e = the wavelength-dependent extinction coefficient in ng-cm/µL

b = the pathlength in cm

The concentration of DNA was recorded to determine the quantity needed in the PCR reaction, and 260/280 values were recorded as a measure of DNA purity. 260/280 values of ~ 1.8 are considered "pure" for DNA. 260/230 values were recorded for RNA, with values of 2.0-2.2 considered "pure". The lense was cleansed between each sample by wiping gently with smooth lint free tissue. Samples were either used immediately for PCR (2.5) or stored at -20°C until needed.

2.5. GENOTYPING. PCR analysis of *Il36r*$^{-/-}$, *Rag1*$^{-/-}$, and *Sigirr*$^{-/-}$ was used to confirm homozygosity of the knockout strains. For *Rag1*$^{-/-}$ mice the PCR protocol used was taken from the JAX® mouse database for strain B6.129S7-Rag1tm1Mom/J, and optimised for efficient use with primers purchased from Taconic Biosciences (Table 2.1). For of *Il36r*$^{-/-}$ mice the PCR protocol was provided by Amgen for the *Il1rl2*^{tm1Hblu} strain, and used primers purchased from Sigma Aldrich (Table 2.1).

Frozen reagents were thawed, and PCR mastermix was made using the Eppendorf® PCR Cooler iceless cold storage system to keep reagents cool. DreamTaq™ Hot Start DNA polymerase and buffers, along with dNTPs, MgCl2, and dH_20 were used in the PCR mastermix. Concentrations and reagents used are listed in Table 2.2. Once mastermixes were prepared they were they were aliquoted in a 20μl volume to PCR tubes. Genomic DNA was then added in a 5μl volume. The contents of the PCR tubes were gently centrifuged and loaded into a Veriti 96 well Thermocycler (Applied Biosystems). Thermocycling protocols are listed in Tables 2.3 and 2.4.

TABLE 2.1: GENOTYPING PRIMERS

	Forward Primer	**Reverse Primer**	**Company**
IL36R	TN53- 5'-GCCCTGAATGAACTGCAGGACG-3' IL1RRP2 - 5'-GCCGCTACACACCACAACCAG – 3'	4TN5 - 5'-CACGGGTAGCCAACGCTATGTC- 3' IL1RRP2 5'-AGTTCAGTAGTCCACTGCCACTC- 3'	Taconic Biosciences
Rag1	OIMR 0189 - 5'TGGATGTGGAATGTGTGCGAG- 3' OIMR 1746 - 5'GAGGTTCCGCTACGACTCTG- 3'	OIMR 3104 - 5' CCGGACAAGTTTTTCATCGT - 3'	Sigma Aldrich
Sigirr	25/10N – 5'-ATCTTTCAGCCACTGCACTGA – 3" 5mAcp – 5' – GTCTGTGACATGGCCCCTAAT – 3'	PGK – 5'-CTGCTCTTTACTGAAGGCTC – 3	Sigma Aldrich

TABLE 2.2 GENOTYPING MASTERMIX REAGENTS

Reagents	***Il36r*$^{-/-}$**	***Rag1*$^{-/-}$**	***Sigirr*$^{-/-}$**	**Company**
ddH20	1X	1X	1X	
Dream Taq PCR Buffer	1.5mM	2mM	2mM	Thermofisher
MgCl2 (25mM)		2mM	2mM	Thermofisher
dNTPs (10mM)	.2mM	.2mM	.2mM	Bioline
Forward Primer (20μM)	.5μM	.5μM	.33μM	Taconic Biosciences/ Sigma Aldrich
Reverse Primer (20μM)	.5μM	.5μM	.33μM	Taconic Biosciences/ Sigma Aldrich
Dream Taq DNA Polymerase	.05U/μl	.01U/μl	.05U/μl	Thermofisher
DNA	50ng	25ng	50ng	

TABLE 2.3: GENOTYPING PCR THERMOCYCLING PROTOCOLS

	IL36R$^{-/-}$ PCR AMPLIFICATION CYCLES		*RAG1*$^{-/-}$ PCR AMPLIFICATION CYCLES	
STEP 1 (HOT START)	**1 X 95°C (15M)**		**1 X 95°C (4M)**	
STEP 2 (DENATURE)	**1 X 94°C (45S)**	**REPEAT STEPS 2-4 30 TIMES**	**1 X 95°C (30S)**	**REPEAT STEPS 2-4 35 TIMES**
STEP3 (ANNEAL)	**1 X 60°C (1M**		**1 X 56°C (30S)**	
STEP 4 (EXTEND)	**1 X 72°C (1M)**		**1 X 72°C (30S)**	
STEP 5 (FINAL EXTENSION)	**1 X 72°C (5M)**		**1 X 72°C (10M)**	
STEP 6 (HOLD)	**1 X 4°C (HOLD)**		**1 X 4°C (HOLD)**	

TABLE 2.4: GENOTYPING PCR THERMOCYCLING PROTOCOL FOR *SIGIRR*-

Sigirr- PCR Amplification Cycles		
STEP 1 **(HOT START)**	1 x 95°C (5m)	
Step 2 (Denature)	1 x 95°C (30s)	1
Step 3 (Anneal)	1 x 65°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	2
Step 3 (Anneal)	1 x 63.5°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	3
Step 3 (Anneal)	1 x 62°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	4
Step 3 (Anneal)	1 x 60.5°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	5
Step 3 (Anneal)	1 x 59°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	6
Step 3 (Anneal)	1 x 57.5°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	7
Step 3 (Anneal)	1 x 56°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	8
Step 3 (Anneal)	1 x 64.5°C (30s)	
Step 4 (Extend)	1 x 72°C (30s)	
Step 2 (Denature)	1 x 95°C (30s)	9
Step 3 (Anneal)	1 x 64.5°C (30s)	
Step 4 (Extend)	1 x 72°C (15m)	(repeat 23 times)
Step 6 (Hold)	1 x 4°C (hold)	

2.6. AGAROSE GEL ELECTROPHORESIS. Following PCR amplification of the *Il36r*$^{-/-}$, *Rag1*$^{-/-}$ and/or *Sigirr*$^{-/-}$ DNA, 20% of the reaction volume mixed with 1μl DNA loading dye (BioLine) was inserted into specific wells on a pre-made 1.5% agarose gel. This gel was made by adding 1.5g Agarose Powder (Sigma Aldrich) to 100ml 1% TAE in a conical flask. This mixture was then heated using a microwave until the agarose powder had dissolved into the TAE, making the gel. After this had cooled slightly, 1μl of Gel Star gel stain (Lonza) was added to the gel liquid. This was then mixed and poured into a mould containing an insert to make wells in the formed gel. Once the gel had cooled and set sufficiently, it was placed in the Gel electrophoresis chamber, with the tank filled with 1X TAE buffer. 5μl of Easy Ladder molecular weight ladder (Bioline) was then added to a well, alongside the 5μl of the amplified DNA premixed a DNA loading dye. The gel electrophoresis was then set to run at the specific voltages for each genotype (Table 2.5) for 30m-1h. Amplicons were then visualized using a GelDoc-It imaging system (BioImaging Systems) and analysed using LabWorks software. Knockout of the specific gene was determined by amplicon size (Table 2.5).

TABLE 2.5: AGAROSE GEL ELECTROPHORESIS VOLTAGES AND EXPECTED AMPLICON BAND SIZES

	Voltage (V)	**Amplicon size (base pairs)**
Il36r$^{-/-}$	90-130	520
Il36r **het**	90-130	390
Rag1$^{-/-}$	60-100	530
Rag1 **het**	60-100	474
Sigirr$^{-/-}$	60-100	450bp
Sigirr^{het}	60-100	147bp

2.7. ELISA. Enzyme-linked Immunosorbent Assays (ELISA) was used to measure the concentration of specific proteins in murine supernatants and human serum samples. The proteins measures and the company they are bought from are listed in Table 2.6.
To perform ELISA analysis Corning® High Binding ELISA plates (Merck, CLS9018) were coated with 50µl/well of appropriate capture antibody diluted in PBS. The concentrations used are listed in Table 2.7. The plate coated in antibody was then sealed in parafilm® (Merck, P7543) and incubated overnight at 4°C. The next morning, the coating antibody was removed from the plate and 200µl/well of ELISA Wash Buffer was added. Details of buffers used are in Table 2.20. This was incubated for 1m and then aspirated off the plate. This was repeated 3 times. 100µl/well of 1X ELISA Assay diluent was then added to "block" plate, to prevent any background binding. This was incubated for a minimum of 1h at room temperature (RT). While the plate was incubating with ELISA diluent, the samples of interest were thawed on ice. During this time, the standards were diluted to their required concentrations using ELISA Assay diluent (Table 2.20). After 1h the plate was washed 3 times, and 50µl/well of standards and samples were added to the appropriate wells. 50µl/well of ELISA Assay diluent was also added to 3 wells to act as a blank for the microplate reader. The plate was then incubated at RT for 2hrs. The plate was then washed 3 times, and 50µl/well of detection antibody previously diluted in Elisa Assay diluent was added. The plate was then incubated for 1h at RT. After 1h, the plate was washed 3 times and 50µl/well of HRP diluted in Elisa Assay diluent was added. The plate was then covered in tinfoil to protect from light and incubated at RT for 30m. The plate was then washed 7 times, and 50µl/well of the substrate TMB was added. The plate was then continually monitored for the development of a blue colour in the wells, this could take up to 30m. Once the standards had developed the desired colour 25µl/well of 1N sulphuric acid was added to stop the reaction. The plate was then immediately analysed using a Synergy MX microplate reader (BioTek) by measuring the absorbance at 450nM.

TABLE 2.6: ELISA KITS

Species	Protein	Company	Catalogue Number
Mouse	IL-2	eBioscience (Invitrogen - Thermo Fischer, UK	# 88-7024-86
Mouse	IFNγ	eBioscience (Invitrogen - Thermo Fischer, UK	# 88-7314-86
Mouse	IL-17a	eBioscience (Invitrogen - Thermo Fischer, UK	# BMS6001TEN
Mouse	IL-13	eBioscience (Invitrogen - Thermo Fischer, UK	# BMS6015TEN
Mouse	IL-9	eBioscience (Invitrogen - Thermo Fischer, UK	# 88-8092-86
Mouse	IL-4	eBioscience (Invitrogen - Thermo Fischer, UK	# BMS613TEN
Mouse	GM- CSF	eBioscience (Invitrogen - Thermo Fischer, UK	# 88-7334-86
Human	IL-36β	BioLegend (London, U.K.)	Discontinued
Human	IL-36γ	BioLegend (London, U.K.)	Discontinued
Human	IL-36RN	BioLegend (London, U.K.)	Discontinued
Human	IL-36α	mybiosource.com (California, USA)	MBS760191
Human	IFNγ	eBioscience (Invitrogen - Thermo Fischer, UK	# BMS228TEN

Table 2.7: Reagents and Concentrations used in ELISAs. Concentrations of antibodies used are specified when available. As kits were used concentrations of antibodies were not always specified, in this case the dilution of the antibody is stated.

Kit	Antibody	Concentration	Elisa Assay Diluent
mIL-2	Capture	2µg/ml	1X ELISA/ELISPOT Diluent
	Detection	2µg/ml	
	Standard	200pg/ml	
mIFNγ	Capture	0.5µg/ml	1X ELISA/ELISPOT Diluent
	Detection	0.5µg/ml	
	Standard	2000pg/ml	

mIL-17a	Capture	2μg/ml	1X ELISA/ELISPOT Diluent
	Detection	250X	
	Standard	500pg/ml	
mIL-13	Capture	2μg/ml	1X ELISA/ELISPOT Diluent
	Detection	1μg/ml	
	Standard	500pg/ml	
mIL-9	Capture	250X	1X ELISA/ELISPOT Diluent
	Detection	250X	
	Standard	4000pg/ml	
mIL-4	Capture	2μg/ml	1X ELISA/ELISPOT Diluent
	Detection	250X	
	Standard	500pg/ml	
mGM-CSF	Capture	250X	1X ELISA/ELISPOT Diluent
	Detection	250X	
	Standard	250pg/ml	
hIL-36β	Capture	400ng/ml	1% BSA in PBS
	Detection	100ng/ml	
	Standard	800pg/ml	
hIL-36γ	Capture	2μg/ml	1% BSA in PBS
	Detection	250ng/ml	
	Standard	1200pg/ml	
hIL-36RN	Capture	4μg/ml	1% BSA in PBS
	Detection	50ng/ml	
	Standard	6000pg/ml	
hIL-36α	Capture	Pre-coated plate	1% BSA in PBS
	Detection	100X	
	Standard	10ng/ml	
hIFNγ	Capture	2μg/ml	ELISA/ELISPOT Diluent
	Detection	250X	
	Standard	500pg/ml	

2.8 CD4+ T_n ISOLATION AND CULTURE

2.8.1. ISOLATION OF MURINE SPLENOCYTES. Following cervical dislocation, the spleen was removed from the abdominal cavity of the chosen mouse. It was then trimmed of any excess fat and connective tissue and stored in cooled PBS on ice until cell isolation commenced. From this point on any isolation and cell culture techniques were performed in a Holten Lamina Air hood (HB2448K).To isolate the splenocytes into a single cell suspension, a 40µM cell strainer was placed onto the top of a 50ml Falcon tube. The spleen was placed in this strainer and the tissue was broken apart by gently mashing it with the removed plunger of a 1ml syringe. The cells were washed through the strainer into the tube with complete RPMI (cRPMI). The cells were then centrifuged at 1300RPM for 5 minutes at RT. The supernatant was discarded, 1ml of Red blood cell (RBC) lysis buffer was added and the cell pellet was re-suspended in this by gently pipetting up and down. The re-suspended cells were then placed in a hot water bath for 2m. The tube was then topped up with cRPMI and centrifuged (1300RPM, 5m, RT). The supernatant was discarded and the cell pellet was re-suspended in 1ml cRPMI. The tube was then topped up with cRPMI to "wash" the cells and once again centrifuged (1300RPM, 5m, RT). The supernatant was discarded and the cells were re-suspended in cRPMI and counted using Trypan Blue exclusion (2.8.5). The cells were stored at 4°C in cRPMI until ready to proceed to CD4+ T_n cell isolation (2.8.4). Details of buffers used are in Table 2.20.

2.8.2. ISOLATION OF MURINE LYMPHOCYTES. Following cervical dislocation the abdominal cavity of the chosen mouse was opened and the intestines were removed. Mesenteric lymph nodes (mLNs) were then identified in the fatty connective tissue stuck to the intestines. These were carefully excised from this tissue and stored in cooled PBS on ice until cell isolation commenced. To isolate the mLNs into a single cell suspension the a 40µM cell strainer was placed onto the top of a 50ml Falcon tube. The mLNs were then placed in this strainer and broken apart by gently mashing them with the removed plunger of a 1ml syringe. The cells were washed through the strainer into the tube with cRPMI. The cells were then centrifuged at 1300rpm for 5 minutes at RT. The supernatant was discarded, 1ml of RBC lysis buffer was added and the cell pellet was re-suspended in this by gently pipetting up and down. The re-suspended cells were then placed in a hot water bath for 1m. The tube was then topped up with cRPMI and centrifuged (1300rpm, 5m, RT). The supernatant was discarded and the cell pellet was re-suspended in 1ml cRPMI. The tube was then topped up with cRPMI to "wash" the cells and once again centrifuged (1300rpm, 5m,

RT). The supernatant was discarded and the cells were re-suspended in cRPMI and counted using Trypan Blue exclusion (2.8.5). The cells were stored at 4°C in cRPMI until ready to proceed to $CD4^+$ T_n cell isolation (2.8.4).

2.8.3. ISOLATION OF MURINE COLONIC LAMINA PROPIA LEUKOCYTES. Following cervical dislocation, the abdominal cavity of the chosen mouse was opened and colons were excised. They were then opened longitudely and washed of faecal contents with PBS. They were then cut in pieces (0.5-1cm), transferred to a 50ml conical tube containing HBSS (Sigma Aldrich) supplemented with 5% FBS (Gibco) and 5mM EDTA (e8008, Sigma Aldrich), and shaken horizontally at 37 °C for 20 min at 200 rpm. The tissue was then washed with HBSS (H8264, Sigma Aldrich) to remove residual EDTA, cut into 1mm pieces, and transferred to a 50-ml conical tube containing HBSS supplemented with 5% FBS, 1.6 mg/ml Collagenase D (11088858001, Roche, Sigma Aldrich), and 40µg/ml DNase I (D4263, Sigma Aldrich). Tube was then horizontally shaken at 37°C for 45 min at 200 rpm. After digestion, the tube was vortexed for 10 seconds to ensure thorough dissociation of remaining intestinal tissue, all content passed through a 100μm cell strainer and cell suspensions pelleted by centrifugation at 2500rpm. Live cells were then counted by Trypan Blue exclusion (2.8.5). Once counted the cells were re-suspended in PBS, and stored at 4°C to await subsequent staining for FACs analysis (2.9).

2.8.4. MOUSE: $CD4^+$ T_n CELL ISOLATION.

Positive selection: Naïve $CD4^+$ T cells were purified from murine splenocytes and mesenteric lymphocytes by positive selection of $CD4^+$ T cells by magnetic beads (CD4 L3T4 Kit, Miltenyi Biotec, 130-117-043) for cell culture experiments. The murine cells were re-suspended in 90µl of cooled MACs buffer (Section) per 10^7 total cells, and 10µl of CD4 (L3T4) MicroBeads per 10^7 total cells was then added. The cells were gently mixed with the beads by pipetting up and down and then placed at 2-8°C for 10m.

Negative selection: For *in vivo* T cell transfer experiments the $CD4^+$ population from murine splenocytes was first enriched using negative selection of $CD4^+$ T cells by magnetic beads (Miltenyi Biotec, 130-104-454) prior to sorting the cells by Flow Cytometry. Splenocytes were re-suspended in 40µl of cooled MACs buffer (Table 2.20) per 10^7 total cells, 10µl/10^7 total cells of CD4+ T Cell Biotin-Antibody Cocktail was then added. The cells were gently mixed with the beads by pipetting up and down and then placed at 2-8°C for 5m. 30µl/10^7 total cells of MACs buffer and 20µl/10^7 total cells of CD4+ T Cell MicroBead Cocktail was added on top of this. Once again, the cells and beads were mixed by pipetting up and down,

and incubated at 2-8°C for 10m. This cocktail magnetically labels all non-$CD4^+$ T cells by using antibodies directed against CD8a, CD11b, CD11c, CD19, CD49b (DX5), CD45R (B220), CD105, Anti-MHC-class II, Ter-119 and TCRγ/δ.

Magnetic Separation. While the cells were incubating the magnet and magnetic columns were prepared for CD4+ T cell separation. An LS Column (Miltenyi Biotec, 130-042-401) was placed in the magnetic field of a MidiMACS™ Separator attached to a MultiStand (Miltenyi Biotec, 130-042-301). 3mls of MACs buffer were passed through this column to prepare it for subsequent separation. This MACs buffer was collected in a 50ml falcon tube designated for "waste". After the 10m incubation period, the positively labelled cells were then brought up to a minimum of 1ml in volume with MACs buffer and placed in the LS column. As the cells traverse through the column, only cells that are $CD4^+$ T cell are retained by the magnet, and only the non-$CD4^+$ cells pass through into the waste tube. Once the cells have passed through, the column is washed by passing 3mls of MACs buffer through it, and the waste tube is subsequently discarded. The LS column containing the labelled $CD4^+$ T cells is removed from the magnet and is placed over the top of a fresh 50ml falcon tube. 5mls of cRPMI are placed in the top of the column and a plunger is then used to push the cRPMI through the column, flushing the $CD4^+$ T cells out with it. For the negatively labelled $CD4^+$ T cells, once the incubation period was complete, the cells were topped up to a 1ml with MACs buffer and transferred to the prewashed LS column. As the cells traverse the column the magnetically labelled non-$CD4^+$ cells are retained, and only the purified $CD4^+$ cells flow through into the awaiting falcon tube. The column is washed with 3mls of MACs buffer and the falcon tubes now containing the isolated $CD4^+$ T_n cells are topped up with cRPMI and centrifuged (1300rpm, 5m, 4°C). These cells are then re-suspended in cRPMI and counted using Trypan blue exclusion (2.8.5). Once counted the cells were re-suspended to the desired volume, and stored at 4°C to await culture (2.8.6) or subsequent staining for FACs sorting (2.9).

2.8.5. CELL COUNTING. Isolated cells were counted selecting for total live cells using Trypan Blue Exclusion (93595, Sigma Aldrich, Merck, Ireland). 90µl of Trypan Blue was added to a well in a 96well plate, and 10µl of the sample was added. This was mixed thoroughly by pipetting up and down and 10µl was then transferred to the chamber of a KOVA™ Glasstic™ (KOVA™ 22270141, Fisher Scientific, Ireland). This slide was then analysed using an Olympus CKX41 culture microscope (Olympus, UK). Live cells (bright white) were distinguished from dead cells (dark blue) and the total number of live cells in 3 large squares (Fig.2.1.) were counted and input to the following formula:

Square 1 = _____

Square 2 = _____

Square 3 = _____

Total Square 1 + Total Square 2 + Total Square 3 = Total Live Cells Counted (TLC)

Plug into formula:

TLC/3 = _____ x 10 x 10^{4} = _____ x 10^{6} x _____ = _____x 10^{6} cells total.

Dilution of Trypan Blue

Amount of mls the cells are resuspended in

Volume of chamber

FIGURE 2.8.: HEMOCYTOMETER LAYOUT

2.8.6. MURINE CD4$^+$ T_n CELL CULTURE. A 96 well round bottomed plate was coated with 100μl/well αCD3ε and αCD28 diluted in PBS. This plate was then incubated at 37^{0}C with 5% CO_2 in a Sanyo CO2 Incubator (MCO-15AC) for a minimum of 3 hours. During this time, the CD4$^+$ T cells were being isolated (2.8.1-2.8.2). After incubation, the αCD3ε and αCD28 should have adhered to the bottom of the wells and become immobilised, the remaining PBS containing soluble αCD3ε/αCD28 was then aspirated from the plate. The plate was then washed by adding 100μl of PBS/well, removing this and tapping dry on absorbent tissue. This was repeated 3 times. The now purified Naïve CD4$^+$ T cells were added to the plate at 2x10^5 cells/well in 100μl of cRPMI. At this point any stimulatory/inhibitory molecules were added to the cells, i.e. αIL-2 (10ug/ml, eBioscince (Thermo Fischer, UK)), IL-36α (10ng/ml-200ng/ml, R&D Systems (Abingdon, UK)), or ATRA (10nM/ml -100nM/ml, Sigma Aldrich, (Merck, UK)); these were diluted in cRPMI and added in a volume of 50μl. Each condition was performed with a minimum of 3 technical replicates. Every well was then topped up to a final volume of 200μl by addition of required amount of cRPMI. The wells were then thoroughly mixed by pipetting up and down and cultured for 48hrs under T_n0 conditions, 72hrs under T_n1 conditions and T_n2 conditions, 96hrs under T_n17 conditions, and 120hrs under iTreg conditions and T_n9 conditions at 37^{0}C with 5% CO_2 (Sanyo CO2 Incubator). CD4$^+$ T_n lineage polarizing cytokines and concentrations are detailed in Table 2.8. All cytokines, antibodies, and stimulatory molecules are specified in Table 2.9.

TABLE 2.8: CD4$^+$ T_n CELL LINEAGE POLARIZING COCKTAILS

	IL-12 (/ml)	**α IFNγ (/ml)**	**IL-4 (/ml)**	**IL-6 (/ml)**	**TGFβ (/ml)**	**αIL-4 (/ml)**	**αCD3 (/ml)**	**αCD28 (/ml)**	**Time in culture**
T_n1	20ng	-	-	-	-	10µg	1µg	3µg	72H
T_n2	-	10µg	10ng	-	-	-	2µg	2µg	72H
T_n17	-	10µg	-	20ng	5ng	5µg	2µg	3µg	96H
T_n9		10µg	20ng	-	5ng	-	1-2µg	2-3µg	120H
iTreg	-	-	-	-	5ng	-	1-2µg	2-3µg	120H

2.8.7. ISOLATION OF HUMAN PBMCS. Human blood was obtained in the form of a buffy pack from the Irish Blood Transfusion Service (IBTS), St. James' Hospital, Dublin. To isolate PBMCs from this, 15mls of lymphoprep (07801 – STEMCELL Technologies) was added to a 50ml falcon tube and 25mls of blood was layered gently on top so as not to disturb the lymphopreps interface. This tube was then centrifuged at 1800rpm for 30m at RT with the acceleration and break turned off. The tube was then gently placed in a rack so as not to disturb the density gradient, and a 1ml pipette was used to remove the PBMCs from the interface between the red blood cells at the bottom of the tube and the lymphoprep at the top. These PBMCs were then placed in a new 50ml falcon tube and 1ml of RBC lysis buffer was added. The PMBCs and RBC lysis buffer was gently mixed by pipetting up and down and then placed in a water bath for 1m. The tube was then topped up with human complete human RPMI (chRPMI) and centrifuged at 1300rpm for 5m at RT with the break and acceleration now turned on (Break = 9, Acceleration = 9). The supernatant was then discarded and the cells re-suspended in 1ml chRPMI. The tube was topped up with chRPMI to wash the cells and centrifuged once more (1300rpm, 5m, RT). The isolated PBMCs were now counted using Trypan Blue exclusion (2.8.5). Once counted the cells were either used immediately or stored at 4°C to await CD4+ purification (2.8.8). Details of buffers used are in Table 2.20.

2.8.8. HUMAN CD4$^+$ T_n CELL ISOLATION. Naïve CD4$^+$ T cells were purified from donor PBMCs using negative selection of CD4$^+$ T cells by magnetic beads (CD4 T cell isolation Kit, human, Miltenyi Biotec - 130-096-533). Purified PBMCs were re-suspended in 40µl of cooled MACs buffer per 10^7 total cells, 10µl CD4$^+$ T Cell Biotin-Antibody Cocktail per 10^7 total cells was then added. The cells were gently mixed with the beads by pipetting up and down and then placed at 2-8°C for 5m. 30µl/10^7 total cells of MACs buffer were added on top of this, alongside 20µl/10^7 total cells of CD4$^+$ T Cell MicroBead Cocktail. Once again, the cells and beads were mixed by pipetting up and down, and incubated for 10m at 2-8°C. This cocktail magnetically labels all the non CD4$^+$ T cells. CD8$^+$ T cells, γ/δ T cells, monocytes, neutrophils, eosinophils, B cells, dendritic cells, NK cells, granulocytes, and erythroid cells are magnetically labelled using a cocktail of biotin-conjugated antibodies against CD8, TCR γ/δ, CD14, CD15, CD16, CD19, CD36, CD56, CD123, and CD235a (Glycophorin A). While the cells were incubating the Magnet and magnetic columns were prepared for CD4$^+$ T cell separation. An LS Column was placed in the magnetic field of a MidiMACS™ Separator attached to a MultiStand (Miltenyi Biotec). 3mls of MACs buffer were passed through this column to prepare it for subsequent separation. This MACs buffer was collected in a 50ml falcon tube and discarded. Once the 10m incubation period was complete, a fresh

50ml falcon tube was placed under the LS column. The PBMCs were then brought up to a minimum of 1ml in volume with MACs buffer and placed in the LS column. As the cells traverse through the column, any cell that is not a CD4+ T cell is retained by the magnet, and only the $CD4^+$ T cells pass through into the 50ml falcon. Once the cells have passed through, the column is washed by passing 3mls of MACs buffer through it. The falcon tube now containing the isolated $CD4^+$ T_N cells is then removed from underneath the column, topped up with chRPMI and centrifuged (1300rpm, 5m, 4°C). These cells are then re-suspended in chRPMI and counted using Trypan blue exclusion (2.8.5). Once counted the cells were re-suspended to the desired volume ($0.8x10^6$/ml), an aliquot was taken to determine the purity of the cells (2.8.9) and the remaining cells were stored at 4°C to await culture (2.8.10).

2.8.9. DETERMINING HUMAN $CD4^+$ T_N CELL PURITY. Once isolated an aliquot of the $CD4^+$ T_N cells was taken to determine cell purity by analysing $CD4^+$ expression by Flow Cytometry (2.9.2). The cells were placed in a 5 ml FACs tube and topped up with PBS. They were then centrifuged at 1300rpm, for 5m at 4°C. The supernatant was discarded and the cells were re-suspended 1ml PBS, to "wash" the cells, and centrifuged once more (1300rpm, 5m, 4°C). The supernatant was discarded and 2μl of human anti-$CD4^+$ PeCy7 was added to the tube in 50μl of PBS, which also contained LIVE/DEAD™ cell viability stain at a 1:500 concentration. Details of fluorophore conjugated antibodies are listed in Table 11. The cells were then thoroughly re-suspended and mixed by vortexing and incubated for 1h at 2-8°C. The cells were then topped up with 1ml PBS and centrifuged (1300rpm, for 5m at 4°C). The supernatant was discarded and the cells were re-suspended in 200-500μl of PBS. $CD4^+$ expression was then LSR/Fortessa (Becton Dickinson Biosciences (BD)) and analysed using FlowJo™ software (Tree Star). Dead cells and debry were excluded from the analysis based on scatter signals and LIVE/DEAD™ fluorescence.

2.8.10. HUMAN $CD4^+$ T_N CELL CULTURE. The purified $CD4^+$ T_N cells were then plated at $0.8x10^5$ cells/well in 80μl chRPMI, and activated by adding 20μl of soluble αCD3ε/αCD28 activation beads (Treg expansion Kit, human, Miltenyi Biotec 130-095-345). Specific wells were then stimulated with IL-36α and/or all trans retinoic acid (ATRA). All antibodies, cytokines and stimulatory molecules used are detailed in Table 2.9. Each condition was performed with a minimum of 3 technical replicates. All wells were brought up to a final volume of 200μl /well by the addition of chRPMI and thoroughly mixed by pipetting. The plate was then incubated for 120hrs at 37°C with 5% CO_2 (Sanyo CO_2 Incubator). Following incubation supernatants were stored at -20°C to await ELISA analysis (2.7), and cells were immediately harvested for analysis by Flow Cytometry (2.9).

TABLE 2.9: CYTOKINES, ANTIBODIES, AND CHEMICAL INHIBITORS/STIMULANTS USED IN CELL CULTURE

Species	Reagent	Company	Catalogue Number
Mouse	αCD3ε (2C11)	eBioscience (Thermo Fischer, UK)	#16-0031-82
Mouse	αCD28 (37.51)	eBioscience (Thermo Fischer, UK)	#16-0281-82
Mouse	IL-12p70	eBioscience (Thermo Fischer, UK)	#14-8121-80
Mouse	αIL-4 (11B1)	eBioscience (Thermo Fischer, UK)	#14-7041-81
Mouse	IL-4	eBioscience (Thermo Fischer, UK)	#14-8041-80
Mouse	αIFNγ	Bio X Cell (Lebanon, USA)	#BE0055
Mouse	IL-6	eBioscience (Thermo Fischer, UK)	#RMIL6I
Human	TGFβ	Immunotools (Germany)	#11343160
Mouse	αIL-2 (JES6-1A12)	eBioscience (Thermo Fischer, UK)	#16-7022-81
Mouse	αCD25 (PC61.5)	eBioscience (Thermo Fischer, UK)	#16-0251-85
Mouse	IL-36α	R&D systems (Abingdon, UK)	#2297-ML-025
Mouse	IL-1β	Gibco™ (Thermo Fischer, UK)	#PMC0814
Mouse	IL-18	R&D systems (Abingdon, UK)	#9319-IL-010
Mouse	IL-33	eBioscience (Thermo Fischer, UK)	#14-8332-62
	ATRA	Sigma Aldrich (Merck, Ireland)	#R2625
Human	IL-36α	R&D systems (Abingdon, UK)	#1078-IL-025
Human	αCD3/αCD28 (Treg expansion kit)	Miltenyi Biotec (Surrey, UK)	#130-095-345

2.8.11. LABELLING OF CD4+ T_n CELLS FOR PROLIFERATION ASSAY. *CFSE:* Following isolation of CD4+ T cells (2.8.6) cells were re-suspended in 10mls of PBS and split into two separate 50mls falcon tubes. 1 tube is to act as a negative control. The other tube is designated for Carboxyfluorescein succinimidyl ester (CFSE) labelling. CFSE labelling is a popular and effective means to monitor lymphocyte proliferation and works by covalently labels long lived intracellular molecules with the fluorescent dye. When a CFSE labelled cell divides, its daughter cells are endowed with half the number of CFSE-tagged molecules. Therefore, each cell division can be evaluated by measuring the decrease in cell fluorescence by flow cytometry.

To perform CFSE labelling the CFSE was first diluted in 15mls of PBS to a concentration of .5mM. This was mixed by inversion and 10mls was discarded. The remaining 5mls was added to 5mls of cells in PBS. CFSE concentration is now at .25mM. The tube is mixed by inversion for 2m, before 3mls of FCS is added to stop the reaction. The cells are pelleted by centrifugation (1300rpm, 5m, RT) and washed in cRPMI. The cells are then counted (2.8.5) and re-suspended in their desired concentration to await plating.

CTV: An alternative dye for CFSE, Cell Trace Violet (CTV), was also used in certain experiments. CTV works in a similar fashion to CFSE. The molecules bind covalently to all free amines on the surface and inside of cells where they are converted to a fluorescent dye. Thus, when a CTV labelled cell divides, its progeny are endowed with half the number of CTV-tagged molecules. Therefore, like CFSE, each cell division can be evaluated by measuring the decrease in cell fluorescence by flow cytometry.

To stain cells with CTV they must first be re-suspended in 5mls of pre-warmed PBS. 2µl of CTV (2mM) is then diluted in 5mls of pre-warmed PBS, mixed by inversion and added to the tube containing the re-suspended cells. CTV is now at a 1mM final concentration. This is once again mixed by inversion and placed in a water bath for 10m. Following this incubation 1ml of FCS is added to the tube to stop the reaction and the cells are placed on ice for 15ml. The cells are pelleted by centrifugation (1300rpm, 5m, RT) and washed in cRPMI. The cells are then counted (2.8.5) and re-suspended in their desired concentration to await plating.

*At the time of plating aliquots of CFSE/CTV labelled cells were taken and fixed (2.9.3) to act as a day 0 CFSE/CTV positive compensation for flow cytometry.

2.8.12. ISOLATING AND MITOMYCIN TREATING APCS. Non-T cell Antigen Presenting Cells (APCs) are used as feeder cells in some tissue culture experiments. For the APCs to just perform a feeder cell role, and not react with any stimulus or other cells in the culture, the APCs were treated with Mitomycin C. Mitomycin c covalently crosslinks DNA, inhibiting DNA synthesis and cell proliferation, therefore allowing our APCs to act as non-responding feeder cells.

To generate these cells splenocytes were isolated from *Rag1*$^{-/-}$ mice (2.8.1). As these cells are T cell deficient they can act as non-T cell APCs without performing further purification. The APCs were then re-suspended at 4x10^{7} cells/ml in PBS and 50µg/ml of Mitomycin C (M4287, Sigma Aldrich) was added. These cells were mixed by inversion and incubated at 37^{0}C with 5% CO_2 (Sanyo CO_2 Incubator) for 30m. After this incubation, the cells were washed 2 times in PBS, counted (2.8.5) and re-suspended to desired concentration in RPMI. APCs were then stored at 4^{0}C to await plating.

2.8.13. LABELLING OF CD4^{+} T$_{H}$ CELLS FOR TREG SUPPRESSION ASSAY AND CULTURE. A Treg suppression assay is a means of measuring the ability of Tregs to supress T effector cell responses. In this assay Tregs are dose dependently titrated into a well of T effector cells, and T effector responses are measured as marker of suppression. In this assay IFNγ was our marker of T effector responses.

In this experiment CD4^{+} T$_{H}$ cells were isolated by negative selection from the spleens and mLNs of donor *wt* and *Il36r*$^{-/-}$ mice (2.8.1-2.8.2). The CD4^{+} T$_{H}$ cells were then counted (2.8.5) and stained for the cell surface markers CD4, CD25, and CD45Rβ (2.9.2). The CD4^{+} cells were then FACs sorted for CD4^{+} CD25^{-} CD45Rβ^{hi} Effector T cell and CD4^{+} CD25^{+} CD45Rβ^{low} Treg cell populations (2.9.6).

Following sort, T effector cells were stained with CFSE and Tregs were stained with CTV (2.8.11). After staining these cells were counted (2.5) and re-suspended at the desired concentrations, and stored at 2-8^{0}C protected from light to await plating.

While the T cells were being stained the non-T cell APCs were being isolated and treated with Mitomycin C (2.8.12). As these cells were not fluorescently stained we were able to distinguish this population by their CFSE-CTV- expression by flow cytometry.

All the cell populations were now ready to be plated. Table 9 shows plating strategy and number of cells/well. Once all cells were added to their respective wells, soluble αCD3 (0.25µg) and IL-36α (200ng) was also added, the wells were mixed by pipetting up and down. The plate was then incubated for 72hrs at 37^{0}C with 5% CO_2(Sanyo CO_2 Incubator). After 72hrs the supernatants are stored at -20^{0}C (2.7) for ELISA and the cells are harvested for staining for flow cytometric analysis (2.9).

TABLE 2.10: PLATING STRATEGY FOR TREG SUPPRESSION ASSAY.

	1	2	3	4	5	6	7	8	9	10
A	75,000 Treg + aCD3 300,000 APCs	75,000 Treg + aCD3 300,000 APCs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs	150,000 T effector + aCD3 300,000 APCs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 75,000 Tregs	150,000 T effector + aCD3 300,000 APCs 25,000 Tregs	150,000 T effector + aCD3 300,000 APCs 12,500 Tregs	150,000 T effector + aCD3 300,000 APCs 75,000 Tregs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 25,000 Tregs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 12,500 Tregs + IL-36 200ng
B	75,000 Treg + aCD3 300,000 APCs	75,000 Treg + aCD3 300,000 APCs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs	150,000 T effector + aCD3 300,000 APCs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 75,000 Tregs	150,000 T effector + aCD3 300,000 APCs 25,000 Tregs	150,000 T effector + aCD3 300,000 APCs 12,500 Tregs	150,000 T effector + aCD3 300,000 APCs 75,000 Tregs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 25,000 Tregs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 12,500 Tregs + IL-36 200ng
C	75,000 Treg + aCD3 300,000 APCs	75,000 Treg + aCD3 300,000 APCs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs	150,000 T effector + aCD3 300,000 APCs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 75,000 Tregs	150,000 T effector + aCD3 300,000 APCs 25,000 Tregs	150,000 T effector + aCD3 300,000 APCs 12,500 Tregs	150,000 T effector + aCD3 300,000 APCs 75,000 Tregs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 25,000 Tregs + IL-36 200ng	150,000 T effector + aCD3 300,000 APCs 12,500 Tregs + IL-36 200ng

2.9. FLOW CYTOMETRY. Flow cytometry was used to determine cell surface and intracellular expression of proteins of interest following cell isolation from *in vivo* experiments, and tissue culture from *ex vivo* and *in vitro* experiments. When analysing the cells isolated from tissues taken from *in vivo* experiments, the cells were first counted (2.8.5) and transferred to a 5ml FACs tube in a 1ml volume diluted in PBS. 50µl of this was taken from each tube and transferred to a "compensation/FMO tube". These cells were later used to create cellular compensation for the staining antibodies used, and for the generation of fluorescence minus one (FMO) controls. For *in vitro* cell culture experiments, all wells from a specific condition were added to its allocated FACs tube. Extra cells had been plated during tissue culture to be used for cellular compensations and FMOs. At this point they were added to their assigned tube.

2.9.1. SAMPLE RE-STIMULATION FOR INTRACELLULAR ANALYSIS. To analyse expression of intracellular cytokines the cells needed first to be re-stimulated to induce cytokine production, and then their secretion of these cytokines needs to be blocked, to allow for detection of the cytokines by flow cytometry. To achieve this, we first re-stimulated the cells with phorbol-12-myristate 13-acetate (PMA) (#79346 - Sigma Aldrich) and Ionomycin (#I9657 - Sigma Aldrich) to induce cytokine production, and added Brefeldin A (BFA) (#00-4506-51 eBioscience) to block protein transport at the endoplasmic reticulum, therefore retaining the induced cytokines inside the cell. The cells were treated with this cocktail in a 500µl volume diluted with RPMI for 4-6 hours at 37°C. Details of re-stimulation conditions for each specific cytokine is listed in Table 10. Following this incubation period the cells were washed twice by adding 1ml of RPMI, centrifuging (1300rpm, 5m, RT), and discarding the supernatant. Cells were then re-suspended in 1ml of PBS and stored at 4°C to wait for cell surface labelling (2.9.2)

TABLE 2.11: RE-STIMULATION CONDITIONS FOR INTRACELLULAR CYTOKINE STAINING BY FLOW CYTOMETRY

Cytokine	PMA (ng/ml)	Ionomycin (µg/ml)	BFA (µg/ml)	Time (H)
IFNγ	10	1	5	4
IL-17a	10	1	5	6
IL-9	100	1	5	6

2.9.2. CELL SURFACE STAINING. To analyse the expression of chosen cell surface markers cells were pelleted by centrifugation (1300rpm, 5m, 4°C). While the cells were spinning down, Live Dead Fixable Aqua Dead Cell stain (LD) (L34957 - Invitrogen) was diluted to a 1:500 volume in PBS and kept protected from light on ice. This kit allows the distinction of live cells from dead cells to allow for accurate analysis. The supernatants were then discarded and the pellets were re-suspended in 50µl of LD. The cells were vortexed and incubated at 2-8°C protected from light for 30m. After this incubation 1ml of PBS+2%FCS was added per tube and the cells were pelleted by centrifugation (1300rpm, 5m, 4°C). During this time, the cell surface antibodies were diluted to their required concentrations (Table 2.12) and kept on ice protected from light. The supernatant was discarded from the cells and they were re-suspended a 1:50 dilution of FC block:PBS. The cells were vortexed and incubated for 10m at RT. 50µl of the cell surface antibodies were then added to the tube. The tubes were vortexed and incubated at 2-8°C protected from light for 30m-1h. 1ml of PBS+2%FCS was added per tube and the cells were pelleted by centrifugation (1300rpm, 5m,4°C). The cells were either re-suspended in 200µl PBS+2%FCS to begin analysis on the flow cytometer, or they proceeded to be fixed and permeabilised for intracellular staining (2.9.3).

2.9.3. FIXATION AND PERMEABILASTION OF CELLS FOR INTRACELLULAR STAINING. A FOXP3 staining buffer set (00-5523-00 - eBioscience/Thermo Fischer, UK) was used to fix and permeabilise cells to facilitate detection of intracellular cytokines and transcription factors. To do this fix/perm concentrate was mixed with fix/perm diluents at a 1:3 ratio. The pelleted cells were re-suspended in 250µl /tube of 1X fix/perm concentrate, vortexed and incubated either at RT for 30m or overnight at 2-8°C protected from light. 2mls of 1X fix/perm buffer was then added per tube, and they cells were pelleted by centrifugation (1300rpm, RT, 5m). The cells were now ready to proceed to intracellular staining (2.9.4). Buffers used are detailed in Table 2.20.

2.9.4. INTRACELLULAR STAINING. The intracellular antibodies were diluted to their required concentrations (Table 2.12) in perm buffer (Table 2.20), and 50µl of this cocktail was added per tube. The cells were vortexed and incubated for 30m at RT protected from light. 2mls of perm buffer was added to each tube and the cells were pelleted by centrifugation (1300rpm, RT, 5m). Samples were then re-suspended in 200µl PBS+2% FCS and stored at 4°C until ready for analysis by flow cytometry (2.9.6).

TABLE 2.12: FLOW CYTOMETRY FLUOROPHORE CONJUGATED ANTIBODIES

Antibody	Clone	Concentration/test	Species	Company	Catalogue #
αCD3ε	2C11	0.5µg 0.25µg	Mouse	eBioscience	11-0031-82 (FITC) 17-0031-82 (APC)
αCD4	GK1.5	0.25µg 0.125µg 0.25µg	Mouse	eBioscience	11-0041-82 (FITC) 12-0041-82 (PE) 25-0041-82 (PeCy7)
αCD4	RPA-T4	0.125µg	Human	eBioscience	45-0049-42 (PerCP-Cy5.5)
αIFNγ	XMG1.2	0.25µg 0.5µg	Mouse	eBioscience	12-7311-82 (PE) 48-7311-82 (ef450)
αIFNγ	4S.B3	0.125µg	Human	eBioscience	12-7319-42 (PE)
αIL-17a	17B7	0.125µg 0.25µg	Mouse	eBioscience	12-7177-81 (PE) 48-7177-82 (ef450)
αFOXP3	NRRF-30; FJK-16S	0.25µg 1µg	Mouse	eBioscience	12-4771-82 (PE); 17-5773-82 (APC)
Anti - α4β7	DATK32	0.25µg	Mouse	eBioscience	17-5887-82 (APC)
Anti - α4β7		1µg	Human	R&D	FAB10078R (af647)
αCCr9	CW-1.2	0.5µg	Mouse	eBioscience	12-1991-82(PE)
αCCr9	CW.1.2	1µg	Human	R&D	565576 (PE)
αCD25	PC61.5	0.125µg	Mouse	eBioscience	12-0251-82 (PE)
αCD45Rβ	C363.16A	0.25µg	Mouse	eBioscience	17-0455-81 (APC)

αCD69	H1.2F3	0.5μg	Mouse	eBioscience	25-0691-82 (PeCy7)
αIL-9	RM9A4: MH9D1	0.06μg 0.06μg	Mouse	eBioscience	50-8091-82 (EF660); 12-7098-42 (PE)

2.9.5. PREPARATION OF COMPENSATIONS AND CONTROLS FOR MULTIPARAMETER FLOW CYTOMETRIC ANALYSIS. In multiparameter flow cytometric analysis, compensation for spectral overlap is necessary. To do this, we used both cellular and bead compensation methods. For cellular compensation, the cells were prepared as described in 9.2-9.4. In this case, the cells were only dyed with the specific antibody-fluorophore conjugate they would be used to compensate. Concentrations of antibodies use is detailed in Table 2.12.

For bead compensation, human (BD, 552842) or mouse (BD, 552845) compensation bead kits were used. To prepare the beads, 1 drop of positive and 1 drop of negative was added to 300µl PBS in a 5ml FACs tube. These were mixed and 100µl of this solution was added to separate tubes. 1µl of each antibody-fluorophore conjugate we wish to compensate was then added to each tube separately (Table 2.12). These were then vortexed and incubate at RT for 30m. 200µl of PBS was then added per tube and the compensations were stored at 4°C to await analysis.

The FMO controls were created throughout the staining process alongside the samples. An FMO means florescent minus one, therefore in each FMO one stain is left out of the staining process. The absence of this one stain allows us to gate the negative space, and any cells from stained samples that enter this gate are therefore positive. An example of this is depicted in Figure 2.2.B.

2.9.6. MULTI-PARAMETER FLOW CYTOMETRIC ANALYSIS AND CELL SORTING. Multi-parameter analysis was performed on an LSR/Fortessa (Becton Dickinson Biosciences (BD)) and analysed using FlowJo software (Tree Star). Cell sorting was performed using a FACs Melody (BD) or MOFLO XDP (Beckman) cell sorter. Initial gating was performed to exclude debry, dead cells and doublets. Gating strategy is depicted in Figure 2.2.A and explained in detail in Table 2.13. A stopping gate of 5-10,000 live single CD4+ positive cells was used for analysis of *in vitro* tissue culture experiments, and whole samples were collected for tissue collected from *in vivo* experiments.

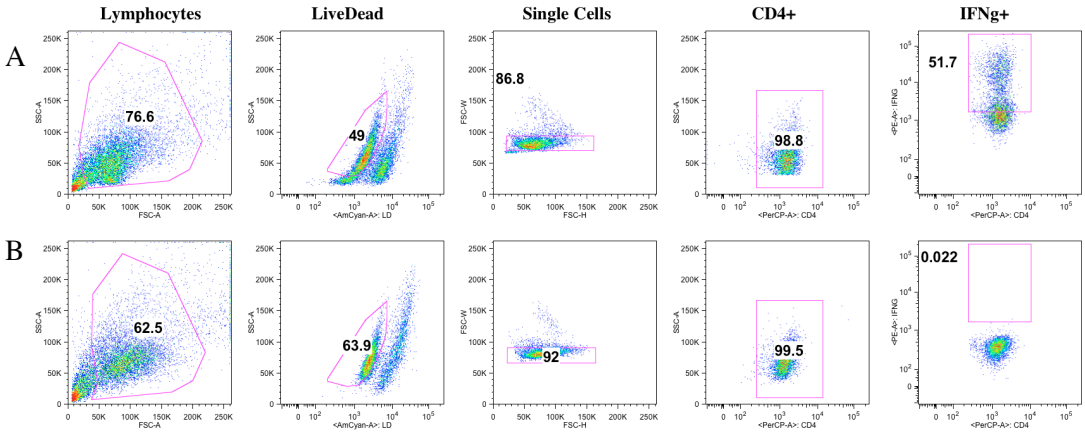

Figure 2.2.: Gating strategy for multi-parametric flow cytometry. A shows sequential gating on lymphocytes --> live cells --> single cells --> CD4+ T cells --> IFNγ+ CD4+ T cells. **B** shows the same sequential gating but for an IFNγ FMO control sample. This sample has no IFNγ antibody staining. Therefore, when we plot IFNγ against CD4, the negative space above the CD4+ population depicts were IFNγ+ stained populations will lie. A detailed description of the gating parameters is shown in table 2.13.

TABLE 2.13: DETAILED DESCRIPTION OF GATING STRATEGY USED IN MULTIPARAMETRIC FLOW CYTOMETRY.

	X axis	Y axis	Gated population
Lymphocytes	FSC-A	SSC-A	This gate encapsulates the lymphocyte population and excludes small cellular debry
LiveDead	LiveDead AmCyan	SSC	This gate captures the live population of lymphocytes and excludes dead cells
Single Cells	FSC-H	FSC-W	This gate captures the single cells in the live lymphocyte population and excludes doublets.
CD4+	CD4 PerCP-A	SSC-A	This gate depicts the CD4+ expressing cells in the single live lymphocyte population and excludes non CD4+ cells.
IFNγ+	CD4 PerCP-A	IFNγ PE-A	This gate depicts the IFNγ expressing cells in the CD4+ single live lymphocyte population.

2.10. T CELL TRANSFER-INDUCED COLITIS. As previous studies from our group reported a potential role for IL-36 in regulating both Th1 and Th17 responses in the inflamed colon during *C. rodentium* induced colitis[394], we decided to analyse the effect of Il36r signalling on the pathogenesis of disease in a model of IBD that is exclusively T cell mediated.

To do this we chose the T cell transfer model of colitis. In this model $CD4^+CD25^-CD45R\beta^{hi}$ T effector cells are transferred into *Rag1*$^{-/-}$, T and B cell deficient hosts. Due to the absence of an adaptive immune response in these hosts, the transferred T cells are under no homeostatic regulation, and proliferate and differentiate into pro-inflammatory T_H cell lineages, classically T_H1 and T_H17, and induce T cell driven intestinal inflammation.

To determine the effect IL-36 signalling has on the induction and perpetuation of GI inflammation splenocytes and lymphocytes were isolated from wt and *Il36r*$^{-/-}$ mice (2.8.1-2). These cells were then enriched for a $CD4^+$ population by magnetic bead isolation (2.8.4) and stained for expression of the cell surface markers CD4, CD25, and CD45Rβ (2.9.2). Once stained the cells were pelleted by centrifugation and re-suspended at $10x10^7$ in ice cold PBS+1%FCS. Cells were stored on ice before, during and after cell sort. Cells to be sorted were gated using FCS-A vs SSC-A to eliminate cellular debry and pick out the lymphocyte population. $CD4^+CD25^-CD45R\beta^{hi}$ were denoted T effector cells, and this population was then FACS-sorted using a Melody (BD).

While the cells were being sorted, the mice were prepared. Age and sex matched *Rag1*$^{-/-}$ mice were split into groups, wt recipients, *Il36r*$^{-/-}$ recipients, and PBS controls. Each mouse in the group then had its ear specifically clipped to allow identification of the specific mice throughout the course of the experiment.

Once the cells were sorted, they were washed in PBS, pelleted by centrifugation (1300rpm, 5m, RT) and re-suspended at $5x10^6$/ml in PBS kept at RT. The weight of each mouse was noted and 100µl of the sorted *wt* and *Il36r*$^{-/-}$ T effector cells were then transferred to their specific hosts by *i.p.* injection. Therefore, each host mouse received 500,000 T effector cells. 100µl of PBS was *i.p.* injected into *Rag1*$^{-/-}$ hosts to act as a control.

Over the course of the next four weeks the weight of the mice was taken at regular intervals to measure the course of the disease. Clinical disease score was measured by % weight loss compared to original weight. Cut off point of was when a mouse lost 20% of original weight or reaching day 28. At takedown, colons and small intestines were excised, weighed and measured, and 5mm samples taken for H&E staining (2.13-2.16). SI tissue was then used for tissue culture explants (2.11), and the remainder of the colon, in addition to the spleens and mesenteric lymph nodes were harvested for multiparameter flow cytometric analysis (2.9).

2.11. EXPLANT OF SMALL INTESTINAL TISSUE. Following the takedown of a T cell induced colitis experiment the small intestine was excised from the abdominal cavity of *Rag1*$^{-/-}$ mice. A 5mm section of this was taken for H&E staining (2.13-2.16) and the remainder was stored on ice in PBS for explants. The SI was then opened logitudely and faecal matter was washed out with PBS. 3 x 3cm sections were then cut for explants a 1 section was then placed in 1 well of a 24 well plate with 3mls of cRPMI. This plate was then incubated at 37°C with 5% CO_2 (Sanyo CO_2 Incubator) for 24hrs. After 24hrs the supernatants were harvested and stored and -20°C for future use in ELISA (2.7) and the tissue was harvested to determine its protein content using the Bicinchonic Acid Protein (BCA) assay (2.12).

2.12. BICINCHONIC ACID PROTEIN (BCA) ASSAY. The BCA protein assay is used to determine the total quantity of protein in a sample. The basic principle of this method is that proteins in the sample can reduce Cu+2 to Cu+1 in an alkaline solution (the biuret reaction). This results in a purple colour formation by bicinchoninic acid and can be measured by a spectrophotometer.

To determine protein quantity using this method we used a BCA Assay kit (Sigma Aldrich, BCA1) following manufactures instructions. First, 19mls of Reagent A was mixed with 0.38mls of Reagent B to make the BCA Working Reagent. This mixed BCA Working Reagent is now a light green colour. Then the standards were prepared, the top standard is 1mg/ml of BSA (Sigma Aldrich, A2153), and this is serially diluted 7 times in the same buffer the sample is stored in in 1.5ml Eppendorf's. 25µl of the standards are then added in duplicate to a 96 well plate. 25µl of the samples are added to the plate in triplicate, and 25µl of the same buffer the samples are stored in are added to the plate in triplicate to act as a negative control. 200µl of BCA Working Reagent is then added to every well. The plate was then continually monitored for the development of a purple colour in the wells, this could take up to 30m at 37°C. Once the standards had developed the desired colour the plate was then immediately analysed using a Synergy MX microplate reader (BioTek) by measuring the absorbance at 562nM.

2.13. TISSUE PROCESSING FOR MICROSCOPY. Approximately 5mm sections of distal colon and small intestine were harvested, fixed in 10% phosphate buffered formalin and incubated for 24-48hrs at room temperature. The samples were then removed from formalin and transferred to 70% ethanol to await tissue processing. This process, uses alcohol, xylene and paraffin, to take the tissue from its fixed to a state where it is completely infiltrated with paraffin wax. For our samples tissue processing was carried out using a Leica EM TP automated tissue processor, and underwent the tissue processing cycle detailed in Table 2.14. Once the tissue was infiltrated with paraffin it was then stored at 20°C until it was ready to be embedded in a paraffin block (2.14)

TABLE 2.14: AUTOMATED TISSUE PROCESSING PROTOCOL

Stage	Steps	Reagent	Time	Temperature
Dehydration	1	70% Ethanol	15m	RT
	2	90% Ethanol	15m	RT
	3	100% Ethanol	15m	RT
	4	100% Ethanol	15m	RT
	5	100% Ethanol	15m	RT
	6	100% Ethanol	15m	RT
Clearing	1	Xylene	20m	RT
	2	Xylene	20m	RT
	3	Xylene	45m	RT
Wax Infiltration	1	Paraffin	30m	60°C
	2	Paraffin	30m	60°C
	3	Paraffin	45m	60°C

2.14. EMBEDDING TISSUE SAMPLES IN PARAFFIN WAX. Processed samples were heated to 55°C by placing in a paraffin bath. Metal trays were cooled on a cold block, and the bottom of the tray was lightly covered in the wax. This tray was then moved to a cold block, and the warmed tissue sample was quickly placed in the setting wax in the desired orientation (vertically). The tray was then filled with hot paraffin, and back of a cassette was placed against it. The tray containing the sample embedded in paraffin was carefully transferred to a cold plate and left to set. Once set the paraffin block was removed from the tray and was stored at room temperature protected from sunlight until ready for cutting (2.15).

2.15. MICROTOME CUTTING OF PARAFFIN EMBEDDED TISSUE SECTIONS. Prior to cutting the paraffin blocks were placed on a cold block and a water bath was heated to 50°C. A new blade (Leica, DB 80 LS) was placed in the microtome (Leica, RM2255) and the microtome was set to trim 25μM sections of the block. The paraffin block was then set in the chuck and was trimmed until the tissue is clearly visible. Excess wax was then cleaned away and the microtome was set to cut 4μM sections from paraffin embedded samples. As many ribbons as needed were then cut from the blocks. These ribbons were placed in 35% IMS in a glass tray, broken into single sections and transferred to the hot water bath. These sections were then mounted on the positive side of a Superfrost Plus adhesion slide (Thermo Scientific, Germany). The slides were placed on a benchtop to dry overnight and stored at room temperature until ready to stain (2.17-2.17).

2.16. HISTOPATHOLOGY. To examine cellular infiltration and over all tissue structure in samples we used Haematoxylin and Eosin (H&E) staining. This staining employs a simple protocol of deparaffinising the tissue, rehydrating it, staining with Haematoxylin (to stain nuclei) and Eosin (to stain the non-nuclear elements of the tissue) (Sigma Aldrich, HT110216), dehydrating the stained tissue and mounting it on a glass side (Superfrost Plus adhesion slide, Thermo Scientific). A detailed protocol of this method is available in Table 2.15. Following staining the tissues were mounted using DPX (Sigma Aldrich, 44581) and a glass cover slide (Applied Biosystems, 4360954) carefully applied to avoid any bubbles in the mountant. The samples were then left to dry overnight, and stored protected from light until ready to be imaged. Images were captured using an Inverted Phase Contrast Fluorescent Microscope (Leica DMLB). Captured images were scored blindly, and graded semi-quantitatively from 0 to 5 based on the established protocol by Prowrie *et al*[409]. Details are listed in Table 2.16.

TABLE 2.15: DEPARAFFINIZATION AND REHYDRATION OF PARRAFIN EMBEDDED SAMPLES FOR HISTOPATHOLOGY AND IMMUNOFLOURESECE, AND H&E STAINING PROTOCOL.

Stage	Reagent	H&E	Immunofluorescence
De-parrafinization	Xylene	20 Dips	10m
Xylene removal	100% ethanol	10 Dips	5m
Xylene removal	90% ethanol	10 Dips	5m
Xylene removal	70% ethanol	10 Dips	5m
Hydration	Tap water	Rinse in tap water to hydrate the tissue	2m
Staining of nucleated cells	Haematoxylin	Leave in stain for 8 minutes	
"Blue-ing"	Tap water	Run under tap water for a few minutes to allow "blueing" of nucleated cells	
Counter-staining of non-nucleated elements	Eosin	Leave in stain for 10minutes	
Rinsing	Tap water	Rinse in tap water to remove excess stain	
Dehydration	70% ethanol	10 Dips	
Dehydration	90% ethanol	10 Dips	
Dehydration	100% ethanol	10 Dips	
"Clearing"	Xylene	10 Dips	
"Clearing"	Xylene	10 Dips	

TABLE 2.16: H&E GRADING

Grade	Description
0	• no changes observed
1	• minimal scattered mucosal inflammatory cell infiltrates • +/- minimal epithelial hyperplasia
2	• mild scattered to diffuse inflammatory cell infiltrates, may extend into the submucosa • associated with erosions • minimal to mild epithelial hyperplasia • minimal to mild mucin depletion from goblet cells
3	• mild to moderate inflammatory cell infiltrates • sometimes transmural • often associated with ulceration • moderate epithelial hyperplasia • moderate mucin depletion
4	• marked inflammatory cell infiltrates • often transmural • often associated with ulceration • marked epithelial hyperplasia • marked mucin depletion
5	• marked transmural inflammation • severe ulceration • loss of intestinal glands

2.16. IMMUNOFLUORESCENCE. Paraffin embedded blocks of colon biopsies from children affected with CD, UC or healthy controls were obtained from Our Lady's Children's Hospital Crumlin (Dublin, 12. Ireland). Blocks were sectioned in a microtome and sections of 5-μm thickness were mounted in Superfrost Plus adhesion slides (Thermo Scientific)(2.15). The sections were then de-paraffinised and rehydrated (Table 2.15) and antigen retrieval was performed using 10mM citrate buffer in a microwave. Details of all buffers used are in Table 2.20. The tissue was then quenched with 0.3% Hydrogen Peroxide (H_2O_2)(Sigma Aldrich, H1009) in water for 15m and washed twice with dH2O (5m each). Free aldehyde groups in the tissue were then blocked by incubating the slides in 1M glycine for 30m. The tissue was then washed 3 times with PBS (5m each) and permeabilised with 1% animal serum in PBS-T for 30-60m. Tissue was probed with anti-human CD3 FITC (UCHT1)(eBioscience, Thermofisher, 11-0038-80) and Anti-IL1RL2 (IL-36R)(ThermoFisher) at dilution 1:1000 at 4°C overnight protected from light. The next day the tissue was washed 3 times with PBS-T with 1% serum (5m each) and the secondary antibody, Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Alexa Fluor 594 (ThermoFisher) was added at a 1:500 dilution with 1% normal blocking serum in PBS-T. This was incubated for 2-3hrs at RT protected from light, and then washed 3 times in PBS-T with 1% serum. The slides were then mounted with SlowFade™ Gold antifade Mountant with DAPI (S36936, ThermoFisher) and left to dry overnight protected from light. Images were taken using a confocal microscope Zeiss LSM700.

2.17. RNA ANALYSIS.

2.17.1. ISOLATION OF RNA. RNA extractions from CD and UC patient, and healthy CTRL samples were carried out using Bioline's RNA extraction kit (London, UK), in accordance with manufacturer's instructions. Tissue was homogenised in PBS and placed 1.5 ml tube. The ground tissue was pelleted by centrifugation (1400rpm, 5, RT). The supernatant was discarded and the pellet was re-suspended in 350µl of Lysis Buffer RLY. An ISOLATE II Filter was placed in a 1.5ml collection tube, the lysate was loaded on top, the tube was then centrifuged (11000g, 1m, 4°C). The filter was then discarded and 350µL of 70% EtOH was added to the collection tube. The tissue was mixed by pipetting up and down 5 times, or until dissolved. The sample was then transferred to an ISOLATE II RNA Mini Column in a 1.5ml collection tube. Columns were then centrifuged (11,00g, 30s, 4°C) and transferred into a new 1.5ml collection tube. 350µl of Membrane Desalting Buffer (MEM) was added to the sample, and it was centrifuged (11,00g, 1m, 4°C) to dry the membrane. 95 µL of DNase I Reaction Mixture was carefully added to the centre of the silica membrane in the column. The samples were them incubated for 15 min. 200 µL of Wash Buffer RW1 was added to the column; the column was then centrifuged (11,000g, 30s, 4°C) and transferred to a new 1.5ml collection tube. 600 µL of Wash Buffer RW2 was added to the column and it was centrifuged at 11,000 g for 30s. The flow-through was discarded and the column was placed back on the same collection tube. 250µl of Wash Buffer RW2 was added, and the column was centrifuged (11,000g, 2m, 4°C) to completely dry the membrane. The column was then placed on a 1.5ml nuclease-free collection tube and 60µl of RNase-free H2O were added to the centre of the silica membrane. The column was then centrifuged (11,000g, 2m, 4°C) and removed from the collection tube and discarded. The concentration of the eluted RNA was measured using NanoDrop® ND-1000 Spectrophotometer and noted (2.4), and tube containing the eluted RNA was then stored at -20°C until ready for retro-transcription-PCR (2.17.2). Details of buffers used are in Table 2.20.

2.17.2. RETRO TRANSCRIPTION. Equal amounts of RNA (0.5-1μg in 10μl RNAse free H2O) were reverse (retro) transcribed using Applied Biosystems high capacity cDNA reverse transcription kit (4368814, Foster City, CA) according to manufacturer's instructions. Table 2.17 details the mastermix used and Table 2.18 shows the thermocycling protocol. 10μl of mastermix was added to 10μl of RNA, the samples were briefly centrifuged and loaded into thermocycler (Veriti, Applied Biosystems). Negative controls were created by adding nuclease-free H2O in place of the reverse transcriptase enzyme or

RNAse inhibitor. Following reverse transcription, PCR tubes were stored at -20°C until ready to perform rtPCR (2.17.3).

TABLE 2.17: RETRO TRANSCRIPTION PCR MASTERMIX

Reagent	Volume With RNase Inhibitor x1	MultiScribeTM Reverse Transcriptase x1	Without RNase Inhibitor x1
10X RT Buffer	2.0µl	2.0µl	2.0µl
25X dNTP Mix (100 mM)	0.8µl	0.8µl	0.8µl
10X RT Random Primers	2.0µl	2.0µl	2.0µl
MultiScribeTM Reverse Transcriptase	1.0µl	—	1.0µl
RNase Inhibitor	1.0µl	1.0µl	—
Nuclease-free H2O	3.2µl	4.2µl	4.2µl
Total per reaction	10.0µl	10.0µl	10.0µl

TABLE 2.18: RETRO-TRANSCRIPTION THERMOCYCLING PROTOCOL

RT PCR Amplification Cycles	
1 x 25oC	10m
1 x 37oC	120m
1 x 85oC	5m
1 x 4oC	hold

2.17.3. RTPCR. Real-time PCR (rtPCR) was performed using SensiFAST Probe Hi-ROX kit (Bioline) incorporating predesigned gene expression assays for genes detailed in Table 2.19. Expression was normalized to 18 s ribosomal RNA. Samples were analysed on an Applied Biosystems 7900HT Fast Real-Time PCR System and relative gene expression levels were obtained by the ΔΔCt method.

TABLE 2.19: PRIMERS FOR QPCR

Gene	Protein	Company	Product number
18S rRNA	18S rRNA	Biosciences	4319413E
IL36A	IL-36α	Applied Biosystems	Hs00205367_g1
IL36B	IL-36β	Applied Biosystems	Hs00758166_g1
IL36G	IL-36γ	Applied Biosystems	Hs00219742_g1
IL36RN	IL-36ra	Applied Biosystems	Hs01104220_g1

2.18. STATISTICS. Data are presented as mean±s.e.m. unless otherwise stated. Statistical significance was determined with two-way analysis of variance (ANOVA), unpaired students t-test, paired students t-test, Mann Whitney t-test, and Spearmans correlation as outlined in the figure legends. Real-time quantitative PCR expression data, following 2-ΔΔCt analysis were subjected to the Mann–Whitney t-test. All statistical tests were performed using Prism6 software (GraphPad Software, La Jolla, CA). A P-value of 0.05 was considered significant.

2.19. BUFFERS: The recipes to all buffers used are detailed in Table 2.20.

TABLE 2.20: RECIPES FOR BUFFERS USED IN METHODS.

Buffer Name	Reagents	Quantity	Company	Catalogue Number
ELISA WASH BUFFER	PBS Tablets	20 tablets	Fischer Scientific	1282-1680
	Tween	1ml	Fischer Scientific	BP337-1680
	dH2O	2l	Available on tap	
1X ELISA/ELISPOT Diluent	5X ELISA/ELISPOT Diluent	10mls	eBioscience (Thermofisher)	00-4202-55
	dH2O	40mls	Available on tap	
ELISA Assay Diluent	BSA	0.5g	Sigma Aldrich	A2153
	dH2O	50mls	Available on tap	
cRPMI	RPMI 1640	500mls	Sigma Aldrich	R8758
	FCS	50mls	Gibco (Merck)	10500-64
	Penicillin-Streptomycin	5ml	Sigma Aldrich	P4333
	2-MERCAPTO	2μl	Sigma Aldrich	M3148
chRPMI	RPMI 1640	500ml	Sigma Aldrich	R8758
	FCS	50ml	Gibco (Merck)	10500-64
	Penicillin-Streptomycin	5ml	Sigma Aldrich	P4333
MACS Buffer	PBS	500ml	Sigma Aldrich	D8537
	FCS	10ml	Gibco (Merck)	10500-64
RBC Lysis	Ammonium Chloride (NH4CL)	0.77g	Fischer Scientific	A/3880/53
	dH2O	100ml	Available on tap	

Permeabilisation Buffer	10X Perm Buffer	5mls	Invitrogen (Thermo Fisher)	00-8333-56
	PBS	45ml	Sigma Aldrich	D8537
PBS-T	PBS	50ml	Sigma Aldrich	D8537
	Triton™	0.1%	Sigma Aldrich	X-100
10mM Citrate Buffer (make sure pH 6.0)	10X Citrate Buffer	5mls	Diapath	T0050
	dH2O	45mls	Available on tap	
1M Glycine	Glycine	3.75g	Fischer Scientific	M-15689
	PBS	46.25mls	Sigma Aldrich	D8537
TAE electrophoresis buffer	Tris base	242 g	Sigma Aldrich	T1503
	Glacial acetic acid	57.1 ml	Sigma Aldrich	A6283
	0.5 M EDTA	100 ml	Sigma Aldrich	E8008
	dH2O	842.9ml	Available on tap	

CHAPTER 3.

IL-36 FAMILY EXPRESSION IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

Chapter 3

IL-36 family expression in patients with Inflammatory Bowel Disease

3.1 INTRODUCTION

Inflammatory Bowel Disease (IBD) encompasses a group of gastro-intestinal disorders characterised by uncontrolled and destructive inflammation of the GI tract . The most common of these disorders are Crohns Disease (CD), defined by inflammation occurring at any point in the GI tract from the mouth to the anus, and Ulcerative colitis (UC), whose disease activity is limited to the colon[64]. Prevalence of this disease is highest in developed western countries, with up to 2 million people in Europe, and 1.6 million in America affected[43,44]. 20-25% of these cases are reported in patients under 20 years old[43,45,46]. Furthermore, recent epidemiological studies have also shown an increase of disease incidence in developing countries, such as in Eastern Europe, Asia South America, and Africa, and is associated with industrialization [43,44]. While the precise causes of IBD remain elusive, such variance in epidemiological patterns highlight the importance of environmental and genetic factors in the development of the disease.

The gastrointestinal system represents most sophisticated immune organ of the entire body. Dynamic layers of mucosal immunoregulation maintain the delicate balance between vigilance and tolerance that is central to GI homeostasis, and it is aberrations in these delicate processes that result in the pathology we see in IBD. Classical treatment of these disorders involves a multi-step approach of GC steroids, immunomodulators, antibiotics and mesalamine compounds[64], all with the ultimate goal of systemic suppression of inflammation and resolution of disease. The GCs work at least in part by redirecting infiltrating monocytes in the inflamed mucosa to regulatory M2 macrophage and $CD103^+$ DC phenotypes. These tolerogenic cells control the inflammatory response by producing anti-inflammatory cytokines that skew T cells in their vicinity towards a Treg lineage, as well as blocking proinflammatory cytokine production by M1 macrophages, T cells and DCs[410]. Despite the efficacy of GCs in modulating the immune response and inducing remission in IBD, there are numerous issues associated with the use of these drugs, including adverse effects, patients resistance, and patients dependence on GC therapy to maintain remission[410].

Therefore it is unsurprising that in recent years, the use of biologics in treating IBD has been gaining much popularity, in particular the use of cytokine targeted therapies, which can allow a much more patient tailored treatment. For instance, Ustekinumab is commonly used in the clinic for treatment of CD[411]. It is a monoclonal antibody targeted against the p40 subunit of IL-12 and IL-23, cytokines key in the polarisation of naïve $CD4^+$ T_n cells to the T_n1 lineage and T_n17 lineages respectively[411]. Thus Ustekinumab neutralises both T_n1 and T_n17 adaptive responses that have been implicated in the pathogenesis of CD. TNFα biologics, infliximab and adalimumab are also well established in their capacity to induce and maintain clinical response and remission, reduce systemic inflammation and induce mucosal healing in patients with IBD[68].

In the past few years, the broader IL-1 family, and the IL-36 cytokine subfamily in particular, has been gaining significant attention for their potent role in modulating the immune response and their association with numerous autoimmune diseases, including IBD[412]. Analysis from adult patient cohorts have shown an increase in these cytokines expression in the colon[377, 394, 397], and have shown it to be upregulated during active inflammation[395]. Similarly, our group have recently reported expression of *IL36A* is elevated in the colonic mucosa of paediatric patients with UC[394]. Aside from this, there is very little information in the literature on the involvement of this cytokine in the pathogenesis of IBD, and in particular, from paediatric patient cohorts. Paediatric and adolescent patients are of particular interest in identifying novel targets for IBD as they often represent the earliest manifestations of chronic life-long diseases, are relatively treatment naïve, and are less likely to have experienced as many confounding factors as adults. Additionally, younger patients are also significantly less likely to suffer from co-morbidities that may be masking the pathogenic mechanisms perpetuating the disease. Therefore, potential therapeutic targets identified through research on paediatric patients may prove beneficial in adult patients, whose confounding factors may have masked the involvement of the specific target.

In this chapter, we will examine the expression of IL-36 family members in a paediatric cohort of IBD patients, and determine the cellular source of IL-36 mediated pathology.

3.2 RESULTS

3.2.1 *IL36A* GENE EXPRESSION IS ELEVATED IN THE COLON OF TREATMENT NAIVE PAEDIATRIC IBD PATIENTS

IL-1 family members have long been implicated in the pathogenesis of IBD[322,324,325,326,338,339,343,344,345,346], and elevated levels of the family members have been shown to correlate with disease severity[327,3468]. In the past few years there have been numerous reports of enhanced levels of IL36 cytokines in the intestines of adult IBD patients[9377,394,397], and our own group has reported upregulated *IL36A* expression in the colonic mucosa of paediatric UC patients[394].

Following on from these initial findings, we sought to determine the gene expression of *IL36* family members in the gut from an extended cohort of treatment naïve paediatric IBD patients. To do this the relative expression of *IL36A*, *IL36G*, *IL36R* and *IL36RN* was measured in rectal biopsies from these patients and compared to normal healthy controls.

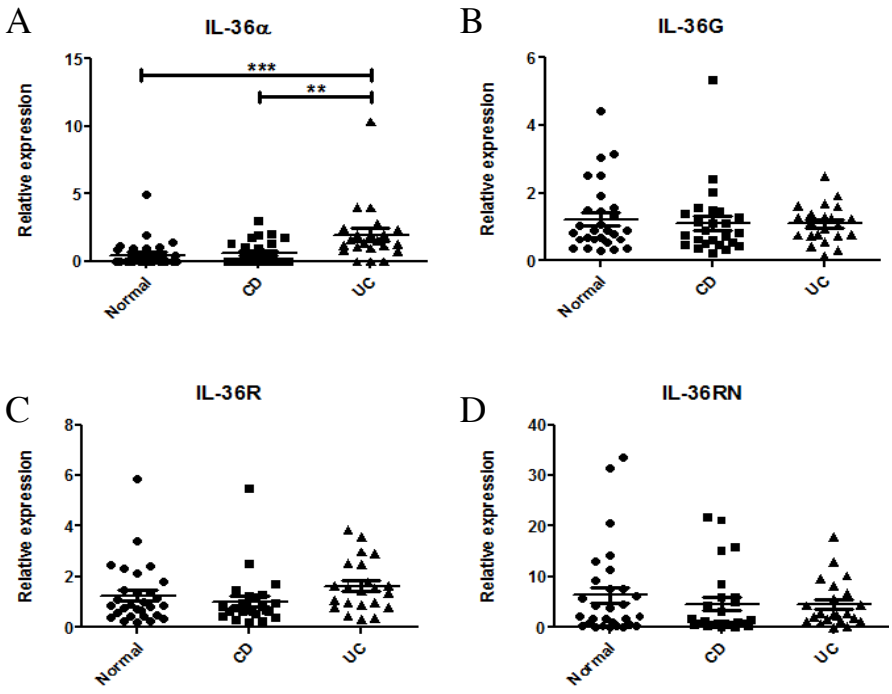

Figure 3.2.1: IL-36 cytokine gene expression in Paediatric IBD samples. Relative expression of IL36a (A), IL36G (B), IL36R (C) and IL36RN (D) was measured in rectal biopsies from treatment naïve paediatric IBD patients or healthy controls by qPCR. CTRL (n=22), CD (n=18) and UC (n=15). Statistical analysis performed by Mann Whitney U Test, **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*. Experiments were carried out by Dr. Martina Gogarty and Dr. Rachel Horan.

Similar to the published findings[394], we see a significant increase in *IL36A* expression from UC patients compared to healthy controls. However, we report for the first time that UC patients also express significantly more *IL36A* than CD patients (Fig.3.2.1A). As the two diseases differ significantly in their pathogenesis and pathological features[64, 345], defining disease specific cytokine profiles may prove useful in determining which patients will benefit the most from specific targeted therapies. *IL36G* expression was evident in CD, UC and control tissues, as was the presence of the *IL36R*. However, there were no significant differences between groups (Fig.3.2.1B & C). Interestingly, there appears to be a trend emerging of decreased expression of *IL36RN* in IBD patients compared to healthy controls (Fig.3.2.1.D). Although it does not reach significance, it is indicative of a potential *IL36A*/*IL36RN* axis in the inflamed mucosa. Taken together these data show that IL-36 family members, *IL36A*, *IL36G*, *IL36R* and *IL36RN* are expressed at the RNA level in the large intestines, but *IL36A* expression is specifically and significantly enhanced in the inflamed mucosa of UC patients upon initial diagnosis, indicating a potential role in the pathogenesis of the disease.

3.2.2 IL36α PROTEIN EXPRESSION IS ELEVATED IN THE SERUM OF TREATMENT NAIVE PAEDIATRIC IBD PATIENTS

IL-1 family member expression is often regulated at the post-transcriptional and post-translational levels[413-415]. We observed elevated expression of *IL36A* in the intestines of UC patients (Fig3.2.1.A); as it is the transcribed protein that elicits the pathogenic effects observed in disease, we decided to next analyse the protein expression of IL-36 family members in our paediatric patient cohort. Initially, to achieve this we determined the amount of IL36α, IL36β, IL36γ, IL36RA and IFNγ protein in the serum of paediatric IBD patients by ELISA.

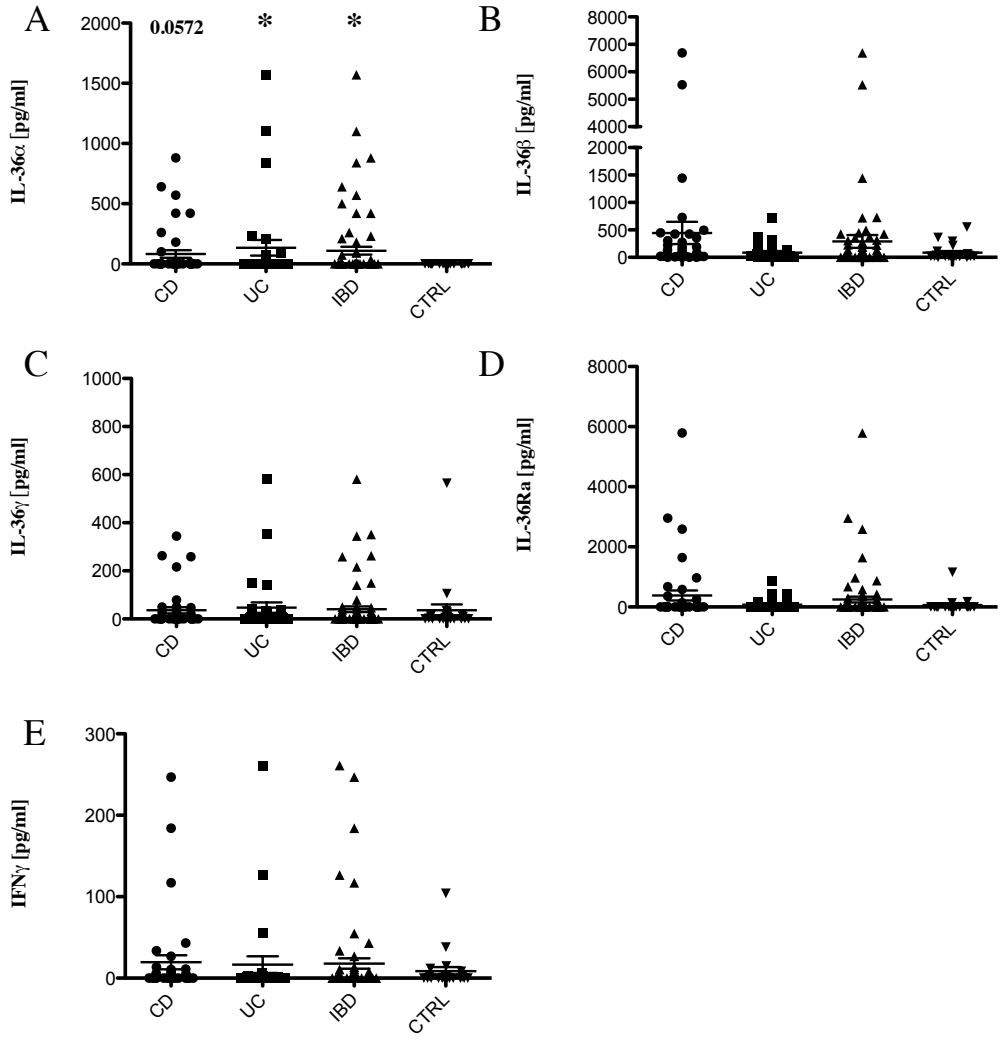

Figure 3.2.2.: IL-36 cytokine protein expression in Paediatric IBD serum samples. Serum samples from paediatric patients with CD or UC were analysed by ELISA for IL-36α (A - n = IBD: 74 CD:42 UC:31 CTRL:23), IL-36β (B - n = IBD: 74 CD:42 UC:31 CTRL:23), IL-36γ (C - n = IBD: 72 CD:41 UC:31 CTRL:23), IL-36Ra (D - n = IBD: 74 CD:42 UC:31 CTRL:23) and IFNγ (E - n = IBD: 42 CD:20 UC:22 CTRL:20). *Statistical analysis performed by Mann Whitney U Test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

In agreement with Fig.3.2.1.A, we see a significant increase IL36α protein in the serum of UC patients compared to controls. Interestingly, expression of this protein is also enhanced in CD patients, but does not reach significance (p=0.057) (Fig.3.2.2.A). Expression of IL36β, IL36γ, and IL36RN is present in the serum of all groups, with no difference observed between them (Fig.3.2.2.B, C & D).

IFNγ protein expression was measured as IL36 has been shown to amplify T_H1 responses[402], which are key in the pathogenesis of IBD. However, while we see a slight increase in the expression of this cytokine in IBD patients compared to controls, this too does not reach significance (Fig.3.2.2.E). Collectively, these results are consistent with our gene expression based data, showing enhanced IL36α expression in the serum of UC patients at a protein level.

3.2.3 IL36α EXPRESSION NEGATIVELY CORRELATES WITH IL36RA

IL36Ra is the negative regulator of IL36 signalling. It functions as a homeostatic molecule, and loss of function mutations in this gene result in the development of the life threatening autoinflammatory disease DITRA[416,417]. Elevated levels of this cytokine have been observed in the mucosa of IBD patients with active inflammation[395], and it is thought to be present to combat IL-36 induced pathology.

As we have observed increased expression of IL36α in IBD patients (Fig.3.2.1A & Fig.3.2.2A), we decided to see if this expression is associated with expression of the IL-36Ra. To do this, statistical analysis was performed using Spearman's Rank Correlation, testing IL36α, IL36β, IL36γ and IFNγ protein expression to that of IL36Ra in UC and CD cohorts. Protein expression of the cytokines was previously measured by ELISA (Fig.3.2.2) and only samples that had detectable levels of cytokines were used in correlation analyses.

While there is no correlation evident between expression of IL36β, IL36γ and IFNγ to IL36Ra (Fig.3.2.3. B, C & D), interestingly, we observe a significant negative correlation between expression of IL36α and IL36Ra (Fig.3.2.3. A). This is indicative of an environment permissive to unregulated IL36α signalling in paediatric IBD patients.

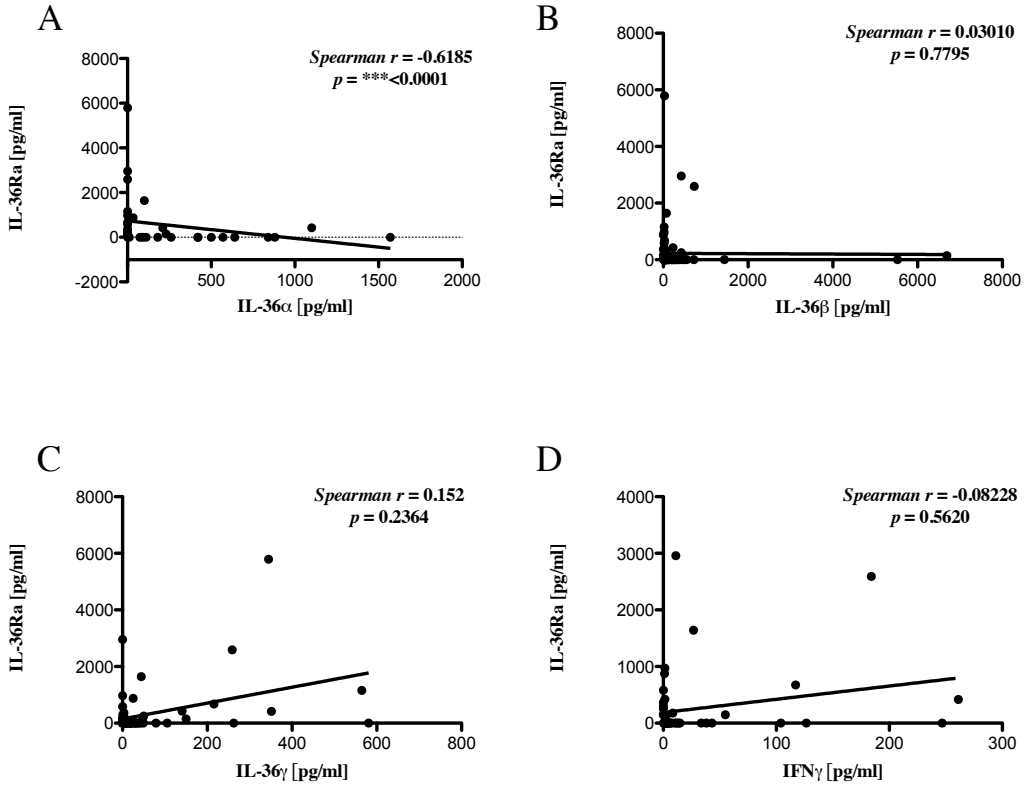

Figure 3.2.3.: IL-36 cytokine protein expression in Paediatric IBD samples. Detectable expression of IL-36α (A – n=36) IL-36β (B – n = 89) IL-36γ (C – n=62) and IFNγ (D – n=52) was compared to then compared to expression of IL-36Ra from paediatric patient serums. *Statistical analysis performed by Spearmans correlation, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.2.4 IL36α PROTEIN EXPRESSION IS ELEVATED IN THE COLONIC MUCOSA OF TREATMENT NAIVE PAEDIATRIC IBD PATIENTS

As we have observed enhanced levels of *IL36A* gene expression in the rectal tissue, as well as protein expression in the serum of IBD patients, we decided to analyse the localization and expression of this protein in the colon. Sections from colonic tissue biopsies were stained using an antibody against human IL36α and imaged using confocal microscopy. This analysis revealed enhanced protein expression of IL36α in the colons of paediatric patients with IBD. In agreement with Scheibe *et al*.'s data from an adult cohort[377], we see a clear increase in the number of IL36α expressing cells in the biopsies from both UC and CD patients compared to healthy controls (Fig.3.2.4A&B). Moreover, the expression of IL36α is localized to cells present in the lamina propria and doesn't appear to be present to significant levels in epithelial cells. These data further confirmed our gene expression analysis at a protein level, and indicate a potential role for IL36α in GI inflammation in IBD.

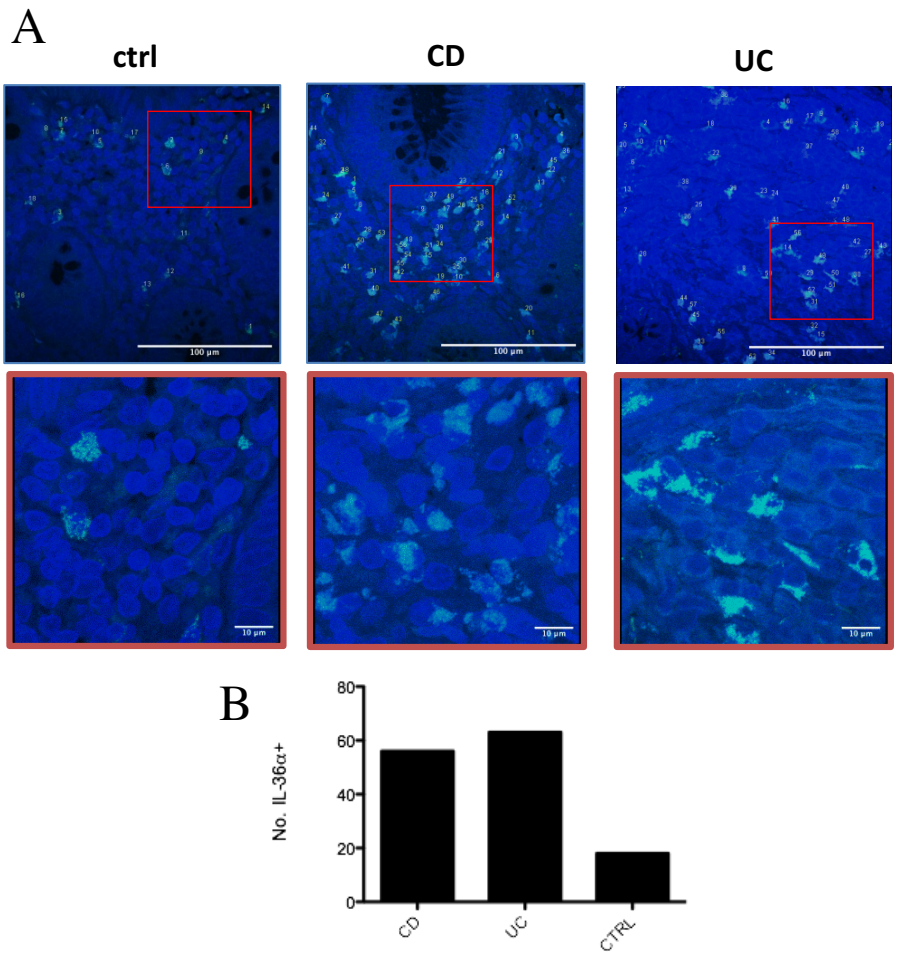

Figure 3.2.4.: IL-36α protein expression is elevated in the colons of Paediatric IBD samples. A - Protein expression of IL36α was measured in colon biopsies from healthy controls and treatment naïve paediatric CD and UC patients by confocal microscopy. The lower panel represents the zoomed in section highlighted by the red box. B - depicts total number of IL36α· cells in each panel. Experiment carried out by Dr. Yasmina E. Hernandez Santana.

3.2.5 IL36R EXPRESSING T CELLS ARE ELEVATED IN THE COLONS OF PAEDIATRIC IBD PATIENTS

Infiltration of T cells to the intestinal mucosa is a key characteristic of IBD, and the pathways regulating their migration, proliferation, and pathogenic differentiation and expansion have long been studied in the hope of finding beneficial targets for therapeutic intervention[205, 235, 240, 249, 286, 288, 337, 352, 357, 394, 407, 418-421]. As we have confirmed enhanced expression of IL36α at both a gene and protein level in the mucosa, we next sought to determine what cells may be responsive to IL-36 cytokines in the inflamed intestinal mucosa in IBD. We previously reported that IL-36R is expressed in lamina propria resident cells in the colons of IBD patients[394]. As effector T cell subsets are strongly associated with the pathogenesis of IBD[62, 398, 407], and have been shown to be responsive to IL-36 cytokine stimulation in preclinical models, we decided to examine the expression of the IL36R on mucosal T cells in our patient cohort. Tissue sections from colon biopsies from UC and CD patients, and non-inflamed controls were stained for the IL36R (PE) and the TCR CD3 (FITC) and imaged using confocal microscopy.

Analysis of these images confirms expression of the IL36R in the mucosa of all groups, and importantly reveals that it is expressed on $CD3^+$ T cells. These data suggest that IL-36 ligands present in the mucosa have the potential to stimulate T cells.
IL-36 stimulation of T cells has previously been shown to enhance T_H1 generation and responses[402], and infiltration of T_H1 cells is a characteristic of CD. As we have previously shown elevated levels of the IL-36α cytokine in the intestines, it may be unsurprising that we now show an increased population of $IL\text{-}36R^+CD3^+$ T cells in colons of UC and CD patients.

Taking these results into consideration with our earlier findings, we now know that there is elevated gene expression of *IL36A* in UC patients, this translates into increased protein expression of this cytokine in the serum and colonic mucosa, in both UC and CD, where there are $IL\text{-}36R^+CD3^+$ T cells receptive to IL-36 signalling, which may act to modulate pathogenic T_H cell responses[262, 394, 402]. Collectively, these data identify a novel potential role for IL-36 in the induction and perpetuation of GI inflammation.

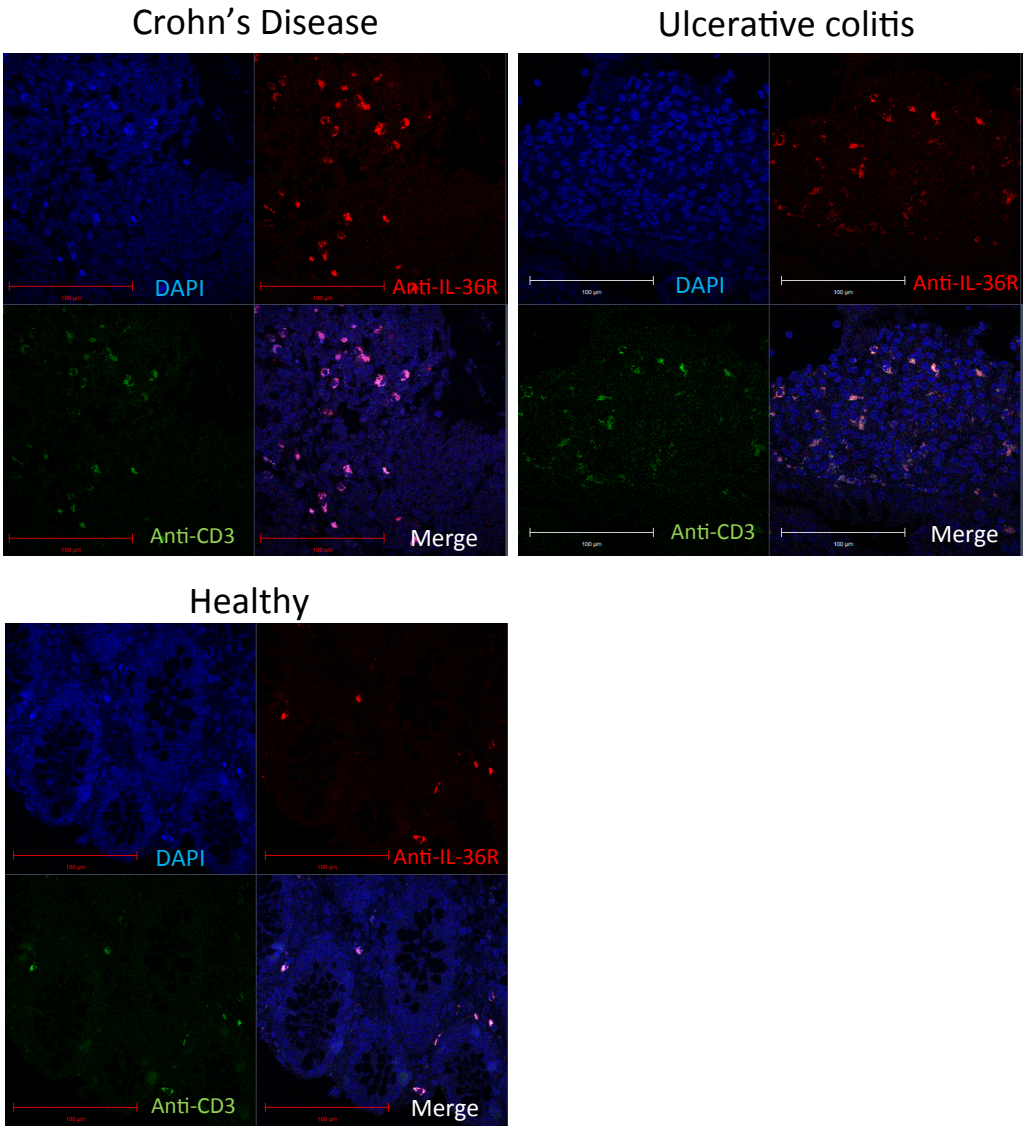

Figure 3.2.5.: IL-36R expression is present on CD3 T cells, and is elevated in the colons of Paediatric IBD samples. Protein expression of the IL36R and CD3 was measured in colon biopsies from healthy controls and treatment naïve paediatric CD and UC patients by confocal microscopy. Experiment conducted in collaboration with Dr. Yasmina E. Hernandez Santana.

3.3 DISCUSSION

While the onset of IBD can occur at any age, approximately 25% of these cases arise in childhood and adolescence. The underlying pathogenesis of the disease in these children appears to be similar to that of adults, involving a complex interaction of genetic, environmental, microbial and immunological factors[43, 45, 46]. However this early onset of IBD is associated with a much more severe disease and encompasses a number of unique features specific to the paediatric IBD population, such as impairment in growth, skeletal development[73-75], and sexual maturation[73]. These problems arise as the children cannot meet the increased nutritional demands associated with childhood and adolescence, due to amplified faecal loss of essential trace elements and protein, poor dietary intake, altered nutrient use, increased metabolic demand, and immunological and endocrine abnormalities[73-75].

Similar to adult onset IBD, paediatric IBD is managed by a multi-step treatment approach of GCs steroids, immunomodulators, antibiotics and mesalamine compounds[64, 73-75]. Yet in recent years, due to their reported efficacy, paediatric gastroenterologists have begun introducing biologics in their treatment plans for these paediatric patients, such anti-TNF treatments, infliximab[81, 82],Adalimumab[83] and certolizumab[84], as well Ustekinumab[85] which targets the p40 subunit of IL-12 and inhibits T_H1 and T_H17 cell generation.

With the accumulating evidence for a role of IL-36 in the pathogenesis of IBD, and the entrance of Spesolimab, a humanized mono-clonal antibody directed against the IL-36R, to several clinical trials in adult IBD populations, we decided to evaluate the role of this cytokine in the pathogenesis of paediatric IBD[422-425].

Early evidence from the literature, has shown that IL-36 agonist expression is upregulated in adult IBD populations, with increased expression of *IL36A* and *IL36G* found in the mucosa[377, 394, 397, 404]. Similarly, we report elevated expression of *IL36A* in the colon of a paediatric UC population, with expression if the cytokine significantly greater than in the ctrl or CD patient groups (Fig.3.2.1.A). This dichotomy in expression has also been observed by Fonseca-Camarillo *et al*. in an adult IBD cohort, and therefore may be indicative of different inflammatory profiles between the disease subgroups[395]. While there is presence of *IL36G*, *IL36R* and *IL36RN* expression in these tissues, there is no significant differences between populations (Fig.3.2.1.B-D). This indicates that IL36α may have initiating role in the inflammation observed in early onset UC.

Our results showing elevated expression of IL36α protein in CD patients, and a significant increase of the cytokine UC patient serum reinforces this potential (Fig.3.2.2.A), as well as the occurrence of the elevated levels of IL36α correlating with decreased amounts of IL36Ra in IBD patients (Fig.3.2.3.A). As other authors have found elevated levels of

IL36α in both CD and UC patients[377, 404], this raises the possibility that the variance we see between CD and UC patients may be an effect of the N numbers used in this study and will be elucidated as more patients are recruited. Furthermore, this result is only seen for IL36α, as protein expression of the other IL36 ligands are present in all groups with no differences observed (Fig.3.2.2.B-D), as well as no significant correlations between their expression and that of IL36Ra (Fig.3.2.3.B-D). Given that IL36γ expression, as well as IL36α, has also been reported to be elevated in adult patients, it is possible that while IL36α plays an early proinflammatory role during the early stages of disease, IL36γ expression only becomes increased once chronicity of inflammation has set in. A further consideration is that a more detailed subset analyses of our paediatric patient cohort may reveal differences in expression of the IL-36 family members in specific patient subsets. This could be done by further stratifying the patients into explicit groups based upon their sex, age, disease severity, disease phenotype, if they present with any other co-morbidities that may be confounding the inflammatory profile, and then performing statistical analysis on IL-36 member expression and correlation with these defined sub-categories.

IFNγ expression was also measured in this patient cohort as it is the signature cytokine of T_H1 cells, and so a key cytokine in the initiation and perpetuation of inflammation in IBD. IL-36 has been shown to promote T_H1 responses *in vitro* and *in vivo*[394, 402], so we postulated there may be elevated IL36 induced IFNγ in these patients' serum. This however, was not the case. While there are increased levels of the cytokine in the UC and CD populations compared to healthy patients, these differences do not reach significance (Fig.3.2.2.E), and do not correlate with IL36Ra expression (Fig.3.2.3.E). Collectively these data show that during inflammation in paediatric IBD, there is a unique environment which may be permissive and specific to unregulated and increased IL36α signalling.

The increased expression and localisation of IL36α protein in the colonic mucosa of these IBD patients further adds to this hypothesis. Importantly we also identify T cells as the cell subset which may be responsive to elevated levels of this proinflammatory cytokine in this setting (Fig.3.2.4). In addition to the epithelium, during intestinal inflammation there is an influx of innate and adaptive immune cells that IL-36 has been shown to stimulate *in vitro*. These include, DCs, macrophages, fibroblasts, and $CD8^+$ and $CD4^+$ T cells[377, 394, 397, 401, 404, 412]. Interestingly, $CD4^+$ T_H cells are the key drivers of the adaptive response during intestinal inflammation, and numerous groups, including our own, have reported a role for IL-36 in modulating their responses[262,394,401,402]. IL-36α[394] and IL-36β[402] promote T_H1 polarization, while IL-36γ enhances T_H9 generation[262, 394] and inhibits iTreg differentiation[262]. Furthermore, *in vivo* deficiency in IL-36R or IL-36 agonist signalling protects against the development of colitis via inhibited pathogenic T cell responses[262, 394, 404]. In keeping with these findings indicating a

regulatory role for IL-36 signalling in mucosal T cell responses in IBD, we observe co-expression of the TCR CD3 and the IL36R in paediatric colonic tissue, and the number of these cells are greatly enhanced in the UC and CD tissues, compared to healthy controls (Fig.3.2.5). This confirms that the elevated cytokine expression of IL36α we see in the serum and colon has the potential to stimulate mucosal T cell responses in the inflamed mucosa.

Furthermore, as it is only an increase in IL36α protein and gene expression we observe in our paediatric population, this may constitute differences observed in between adult and paediatric cytokine profiles, and prove informative for future therapeutic interventions. For example, while targeting the IL36R with monoclonal antibodies, such as Spesolimab, is useful in global IL36 signalling blockade, there are several potential problems that may arise through this approach. Firstly, the impact that global IL-36 family blockade will have on the microbiome is one potential factor which requires consideration. IL-36 ligand signalling has been shown essential in maintaining microbial homeostasis in the intestines, and dysregulation in IL-36 signalling has resulted in increased incidence of obesity and metabolic dysfunction[396], reduced protection and clearance of pathogenic bacteria[426] and diminished levels of pro-resolving cytokines and AMPs during GI inflammation[397, 399].

Secondly, it is important to consider the possible effect that inhibiting IL36 signalling will have on mucosal resolution and healing. While there are many reports highlighting the pathogenic role for IL36 signalling in the intestines[262, 394], there are also numerous studies showing the importance of IL36R signalling in the resolution of mucosal inflammation[377, 397, 399]. Collectively these studies show that IL36 amplifies the immune response in the acute stage of colitis, causing the damage associated with IBD, but as the disease resolves, IL36 signalling is necessary to promote mucosal barrier repair and wound healing.

Interestingly, as we only see an increase of IL36α in out paediatric cohort, and not IL36γ, as other authors report in adult populations, this may be indicative a temporal and dichotomous IL36 response. IL36α promoting the generation and perpetuation of pro-inflammatory responses in the in the intestines early in disease, and IL36γ then inducing mucosal healing and GI homeostasis later on into adulthood.

If this is the case, targeting IL36R signalling in adult IBD may not be the most beneficial option for patients. In this scenario, while some inflammation may be attenuated by blocking the IL36R, the capacity for GI epithelial barrier repair and healing will also be greatly inhibited, and may leave these patients vulnerable to relapse and colonisation by pathogenic bacteria. However, as elevated levels of possible pro-resolving IL36γ are not evident our paediatric cohort, treating these patients with Spesolimab may be in fact

attenuate inflammation before IL36γ mediated resolution is necessary, effectively stopping the perpetuation of inflammation in its tracks. Alternatively, the development of IL36 ligand specific targeted therapies may be the most beneficial to all IBD cohorts, as treatment can be tailored to the individual patient's cytokine profile, while preserving intact IL36R signalling and potentially any pro-resolving effects.

In this chapter, we have shown evidence for a potential role for IL36α specific signalling in the pathogenesis of paediatric IBD. In these patients, IL36α is elevated both in the serum and at the site of inflammation, and appears to be unregulated by the natural antagonist of IL36R signalling, IL36Ra. Whatsmore, the localisation of this cytokine in the intestines, coincides with presence of IL36R$^+$ T cells, suggesting IL36α may be mediating T cell responses in the inflamed mucosa. Aberrant T cell responses are a hallmark of IBD and key in inducing the inflammation and mucosal damage associated with the disease, and represent major targets in the clinic. With the continued association of IL36 family members with the pathogenesis of IBD, further studies are required to elucidate the mechanisms and functionality of this family's signalling on the mucosal immune response, and determine their validity as a target for therapeutic intervention. Such investigations form the next steps in this thesis.

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CHAPTER 4.

THE EFFECT OF IL-36 ON $CD4^+$ T CELL ACTIVATION AND DIFFERENTIATION

Chapter 4

The effect of IL-36 on CD4+ T_H cell activation and differentiation

4.1 INTRODUCTION

Infiltration of CD4+ T_H cells into the intestinal mucosa is a defining and pathogenic characteristic of IBD. As discussed in Chapter 1, enriched populations of these cells are found in the inflamed regions of the intestines in IBD patients, and are believed to be the key initiators in the pathogenesis of the GI inflammation[201, 202]. Classically, T_H1 and T_H17 cells have been associated with the onset of CD, whereas atypical T_H2 and T_H9 cells are implicated in the pathogenesis of UC. Furthermore, Single Nucleotide Polymorphisms (SNPs) have been found in the genes encoding specific CD4+ T_H lineage associated cytokines, chemokines and transcription factors in IBD patients, highlighting the importance of these cells in mucosal inflammation. SNPs in the *IL12B* and *IL23R* genes, that code the IL12 and IL23R proteins, key in T_H1 and T_H17 differentiation, are found in CD patients (Table 1.2). IBD patients have also exhibited SNPs in *IL4*, a key T_H2/ T_H9 differentiation cytokine. Furthermore, numerous IBD associated polymorphisms have been identified in genes important in Treg generation and function, such as *IL2*, *IL2R* and *IL10* (Table 1.2), indicating that loss of function of these suppressive cells also plays a strong role in the pathogenesis of the disease.

Regulation of CD4+ T_H cells relies strongly upon intricate cytokine orchestration of their activation, differentiation, lineage commitment and trafficking[427]. As previously described, the IL-1 superfamily of cytokines are master regulatory proteins in this delicate system, and aberrations in their signalling can contribute to many autoimmune and auto-inflammatory diseases[318]. In regards to IBD, every member of this family has been implicated in its pathogenesis, with some exhibiting a protective role, others promoting pathogenic activity, and many eliciting a dichotomy between the two[320, 321, 323, 358, 428].

Functionally, many of these effects IL-1 family members exert in the mucosa derive from their key instructive roles in CD4+ T_H cell differentiation. IL-1β is well known as a key cytokine necessary to induce T_H17 cell polarisation and responses[172, 429, 430], which have been associated with pathogenesis of CD[351, 414, 431, 432]. Interestingly, recent reports have shown that IL-33 can also coordinate with IL-12 and enhance T_H1 responses[433], showing the importance of the specific cytokine milea in the microenvironment to induce T_H cell responses and their associated inflammatory cascades. In that regard, the presence of negative regulators of IL-1 signalling in the microenvironment is also crucial in modulating intestinal homeostasis.

Absence of the IL-1 orphan receptor SIGIRR results in a more severe form of colitis *in vivo*, associated with enhanced T_H1 and T_H17 responses[434].

In recent years, T_H9 cells have been implicated in the pathogenesis of IBD[258, 263, 420], with various preclinical studies highlighting the pathogenicity of these T_H cells in murine models of colitis[262, 263]. These cells are conventionally polarized upon IL-4 and TGFß stimulation, yet there is emerging evidence that IL-1 members can replace IL-4 to induce the T_H9 phenotype. IL-1α, IL-1β, IL-18, and IL-33 have all been shown to work in combination with TGFß and induce IL-9 to equivalent levels of IL-4 *in vitro*[258, 263, 420, 435].

The IL-36 members of the IL-1 family have been implicated as potent drivers of $CD4^+$ T_H cell activation, polarization and enhanced cytokine production[402], with numerous studies demonstrating a role for IL-36 in modulating T_H1, T_H9, iTreg and T_H17 responses[262, 394, 402, 436]. Appropriate regulation of these cytokines is crucial in the maintenance of immune homeostasis, and loss of function mutations in the *IL-36RN* gene result in the development of the life-threatening autoinflammatory disorders, DITRA (deficiency of IL-36 receptor antagonist)[417] and GPP (generalised pustular psoriasis)[416]. Furthermore, over-expression of the IL-36 agonists has been associated with rheumatoid arthritis, disorders of the lung and kidneys, and IBD[394, 437, 438]. Importantly, there are increasing reports emerging highlighting a role for these cytokines modulating the innate and adaptive response in murine models of IBD[262, 377, 394, 397, 404].

In Chapter 1 we demonstrated that expression of IL-36α is significantly increased in both paediatric CD and UC patients compared to CTRL patients, and has the potential to act on $IL\text{-}36R^+$ $CD3^+$ T cells in the mucosa. Interestingly, among the IBD patients with detectable levels of IL-36α expression, this enhancement was negatively correlated with expression of IL-36Ra indicating a permissive environment for unregulated IL-36 signalling among some IBD patients. This is of particular interest when we compare this expression profile to that of IL-1. Kleiner *et al*. conducted a large analysis comparing cytokine and chemokine profiles between paediatric IBD patients and healthy controls and found significantly elevated levels of proinflammatory IL-1β in patient serum[419]. However, the authors also observe an increase in anti-inflammatory IL-1Ra, indicating that the body's natural defences against superfluous IL-1 signalling are functioning, and preventing IL-1β mediated autoimmunity[419].

IL-1 has been shown to promote the generation of proinflammatory T_H1 and T_H17 cells in preclinical models of IBD, and treatment with anti-IL-1 antibodies, recombinant IL-1Ra proteins, and deletion of the *Il-1r*, *Il-1α* and *Il-1β* genes have all proven efficacious in treating the disease via decreased $CD4^+$ T_H cell pathogenicity[328, 439-442]. However, while IL-1

targeting in the clinic has proven successful in some cases of systemic inflammation[330, 443], this is not the case with IBD[444, 445].

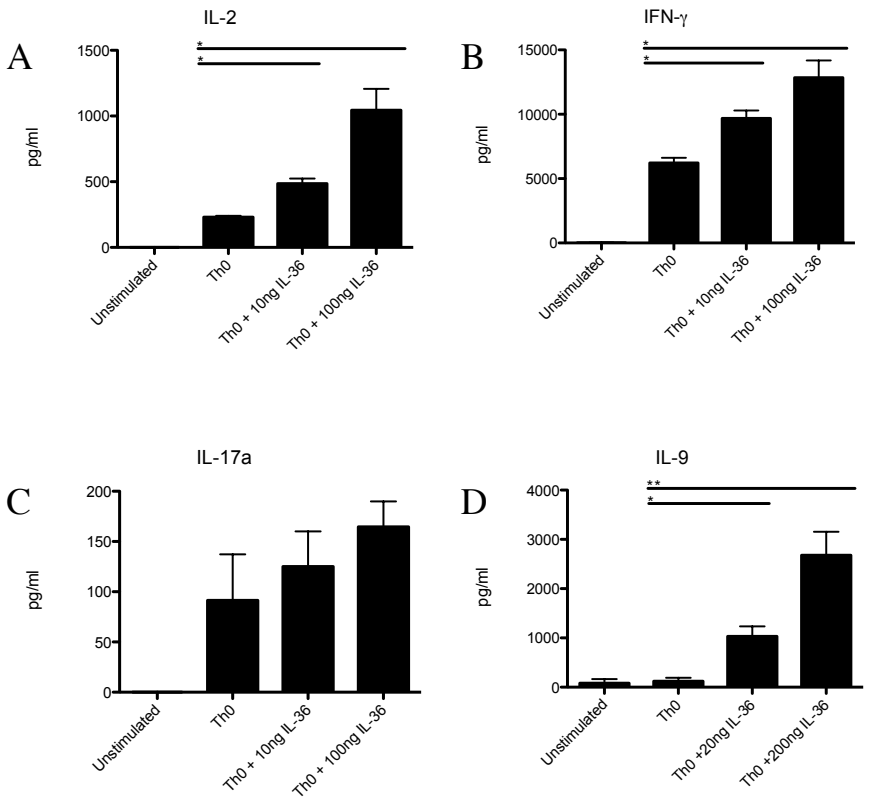
Therefore, considering the possible unregulated IL36α signalling we observed in the serum of paediatric patients, and the increased number of IL36R$^+$ T cells in the colons of IBD patients, as well as the reported role for IL-36 in proinflammatory CD4$^+$ T_H cell differentiation[362, 394, 402], we decided to evaluate the effect of IL-36 on CD4$^+$ T_H cell responses associated with IBD.

4.2 RESULTS

4.2.1 THE EFFECTS OF IL-36α ON CD4+ T_H CELL CYTOKINE PRODUCTION *IN VITRO*

A crucial component of normal adaptive immunity is cytokine modulation of T cell responses. Imbalances in this fragile network have been found to contribute to many autoimmune and autoinflammatory diseases, including Crohn's Disease and Ulcerative Colitis[326, 407, 446]. Therefore, elucidating the responsible cytokines, and how they influence T cell activation, provide attractive candidates for therapy.

To investigate the role of IL-36 in the CD4+ T_H cell response, purified CD4+ $T_H$cells were analysed for of IL-2, IFNγ (T_H1 signature) (Fig. 4.2.1A & B), IL-17a (T_H17 signature) (Fig. 4.2.1C), IL-9 (T_H9 signature) (Fig. 4.2.1D) and IL-13 (T_H2 signature) (Fig. 4.2.1.E) cytokine production when stimulated with varying concentrations of IL-36α (10ng/ml, 20ng/ml, 100ng/ml & 200ng/ml).



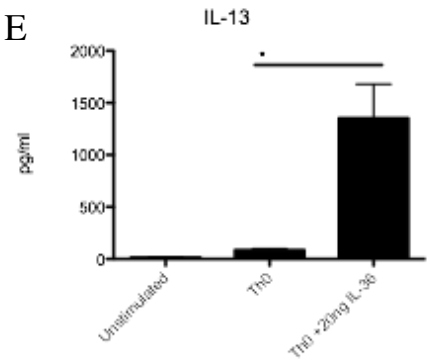
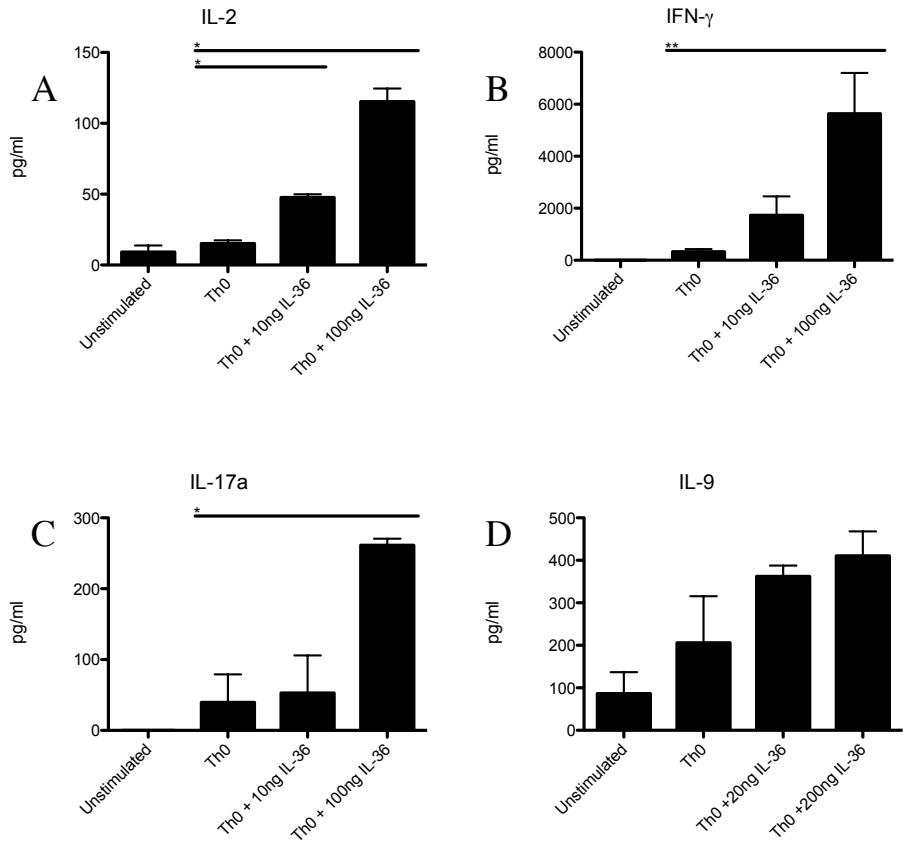


Figure 4.2.1.: The effects of IL-36α on CD4+ $T_н$ cell activation and responses under full activation conditions. Purified CD4+ T cells were cultured in the presence or absence of IL-36α at 37°C under full (0.5μg/ml αCD3ε;4μg/ml αCD28) activation conditions. At 24hr and 72hr time points supernatants were analysed for IL-2 (A) and IFNγ (B), IL-17a (C), IL-19 (D), and IL-13 (E) respectively. These data suggest that IL-36α promotes CD4+ T cell responses *in vitro*. Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Analysis of these results demonstrated that IL-36α stimulation of CD4+ $T_н0$ cells promotes a dose dependent increase in both IL-2 and IFNγ expression (Fig. 4.2.1A & B), suggesting a role for IL-36α in $T_н1$ polarization. Furthermore, a significant increase in IL-9 and IL-13 was also observed (Fig. 4.2.1 D & E), indicating a novel role for IL-36α in driving Type 2 immune responses, whereas IL-17a expression was not significantly altered under these conditions (Fig. 4.2.1 C). This enhancement in both Type 1 and Type 2 responses is of interest in terms of IBD as CD is associated with a predominantly Type1 $T_н1$ response, and UC is associated to a Type 2 $T_н2$, and more recently $T_н9$ response. This broad increase in CD4+ T cell responses suggests IL-36 may represent a suitable target for IBD patients as a whole, and not as a disease specific subtype for treatment. It is also noteworthy that IL-36α stimulation resulted in significantly increased IL-2 expression after 24 hrs. Apart from its role in $T_н1$ polarization, IL-2 is also extensively characterized as a T cell growth factor and potently enhances T cell activation and expansion, while also modulating the differentiation of other $T_н$ subsets. For instance, *in vitro* IL-2 enhances the expansion and polarization of $T_н1$ and iTreg cells, but conversely suppresses the differentiation of $T_н17$ cells[187, 447]. This observation led us then to examine more closely how IL-36α stimulation affects $T_н$ cell activation.

4.2.2 THE EFFECTS OF IL-36α ON CD4+ T_H CELL ACTIVATION *IN VITRO*

T Cell Receptor (TCR) signalling is a critical component in T_H cell activation. For full T_H cell activation to be achieved, stimulation of both the TCR (CD3) and the TCR-co-stimulatory molecule (CD28) needs to occur[448]. As our results demonstrated potent effects of IL-36α stimulation on T_H0 cell cytokine production under full T_H cell activation (Fig.4.2.1) we next decided to evaluate the effects of IL-36α stimulation under partial (αCD3ε alone) activation conditions, to determine whether IL-36 could act in a co-stimulatory fashion. To do this CD4+ T cells were magnetically purified from the spleens of *wt* mice and activated with 0.5μg/ml αCD3ε and varying amounts of IL-36α for 24-72hrs, after which IL-2, IFNγ, IL-17a, IL-9 and IL-13 were measured by ELISA.



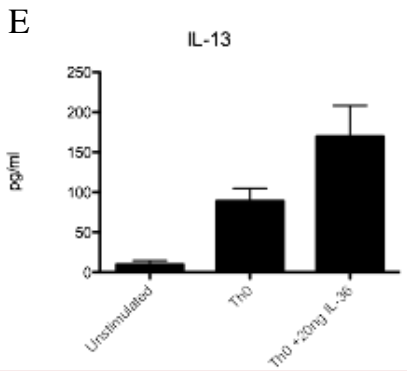
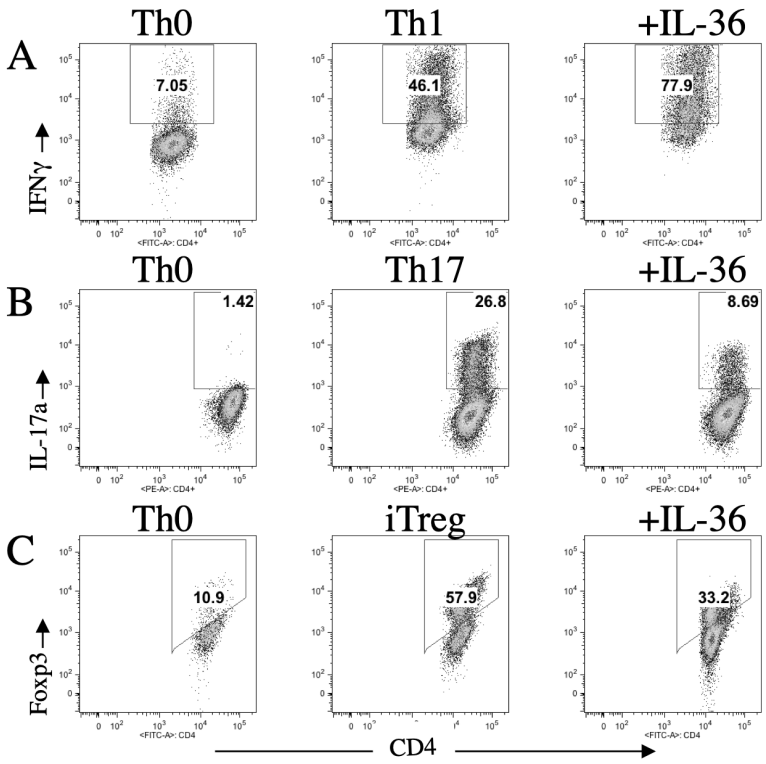


Figure 4.2.2.: The effects of IL-36α on CD4+ T_N cell activation and responses under partial activation conditions. Purified CD4+ T cells were cultured in the presence or absence of IL-36α at 37°C under partial (0.5μg/ml αCD3ε) activation conditions. At 24hr and 72hr time points supernatants were analysed for IL-2 (A) and IFNγ (B), IL-17a (C), IL-9 (D), and IL-13 (E) respectively. These data suggest that IL-36α promotes CD4+ T cell responses and can act as a co-stimulatory molecule for T cell activation *in-vitro*. Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Significantly, we observed an increase in IL-2, IFNγ, IL-17a, and IL-13 in T_N0 cells that had undergone partial activation using αCD3ε alone, thus demonstrating that the effects of IL-36α on T_N cell activation can occur in the absence of CD28 co-stimulation (Fig.4.2.2 A, B, C & E), indicating a novel role for IL-36 as an alternative costimulatory pathway in CD4+ T_N cell activation. While we do see an increase in IL-9 output from these cells, it does not achieve significance (Fig.4.2.2.F). Collectively, these data demonstrate a role for IL-36 ligands as broad regulators of CD4+ T_N cell activation. The ability of IL-36α to activate CD4+ T_N cells and induce various lineage specific signature cytokines indicates a potential ability of IL-36α to promote pathogenic T cell responses in environments with suboptimal stimulus. While this may be beneficial in cases of infection and injury, where a strong and rapid response is necessary, it may also highlight the detrimental significance of this cytokine in the initiation and perpetuation of autoimmunity.

4.2.3 IL-36α DIFFERENTIALLY REGULATES MURINE CD4+ T_H CELL RESPONSES

Having shown that IL-36 cytokines can potently enhance CD4+ T_H cell activation under non-polarizing conditions, we next sought to investigate whether these cytokines may affect the generation of differentiated T_H subsets, which are known to play a central role in autoimmune diseases such as IBD[240, 407]. To date, elevated T_H1 and T_H17 subsets have been associated with CD, whereas 'atypical' T_H2 and T_H9 subsets have been linked to UC[62, 177, 179, 266, 407, 420, 421]. In contrast, iTreg cells promote immune homeostasis in the gut[420, 446, 449]. Furthermore, related IL-1 family members are established as potent modulators of $T_H$cell responses and differentiation *in vitro* and *in vivo*[450]. Therefore, in order to evaluate the role of IL-36 in such settings, purified CD4+ T_H cells were differentiated to the different T_H cell lineages with the addition of instructive cytokines and cultured in the presence or absence of IL-36α.



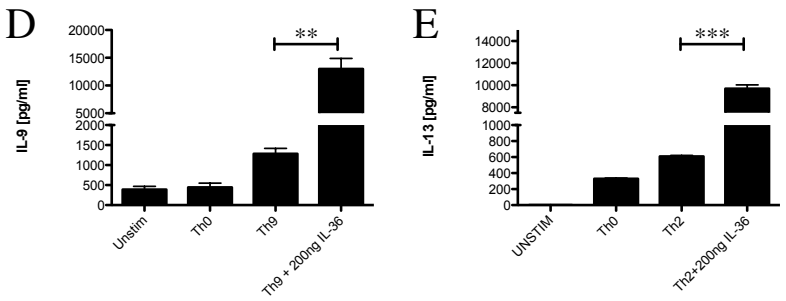


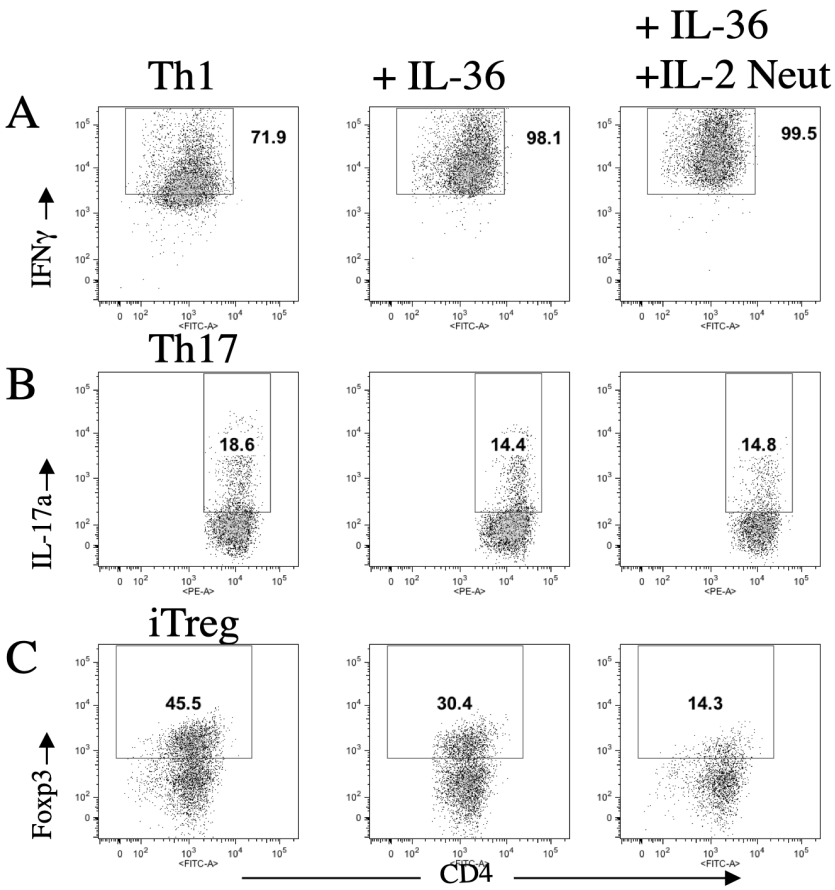
Figure 4.2.3.: IL-36α differentially regulates murine CD4$^+$ T$_H$ cell responses. Magnetically purified CD4$^+$ T cells derived from *wt* mice splenocytes activated with plate-bound αCD3ε /αCD28 (2μg/ml, 2μg/ml), were differentiated to T$_H$1 (20ng/ml IL-12, 10μg/ml αIL-4), T$_H$17 (10μg/ml αIFNγ, 5μg/ml αIL-4, 20ng/ml IL-6), iTreg (5ng/ml TGFß) T$_H$9 (10μg/ml αIFNγ, 20ng/ml IL-4, 5ng/ml TGFß), and T$_H$2 (10ng/ml IL-4, 10μg/ml αIFNγ)) phenotypes, and stimulated with increasing amounts of IL-36α (20ng/ml, 200ng/ml) prior to incubation at 37^{o}C for 72hr - 120hrs. Following incubation supernatants were harvested for ELISA and cells were analysed by intracellular flow cytometry for signature cytokine expression (T$_H$1 - IFNγ (A); T$_H$2 - IL-13 (E); T$_H$17 - IL-17a (B); iTreg - FOXP3 (C); T$_H$9 - IL-9 (D). Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

These results revealed that upon stimulation with IL-36α, T$_H$1, T$_H$9, and T$_H$2 cells all increase signature cytokine production (Fig.4.2.3A, D & E), whereas, in contrast iTreg and T$_H$17 cells signature cytokine output is inhibited (Fig.4.2.3 C & B). Taken together, these data demonstrate an important role for IL-36 ligands in promoting proinflammatory CD4$^+$T$_H$ cell differentiation towards effector subsets important in the pathogenesis of both CD and UC, while potentially further promoting a pro-inflammatory environment by the synergistic inhibition of iTreg and T$_H$17 cell generation.

4.2.4 IL-36α REGULATION OF MURINE CD4+ T_H CELLS OCCURS INDEPENDENTLY OF EARLY IL-2 INDUCTION

Having demonstrated the proficiency of IL-36α in promoting CD4+ T_H cell responses and differentiation we next sought to evaluate the mechanisms by which the cytokine is doing so. One possible explanation of the enhanced proinflammatory effects observed, is the fact that IL-36α has been shown to promote early IL-2 expression upon T_H activation (Fig.4.2.1A). IL-2 is an important instructive cytokine in T cell maturation, proliferation and lineage polarization and is expressed early upon T_H cell activation[447, 451-453].

Therefore, we evaluated if the observed effects of IL-36α in the different CD4+ T_H cell lineages was due to the early influences of increased IL-2 expression. Purified CD4+ T_H cells, differentiated to T_H1, T_H9 or T_H2 lineages were stimulated with IL-36α, with and without IL-2/IL-2R neutralizing antibodies (αIL-2/αCD25).



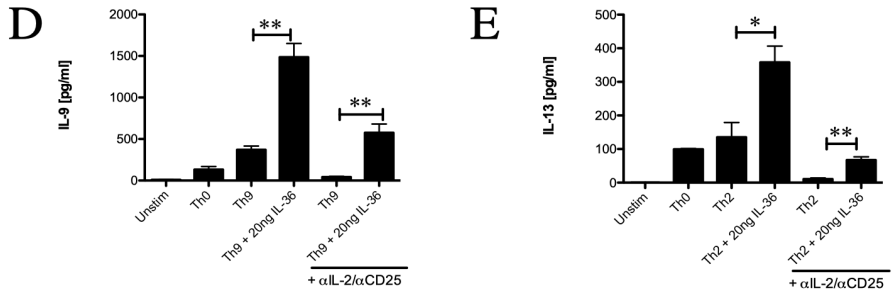


Figure 4.2.4.: IL-36α regulation of murine CD4$^+$ T$_H$ cells occurs independently of early IL-2 induction. Purified CD4$^+$ T cells activated with plate-bound αCD3ε(2μg/ml) & αCD28(2μg/ml), differentiated to T$_H$1 (20ng/ml IL-12, 10μg/ml αIL-4), T$_H$17 (10μg/ml αIFNγ, 5μg/ml αIL-4, 20ng/ml IL-6), iTreg (5ng/ml TGFß) T$_H$9 (10μg/ml αIFNγ, 20ng/ml IL-4, 5ng/ml TGFß), and T$_H$2 (10ng/ml IL-4, 10μg/ml αIFNγ)) phenotypes, and stimulated with increasing amounts of IL-36**α** (20ng/ml, 200ng/ml)with and without IL-2 neutralizing antibodies (αCD25 - 10μg/ml; αIL-2 10μg/ml). At 72h-120h T$_H$1, T$_H$17 and iTreg cells were analysed by FACs for IFNγ (A), IL-17a (B) and FOXP3 (C) respectively and T$_H$9 and T$_H$2 supernatants were analysed for IL-9 (D), and IL-13 (E) expression respectively by ELISA. Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

At 72hrs T$_H$1 cells were analysed by FACs for IFNγ expression and demonstrated an enhanced IFNγ$^+$ population when stimulated with IL-36α. Additionally, this effect of IL-36α was maintained even upon neutralization of IL-2 signalling (Fig.4.2.4.A). Similarly, T$_H$9 and T$_H$2 cells significantly increased their signature cytokine output upon IL-36α stimulation, and although neutralization of IL-2 resulted in decreased IL-9 and IL-13 expression across all conditions, once more enhanced differentiation in the presence of IL-36α was maintained upon neutralization of IL-2 signalling (Fig.4.2.4 D & E). As previously observed (Fig.4.2.3.B & C), IL-36α stimulation of iTregs and T$_H$17 cells results in inhibited levels of their signature transcription factor, FOXP3, and signature cytokine, IL-17a respectively (Fig.4.2.4.B & C). For T$_H$17s, this effect is maintained with the neutralization of IL-2 (Fig.4.2.4.B), but for Tregs this inhibitory effect is further enhanced upon IL-2 neutralization (Fig.4.2.4.C). These results can be explained by the important role of IL-2 in early T$_H$ cell priming[451], in particular its importance in Treg polarization and expansion[447], In addition, as IL-2 is key in facilitating the suppressive effect of Treg on T$_H$ effector cells[454], and IL-36α can induce potent proinflammatory effects from T$_H$ lineages in the absence of initial IL-2-dependant T$_H$ cell priming, this raises the possibility that in environments of very little immune stimulation IL-36α signalling can independently drive T$_H$ cell differentiation that goes unchecked by Treg cell modulation.

4.2.5 THE ABILITY OF TREGS TO SUPPRESS EFFECTOR T CELL RESPONSES *IN VITRO* IS LOST IN THE PRESENCE OF IL-36α

Tregs play an essential role in the orchestration of immune response to foreign and self-antigens in the body and play a critical role in maintaining immune tolerance at mucosal sites such as the gut[455]. They have been shown to counterbalance immunity by suppression of proliferation (at least partially through IL-2 neutralization), survival, maturation, differentiation, and effector function of $CD4^+$ T_H cells[454]. Our previous studies have demonstrated that IL-36α stimulation of inducible Tregs (iTregs) inhibits the induction of FOXP3 expression (Fig.4.2.3 C) as well as promoting enhanced IFNγ expression by effector $T_H$cells that is IL-2-independent (Fig.4.2.4.A). Therefore, we sought to investigate whether IL-36 cytokines can affect Treg suppressive function and either cause Treg to lose their suppressive ability and/or facilitate $CD4^+$ T_H effector cells to escape suppression. The first step in answering this question was to determine if Treg suppression was maintained in the presence of exogenously added IL-36α. This was accomplished by performing an *in vitro* Treg Suppression Assay.

$CD4^+$ $CD25^-$ $CD45R\beta^{hi}$ T effector cells were FACs sorted and labelled with CFSE to allow for future population identification and analysis of proliferation (Fig.4.2.5. A – T effector population). These were activated with soluble αCD3ε and placed in culture at a 1:2 ratio with magnetically purified T cell depleted splenocytes that were treated with Mitomycin to act as antigen presenting cells (APCs) (Fig.4.2.5 A – APC population). This provides an ideal environment to allow T effector cell proliferation and expansion. $CD4^+$ $CD25^+$ $CD45R\beta^{low}$ Treg cells were also FACs sorted; these were stained with CTV as way to identify the population and analyse their proliferation (Fig.4.2.5 A – Treg population). The stained Treg cells were then added to the co-culture of T effector cells and APCs, at a 2:1 ratio of T effector:Treg. The added Treg cells should then inhibit T effector responses and proliferation through suppressive mechanisms. We then added exogenous IL-36α and incubated the cells at 37°C for 72hrs.

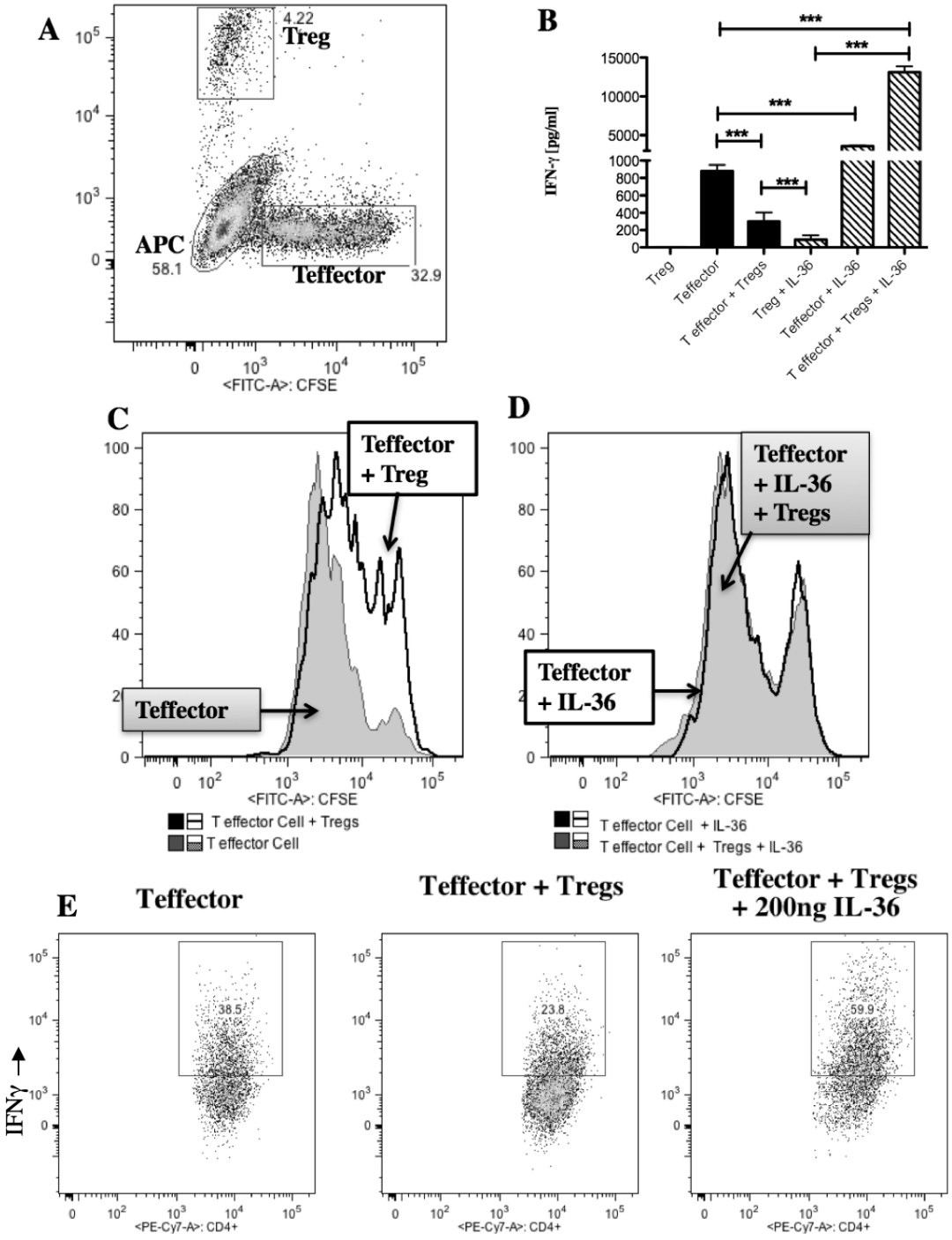


Figure 4.2.5.: IL-36α stimulations allows CD4+ T effector cells to escape from suppression by Tregs. CD4+ CD25+ CD45^{lo} Effector T cell and CD4+ CD25+ CD45Rβ^{low} Treg cell populations were FACs sorted. To track the individual populations, T effector cells were stained with CFSE and Tregs were stained with CTV. These cells were then co-cultured at a 2:1 T effector:Treg ratio in the presence of αCD3 (0.25μg/ml) and magnetically purified Mitomycin treated feeder APCs, with and without IL-36α stimulation for 72hrs at 37°C (A). After 72hrs the T effector population is identified by CFSE expression, the Treg population is identified by CTV expression, and the APC population is identified by negativity of CFSE and CTV (A). Tregs are known to suppress T effector cell proliferation and responses, therefore we measured the proliferation of the T effector cells was using FACs analysis by gating on the CFSE+ cells specifically, and also measured Treg proliferation was by gating on the CTV+ cells (C). The effect of IL-36α on their respective proliferative capacity is measured in D. As IFNγ output is indicative of Treg suppression of T effector populations, this was measured in the supernatant of the co-cultured cells using ELISA (B), and was also measured on the CFSE+ T effector population using FACs (E). Data from 1 independent experiment. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

FACs analysis of the T effector population shows that while Tregs suppress IFNγ production from $CD4^+$ T effector cells, this suppression is lost, and IFNγ output is actually enhanced, in the presence of IL-36α (Fig.4.2.5 B& E). Similarly, analysis of effector $CD4^+$ T cell proliferation using CFSE dilution showed that IL-36α stimulation also results in a loss of Treg suppression of effector cell proliferation (Fig.4.2.5 C & D). Therefore, these data confirm that IL-36α stimulation does cause the loss of Treg suppression on $CD4^+$ T effector cells and raises the question of whether IL-36α is exerting these effects on T effector and/or Treg cells or indeed indirectly through acting on APCs.

In cases of IBD, low levels of $FOXP3^+$ Treg cells have been reported in the peripheral blood of patients[80]. Taking this into account alongside our findings of IL-36α inhibiting $FOXP3^+$ Treg generation and overcoming Treg suppression *in vitro*, may indicate that IL-36 could play a dual role in the loss of $CD4^+$ T cell tolerance in IBD through both inhibiting the suppressive capacity of Tregs as well as an enhancing pro-inflammatory T_H1 responses in patients.

4.2.6 IL-36α INTERRUPTS iTREG DIFFERENTIATION AND REPROGRAMS THE CELLS TOWARDS A T_H9 PHENOTYPE

Considering the inhibitory effect of IL-36 on iTreg generation alongside the potent effects of IL-36α on proinflammatory T cell differentiation previously demonstrated, we next sought to examine the potential for IL-36α to skew $CD4^+$ T_H cells under iTreg polarizing conditions towards proinflammatory $CD4^+$ T_H cell lineages.

Purified $CD4^+$ T_H cells were cultured under iTreg conditions with various concentrations of IL-36α. At 108hrs the iTreg cells were analysed by FACs for FOXP3 expression and demonstrated a reduced $FOXP3^+$ population when stimulated with IL-36α in a dose dependent manner (Fig.4.2.6.A). We then analysed the supernatants from these cells for proinflammatory cytokine expression. Interestingly, we see a dose dependent increase in IFNγ and GM-CSF from CD4 T_H cells under iTreg conditions when stimulated with IL-36α, indicating that IL-36α may be redirecting the differentiation of these cells towards a pro-inflammatory T_H1 phenotype.

In addition to enhancing T_H1 responses under iTreg conditions we also observed a significant increase in the T_H2 and T_H9 signature cytokines, IL-4 and IL-9 respectively, following IL-36α stimulation (Fig.4.2.6.D & E). Together, these data indicate that IL-36α may have a functional role in $CD4^+$ T_H cell polarization and plasticity.

There are already well documented reports of plasticity between Tregs and T_H17 cells in certain disease states, such as multiple sclerosis (MS), rheumatoid arthritis (RA), collagen-induced arthritis (CIA), systemic sclerosis, psoriasis and Type 1 Diabetes (T1D)[456]. In these cases, this is primarily due to the necessity for TGFß in both of their differentiation, then the other cytokines present in the micro-environment skew the cells towards one of towards either lineage[456]. TGFß is also crucial in T_H9 differentiation in conjunction with IL-4. Interestingly, as we observed that IL-36α induces both IL-4 (Fig.4.2.6.D) and IL-9 (Fig.4.2.6.E) from $CD4^+$ cells under iTreg conditions, where TGFß is present, this suggests that IL-36α may also be skewing the cells towards a T_H9 phenotype, as well as T_H1 phenotype.

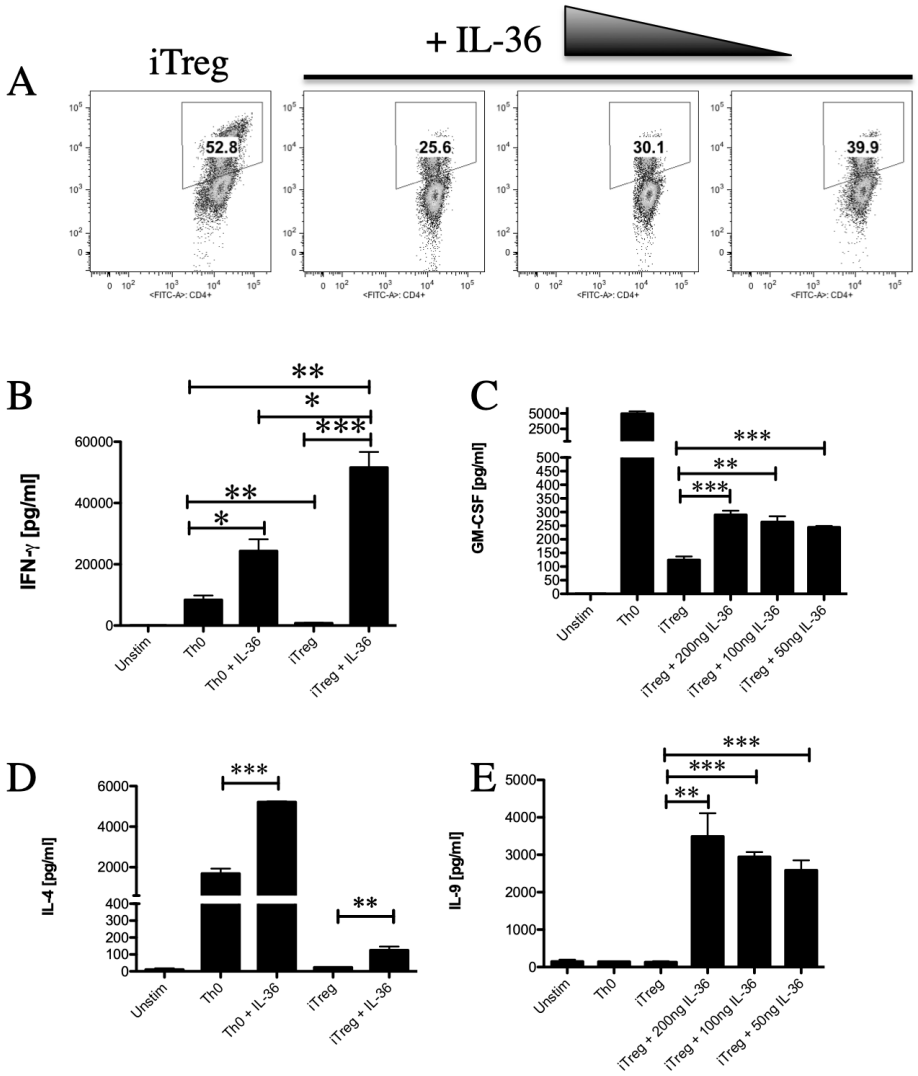


Figure 4.2.6.: IL-36α inhibits iTreg generation and promotes IL-9 expression under iTreg polarizing conditions. Purified $CD4^+$ T cells activated with plate-bound αCD3ε(2μg/ml) & αCD28(2μg/ml), were differentiated to an iTreg (5ng/ml TGFß) phenotype, and stimulated with varying amounts of IL-36α (20ng/ml, 50ng/ml, 200ng/ml). At 72h-120h iTreg cells were analysed by FACs for FOXP3 expression (A) and supernatants were analysed for IFNγ (B), GM-CSF(C), IL-4(D) and IL-9(E) expression by ELISA. Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.2.7 IL-36α CAN REPLACE IL-4 IN THE INSTRUCTION OF T_H9 DIFFERENTIATION

As mentioned above, IL-4 has been demonstrated to act as an important instructive cytokine in the differentiation of T_H9 cells. Having observed such a potent induction of IL-9 and IL-4 under iTreg conditions in the presence of IL-36α (Fig.4.2.6.D & E), we next decided to analyse both cytokines in their ability to induce IL-9 expression. To address this, $CD4^+$ T_H cells were cultured with TGFß and either IL-4, to induce a classical T_H9 population, or with IL-36α alone. After 72h IL-9 was measured by ELISA, and while IL-4 did induce IL-9 as expected, IL-36α alone induced a significantly greater amount, in a dose dependent manner (Fig.4.2.7.A & C). This effect was maintained after 120h (Fig.4.2.7.B), and can be seen by FACs and ELISA (Fig. 4.2.7.A-C). This suggests a novel role for IL-36α as an instructive cytokine, more potent than IL-4, in the differentiation of T_H9 cells.

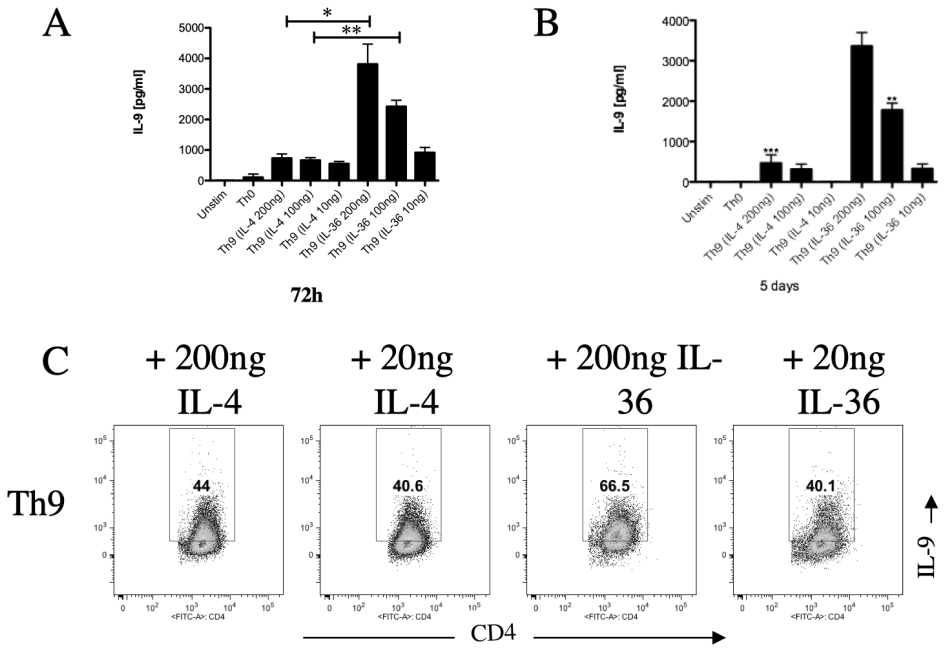
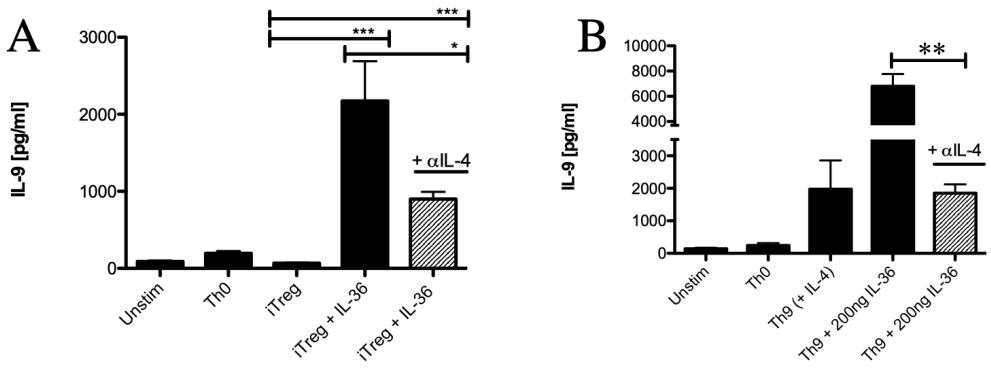


Figure 4.2.7.: IL-36α promotes T_H9 differentiation more effectively than IL-4. Purified $CD4^+$ T cells activated with plate-bound αCD3ε(2µg/ml) & αCD28(2µg/ml) (A & B), as well as Mitomycin inactivated APCs (C), were differentiated to a T_H9 phenotype (10µg/ml αIFNγ, 5ng/ml TGFß), and stimulated with IL-36α (10ng/ml, 20ng/ml, 100ng/ml, 200ng/ml) or IL-4 (10ng/ml, 20ng/ml, 100ng/ml, 200ng/ml). At 72h-120h iTreg cells were analysed by FACs for IL-9 (B) and supernatants were analysed for IL-9 (A & B). Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.2.8 THERE IS A IL-36/IL-4 AXIS INVOLVED IN THE CAPACITY OF IL-36α TO INHIBIT TREGS AND ENHANCE T_H9S

IL-4R signalling is coming to light as an prominent factor in FOXP3+ Treg plasticity[456]. While some studies have shown IL-4 signalling is important in maintaining the Treg compartment in inflammation[457, 458], others have shown an inhibitory role for this cytokine in Treg generation and expansion[165]. As we have demonstrated that IL-36 has the capacity to inhibit Treg generation, as well as act to enhance T_H9 responses, we next sought to evaluate whether IL-36s effects on Treg and T_H9 polarization was IL-4 related.

To achieve this CD4+ T cells were cultured under iTreg (TGFß) and T_H9 (αIFN γ , + TGFß +/- IL-4) differentiating conditions and stimulated with IL-36α in the presence or absence of anti-IL-4 antibodies. After 5 days, IL-9 was measured by ELISA. While IL-36α continues to induce elevated levels of the cytokine from iTregs, this effect is significantly reduced when IL-4 is neutralised (Fig.4.2.8.A). Similarly, IL-4 blockade also inhibited IL-36α driven IL-9 production from T_H9 cells (Fig.4.2.8.B). Interestingly, IL-36α also loses its iTreg inhibitory capacity when IL-4 is neutralised, with iTregs exhibiting normal levels of FOXP3, even in the presence of IL-36α (Fig.4.2.8.C). Furthermore, the combination of IL-36α and αIL-4 actually increases the FOXP3 population under T_H9 conditions indicating that IL-36α induced IL-4 expression plays a key role in modulating iTreg versus T_H9 responses.



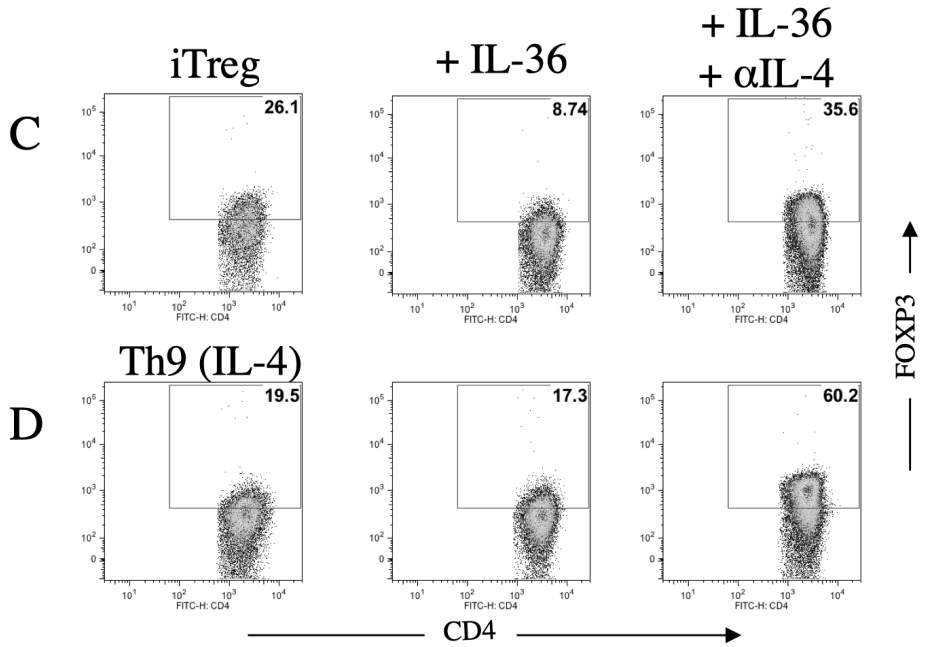


Figure 4.2.8.: IL-36α works in synergy with IL-4 to inhibit FOXP3+ iTreg cells and promote T_H9 cell generation. Purified $CD4^+$ T cells activated with plate-bound αCD3ε(2μg/ml) & αCD28(2μg/ml) (A & C), as well as Mitomycin inactivated APCs (B &D), were differentiated to an iTreg (5ng TGFß) or TH9 phenotype (10μg/ml αIFNγ, 5ng/ml TGFß), and stimulated with IL-36**α** (200ng/ml) or IL-4 (20ng/ml) with and without anti-IL-4 (10ug/ml). At 72h-120h iTreg and T_H9 cells were analysed by FACs for FOXP3 (C & D) and supernatants were analysed for IL-9 (A & B). Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.2.9 THE IL-1 ORPHAN RECEPTOR SIGIRR NEGATIVELY REGULATES IL-36α INDUCED T_H1 RESPONSES.

SIGIRR is an IL-1 orphan receptor that has shown to negatively regulate IL-1 signalling[318]. There are various reports highlighting its ability to regulate the adaptive response in colitis, with studies demonstrating its ability to inhibit T_H1 and $T_H17$responses[434]. As IL-36α is a strong modulator of $CD4^+$ T_H responses we decided to examine what effect SIGIRR has on IL-36α's ability to regulate T_H1 and Treg responses. To do this purified $CD4^+$ T_H cells from *wt* and *Sigir*$^{-/-}$ mice were differentiated to T_H1, and iTreg lineages were stimulated with IL-36α.

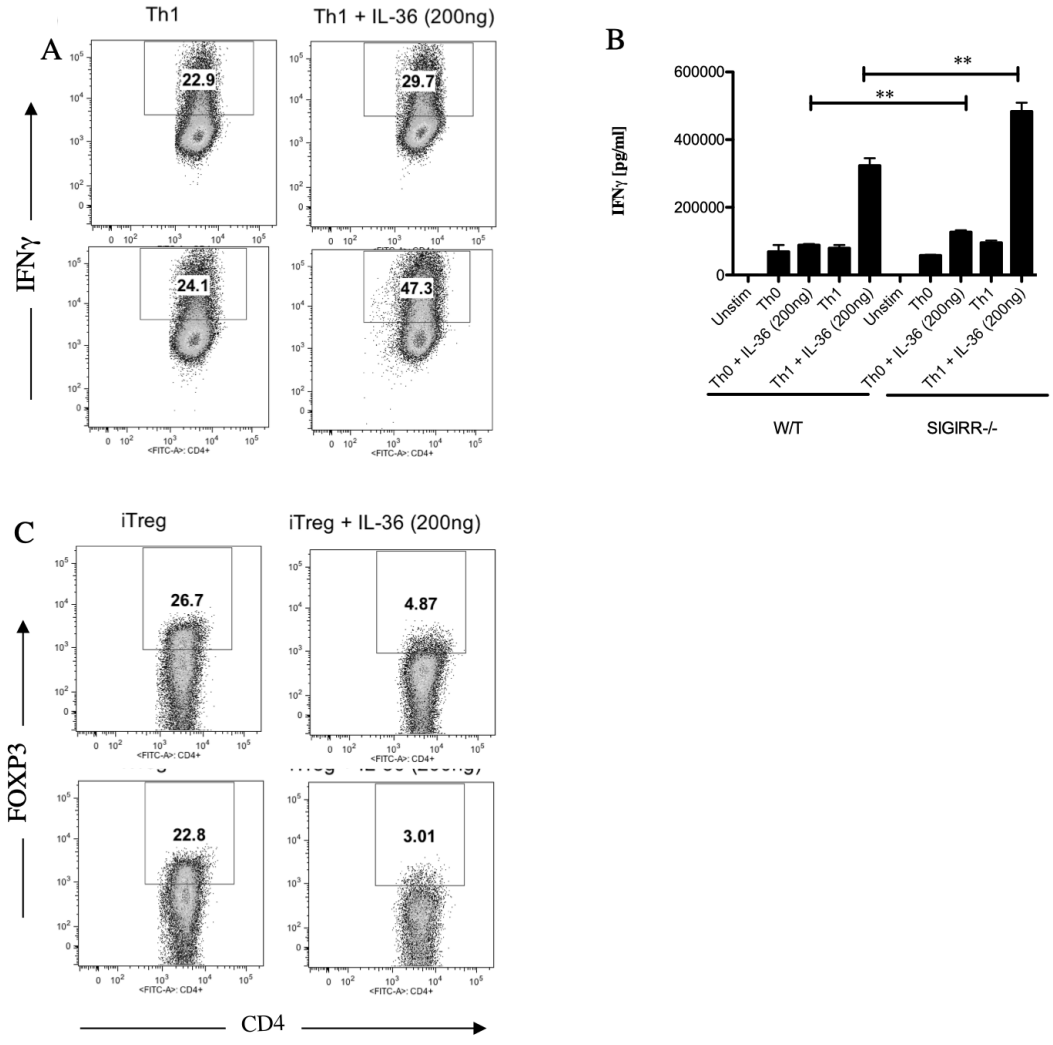


Figure 4.2.9.: SIGIRR negatively regulates IL-36α induced T_H1 responses. Purified $CD4^+$ T cells activated with plate-bound αCD3ε(2μg/ml) & αCD28(2μg/ml), differentiated to T_H1 (20ng/ml IL-12, 10μg/ml αIL-4) & iTreg (5ng/ml TGFß) phenotypes, and stimulated with IL-36α (200ng/ml). At 72h-120h T_H1 and iTreg cells were analysed by FACs for IFNγ (A) and FOXP3 (C), while T_H1 supernatants were analysed for IFNγ expression by ELISA (B). Data representative of a minimum of 2 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

After 72 hours IFNγ expression was measured by FACS (Fig.4.2.9.A). Both *wt* and *Sigir*$^{-/-}$ T_H1 cells express similar amounts of IFNγ, demonstrating that *Sigir*$^{-/-}$ and *wt* CD4$^+$ T_H cells possess the same capacity to differentiate to T_H1 cells under the same skewing condition. However, when stimulated with IL-36α, *Sigir*$^{-/-}$ T_H1 cells express significantly more IFNγ than *wt*s under the same stimulus. These results are recapitulated by ELISA (Fig.4.2.9.B), and suggest that IL-36α's ability to induce a T_H1 phenotype is negatively regulated by SIGIRR.

After 120hrs, FOXP3 expression was measured from Tregs. Once again, there is no difference in FOXP3 expression observed between *wt*s and *Sigir*$^{-/-}$ indicating that SIGIRR does not influence the generation of iTregs *in vitro*. Furthermore, IL-36α's ability to inhibit FOXP3 expression in Tregs is not effected by deficiency in SIGIRR signalling (Fig.4.2.9.C). Collectively these results demonstrate that SIGIRR negatively regulates IL-36α induced T_H1 responses.

4.3 DISCUSSION

As global incidence of Inflammatory Bowel Disease continues to rise, with 0.3% of the population suffering from this debilitating disease[43], there is a pressing need for the development of new more efficacious targeted therapies. With the importance of the IL-36 family of cytokines emerging in the regulation of adaptive immune responses, in addition to their reported involvement in numerous autoimmune and auto-inflammatory disorders, such as IBD, this cytokine family may provide a potential novel and specific target[412]. Our data from chapter 3, showing enhanced expression IL-36α in the serum and colons of IBD patients, as well as increased numbers of IL-36R$^+$CD3$^+$ T cells in their colon further validated evaluating this cytokine in the adaptive immune response associated with IBD.

The pathologic activity of IL-36 family members in inflammatory diseases has been previously been associated with inappropriate T_H1 or T_H17 responses[394, 402]. In agreement, this study has found that IL-36α exerts potent regulatory effects on CD4$^+$ T_H cells. From initial stimulation of naïve T_H0 cells, IL-36α induces markedly enhanced expression of a number of different cytokines, such as IL-2, a key cytokine in $T_H$cell activation and polarization (Fig.4.2.1-2.A) [451-453], and signature cytokines of CD4$^+$ T_H cell lineage commitment, IFNγ (T_H1), IL-17a (T_H17) and IL-13 (T_H2)(Fig.4.2.1-2. B, C & D)[459]. Interestingly, this effect on IL-2 and IFNγ is maintained when only partial T_H cell activation is induced with stimulation of just the TCR (CD3), without stimulation of the CD28 co-stimulatory molecule (Fig.4.2.2.A&B). Thus, suggesting that IL-36 signalling may act as an alternative pathway in early T_H cell activation and T_H1 lineage priming. The possible important role for IL-36α in driving T_H1 responses is further strengthened when we examine the effects of the cytokine on differentiated T_H1 cells, and in agreement with Vigne *et al.*[402] we see a marked enhancement of IFNγ production, resulting in 99% of the total CD4$^+$ T_H cell population expressing IFNγ (Fig.4.2.3.A) and a decrease in IL-17a producing T_H17 cells (Fig 4.2.3.B). However, in contrast to their findings, we have found that *in vitro* this effect on T_H1s is IL-2-independent (Fig.4.2.4.A)[402]. As optimal IL-2 production in CD4$^+$ T_H cells is CD28-mediated[451], this finding further promotes the concept of IL-36 acting as an alternative co-stimulatory molecule in T_H cell activation and lineage priming

Furthermore, in addition to enhancing both T_H2 and T_H9 generation (Fig 4.2.1-3.D & E), IL-36α also mediates these effects in an IL-2-independent manner (Fig.4.2.4.D&E), indicating a specific role for IL-36 in both Type 1 and Type 2 adaptive immunity.

These effects of IL-36 enhancing T_H9 cell responses was recently confirmed by Harusato *et al*., who found that in inflamed colonic murine mucosa, IL-36α enhances T_H9 activity while synergistically inhibiting the development Tregs, therefore inhibiting the resolution of intestinal inflammation[292]. Similarly, we also observed an inhibitory effect of IL-36α on iTregs (Fig.4.2.3.C), leading us to the question whether in an inflamed system, IL-36α can cause T_H effector cells to escape from Treg suppression, consequently resulting in the type of unregulated inflammation we see in IBD. Based on our findings, the answer it seems is yes. When T_H effector cells are co-cultured with Tregs and stimulated with endogenous IL-36α, suppression is broken, proliferation is no longer impeded (Fig.4.2.5.C & D), and IFNγ output is significantly enhanced (Fig.4.2.5.B & E). There are three possible mechanisms by which IL-36 may be causing these effects: 1) IL-36 is acting directly on the Tregs and inhibiting their suppressive effects and/or 2) IL-36 is stimulating the T effector cells so strongly that they overcome suppression. 3) Alternatively, there is also the possibility that the IL-36 may be acting on the T cell depleted APCs and these in turn are causing the escape from Treg expression we have observed. Further studies using *Il36r* deficient cell subsets will address which cell type(s) are the key responders to IL-36 cytokines in breaking Treg suppression.

Interestingly, we also observed a slight increase in expression of IFNγ from nTregs stimulated with IL-36α *ex vivo*, indicating a potential role for IL-36α in nTreg plasticity (Fig.4.2.5.B). Dominguez-Villar *et al*. demonstrated that healthy human nTregs acquire a T_H1-like phenotype *ex vivo* when cultured in the presence of IL-12[460]. Furthermore, these now IFNγ producing T_H1-like Tregs exhibit a reduced suppressive activity[460]. Taking this data into consideration with results showing IL-36 allows T_H effector cells to evade suppression by nTregs, perhaps IL-36 is enhancing sensitivity to IL-12 to elicit this inhibitory effect on Tregs? If so, this would also explain the potent effect IL-36 stimulation has on the generation of T_H1 responses (Fig.4.2.3.A) and warrants further investigation. Koenecke *et al*. also witness induction IFNγ production by nTregs when stimulated with IL-12 in a dose-dependent manner *ex vivo*[461]. Furthermore, the group also report that $Foxp3^+$ Treg cells readily produced IFNγ *in vivo* during a T_H1-dominated immune response to intracellular bacteria (L. monocytogenes)[461]. Collectively these data suggest that in a T_H1 dominant environment Treg cells can readily produce IFNγ. Whether the function of this is to induce Treg cells with optimized migratory properties for the suppression of T_H1 responses as Koch *et al*.[462] suggests, or for the Treg cells to adopt a T_H1 effector phenotype, as suggested by Oldenhove et al.[463] still needs to be clarified.

We also focused on what effect IL-36α stimulation was having on the generation of inducible Tregs. *In vitro* studies revealed that IL-36α synergistically inhibits FOXP3

expression and enhances secretion of proinflammatory cytokines, IFNγ, IL-9, IL-4 and GM-CSF, from these cells (Fig.4.2.6). As these cytokines are signature cytokines of proinflammatory T_H cell subsets (IFNγ, IL-4 and IL-9), and can be key in driving their differentiation (IL-4-T_H2/T_H9), this proposes a possible instructive role for IL-36α in T_H cell generation and plasticity.

Over the past few years there has been emerging reports of Treg plasticity in various autoimmune diseases[456]. In these cases, the Tregs begin to express the lineage defining factors of the particular $CD4^+$ T_H cell lineage that is causing disease, and have been named T helper cell like (T_H-like) Tregs. For example, Tregs in patients with T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis expressed IFNγ, Tbet, and CXCR3[464], and become T_H1-like Tregs, and tTregs from atopic dermatitis (AD) patients were shown to express IL-13, becoming T_H2-like Tregs[465].

The role of these T_H-like Tregs has yet to be elucidated, with some studies demonstrating remarkable inhibitory effects on their associated pathogenic immune responses[457, 466], while others show they perpetuate disease and exacerbate disease[465]. As our results have shown IL-36α's ability to drive proinflammatory T_H1, T_H2, and T_H 9 cell differentiation, this secretion of proinflammatory cytokines under iTreg conditions (IFNγ, IL-4, IL-9) and nTreg conditions (IFNγ) may indicate a role for IL-36α in promoting Treg plasticity and warrants further investigation through fate-mapping studies.

Furthermore, in contrast to Harusato *et al*.'s reported role for IL-36 mediated IL-2/IL-4 signalling in the T_H9/iTreg axis[262],we report a novel role, where IL-36α induces T_H9 generation with greater potency than IL-4 alone (Fig.4.2.7), and can also replace IL-4 as a T_H9 differentiating cytokine (Fig.4.2.8.B), similar to other IL-1 family members, IL-1α, IL-1β, IL-18, and IL-33[258, 263, 420, 435].. However, IL-36αs inhibitory capacity on Treg inhibition is indeed IL-4 dependant, in agreement with the authors (Fig.4.2.8.C). This also confers with Dardalhon *et al*., who demonstrate that IL-4 inhibits TGFß induced iTregs. In their study IL-4 synergises with TGFß to promote T_H9 cell generation[165], an effect we also observe with IL-36α stimulation under iTregs conditions (Fig.4.2.6.E & Fig.4.2.8.A). However, in our case this effect is maintained even when IL-4 is neutralised, although to a lesser extent than when IL-4 and IL-36α are present together (Fig.4.2.8.A). This eludes to the potential capacity for IL-36α to synergise with TGFß and redirect these cells towards a T_H9 phenotype, and also induce IL-4 production from these cells further amplifying this effect.

Interestingly, neutralizing IL-4 also diminishes the enhanced IL-9 production observed from IL-36α stimulated T_H9 cells, and reduces it to similar levels as conventionally differentiated T_H9 cells (Fig.4.2.8.B). Collectively these data indicate that IL-36α induces IL-9 and represses FOXP3 through an IL-36/IL-4 amplification loop. The fact that we see an

increase in FOXP3 expression from IL-36α stimulated T_H9 cells when IL-4 is neutralised further indicates the potent effects of IL-4 as an inhibitor of iTreg differentiation (Fig.4.2.8.1.D). In agreement, Pace *et al*. demonstrate that IL-4 stimulation allows T_H effector cells to evade suppression by Tregs *ex vivo*[467], similar to the results we observed of IL-36 facilitating T_H effector cells to break Treg-mediated suppression.

Interestingly, Treg suppression of T_H effector cells has been largely associated with downregulation of CD28-mediated production of IL-2 via CTLA-4 signalling. Conversely, potent CD28 signalling has been proven to break this suppression[454, 468]. Therefore, these data present CD28 as an important checkpoint molecule in the balance between resistance and susceptibility to Treg suppression of T_H effector cells. Intriguingly, our results demonstrate that IL-36α stimulation of $CD4^+$ T_H effector cells circumvents the necessity for CD28-mediated activation (Fig.4.2.2). As such, perhaps this proposed alternate costimulatory pathway allows enhanced activation of T_H effector cells and so evasion of suppression by Tregs (Fig.4.2.5). This kind of unregulated T cell activity could explain the uncontrolled immune response observed in numerous autoimmune diseases, such as IBD, and offers a potential target for therapy.

Interestingly, preliminary data indicates that IL-36αs ability to enhance T_H1 generation is negatively regulated by the IL-1 orphan receptor SIGIRR (Fig.4.2.9). This receptor has been demonstrated to regulate T_H1 responses in the mucosa, and its absence has been shown to exacerbate disease in murine colitis[434]. Furthermore, reports demonstrate that SIGIRR regulates T_H2 and T_H17 responses[434, 469]. Although, SIGIRR did not regulate IL-36αs inhibitory effect on iTreg cells, it would be worthwhile to determine if SIGIRR regulates other IL-36α induced $CD4^+$ T_H lineage responses.

Collectively, these data identify a prominent role for IL-36α in broadly driving the pathogenic activity of T_H cell responses. This capacity of IL-36α appears to stem from early effects in $CD4^+$ T_H cell activation and lineage priming, resulting in an enhancement of pro-inflammatory phenotypes that are also potentially left unregulated due to the synergistic inhibition of Treg activity, and T_H17 responses, that have reported pro-homeostatic functions in the gut[470, 471], thus resulting in massive disruption of normal immune homeostasis.

The maintenance of a functional immune system is a multistep process that involves strict regulation T effector cell responses from their very generation in the thymus to their effector responses at sites of infection or inflammation. With increasing interest in the use of biologics as therapies, in particular for autoimmune disease, it is important to understand the exact stage and source of disruption to immune homeostasis. As IL-36 family members are continually being associated with increasing numbers of autoimmune and auto-inflammatory

disorders[412] elucidating the pathologic activity of these cytokines may prove beneficial in designing effective therapies.

CHAPTER 5.

THE EFFECTS OF IL-36 ON THE GENERATION OF PATHOGENIC T CELL RESPONSES IN IBD

Chapter 5

The effects of IL-36 on the generation of pathogenic T cell responses in IBD

5.1 INTRODUCTION

Inflammatory bowel diseases, including CD and UC, represents a significant socioeconomic burden to western and newly industrialized countries[43, 472]. This is due to their early onset, chronic and progressive disease course, in addition to frequent hospitalizations and surgical operations made necessary by the severity of the disease[43, 45, 62, 75, 472]. As previously mentioned, aberrant infiltration of $CD4^+$ T_H cells to the intestinal mucosa is a characteristic of the disease, and epidemiological and clinical observations in humans, as well preclinical studies in murine models of IBD suggest that these $CD4^+$ T_H cells are master orchestrators of the intestinal inflammation observed in IBD[43, 406, 472]. Evidence supporting this pathogenic role for $CD4^+$ T_H cells comes from studies reporting enhanced recruitment of T cells from the systemic circulation to the gut[67, 473, 474], increased cell cycling[475], and resistance against cell death[476] in IBD patients. Furthermore, IBD develops when T effector cells are transferred into lymphopenic hosts, such as in the T cell transfer model of colitis[477], and ameliorates with the development of lymphopenia, for example, when a patient develops HIV[478] or undergoes a bone marrow transplant[479]. Moreover, strategies blocking T cell functioning and migration to the intestines have proven effective in alleviating disease in murine models of colitis[225], and are an attractive target for the development of new biologic therapies.

One such biologic proving successful in the clinic is the use of TNFα inhibitors. Administration of the anti-TNFα antibodies, adalimumab and infliximab, have been shown to induce lamina propia (LP) T cell apoptosis, and induce and maintain clinical remission in up to 70% of IBD patients[78, 203, 230, 480-482]. The efficacy of these drugs observed is dependent on induction of apoptosis in the LP T cells, and is proven as enterocept, a fusion protein that neutralizes membrane-bound and soluble TNF, fails to induce LP T cell apoptosis, and is unbeneficial in CD[483].

Targeting T cell migration to the gut is also gaining popularity. In particular, with the success of Vedolizumab, monoclonal antibody that targets the α4β7 integrin, and

therefore CD4+ T cell migration to the intestines. This antibody is proving beneficial in the clinic, with short-term response rates of 29% in CD, and 66% in UC[484, 485]. New compounds blocking chemokine mediated T cell gut homing are also under investigation, with CCR9 and CXCL10 blockade exhibiting clinical efficacy in CD and UC respectively[486, 487].

Another approach is inhibiting the differentiation of pathogenic CD4+ T_H cell lineages. Ustekinumab, a monoclonal antibody targeted against the p40 subunit of IL-12 and IL-23, effectively blocks the polarization of T_H1 and T_H17 cells and has shown efficacy in the clinic in the treatment of IBD[431], with clinical response rates of 63% and clinical remission rates of 41% reported. However, despite all of these promising results, IBD is an extremely heterogeneous disease, that has a multitude of innate and adaptive mediators of T cell responses at play in its pathogenesis[446]. The development of novel biologics to target key T cells responses, such as activation and differentiation, migration and apoptosis, will be key in developing effective treatment regimens tailored for each patient's specific immune response.

In recent years, the IL-36 family has been implicated in the pathogenesis of IBD, although these reports vary between perpetuating intestinal inflammation on one hand[394], and facilitating mucosal resolution of this inflammation on the other[377]. Earlier findings from our own group have revealed elevated levels of IL-36 cytokines in the mucosa of IBD patients, and reduced T_H1 responses in the absence of *Il36r* signalling in preclinical studies[394], suggesting a pathogenic role for this cytokine family in the intestines. In keeping with these findings, our results in Chapter 3 have identified enhanced amounts IL-36α in paediatric IBD patients and the potential for this cytokine to act on T cells in the mucosa. Following on from this, in Chapter 4 we have shown that its signalling promotes the activation and generation of pathogenic CD4+ T_H cell responses associated with IBD, thereby further agreeing with the literature supporting a pathogenic role for this cytokine in IBD. Furthermore, multiple clinical trials in IBD patients have commenced evaluating the efficacy of blocking IL-36R signalling with the administration of Spesolimab, and the results of these studies are eagerly awaited[422, 423, 425].

Following on from these initial findings, in this chapter, we will examine the effects of IL-36 on generation of pathogenic mucosal T_H cell responses in IBD *in vivo*, elucidate the underlying mechanisms of these effects, in addition to determining how IL-36α signalling impacts the generation of human colitogenic CD4+ T_H cells. Mechanistic studies like these may shed some light on the functional effect of potential biologics like Spesolimab, and offer other avenues to explore as potential areas for improved therapeutic intervention.

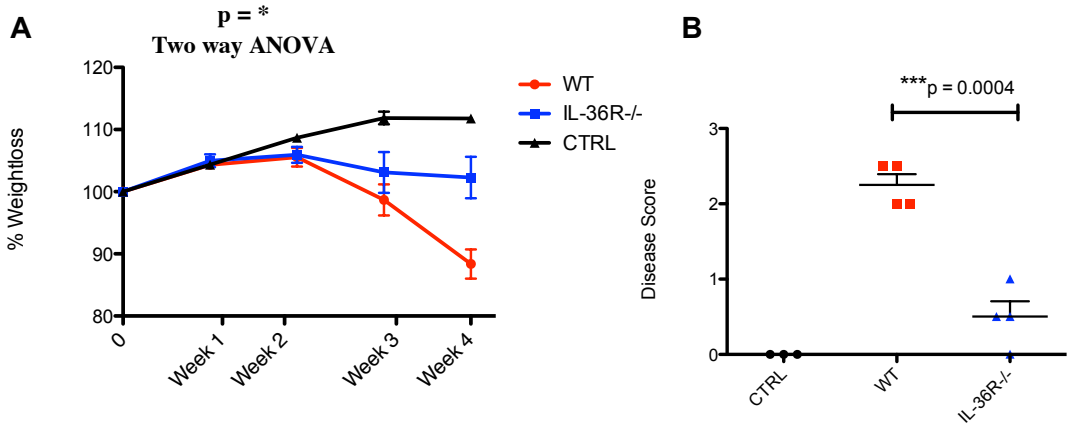
5.2 RESULTS

5.2.1 IL36R EXPRESSION REGULATES THE DEVELOPMENT OF CD4+ T_H CELL MEDIATED COLITIS

The generation and infiltration of unregulated pathogenic CD4+ T_H cells in the mucosa is characteristic of IBD[64]. As our results thus far have indicated an over expression of IL-36α in IBD patients, as well as a potent role for IL-36 signalling in proinflammatory CD4+ T_H responses we began to explore the effect of IL-36R signalling on pathogenic CD4+ T_H cell responses *in vivo*.

To achieve this, we used the T cell transfer model of colitis, which exploits the T cell deficiency in *Rag1*$^{-/-}$ mice to initiate and sustain adaptive driven mucosal inflammation when CD4+CD25-CD45Rβ^{high} effector T cells are transferred from donor *wt* mice. Due to the absence of lymphocytes in the recipient, these transferred cells then undergo homeostatic and intestinal microbiome driven expansion and overtime cause severe colitis to develop as there are no Treg cells present to suppress their activity[477].

In this experiment, CD4+ CD25 CD45Rβ^{hi} T effector cells were FACs sorted from *wt* and *Il36r*$^{-/-}$ mice and transferred to *Rag1*$^{-/-}$ recipient mice by *i.p.* injection. Over the course of the next 4 weeks, colitis develops and disease activity is measured by weight-loss. At takedown, the colons and small intestine were taken for H&E staining to measure histopathologic severity of disease. As the T cell transfer model of colitis is associated with eliciting a pathogenic T_H1/ T_H17 response in the mucosa explants of SI were analysed for production of their signature cytokines, IFNγ and IL-17a respectively.



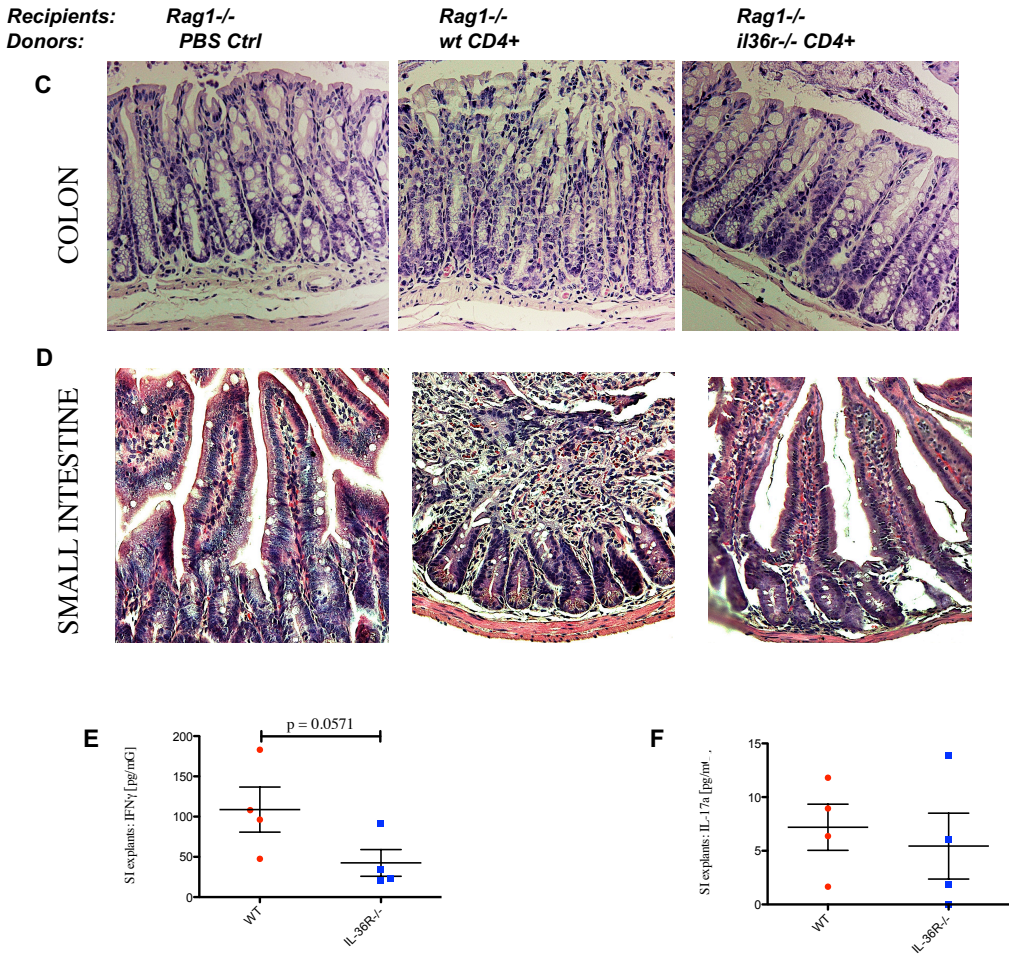


Figure 5.2.1.: Absence of *Il36r* expression protects against the development of colitis in a T cell transfer model. FACs sorted $CD4^+CD25^-CD45R\beta^{lo}$ effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by IP injection to *Rag1*$^{-/-}$ recipient mice. Weight was taken over the course of the following 4 weeks as a measure of disease progression (A). (A) shows the combined weightloss from two separate experiments. N = (*wt* recipients 10), (*Il36r*$^{-/-}$ recipients = 10), (PBS (*Ctrl*) recipients = 5). At takedown colons (B & C) and small intestines were harvested for H&E analysis (D). Explants of small intestines were cultured for 24hrs at 37°C and IFNγ (E) and IL-17a (F) were measured by ELISA. (B – F, N = 4 *wt*, 4 *Il36r*$^{-/-}$, 3 *Ctrl*. *Statistical analysis performed by student's t test and two way anova, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

As expected, mice that received *wt* T_H cells, exhibited a significant decrease in weight over the course of the experiment (Fig.5.2.1. A), However, *Il36r*$^{-/-}$ $CD4^+$ T cell recipients appeared relatively protected from the onset of disease, exhibiting weightloss pattern similar to *ctrl* mice (Fig.5.2.1. A). This protection was also evident when we examined H&E staining from the colon and SI of *Il36r*$^{-/-}$ recipients. Whereas *wt* recipients exhibit a significantly enhanced histopathologic disease score, with significantly increased intestinal crypt architecture destruction and cellular infiltration detected in the colonic (Fig.5.2.1. B & D) and small intestinal mucosa (Fig.5.2.1. C). Explant cultures from the SI of these mice also showed that *Il36r*$^{-/-}$ $CD4^+$ T cell recipients secreted less IFNγ than *wt* recipients, although this was not the case for IL-17a (Fig.5.2.1. E & F). As the T cell transfer model of colitis is mediated by T_H1 induced intestinal damage, and we have previously shown that IL-36α promotes the

generation of T_H1 cells (Chapter 4), these results raise the possibility that IL-36 may be promoting the development of colitis and intestinal damage via its effects on $CD4^+$ T_H cell responses in the mucosa.

5.2.2 IL-36 REGULATES THE GENERATION OF PATHOGENIC MUCOSAL T CELL RESPONSES IN IBD *IN VIVO*

IFNγ is one of the key cytokines in the pathogenesis of IBD. It plays a central role in the generation and endurance of mucosal inflammation in many clinical models of disease, including T cell transfer colitis, and is key to the induction and perpetuation of CD[206, 240, 477, 488]. Our results thus far have indicated a central role for IL-36 signalling in the induction of T_H1 responses from $CD4^+$ T_H cells *in vitro* (Chapter 4) and in promoting the pathogenesis of colitis *in vivo* (Fig.5.2.1*)*. When taken into consideration with our earlier demonstration for the capacity of IL-36α to significantly promote T_H1 generation (Fig.4.2.3.A), whilst concomitantly inhibiting Treg generation and suppressive functioning (Fig.4.2.3.A & Fig.4.2.5), this led us to question does IL-36 regulation of $CD4^+$ T cell responses have the capacity to initiate and perpetuate IBD *in vivo*?

To answer this question, we used the T cell transfer model of colitis. $CD4^+$ $CD25^-$ $CD45R\beta^{hi}$ T effector cells were FACs sorted from *wt* and *Il36r*$^{-/-}$ mice and were adoptively transferred to *Rag1*$^{-/-}$ recipient mice. After 4 weeks' spleens, mLNs, and colons were harvested for FACs analysis of IFNγ, IL-17a and FOXP3 production by transferred $CD4^+$ T_H cells.

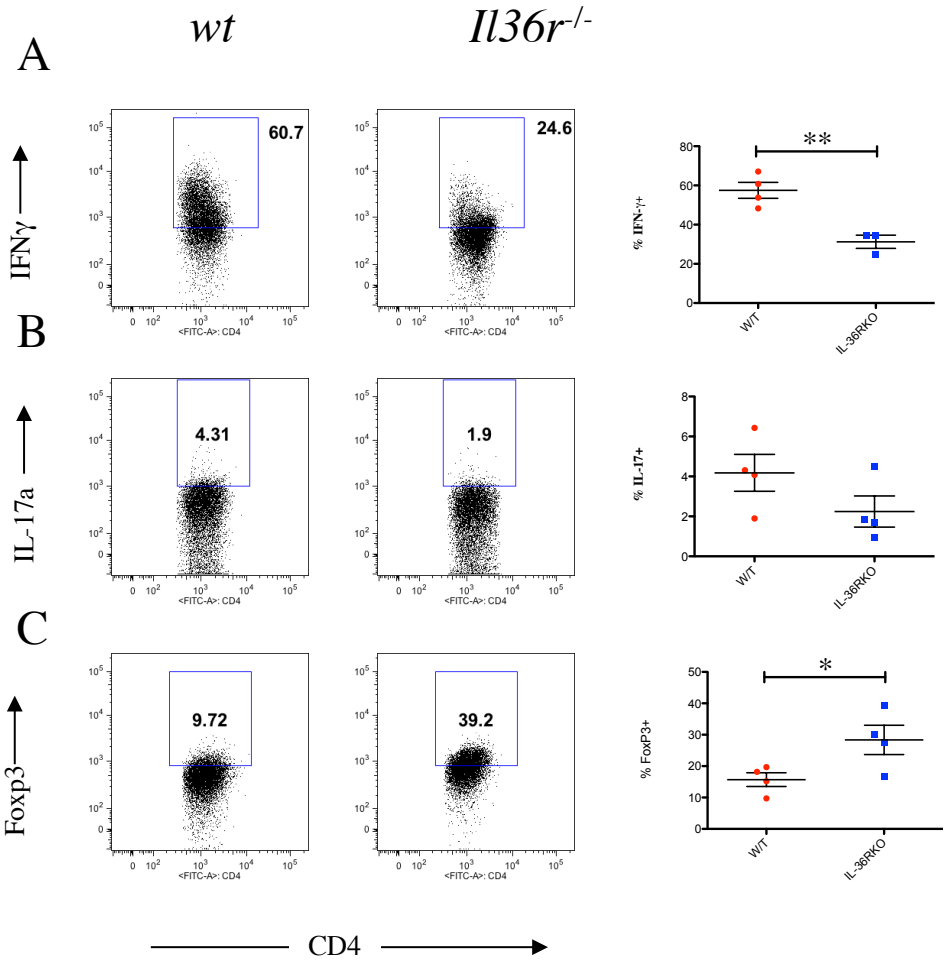


Figure 5.2.2.1.: *Il36r* expression regulates the generation of pathogenic CD4+ T cells in the spleen in a T cell dependant model of colitis. FACs sorted $CD4^{+}CD25^{-}CD45RB^{hi}$ effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by i.p injection to *Rag1*$^{-/-}$ recipient mice. At takedown (day 28) spleens were taken for FACs analysis of IFNγ (A), IL-17a (B) and FOXP3 (C). N = 4 *wt*, 4 *Il36r-/-*. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

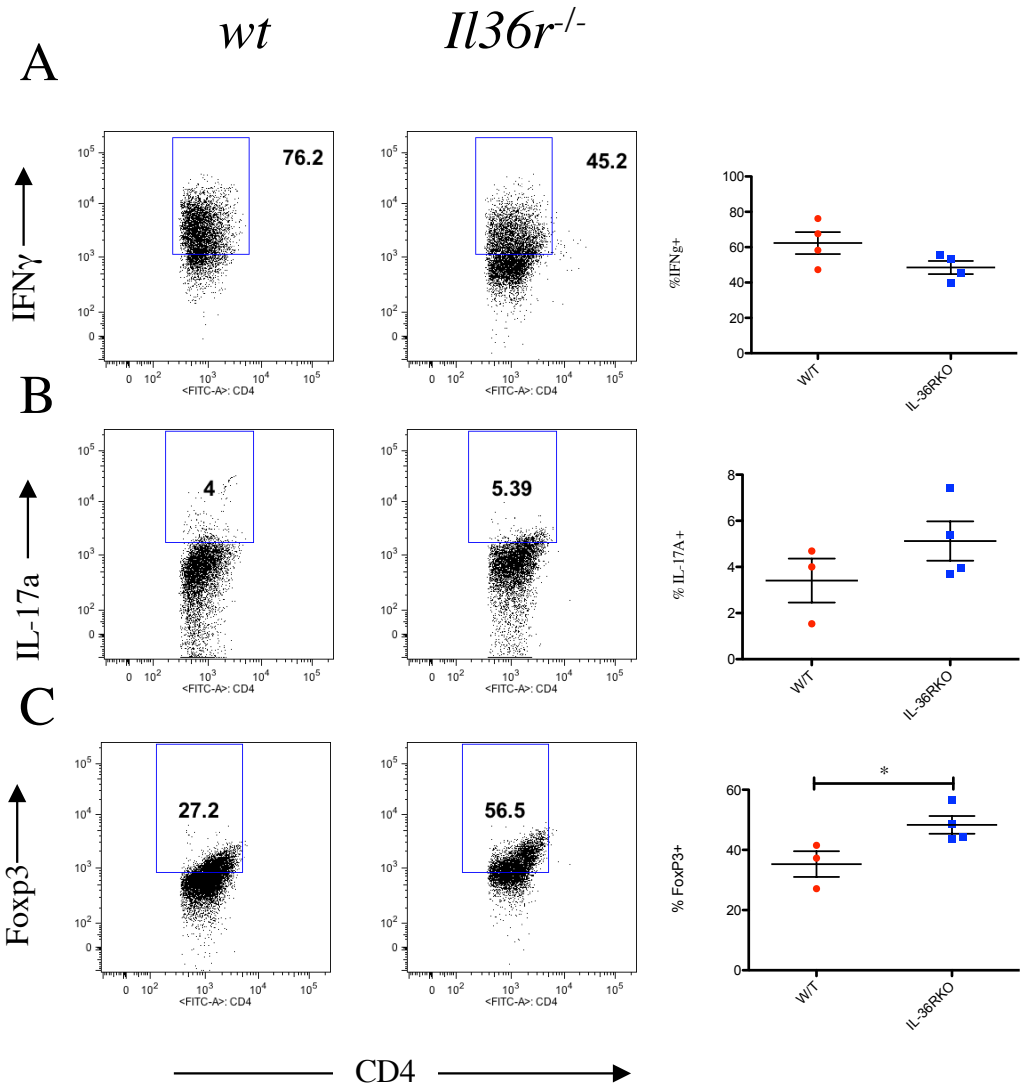


Figure 5.2.2.2: *Il36r* expression regulates the generation of pathogenic CD4+ T cells in the mesenteric lymph nodes in T cell dependant model of colitis. FACs sorted CD4$^+$CD25$^-$CD45β^{lo} effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by IP injection to *Rag1*$^{-/-}$ recipient mice. At takedown (day 28) mesenteric lymph nodes were taken for FACs analysis of IFNγ (A), IL-17a (B) and FOXP3 (C).). A - N = 4 *wt*, 4 *Il36r-/-*, B&C – N *=3 wt, 4 Il36r-/-. Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

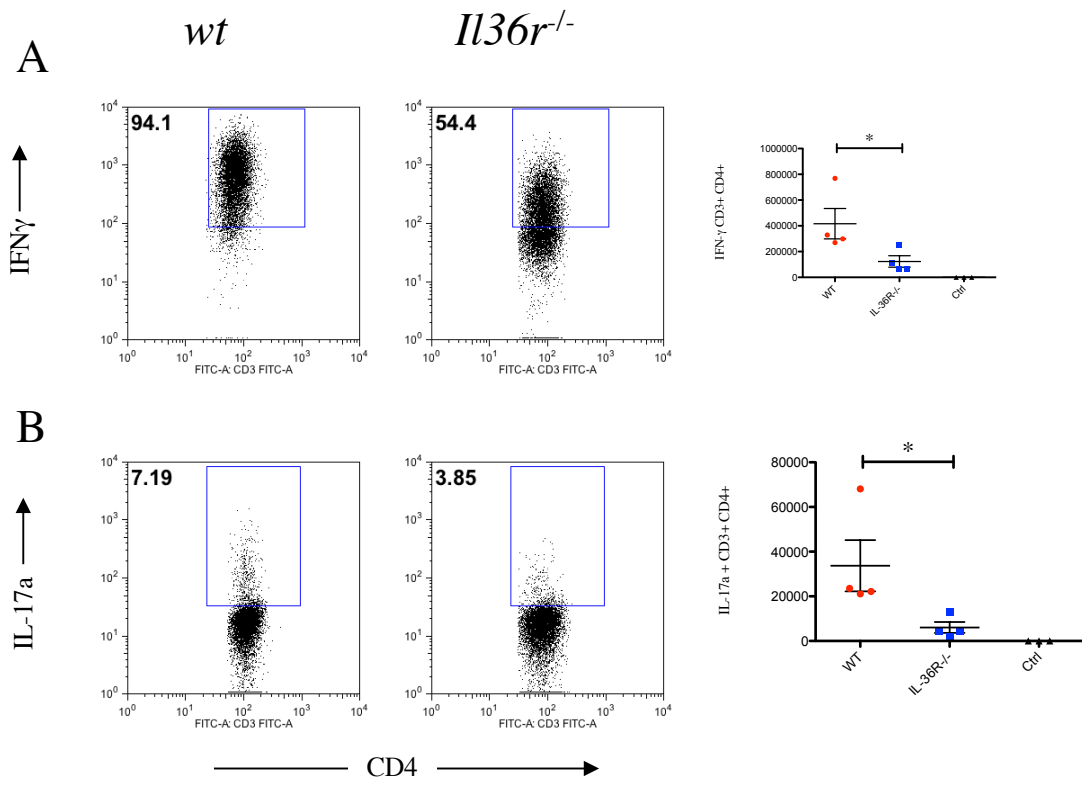


Figure 5.2.2.3.: ***Il36r*** **expression regulates the generation of pathogenic CD4+ T cells in the colon in T cell dependant model of colitis.** FACs sorted $CD4^+CD25^-CD45R\beta^+$ effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by IP injection to *Rag1*$^{-/-}$ recipient mice. At takedown (day 28) colon tissues were taken for FACs analysis of IFNγ (A), IL-17a (B).). N = 4 *wt*, 4 *Il36r-/-*, 3 *Ctrl*. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

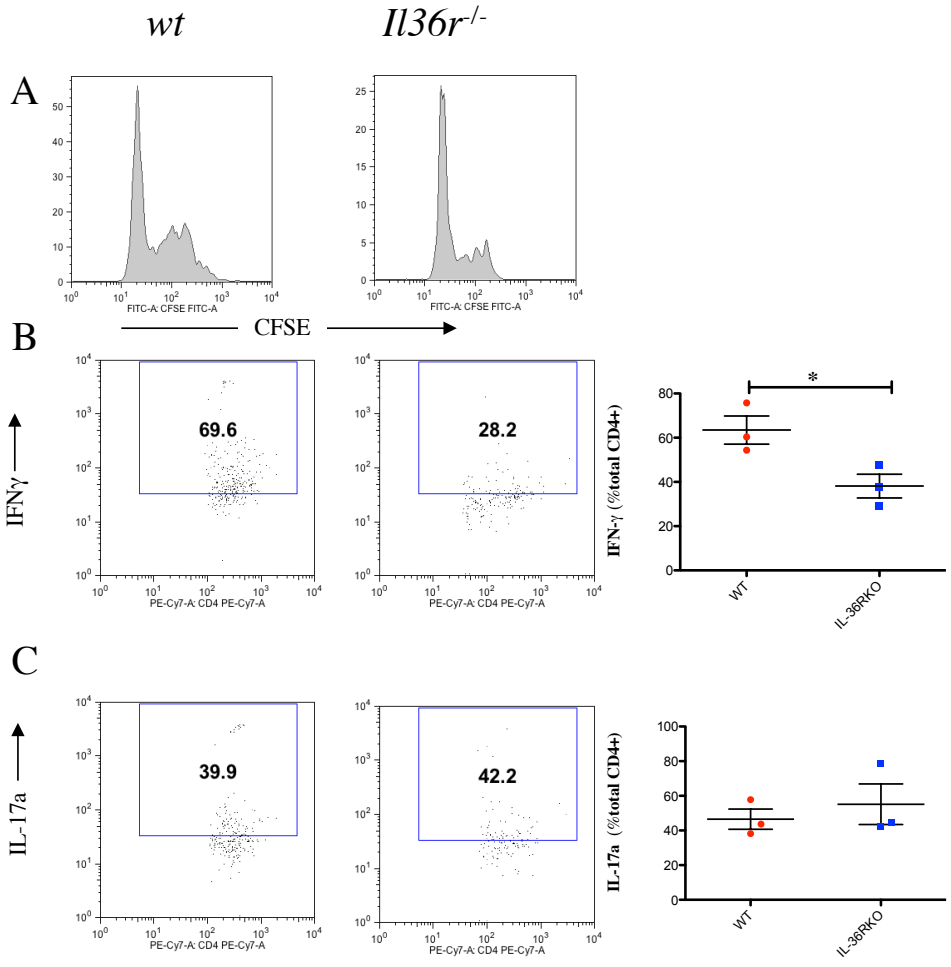
Interestingly our T cell results mirror that of what we have seen *in vitro*. As expected, mice that received *wt* T_H cells, whilst displaying a significant decrease in weight over the course of the experiment (Fig.5.2.1. A), also exhibit a significantly greater population of $IFN\gamma^+$ cells in the spleens (5.2.2.1.A) and colons (5.2.2.3.A) compared to *Il36r*$^{-/-}$ recipients. While this does not reach significance in the LNs (Fig.5.2.2.2.A) there is an increase in $IFN\gamma^+$ cells present. Furthermore, as there is no difference in IL-17a production between groups (Fig.5.2.2.1-3.B), this would suggest that IL-36 mediated T cell induced colitis is T_H1 dependent.

Interestingly, as we seen in Fig.5.2.1.A, Il*36r*$^{-/-}$ recipients appear protected from the onset of disease, an occurrence we hypothesized may be due to due reduced IFNγ output and T_H1 generation, but may be in fact be due to enhanced polarization and expansion of peripherally induced Tregs (pTregs). In the T cell transfer model of colitis $FOXP3^+$ Treg cells have been shown to develop in the post-thymic compartment from $FOXP3^-$ naïve $CD4^+$ T cells[489]. However, the low levels of these cells present fail to prevent and resolve the T effector induced colitis.

In this study, when IL-36R signalling is absent we see a significantly greater number of FOXP3^{+} cells in the periphery (Fig.5.2.2.1. C & Fig.5.2.2.2. C.). Taking this into consideration with our earlier results showing the ability of IL-36α to inhibit iTreg generation *in vitro*, as well its capacity to allow T effector cells overcome suppression by nTregs *ex vivo*, these results indicate that IL-36 has the potential to elicit and drive colitis with a dual mechanism promoting the expansion and polarization of pro-inflammatory T_H1 responses on one hand, and simultaneously inhibiting the generation of anti-inflammatory Tregs on the other.

5.2.3 IL36R EXPRESSION REGULATES THE EARLY GENERATION OF PATHOGENIC CD4+ T_H CELL RESPONSES ASSOCIATED WITH IBD *IN VIVO*.

As our findings in Chapter 4 have shown that IL-36α promotes the activation and generation of pathogenic CD4+ T_H cell lineages (Fig.4.2.1 & Fig.4.2.3), even in environments with suboptimal stimulation (Fig.4.2.2), we decided to evaluate if IL-36s colitogenic properties were due to early effects on CD4+ T_H cell activation and promoting proliferation *in vivo*. To achieve this, we used *Rag1*-/- mice for adoptive transfer studies, as these mice form the basis for an established model of T cell mediated colitis and allow us to examine CD4+ T_H cell activation and responses *in vivo*. CD4+ T cells isolated from *wt* and *Il36r*-/- mice, stained with CFSE, and transferred by *i.p* injection to *Rag1*-/- recipients. After 5 days, the mLNs were harvested for FACs analysis of IFNγ, IL-17a, FOXP3 and proliferation (CFSE).



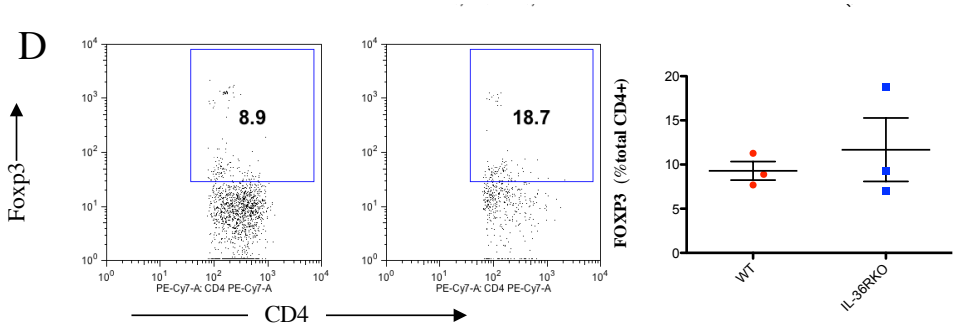


Figure 5.2.3.: *Il36r* expression regulates the early generation of pathogenic CD4$^+$ T$_н$ cells in the LN in a T cell transfer model. FACs sorted CD4$^+$CD25$^-$CD45Rβ^{hi} effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice, were stained with CFSE and transferred by IP injection to *Rag1*$^{-/-}$ recipient mice. At takedown (day 5) LN were taken for FACs analysis of CFSE (A) IFNγ (B), IL-17a (C) and FOXP3 (D). N = 3 *wt*, 3 *Il36r-/-*. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Levels of CFSE dilution of transferred T$_н$ cells was similar irrespective of *Il36r* expression (Fig.5.2.3A), indicating that IL-36 does not play a significant role in driving CD4$^+$ T$_н$ cell proliferation *in vivo*. However, we did observe a significantly enhanced population of IFNγ$^+$ cells in mLNs of *wt* recipients when compared to *Il36r*$^{-/-}$ recipients (Fig.5.2.3.A) demonstrating a potent ability of IL-36 to induce pro-inflammatory T$_н$1 cells very early after transfer and prior to disease onset. No differences in IL-17a or FOXP3 expression by these cells were observed between groups (Fig.5.2.3A), once again indicating that IL-36 may mediate colitis via enhanced T$_н$1 responses, and it can initiate this process from initial CD4$^+$ T$_н$ cell expansion in the periphery.

5.2.4 ABSENCE OF IL36R SIGNALLING RESULTS IN SPLENOMEGALY AND LYMPHADENOPATHY IN A T CELL TRANSFER MODEL OF COLITIS

The T cell transfer model of colitis relies upon 3 key mechanisms to initiate and perpetuate colitis. 1. The $CD4^+$ T cells adoptively transferred must be pure $CD4^+$ $CD45R\beta^{high}$ $CD25^{LOW}$ T effector cells capable of initiating inflammation. 2. Once transferred these cells must migrate to the periphery, where they will proliferate, expand, and differentiate into the proinflammatory lineages known to induce disease. 3. These now colitogenic T effector cells must exit the periphery and migrate to the intestines where they will initiate inflammation and mucosal destruction. If any of these 3 components fail, colitis will not develop[477,490]. Our results so far have shown that when IL-36R signalling is present, *wt* T effector cells facilitate disease onset and perpetuation in association with the enhanced generation of T_H1 cells in the periphery and colon. As the *Il36r*$^{-/-}$ recipients exhibit the opposite effects, with diminished numbers of $IFN\gamma^+$ cells and indeed an enhanced induction of $FOXP3^+$ expression, it is possible that these effects represent the primary mechanism through which IL-36 promotes T cell driven colitis. Interestingly however, whilst harvesting the tissues from the *wt* and *Il36r*$^{-/-}$ recipient mice for FACs analysis we also observed splenomegaly and lymphadenopathy in *Il36r*$^{-/-}$ T_H recipient mice (Fig.5.2.4 A & C). As expected, we also observed a shortening and increased weight of the colon from *wt* $CD4^+$ T cell recipients (Fig.5.2.4.E), which is a typical occurrence with intestinal inflammation due to intestinal architecture destruction alongside an accumulation of lymphocytes[491]. Surprisingly though, there was significant increase in the size of spleens and mLNs collected from the *Il36r*$^{-/-}$ T cell recipients (Fig.5.2.4.A & C). This correlated with an increase in overall live cell numbers harvested from these tissues (Fig.5.2.4. B & D). The opposite was seen for the colon, with significantly less total live cells present in *Il36r*$^{-/-}$ recipients compared to *wt* recipients when measured by Trypan blue exclusion (Fig.5.2.4. F). These observations are indicative of two possibilities: 1. IL-36 signalling promotes $CD4^+$ T_H cell gut homing, 2. IL-36 signalling is necessary for $CD4^+$ T cell egress from the periphery. Collectively these data suggest a role for IL-36 in $CD4^+$ T_H cell mediated pathology in IBD. IL-36 signalling promotes the generation and expansion of pathogenic T_H1 cells, and possibly their exit from the periphery and trafficking to the mucosa leading to the induction and perpetuation of inflammation in the intestines.

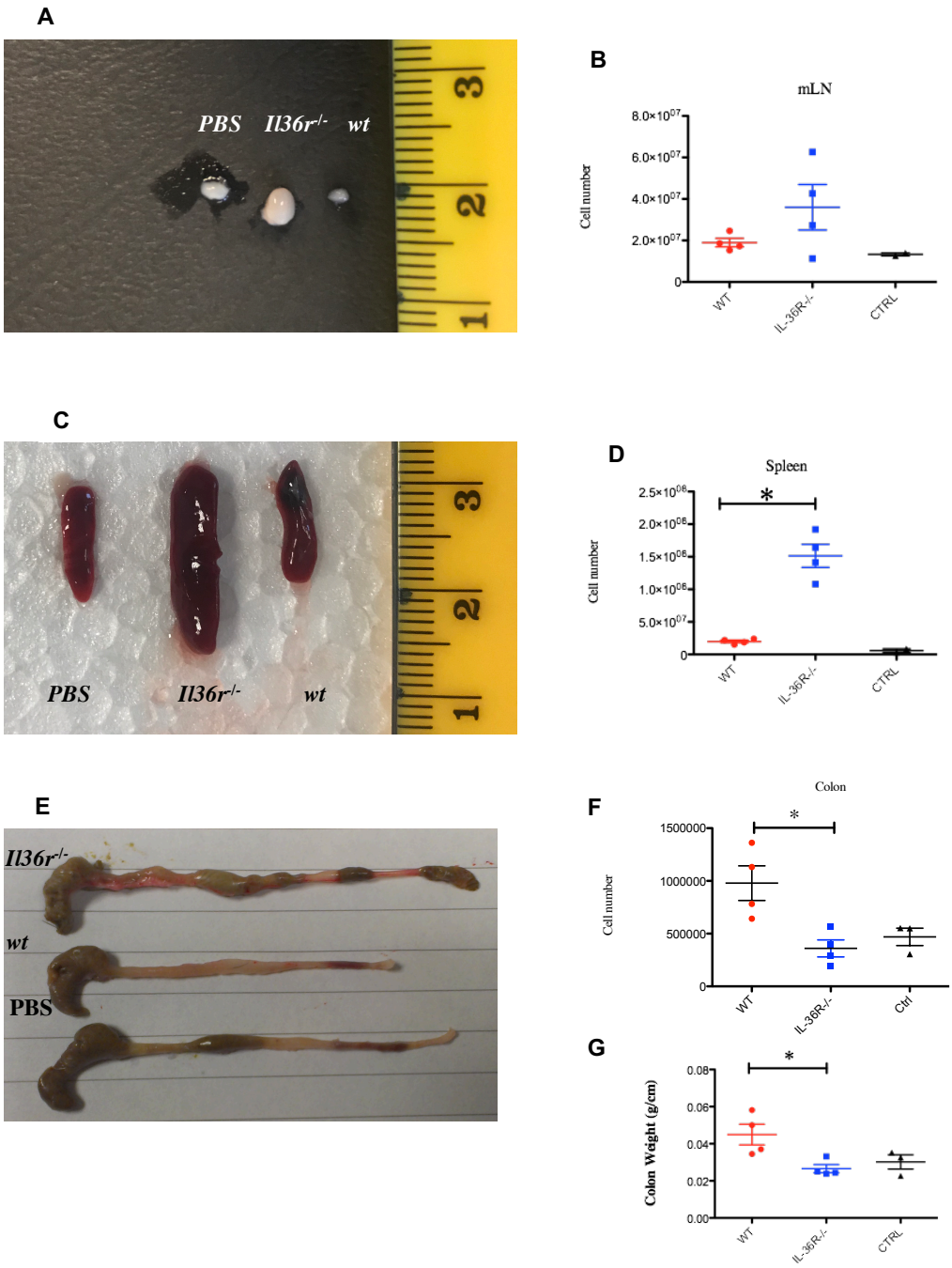


Figure 5.2.4.: ***Il36r recipient mice*** **exhibit splenomegaly and lymphadenopathy in a T cell transfer model of colitis.** FACs sorted CD4$^+$CD25$^+$CD45Rβ^{lo} effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by IP injection to *Rag1*$^{-/-}$ recipient mice. At takedown (day 28) mesenteric lymph nodes (A), spleens (C) and colons (E) were harvested, cell numbers were counted (B, D, & F) and colons were measured and weighed (G). N = 4 *wt*, 4 *Il36r-/-*, 3 *Ctrl*. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

5.2.5 IL-36 MEDIATES PATHOGENIC MUCOSAL T CELL GUT HOMING IN IBD *IN VIVO*

Infiltration of $CD4^+$ T_H cells to the mucosa is a characteristic of IBD, and therapeutically inhibiting this this mechanism has long been considered[43, 201]. Recently Vedolizumab, a monoclonal antibody against the α4β7 integrin has been gaining attention for its efficacy in treatment of patients with IBD, with remission rated of up to 44% reported from the GEMINI trials[474]. While this leaves a large cohort of patients with unmet clinical needs, it also highlights the success of targeting $CD4^+$ T_H cell gut homing in the setting of IBD. As our data has suggested a possible role for IL-36 in the generation of IBD associated pathogenic T_H cells and their migration to the gut we decided to evaluate the effect of IL-36 on mechanisms associated with $CD4^+$ T cell gut homing.

To do this, we once again used the T cell transfer model of colitis, evaluating the differences in the $CD4^+$ T_H cell populations in the mLNs, spleen and colons, of *wt* T effector and *Il36r*$^{-/-}$ T effector recipient *Rag1*$^{-/-}$ mice, and the expression of gut homing markers from these cells in the spleen and colon.

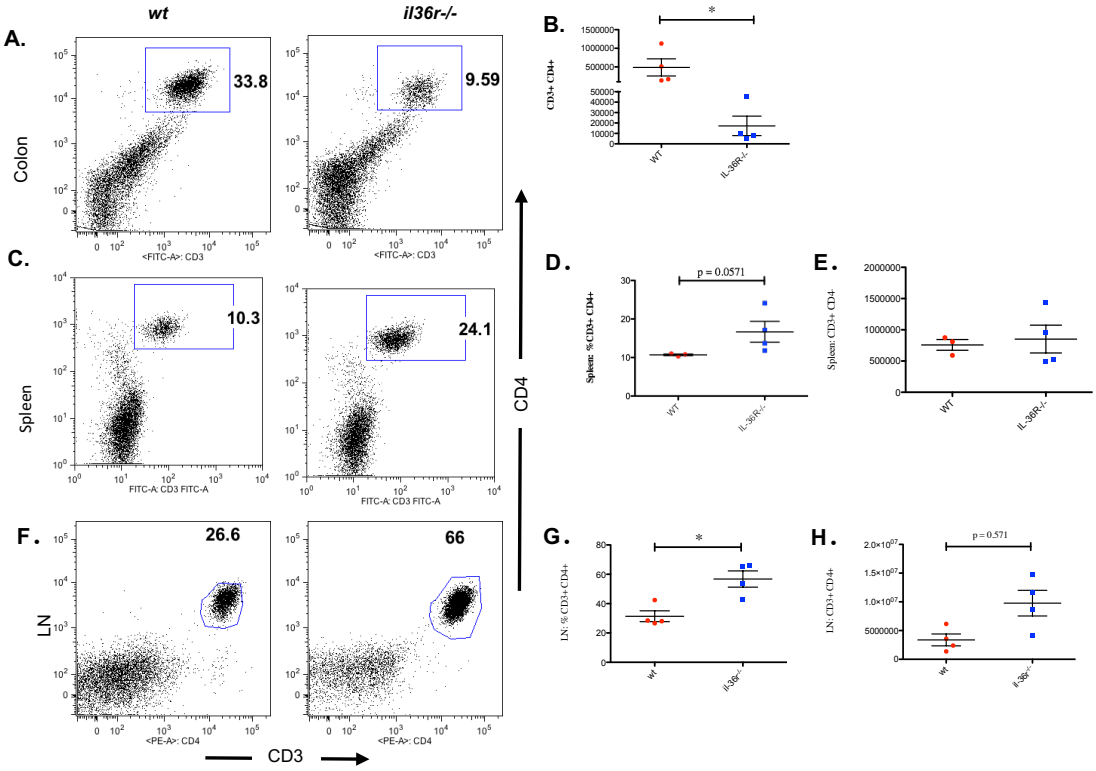


Figure 5.2.5.1.: *Il36r* expression promotes the migration of $CD4^+$ T cells from the periphery to the intestines. FACs sorted $CD4^+CD25^+CD45R\beta^{lo}$ effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by IP injection to *Rag1*$^{-/-}$ recipient mice. At takedown (day 28) colon (A-B), spleen (C-E), and mesenteric lymph nodes (F-H) were harvested for FACs analysis of $CD3^+$ and $CD4^+$ T cells. Due to low levels of lymphocytes isolated from the colons the cells were pooled for representative FACs analysis (A) The $\%CD3^+CD4^+$ cells from the pooled samples was then used to calculate the total number $CD3^+$ $CD4^+$ cells for each mouse using their individual Trypan blue cell count data obtained before they were pooled (B). N = 4 *wt*, 4 *Il36r-/-*, 3 *Ctrl*. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

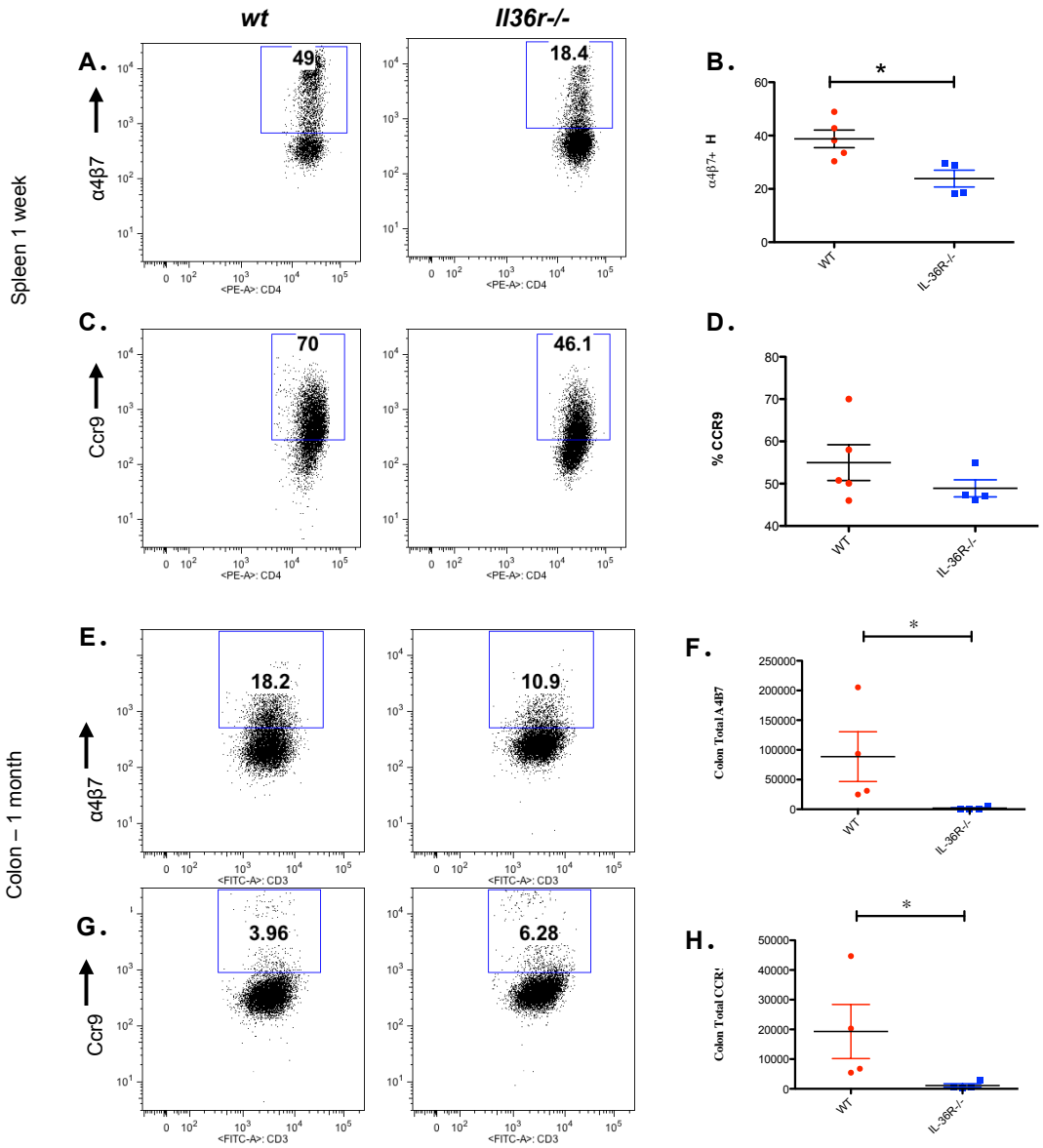


Figure 5.2.5.2: *Il36r* expression promotes the expression of gut homing molecules on CD4⁺ T cells. FACs sorted CD4⁺CD25⁻CD45Rβ^{hi} effector T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by IP injection to *Rag1*$^{-/-}$ recipient mice. At takedown spleens (A-D) and colons (E-H) were harvested for FACs analysis of α4β7 (A, B, E, & F) and Ccr9 (C, D, G & H) on CD4+ T cells. A –D, n = 5 *wt*, 4 *Il36r*-/-, E-H, n = 4 *wt*, 4 *Il36r*-/-. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

These results revealed a significantly higher total CD3⁺ CD4⁺ T$_H$ cell numbers in the colon of *wt* recipient mice (Fig.5.2.5.1 A & B), in comparison to mice which received *Il36r*$^{-/-}$ T cells. In contrast, Il*36r*$^{-/-}$ T cell recipient mice displayed increased percentage and number of CD4⁺ expressing T$_H$ cells in the mLNs when compared to *wt* CD4⁺ T$_H$ cell recipients (Fig.5.2.5.1 F, G & H), and this increase in percentage, but not number, of CD4⁺ T$_H$ cells is also evident in the spleen (Fig.5.2.5.1 C, D & E). These data indicate that IL-36R expression plays an

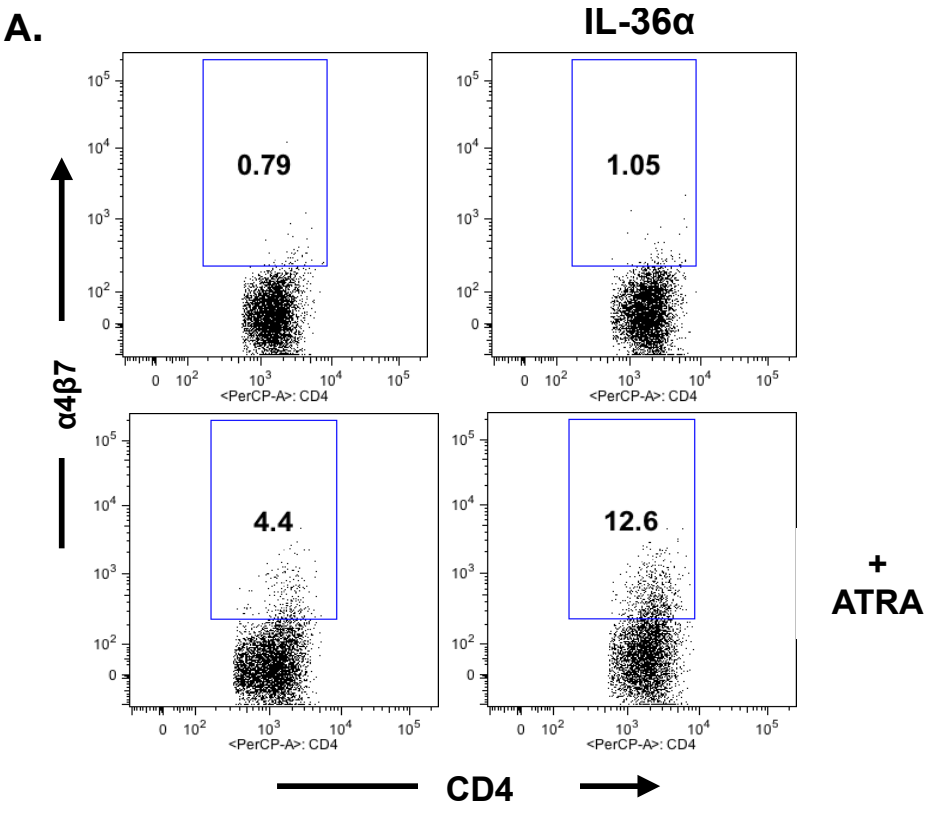
important role in promoting the ability of $CD4^+$ T_H cells to infiltrate the colon. Interestingly, we also observed relatively high expression of the gut homing integrin, α4β7 and chemokine receptor, CCR9, on $CD4^+$ T_H cells isolated from spleens of *wt* recipients, whereas this effect was diminished on *Il36r*$^{-/-}$ $CD4^+$ T_H cells. (Fig.5.2.5.2 A-D) 1 week post transfer. This observation was also seen in the colon, where number of T_H cells expressing both gut homing markers were significantly lower in *Il36r*$^{-/-}$ recipients after 1 month (Fig.5.2.5.2 F & H).

Taken together, these data strengthen the hypothesis for a central role for IL-36 in the pathogenesis of IBD, with multiple mechanisms promoting the pathogenesis of disease, including the promotion of polarisation of proinflammatory $CD4^+$ T_H cell subsets, inhibition of regulatory T cell generation and functioning, and enhancing migratory capacity of pathogenic $CD4^+$ T_H cells to the intestines.

5.2.6 IL-36α MEDIATES THE EXPRESSION OF GUT HOMING MOLECULES ON T_H CELLS *IN VITRO*

Following on from our finding of enhanced expression of α4β7and CCR9 from *wt* T_H cells compared to *Il36r*$^{-/-}$ T_H cells isolated from the periphery and colon of *Rag1*$^{-/-}$ mice during colitis, we decided to compare the effects of IL-36 signalling on induction of a T_H cell gut homing phenotype to Retinoic Acid. Retinoic acid (RA), a vitamin A metabolite, has been widely described as the master regulator of T cell homing to the gut[492]. Interaction of this metabolite with CD4$^+$ T_H cells upregulates the expression of the α4β7 integrin and CCR9 chemokine receptor to facilitate trafficking to the mucosa[492]. In the gut, Vitamin A is converted to RA by DCs from the Peyer's patches and mesenteric LNs[493], CD103$^+$ DCs that are found in the small intestine[494-497], and at the sites of inflammation[498].

To compare the effects of IL-36α and Retinoic acid on CD4$^+$ T_H cell gut homing, CD4$^+$ T_H cells were isolated from *wt* mice and stimulated with IL-36α and all trans Retinoic Acid (ATRA) for 72hrs. α4β7 and CCR9 expression was then measured by FACs.



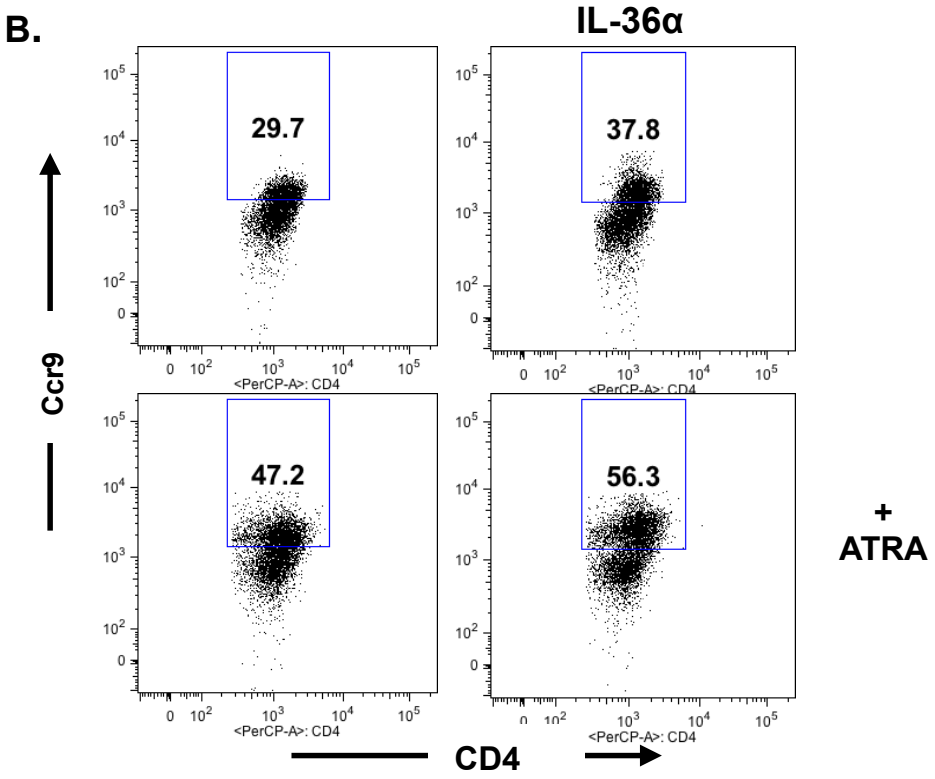


Figure 5.2.6. : IL-36α promotes the expression of gut homing molecules on CD4+ T cells. Purified $CD4^+$ T cells were cultured in the presence or absence of IL-36α(200ng/ml) and ATRA (200nM) at 37°C under full (1μg/ml αCD3ε;3μg/ml αCD28) activation conditions. At 72hr supernatants were analysed for α4β7 (A) and CCR9 (B). Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

As expected, ATRA upregulated expression of both gut homing molecules (Fig.5.2.6 A & B). CCR9 expression was enhanced by IL-36α stimulation alone, but was further enhanced when combined with ATRA. These combined effects were also greater than the effect of ATRA alone (Fig.5.2.6 B). Similarly, the combination of IL-36α/ATRA induced greater expression of α4β7 than that of IL-36α or ATRA separately (Fig.5.2.6 A). These data indicate that IL-36α stimulation of $CD4^+$ T_N cells imprints a gut homing phenotype, and when combined with ATRA, which is naturally present in the gut, does this to greater effect.

5.2.7 IL-36α MEDIATES THE EXPRESSION OF GUT HOMING MOLECULES ON T_H CELL LINEAGES CELLS *IN VITRO*

As well as instructing gut homing capacity, in the intestines, ATRA is known to promote oral tolerance and maintain immune homeostasis by enhancing Treg generation and suppressive functioning. The metabolite interacts directly with the Tregs themselves to promote FOXP3 stability, prevent Treg plasticity and expand their population[499, 500]. Indirectly, ATRA interacts with $CD44^{hi}$ memory T cells, to inhibit their production of IL-4, IL-21 and IFNγ, cytokines that prevent the polarisation of naïve T cells into Tregs and pharmacological doses of ATRA (10nM +) have been shown to inhibit T_H17 and T_H1 polarisation[501-503].

Taking into consideration our data demonstrating IL-36α promotes the generation of pathogenic $CD4^+$ T_H cell subsets associated with IBD (Fig.4.2.3 A, D & E), the protection from colitis when IL-36R signalling is absent on $CD4^+$ T_H cells (Fig.5.2.2.4), as well as their diminished capabilities to expand a T_H1 population and migrate to the intestines (Fig.5.2.2.1-2. A & Fig.5.2.3), we began to question:

1. How does IL-36 effect the expression of gut homing molecules on the T_H lineages associated with IBD?
2. How does ATRA effect this?

To answer these questions $CD4^+$ T_H cells were isolated from *wt* mice, skewed towards T_H1, iTreg, T_H17, and T_H9 phenotypes and stimulated with IL-36α and ATRA. After 72-120hrs α4β7 and CCR9 expression was measured by FACs and compared to signature cytokine expression.

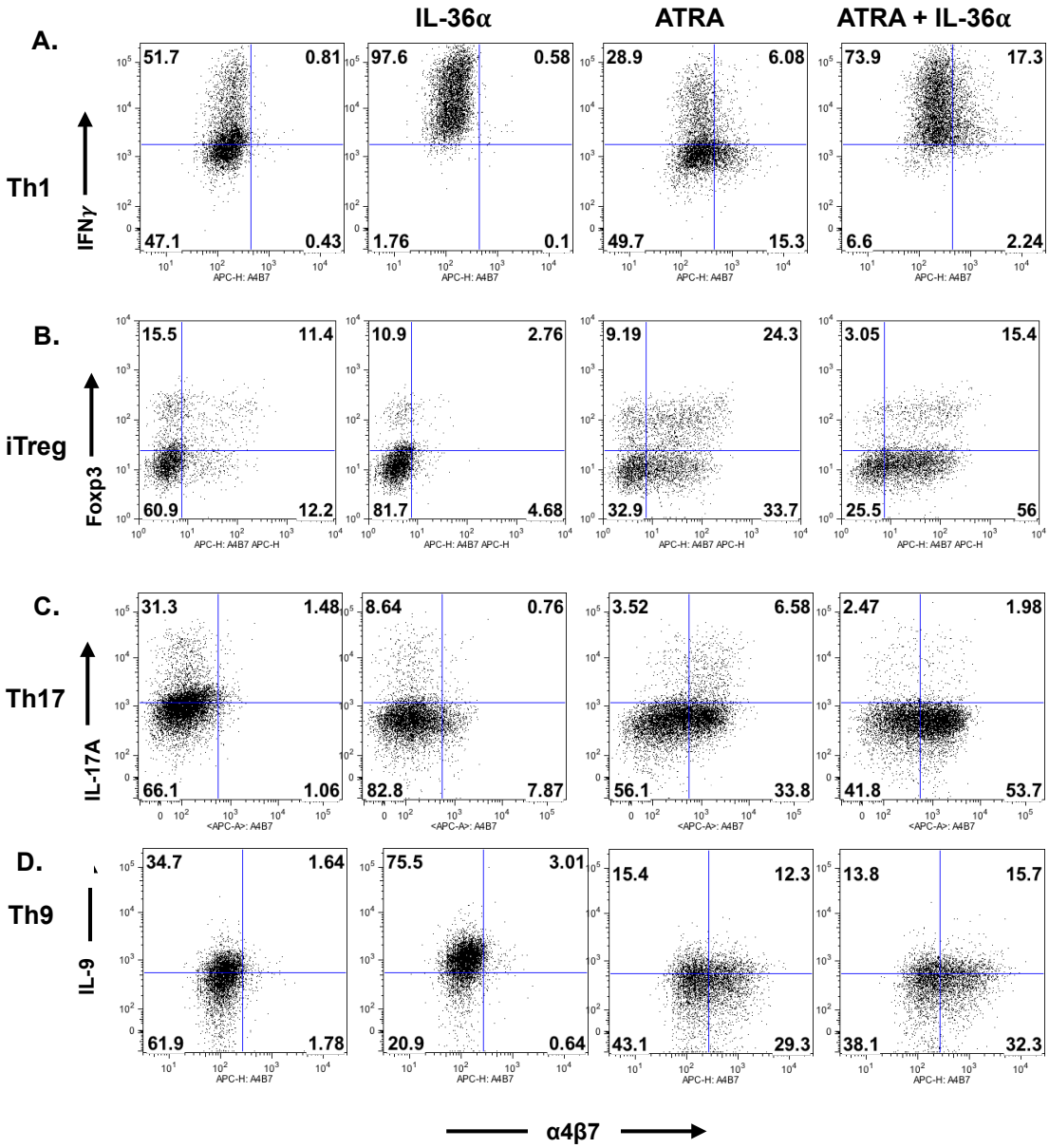


Figure 5.2.7.1: IL-36 regulated the expression of α4β7 on different CD4+ T cell lineages. Magnetically purified CD4+ T cells activated with plate-bound αCD3ε /αCD28 (1μg/ml, 3μg/ml), were differentiated to T_H1 (20ng/ml IL-12, 10μg/ml αIL-4), T_H17 (10μg/ml αIFNγ, 5μg/ml αIL-4, 20ng/ml IL-6), iTreg (5ng/ml TGFB) T_H9 (10μg/ml αIFNγ, 20ng/ml IL-4, 5ng/ml TGFB), phenotypes, and stimulated wit IL-36α (200ng/ml) +/- ATRA (200nM) prior to incubation at 37°C for 72hr - 120hrs. Following incubation cells were analysed by intracellular flow cytometry for signature cytokine and α4β7 expression (T_H1 - IFNγ (A); iTreg - FOXP3 (B); Th17 – IL-17a (C) and T_H9 - IL-9 (D)). Data representative of a minimum of 2 independent repeats.

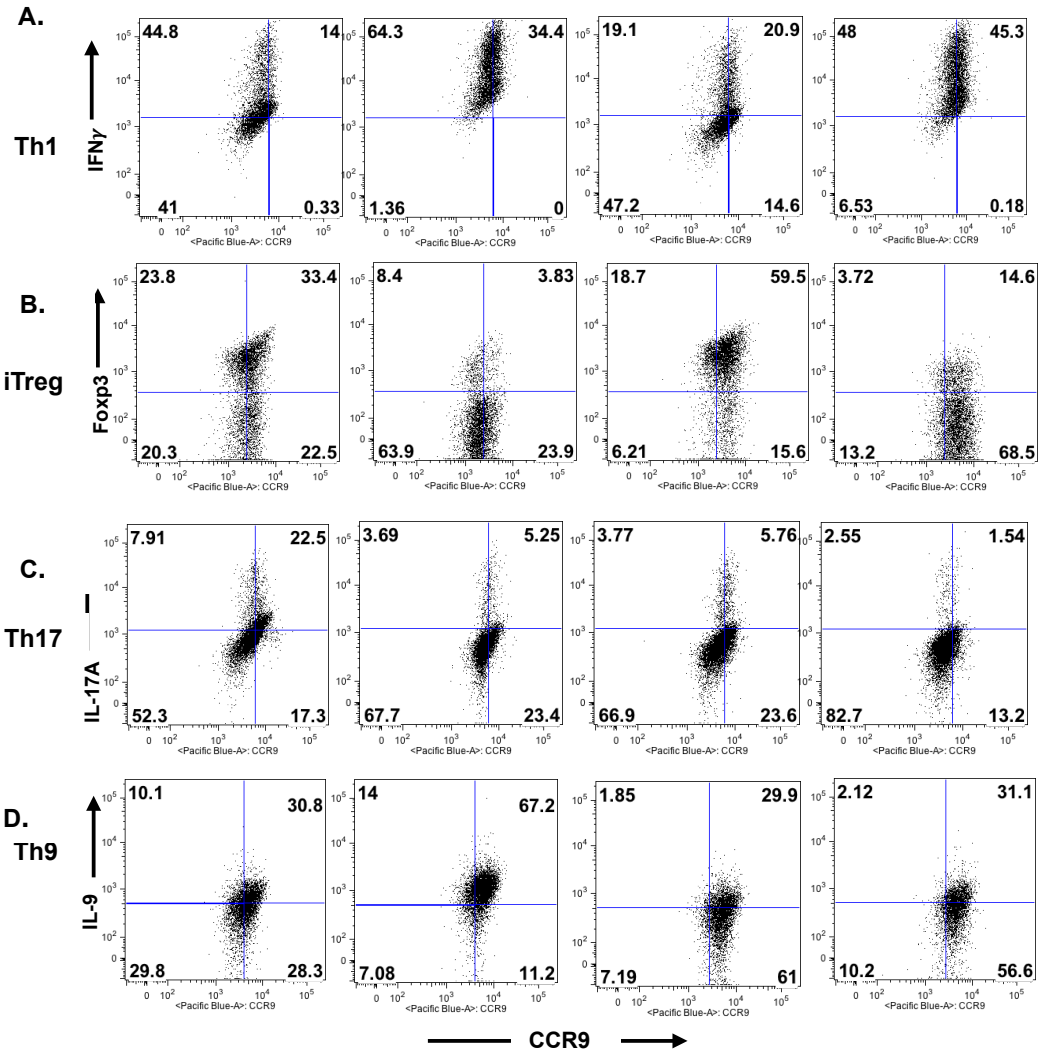


Figure 5.2.7.2-: IL-36 regulated the expression of CCR9 on different CD4+ T cell lineages. Magnetically purified CD4+ T cells activated with plate-bound αCD3ε /αCD28 (1μg/ml, 3μg/ml), were differentiated to T_H1 (20ng/ml IL-12, 10μg/ml αIL-4), T_H17 (10μg/ml αIFNγ, 5μg/ml αIL-4, 20ng/ml IL-6), iTreg (5ng/ml TGFB) T_H9 (10μg/ml αIFNγ, 20ng/ml IL-4, 5ng/ml TGFB), phenotypes, and stimulated wit IL-36α (200ng/ml) +/- ATRA (200nM) prior to incubation at 37°C for 72hr - 120hrs. Following incubation cells were analysed by intracellular flow cytometry for signature cytokine and Ccr9 (T_H1 - IFNγ (A); iTreg - FOXP3 (B); Th17 – IL-17a (C) and T_H9 - IL-9 (D)). Data representative of a minimum of 2 independent repeats.

After 72hrs we observed an increase in IFNγ from the T_H1 population stimulated with IL-36α alone. However, as previously reported, T_H1 cell differentiation in the presence of ATRA resulted in a marked reduction of IFNγ+ cells, and an increase in α4β7+ cells, suggesting the cells are being redirected away from a T_H1 phenotype but gaining gut homing potential. Interestingly, when these T_H1 cells are stimulated with IL-36α in the presence of ATRA, the cells maintained their enhanced T_H1 population, and a subset of these IFNγ+ also express α4β7 (Fig.5.2.7.1 A). We see a similar expression pattern for CCR9, although IL-36α stimulation alone, promoted both IFNγ+ and CCR9+ expression on these cells (Fig.5.2.7.2

A). These results suggest ATRA and IL-36α have opposing effects on T_H1 differentiation, but synergistically promote the generation of proinflammatory T_H1 cells with gut homing potential (Fig.5.2.7.1 A & Fig.5.2.7.2 A).

Under iTreg conditions we see opposing results. IL-36α inhibited the FOXP3$^+$ population, whilst ATRA enhanced it, as well as α4β7 expression. Intriguingly, IL-36α /ATRA costimulation, resulted in a diminished population of FOXP3$^+$ Tregs, but enhanced the expansion of a population of α4β7$^+$ FOXP3$^-$ T cells (Fig.5.2.7.1 B). Once again, we observed a similar expression pattern for CCR9 (Fig.5.2.7.2 B). These results demonstrate that ATRA and IL-36α have opposing effects on CD4$^+$ T cells, and synergistically enhance the generation of proinflammatory FOXP3$^-$ cells with gut homing potential (Fig.5.2.7.1.B & Fig.5.2.7.2.B).

The differentiation of T_H17 cells are inhibited independently by both ATRA and IL-36α (Fig.5.2.7.1. C). However, while IL-36α inhibited IL-17a expression alone, it did not possess the capacity to induce gut homing markers under T_H17 conditions (Fig.5.2.7.1-2.C). ATRA, on the other hand, both inhibited IL-17a expression and simultaneously promotes α4β7 expression (Fig.5.2.7.1.C) under T_H17 conditions. What's more, IL-36α/ATRA costimulation of T_H17 cells enhanced the expansion of an IL-17a$^-$ α4β7$^+$ population further (Fig.5.2.7.1.C). However, unlike T_H1 and iTreg cells, we did not see a similar expression pattern for CCR9. In fact, we observed a decrease in CCR9$^+$ cells following both IL-36α and ATRA stimulation, and the CCR9$^+$ population is further diminished by IL-36α/ATRA costimulation (Fig.5.2.7.2.C). Collectively, these results show that IL-36α and ATRA work similarly under T_H17 conditions, both molecules independently inhibited the generation of IL-17a cells, and work together to promote the generation of an enhanced IL-17a$^-$ α4β7$^+$ gut homing T_H cell.

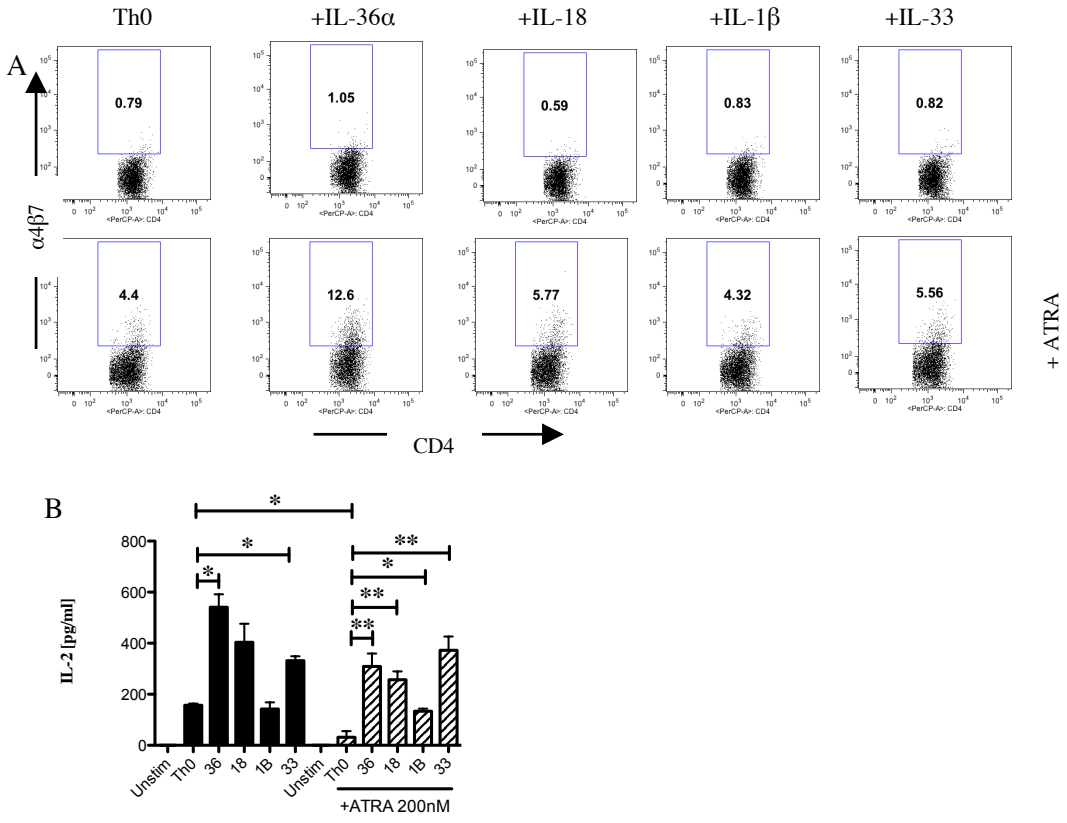
For T_H9s we see a similar pattern to T_H1 cells. IL-36α alone enhanced IL-9 expression, whereas ATRA alone inhibited IL-9 but enhances α4β7. However, unlike T_H1 cells, IL-36α stimulation in presence of ATRA present does not break ATRA mediated suppression of T_H9 generation, but it does further enhance α4β7 expression (Fig.5.2.7.1.D). Interestingly, IL-36α stimulation alone induced a large population of CCR9$^-$IL-9$^+$ cells, and when ATRA is included, these cells lose their enhanced IL-9 expression but maintain their CCR9 expression (Fig.5.2.7.2.D). These data indicate that independent IL-36α stimulation facilitates the generation of a gut homing T_H9 population, but ATRA maintains its immunomodulatory effects and inhibits this response, whilst promoting an IL-9$^-$ gut homing CD4$^+$ T_H cell population.

Collectively these data show there are opposing mechanisms at play for IL-36α and ATRA in the generation of T_H cell responses associated with IBD and that, for the most part,

IL-36 can overcome the immunosuppressive effects of retinoic acid while promoting the acquisition of a gut homing phenotype.

5.2.8 IL-36α PROMOTES THE GENERATION OF PRO-INFLAMMATORY GUT HOMING T_H CELL LINEAGES CELLS MORE POTENTLY THAT OTHER IL-1 FAMILY MEMBERS *IN VITRO*.

As we have mentioned above ATRA promotes GI homeostasis by modulating T_H cell responses in the mucosa, promoting the generation of regulatory T cells and synergistically inhibiting the polarisation of proinflammatory T_H cell subsets[499, 500, 504-507 501-503]. Similarly, IL-1 family cytokines are known to play key roles in modulating CD4+ T_H cell responses, however in contrast to ATRA, many promote the polarisation of proinflammatory T_H cell lineages[172, 258, 263, 420, 429, 430, 433, 435]. As we have just demonstrated that the IL-1 family member IL-36 can overcome ATRA suppression and promote the generation of proinflammatory T_H cells with a gut homing phenotype (Fig.5.2.7), we decided to evaluate the effect of other IL-1 family members on T_H cell gut homing. To achieve this, CD4+ T_H cells were isolated from *wt* mice, skewed towards a T_H0 or T_H1, phenotype and stimulated with IL-36α, IL-18, IL-1 β, IL-33 and ATRA. After 72 α4β7and IFNγ expression was measured by FACs, and IL-2 was measured by ELISA.



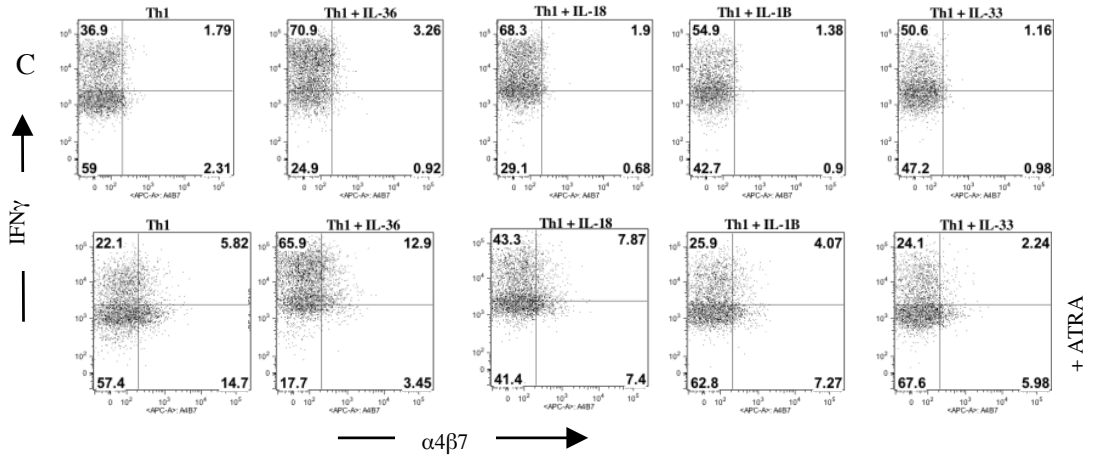


Figure 5.2.8.: IL-36α enhances the generation of α4β7+ $T_н$ cells more potently than other IL-1 family members. Magnetically purified $CD4^+$ T cells activated with plate-bound αCD3ε /αCD28 (1μg/ml, 3μg/ml), were stimulated wit IL-36α (200ng/ml), IL-18 (200ng/ml) IL-1 β (200ng/ml), or IL-33 (200ng/ml) +/- ATRA (200nM), with or without $T_н1$ skewing cytokines (20ng/ml IL-12, 10μg/ml αIL-4), prior to incubation at 37^oC for 72hr. Following incubation $T_н0$ cells were analysed by flow cytometry for α4β7 expression (A), and by ELISA for IL-2 expression (B). $T_н1$ cells were analysed for expression of signature cytokine (IFNγ) and α4β7 by FACs. Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

After 72hrs there is very little expression of α4β7 observed from $T_н0$ cells, even when stimulated with the IL-1 family member cytokines(Fig.5.2.8.A). When these cells are treated with ATRA, we see an increase in α4β7 expression, which is once again enhanced by IL-36α stimulation. IL-18 and IL-33 appear to slightly enhance α4β7 expression on $T_н0$ cells under these conditions, but IL-1β appears to have no effect (Fig.5.2.8A). Therefore IL-36α appears to be the most potent IL-1 family member in inducing α4β7 expression from naïve $T_н$ cells. Interestingly, IL-36α also induces the most IL-2 from naïve $T_н$ cells compared to other IL-1 family members, although IL-33 does also induce a significantly elevated amount. ATRA treatment inhibits IL-2 production basally from naïve T cells, yet the IL-1 members maintain their stimulatory effect, and IL-36α, IL-18 and IL-33 significantly enhance IL-2 output (Fig.5.2.8.B).

For the $T_н1$ population we observed an increase in IFNγ when the cells were stimulated with IL-36α alone (Fig.5.2.8.C). However, as previously reported, $T_н1$ cell differentiation in the presence of ATRA resulted in a marked reduction of $IFN\gamma^+$ cells, and an increase in $\alpha4\beta7^+$ cells, once again suggesting the cells are being redirected away from a $T_н1$ phenotype and gaining gut homing potential. Once again, when these $T_н1$ cells are co-stimulated with IL-36α and ATRA, the cells maintained their enhanced $T_н1$ population, and a subset of these $IFN\gamma^+$ also express α4β7 (Fig.5.2.8.C), suggesting that IL-36α promotes the generation of gut homing $T_н1$ cells. We see a similar profile for $T_н1$ cells stimulated with IL-18. IL-18

stimulation alones enhanced the population of IFNγ+cells, the addition of ATRA inhibited the IFNγ+ population, but enhanced a population of IFNγ+ α4β7+ T_H1 cells. However, this population of IL-18 induced IFNγ+ α4β7+ is smaller than the IL-36α induced IFNγ+ α4β7+ population. IL-1β and IL-33 stimulation of T_H1 cells did enhance IFNγ+population, but neither induced α4β7 expression independently, nor were they capable of evading ATRA mediated IFNγ+ suppression (Fig.5.2.8.C). Collectively, these data demonstrate that IL-36α is the most potent IL-1 family member in inducing an enhanced population of T_H1 cells with a gut homing potential.

5.2.9 IL-36R SIGNALLING REGULATES THE EXPRESSION OF T_H CELL ACTIVATION AND GUT HOMING MARKER CD69

Following on from our observations of splenomegaly and lymphadenopathy in *Il36r*$^{-/-}$ recipient mice after T cell transfer (Fig.5.2.4.A&B), we postulated that this may be due to IL-36 enhancing T_H cell migration to the intestines or regulating T_H cell egress from the periphery. Having demonstrated that IL-36 has the capacity to promote the generation of pathogenic T_H cells with gut homing properties (Fig.5.2.5.2, Fig.5.2.6, Fig.5.2.7.1-2), we next decided to evaluate the role of IL-36 in T_H cell retention and egress from the periphery.

In addition to the expression of gut homing mediators, such as α4β7 and CCR9, T_H cell expression of the early activation marker CD69 has also been shown to regulate their migration to the intestines[508]. CD69 deficiency results in an increased accumulation of T effector cells in the intestines in T cell transfer colitis, associated with an enhanced expression of chemokine receptors genes in the T cells[508]. Furthermore, a CD69/Sphingosine-1 phosphate ($S1P_1$) axis plays a role in T cell egress from lymphoid tissues. CD69 upregulation on lymphocytes inhibits S1P1's chemotactic function and results in the cells retention in the lymphoid tissue[509]. As we observed exceptionally high numbers of T_H cells being retained in the periphery of *Il36r*$^{-/-}$ CD4$^+$ T cell recipient mice (Fig.5.2.3.1) we decided to carry out a preliminary analysis of CD69 expression on these cells.

To do this, we once again used the T cell transfer model of colitis, evaluating CD69 expression on T_H cells harvested from the spleens of *wt* and *Il36r*$^{-/-}$ T_H cell recipient *Rag1*$^{-/-}$ mice 1 week after transfer.

Interestingly we observed an increase in CD69 expression from *Il36r*$^{-/-}$ T_H cells compared to their *wt* counterparts, indicating that IL-36R signalling may be important in T cell egress. This may be due to an inhibitory effect on CD69 expression, or an enhancing effect on $S1P_1$ expression, and warrants further investigation.

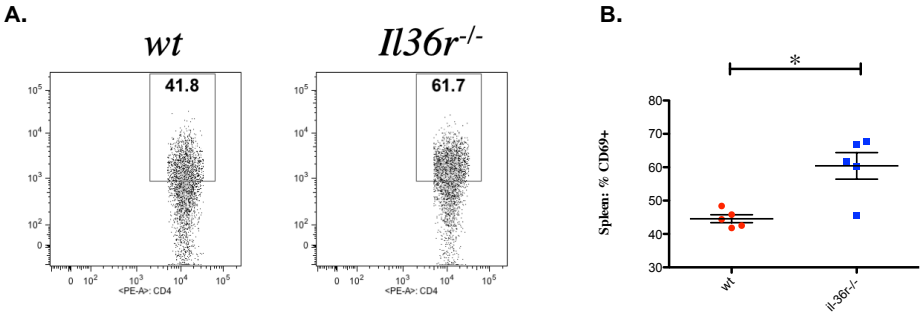
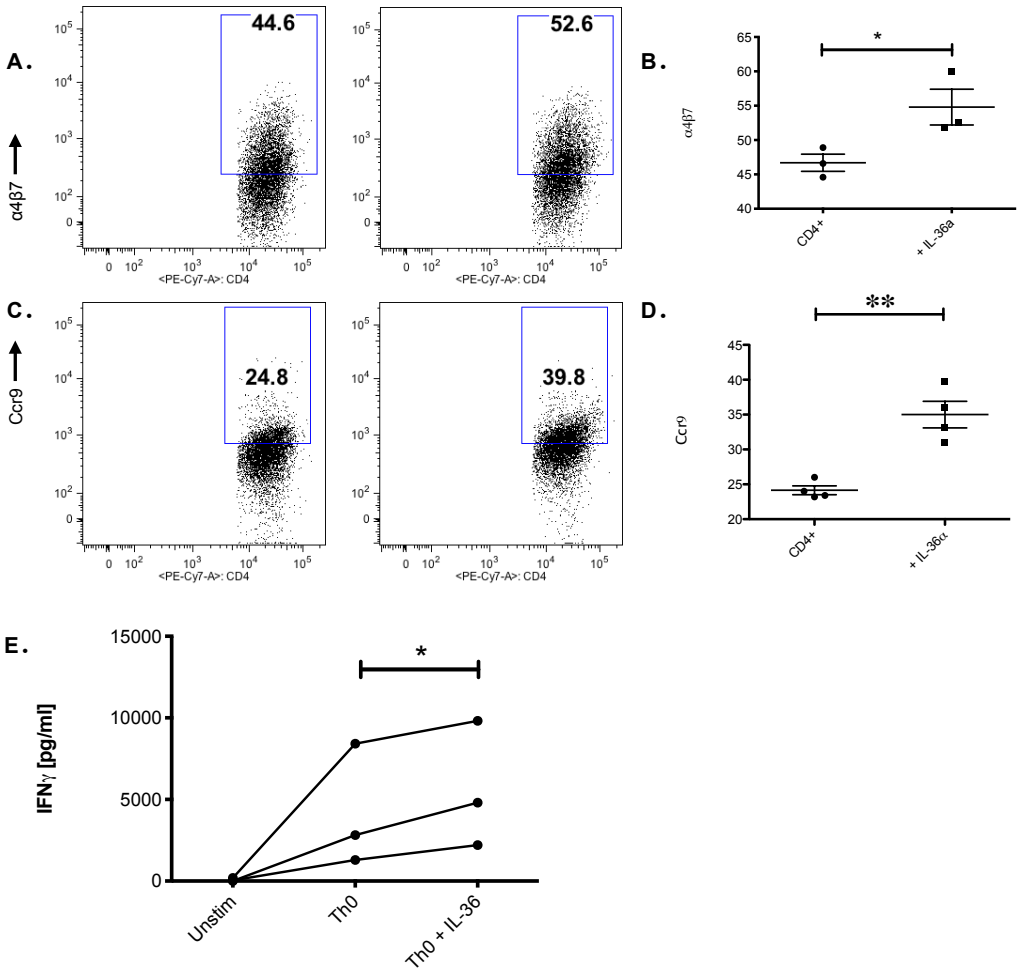


Figure 5.2.9.: *Il36r* expression promotes the expression of CD69 on CD4$^+$ T cells. Magnetically sorted CD4$^+$ T cells from donor *wt* and *Il36r*$^{-/-}$ mice were transferred by IP injection to *Rag1*$^{-/-}$ recipient mice for 1 week. At takedown spleens were harvested for FACs analysis of CD69 on CD4$^+$ T cells. N = 5 *wt*, 5 *Il36r-/-*. *Statistical analysis performed by student's t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

5.2.10 IL-36α PROMOTES THE GENERATION OF HUMAN PATHOGENIC GUT HOMING T_H CELLS

As previously mentioned, Vedolizumab has shown significant efficacy in the treatment of IBD, by inhibiting T cell migration to the colon by blocking MAdCAM/α4β7 interactions[474, 510]. As we have gained significant evidence for a role for IL-36 enhancing murine colitis via enhanced migration of pathogenic T_H1 cells to the gut, we wanted to determine what effect IL-36 has on human $CD4^+$ T cells, to determine if it induces a similar proinflammatory phenotype type in these cells, and if so, provide further validation of its potential as a therapeutic target. To address this question $CD4^+$ T_H cells were isolated from human PBMCs, stained with CFSE, activated with anti-CD3/anti-CD28 activation beads and stimulated in the presence or absence of IL-36α for 120hours. α4β7, CCR9 and CFSE was then measured by FACs, and IFNγ was measured by ELISA.



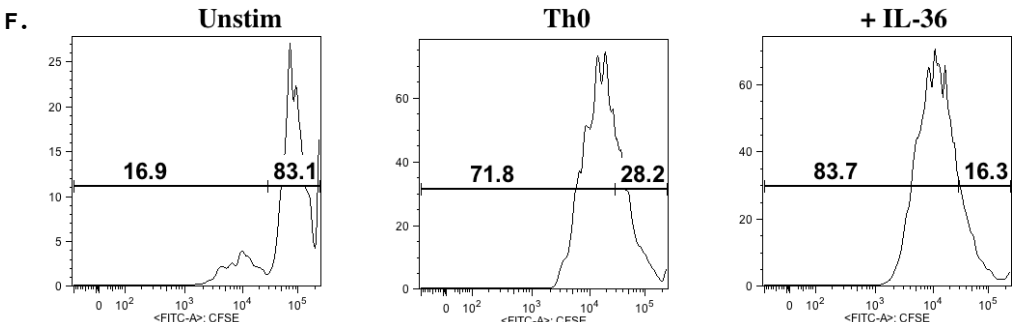


Figure 5.2.10.: IL-36α promotes the generation of human IFNγ+ gut homing T cells. CD4+ T cells were magnetically isolated from human PBMCs and activated with antiCD3/antiCD28 beads. Cells were stimulated with IL-36α (100ng/ml) and placed in culture for 120hrs at 37°C. Following incubation supernatants were analysed for IFNγ by ELISA (E) and cells were analysed for α4β7 (A), CCR9 (C) and CFSE by FACs. Data representative of a minimum of 3 independent repeats. *Statistical analysis performed by student's t test, (B&D) and paired t test (E) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Interestingly, we observed an increase in α4β7 (Fig.5.2.10.A&B), CCR9 (Fig.5.2.10.C&D), IFNγ (Fig.5.2.10.E) expression, as well as marginally enhanced proliferation (Fig.5.2.10.F), when these cells are stimulated with IL-36α. These results replicate our observations using murine T_H cells in proposing a role for IL-36α in the generation of pathogenic T_H cells with the capacity to home to the gut. Taking this into account, along with the critical role for IL-36 in increasing the severity of disease in our T cells transfer model of colitis, these results propose IL-36 dependent T cell responses as an attractive candidate for therapeutic intervention in IBD, as well as in other possible IL-36 mediated diseases.

5.3 DISCUSSION

Cytokines are considered key constituents in the immunopathogenesis of IBD and play an important role in defining the persistence versus the resolution of intestinal inflammation. GI homeostasis is achieved by complex orchestration of pro and anti-inflammatory effector cytokines at the mucosal surface, and consequently targeting these inflammatory modulators has offered an attractive potential therapy for patients. There have already been some notable successes using this approach, with the use of anti-TNFα biologics, infliximab, adalimumab, and certolizumab[65,66] as well as Ustekinumab, which inhibits T_H1/ T_H17 generation[67,411]. Furthermore, targeting T cell gut homing has been gaining significant popularity. Vedolizumab, has shown considerable efficacy inhibiting T cell migration in the clinic[67,484,485], and has paved the way for the development of novel biologics exploiting T cell chemoattractant mechansisms[486,487,511]. Yet, with each of these approaches there remains a significant cohort of patients who do not respond and may benefit from the use of alternative immune targeted therapeutic techniques. One, which is gaining significant interest in the clinic and has recently entered clinical trials is Spesolimab, which targets IL-36R signalling[423]. However, the potential efficacy of this approach remains to be determined as there are dichotomous reports in the literature as to whether this cytokine family promotes the perpetuation or resolution of intestinal inflammation.

Our findings from chapter 3 and 4, indicate a pathogenic role for IL-36 in the immuno-pathogenesis of IBD, reporting a compelling role for the cytokine family in the generation of proinflammatory T_H1, T_H2 and T_H9 cell responses *in vitro*. These cells have all been implicated in the perpetuation of colitis, and regulating their responses may be key in effectively treating the disease[205,235,262,264,407,477].

Recently, Denning *et al*. reported that recipients of *Il-36r*$^{-/-}$ effector CD4$^+$ T cells exhibited a significantly milder form of disease compared to recipients of *wt* CD4$^+$T cells in the T cell transfer model of colitis[262], a form of murine colitis that mimics human CD. In agreement, this study also confirmed protection from the development of colitis in *Rag1*$^{-/-}$ mice that had received *Il-36r*$^{-/-}$T effector cells (Fig.5.2.1.). The *Il-36r*$^{-/-}$ recipient mice exhibited significantly less weight loss compared to *wt* counterparts, as well as maintaining the integrity of their intestinal crypt architecture, and displaying reduced cellular infiltration to the mucosa, in addition to producing significantly less IFNγ (Fig.5.2.1.A-E). We further advanced these findings, through a comprehensive deeper mechanistic analysis, demonstrating that this protection from disease appears to be due to a reduced population of T_H1 cells in the periphery and colon (5.2.2.1-3A), alongside an increased population of FOXP3$^+$T cells the periphery (Fig.5.2.2.1-2C). Interestingly, these effects can be seen from

as little as 5 days after T cell transfer, with the enhanced IFNγ$^+$ population evident at this early initiating stage of disease (Fig.5.2.3.1-3C).

Another, particularly striking finding was the incidence of splenomegaly and lymphadenopathy in *Il36r*$^{-/-}$ T cell recipient mice. Upon harvesting these tissues for analysis there was a significant difference in their sizes between groups. *Il-36r*$^{-/-}$ recipients mLNs and spleens were considerably larger those from *wt* or PBS recipients (Fig.5.2.4.A&C), and consisted of significantly more CD4$^+$ $T_н$ cells (Fig.5.2.5.1. C-F). Conversely, although the colons of *Il-36r*$^{-/-}$ recipients were larger than *wt* recipients (Fig.5.2.4.C), they contained significantly less CD4$^+$ $T_н$ cells (Fig.5.2.5.1A&B), indicating there are less infiltrating lymphocytes present. In order for CD4$^+$ $T_н$ cells to reach the intestines and initiate and perpetuate inflammation in IBD two things must happen, 1. The cells must exit the lymphoid tissue, and 2. The cells must receive and respond to a chemoattractant stimulus to initiate trafficking to the mucosa[490], both of which *Il-36r*$^{-/-}$ cells appear not to do. Therefore, there appears to be multiple mechanisms through which IL-36 may be exploiting to promote the pathogenesis of IBD. Firstly IL-36 cytokines can instruct the generation of pathogenic CD4$^+$ $T_н$ cell responses while inhibiting the generation of iTregs. Secondly, IL-36 also appears to play a role in either or both T cell egress from the lymphoid tissue and T cell gut homing to the intestines. It is unclear from our studies to date whether these mechanisms occur independently or are intrinsically linked.

Il36r$^{-/-}$ $T_н$ cells express significantly less of the gut homing integrin α4β7 than *wt* cells in the periphery, corresponding with significantly less α4β7$^+$ and CCR9$^+$ T cells infiltrating the colonic tissue (Fig.5.2.5.2). α4β7 and CCR9 expression is imprinted on lymphocytes by the vitamin A metabolite, Retinoic Acid (RA/ATRA)[493, 512], via RARα signalling[494, 513, 514]. Interestingly, we see that IL-36 has the capability to induce expression of both of these molecules independently on CD4$^+$ $T_н$ cells, but when used in synergy with ATRA enhances both α4β7 and CCR9 expression to a much greater extent (Fig.5.2.6.). RA is produced by innate cells in the mucosa[493-498] and is well known to promote gut homeostasis by enhancing regulatory T cell population expansion and immunosuppressive functioning[499, 500], whilst simultaneously inhibiting the generation of pathogenic T cell lineages[501-507]. Our data largely agrees with these reports, as we see increased α4β7$^+$ CCR9$^+$ Treg populations, and diminished $T_н1$, $T_н17$ and $T_н9$ generation following ATRA stimulation (Fig.5.2.7.1 & Fig.5.2.7.2). Conversely, in agreement with previous reports, we demonstrate that IL-36 promotes the generation of $T_н1$ and $T_н9$ cells, which are associated with CD and UC respectively[262, 402]. Remarkably, we report for the first time a novel mechanism in which ATRA/IL-36α costimulation results in the generation of pathogenic $T_н1$ cells with gut homing properties (Fig.5.2.7.1 A & Fig.5.2.7.2 A). Furthermore, IL-36α elicits this effect

more potently than other IL-1 family members, although IL-18 can facilitate the generation of these colitogenic cells, albeit to a lesser extent (Fig.5.2.8.C). Whatsmore, this costimulation resulted in $CD4^+$ T_H cells under iTreg conditions being redirected from $FOXP3^+$ Treg generation, and towards an $\alpha4\beta7^+CCR^+FOXP3^-$ negative phenotype (Fig.5.2.7.1 B & Fig.5.2.7.2 B). As ATRA is present in the gut in the steady state, these data suggest that when IL-36α stimulation of $CD4^+$ T cells occurs in this environment there is an impressive potential for the enhanced generation of proinflammatory T_H cells primed to home to the intestines and likely instigate and perpetuate inflammation once there.

Furthermore, $CD4^+$ T_H cells deficient in IL-36R signalling also displayed an increase in CD69 expression (Fig.5.2.9). CD69 is known to regulate T cell egress from the lymphoid tissue via inhibition of $S1P_1$ signalling[509]. Therefore, IL-36 may promote T_H cell trafficking from the mLNs by influencing the CD69/$S1P_1$ axis. In support of this concept, Radulovic *et al*. reported increased T cell accumulation in colonic lamina propia of *CD69*$^{-/-}$ mice in DSS induced colitis, resulting in a more severe disease compared to *wt mice*[508], and this accelerated colitis is also observed when *CD69*$^{-/-}$ T effector cells are transferred to *Rag1*$^{-/-}$ mice[515]. In contrast, we see protection from colitis in our *Il36r*$^{-/-}$ mice (Fig.5.2.1), indicating that IL-36R signalling may be downregulating CD69 expression. In this regard, it is also noteworthy that CD69 expression has also been shown to enhance $FOXP3^+$ Treg differentiation, expansion, and suppressive functioning[515-517], mechanisms which IL-36 stimulation has been shown to inhibit (Fig.4.2.3.C, Fig.5.2.2.1 C & Fig.5.2.2.2 C). Taking these reports into consideration, it seems suitable to propose a role for IL-36 in the regulation of CD69 expression in the context of intestinal inflammation. As CD69s profound effect on the generation and regulation of suppressive Tregs is coming to light[515-517], this offers another possible mechanism as how IL-36 signalling is promoting IBD, and substantiates its choice as a therapeutic target. Furthermore, $CD69^+$ Tregs have been shown to attenuate inflammation in SLE[518], a disorder that IL-36 signalling has also been implicated in the pathogenesis[393, 519, 520]. Therefore inhibiting IL-36 mediated downregulation of CD69 may be beneficial in other inflammatory conditions too.

Finally, the increased expression of α4β7, CCR9 and IFNγ from human $CD4^+$ T_H cells stimulated with IL-36α (Fig.5.2.10. A, C & E) advances the validation for targeting IL-36R signalling in the clinic. In light of these findings, alongside previous reports in the literature, it is perhaps unsurprising that efforts to target the IL-36 axis using Spesolimab (BI655130), a monoclonal antibody that is directed against the IL-36R, have entered the clinic for both CD and UC patients. In an ongoing study, the efficacy of Spesolimab in promoting healing of perianal fistulas is being analysed in patients with fistulizing Crohn's Disease (NCT03752970)[422]. The clinical activity of the drug is also being assessed in UC

patients who have previously failed other biological therapy (NCT03482635)[423] and the potential use of Spesolimab as an add on to TNFα therapy is also under investigation in UC patients (NCT03123120)[424]. Furthermore, the safety and efficacy of Spesolimab use, long term, is being analysed in an open-label phase II trial in UC patients who have effectively completed previous trials (NCT03648541)[425].

Our data indicates that targeting IL-36 signalling via IL-36R blockade would be beneficial in IBD by preventing the generation and expansion of pathogenic $CD4^+$ T_H cell subsets associated with the disease pathogenesis, and inhibiting their migration to the mucosa. As our results have highlighted IL-36α a potent generator of colitogenic $CD4^+$ T_H cells, and others report IL-36γ key in the resolution of intestinal inflammation[397] [426], this dichotomy may be beneficial in elucidating patient cohorts who may benefit most from IL-36R targeted therapy. Where patients present with elevated levels of IL-36α, this may indicate active inflammation, and would benefit from IL-36R blockade. But if IL-36γ is elevated this may indicate there is homeostatic resolution occurring, and it may be best to employ an alternate therapy.

CHAPTER 6.

GENERAL DISCUSSION

Chapter 6

6.1 General Discussion

The global burden of inflammatory bowel disease is on the rise. In the last 20 years the number of individuals with IBD has almost doubled, with reported incidence increasing from 3.7 million in 1990, to 6.8 million in 2017[521]. Approximately 25% of these patients reside in the USA, who report the highest prevalence globally, with 464.5 cases reported per 100,000 population. The UK exhibits the second highest prevalence globally, and the highest prevalence in Europe, with 373 individuals per 100,000 diagnosed with IBD. This increase in prevalence has been associated with a rise in urbanisation, coinciding with increased incidence of IBD reported in newly industrialised countries such as Asia, Oceania and sub-Saharan Africa[521]. Epidemiologically, these trends indicate that lifestyle factors associated with urbanisation, a higher socio-economic status, a shift to a western diet, and an increase in sanitisation, bear a significant influence on the development of IBD. Socio-economically, these increases place a substantial burden on the healthcare systems in their respective countries. As IBD is a chronic relapsing disorder, these patients will require life long and costly care[521]. Furthermore, this burden on the economy is not limited to healthcare, as a German study revealed that 9% of employees with IBD required rehabilitation, and 3% were granted a disability pension. Strikingly, 50% of the total social costs of IBD in Germany are associated with early retirement or sick leave, highlighting the burden IBD places on the economy as a whole[522].

According to the immune disease development model, a delay in exposure to common infectious agents during childhood is associated with the increase in IBD in the individuals with a higher socio-economic status[521], and this may allude to the increase in prevalence of IBD in children and adolescents. Currently, approximately 20-25% of all IBD cases arise in children and adolescents[43, 45, 46], with children diagnosed before their 10th birthdays exhibiting a particularly severe phenotype[50-52]. This inflammation persists throughout the patient's life and has a significant impact on their physical, psychological and academic well-being[50-52].

However, recent epidemiological findings report a decrease in IBD associated mortality, and this is likely due to improvements in therapeutics, with increased use and earlier introduction of immunomodulators, and a larger selection biological agents[523, 524]. These

biologics, such as anti-TNF and anti-IL-12/23 therapies, are already proving significantly beneficial to patients, with increased rates of remission, and mucosal healing reported[68-71]. Furthermore, these modes of cytokine based therapies, show the power of precision based medicine in manipulating the immune response to regulate disease. However, as with all medicines, there is no "one size fit all" for patients and these therapies need to be prescribed based on each IBD patient's specific inflammatory profile. Furthermore, as more and more immune targets are being associated with IBD, and with the maintenance on GI homeostasis, there is a pressing need to validate these targets of their potential to improve disease outcomes. To that end, this study has demonstrated that the IL-36 family of cytokines are potent regulators of the adaptive immune response associated with the onset and maintenance of intestinal inflammation and further promotes the rationale for targeting this cytokine family in the clinic, for adult and paediatric, UC and CD cohorts alike.

In keeping with early reports from the literature, our initial investigations revealed a significant increase in expression of *IL36A* in the colon of a paediatric UC populations compared to healthy controls (Fig.3.2.1.A). Interestingly, we did not see this enhanced expression in our paediatric CD cohort, complementing similar findings by Fonseca-Camarillo *et al*. in an adult IBD cohort[395]. We see this same protein expression profile in the serum of IBD patients (Fig.3.2.2.A), with UC patients producing significantly enhanced amounts of IL-36α. However, unlike our gene expression data at the colonic mucosa, CD patients do appear to produce more of the cytokine in the periphery than healthy controls, and just miss achieving significance (p=0.0572). Therefore, these data are indicative of two possibilities, 1. There is a dichotomy in expression of IL-36α in the inflamed intestines between IBD subsets and 2. Our population size isn't large enough to give an accurate portrayal of what is truly being upregulated in the intestines. This is a possibility, given that others have reported elevated levels of *IL36A* gene expression in both CD and UC adult patient cohorts[377, 404]. It is also possible that the differences observed in our study, may reflect phenotypical differences between paediatric and adult inflammatory profiles in IBD. Another thing to consider, is that our healthy control population is comprised of patients who were taken in for diagnostic testing as "query IBD" and subsequently diagnosed as negative for disease. Therefore, we could assume that at some point or other these children displayed some form of GI inflammation and this may explain the levels of IL-36α we see in our control population. If there was no inflammation at all in these patients this may allow the CD population to achieve significance. While obtaining colonic tissue from a child with no GI issues to compare gene expression profiles would not be feasible, or ethical, comparing serum levels of IL-36 from children admitted for non-GI testing may prove to be a more accurate control in this case.

Interestingly, serum levels of IL-36α and IL-36Ra reveal a negative correlation in IBD patients (Fig.3.2.3), indicating that in these patients there is an environment permissive to IL-36α signalling. These results are also of relevance considering a recent report by Fonseca-Camarillo *et al*.. In the inflamed mucosa of UC patients, they observed temporal and opposing expression of IL-36Ra and IL-38. In their study, they reported enhanced *IL-36RN* expression in the colonic mucosa of patients with active disease[395], whereas *IL38* expression levels were enhanced in the inactive disease cohort[395]. These finding suggest that IL-38 may have a resolving role in UC, and perhaps elude to a potential mechanism where IL-36Ra modulates active inflammation and IL-38 promotes resolution and homeostasis. Furthermore, these data potentially present a unique IL-36Ra/IL-38 signalling axis that serves to modulate IL-36 agonist signalling in a disease subtype, and stage specific manner, and suggest an innovative target for the therapeutic treatment of IBD and GI tract inflammation. Therefore, it would be interesting to measure IL-38 expression in our paediatric cohort and see is there a similar trend. Notably, we show that the elevated levels of IL-36α protein detected are localised in the colonic mucosa, an important immune inductive site, and perhaps more importantly, we identify colonic T cells as the immune cell subset that may be responsive to IL-36R ligand signalling in the inflamed mucosa (Fig.3.2.4-5).

Taking these data into consideration with the known involvement of $CD4^+ T_н$ cells in the pathogenesis of IBD, this led to examine the potential effects IL-36 signalling has on $CD4^+ T_н$ cell responses. There has already been some detailed studies in the literature, reporting the potent effect IL-36 has on inducing $T_н1$ responses[402], which we also report. But in the last few years IL-36 has been emerging as a prominent regulator of numerous $T_н$cell responses, with Harusato *et al*. showing IL-36α enhances $T_н9$ responses[262], and Russell *et al*. showing an enhancement in $T_н2$[394]. Conversely, other studies demonstrate IL-36 signalling as negative regulator of $T_н17$[394, 402], and tolerogenic Treg responses[262].

Our data are broadly in agreement with these findings (Fig.4.2.3), and further enhance the hypothesis that IL-36α can act as a master regulator of $CD4^+$ T helper cells responses. To add to the current knowledge on IL-36s effects on these cells, we report for the first time that IL-36α can elicit these effects in conditions of sub-optimal activation, i.e., in the absence of CD28 costimulation (Fig.4.2.2), and independently of IL-2 (Fig.4.2.4). As optimal IL-2 production in $CD4^+ T_н$ cells is CD28-mediated[451], collectively these findings indicate that IL-36 can act as an alternative co-stimulatory molecule in $T_н$ cell activation and lineage priming. Interestingly, we also report for the first time that IL-36α can synchronise with TGFβ and act as a substitute for IL-4 in the polarization of $T_н9$ cells, like other IL-1

family members[258, 263, 420, 435], but can also amplify T_H9 differentiation in concert with IL-4 (Fig.4.2.7). We also demonstrate that IL-36αs inhibitory capacity on Treg polarization is IL-4 mediated (Fig.4.2.8), and this fits in with the current literature showing that IL-4 synergizes with TGFβ to redirect T cells to a T_H9 phenotype, rather than a Treg phenotype[165]. Accordingly, we see an upregulation of IL-4 and IL-9 under Treg polarizing conditions, implying that IL-36 may be modulating T_H9/Treg polarisation via this mechanism.

Furthermore, we demonstrate that IL-36α facilitates T_H effector evasion of suppression by nTregs (Fig.4.2.5), indicating that in an inflamed system IL-36 signalling can promote the kind of unregulated inflammation we see in IBD. This agrees with Harusato *et al*., who demonstrate that in the inflamed colonic murine mucosa, IL-36α synergistically inhibits the development Tregs and enhances generation of T_H9 responses, thereby inhibiting the resolution of intestinal inflammation[262]. While we did not measure IL-9 production in the supernatants of these co-cultured cells, we did observe an induction of IFNγ from the both nTregs and nTregs + T_H effector cells cultured with IL-36α (Fig.4.2.5.B), indicating IL-36α may be promoting Treg plasticity. Various reports have shown that IL-12 mediates the generation of IFNγ producing "T_H1-like" Tregs both *in vivo* and *ex vivo*[460, 461], and given the potent effect IL-36α has on the generation of T_H1 responses, it seems plausible that IL-36α may be mediating both of these effects by enhancing the sensitivity of T_H cells to IL-12.

Furthermore, IL-12 stimulated Tregs show a reduced suppressive capacity *ex vivo*[460], as do our IL-36α stimulated Tregs (Fig.4.2.5). As we have have previously mentioned, Treg suppression of T_H effector cells has been largely associated with downregulation of CD28-mediated production of IL-2 via CTLA-4 signalling, and potent CD28 signalling breaks this suppression[454, 468]. Collectively these studies indicate that CD28 is an important checkpoint molecule in the balance between resistance and susceptibility to Treg mediated suppression of T_H effector cells. Importantly, our results demonstrate that IL-36α stimulation of $CD4^+$ T_H effector cells circumvents the necessity for CD28-mediated activation (Fig.4.2.2). Interestingly, CTLA-4 is only transiently expressed on $CD4^+$ $T_H$cells following CD3/CD28 mediated T cell activation[525, 526]. Therefore, CD3/IL-36 activated T_H effector cells may not induce expression of CTLA-4, thereby evading suppression by CTLA-4 signalling. Furthermore, CTLA-4 has also been shown to inhibit T_H1 responses[527], therefore if IL-36 is indeed activating an alternate co-stimulatory pathway, this would simultaneously enhance activation of T_H effector cells, promote their polarisation towards a T_H1 lineage and aid in their evasion of suppression by Tregs (Fig.6.1.).

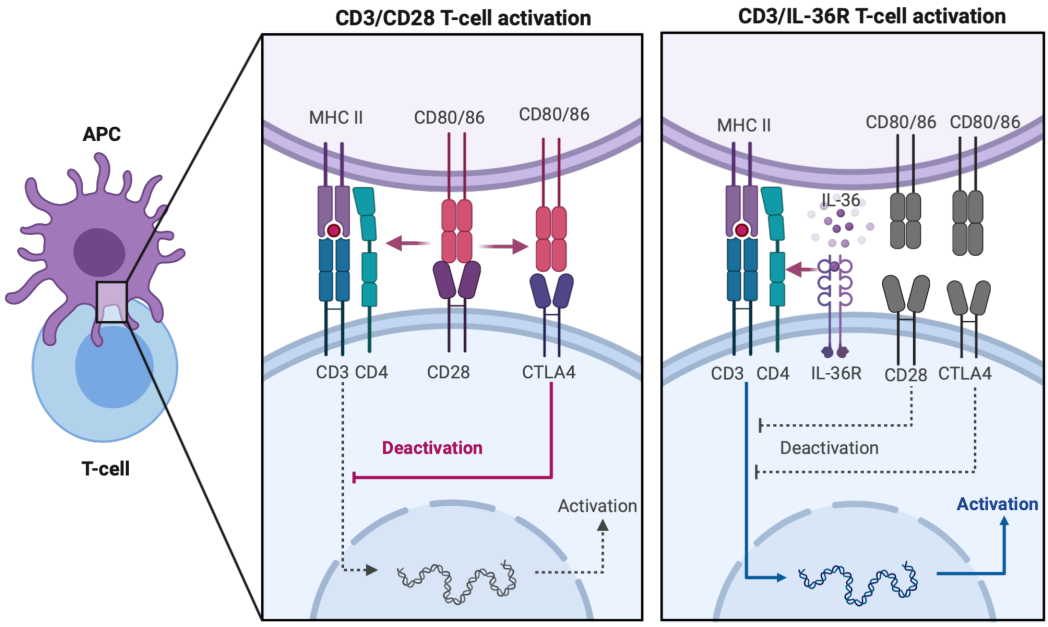


Figure 6.1.: Proposed alternate IL-36 mediated T_H cell activation. Optimal T_H cell activation occurs when the TCR CD3 and co-stimulatory receptor CD28 engage with their ligands, MHCII and CD80/86, on an APC. This initiates T_H cell activation and proliferation. However, engagement of CD28 and subsequent T_H cell activation, results in the upregulation of CTLA-4. This competes with CD28 and binds CD80/CD86, inhibiting further activation and proliferation of the T cell. In our alternate T cell activation pathway, CD3 engages with MHCII, and IL-36R activation by IL-36 ligands, acts as the co-stimulatory signal needed to activate the T_H cell. In this circumstance, CD28 is not engaged, thereby CTLA-4 expression is not induced. This allows unregulated activation and proliferation of the T_H cells. *Created with Biorender.com*.

Although this kind of unregulated activation of T_H effector cells is detrimental in the context of autoimmunity, it can be beneficial in other settings. For instance, in the case of cancer, where tumour cells evade immune recognition and destruction[528]. These cancer cells downregulate expression of tumour antigens, and MHC, so they evade detection by immune cells in the microenvironment, and can manipulate tolerogenic cells, such as Tregs and regulatory B cells (Bregs), to prevent immunopathology. Furthermore, effector T cells in the tumour environment become exhausted and begin to express inhibitory checkpoint inhibitors, such as CTLA-4, which subsequently downregulate the T effector cell response.

Therefore, it is not surprising that there has already been some interest in the development of antibodies inhibiting these immunosuppressive receptors for the treatment of cancer[528]. While blockade of tumour checkpoint inhibitors has had a great impact on the long term survival of some cancer patients, response rates are low. Therefore, as IL-36 may act independent of CD28 activation and CTLA-4 inhibition, it may serve as a promising target for rebooting the immune response in cancer. Furthermore, as CTLA-4 may also remove CD80 and CD86 (including their cytoplasmic domains) from the cell surfaces of APCs via trans-endocytosis[529], IL-36 may serve to enhance the immune response even in environments where CD28 stimulation is low. In keeping with this, Qu *et al*., recently demonstrated that a combination of CTLA-4 mAbs and IL-36 led to an increase in proliferation and IFNγ production by $CD4^+$ and $CD8^+$ T cells in a murine melanoma model, when compared to single therapy with CTLA-4 mAbs or IL36 alone[436]. Furthermore, high expression of IL-36α in the colon has been reported to be beneficial for survival in colorectal cancer (CRC) patients[530], and IL-36γ has been shown to promote the inflammatory response in CRC, while IL-36Ra has been associated with intra-tumoural expression of CTLA-4[531]. Collectively, these studies highlight the importance of IL-36 mediated amplification of the immune response in overcoming T cell exhaustion and enhancing tumour targeted immunopathology.

However, as previously stated, this IL-36 mediated amplified adaptive response is double edged sword, while beneficial in eliciting enhanced immune responses when necessary, such as in the case of cancer, it can cause detrimental destruction in the case of autoimmunity. We witness this *in vivo*, using the T cell transfer model of colitis. In agreement with Denning *et al*., *Rag1*$^{-/-}$ mice who were reconstituted with *Il-36r*$^{-/-}$ T effector cells, exhibit protection from the development of colitis (Fig.5.2.1). In this model, IL-36R signalling appears to simultaneously enhance the generation and proliferation of a pro-inflammatory T_H1 mediated response, and inhibit the generation of peripherally induced Tregs (Fig.5.2.2.1-3). This unregulated $T_H$effector cell response is evident as early as 5 days after transfer (Fig.5.2.3.1), implying that IL-36 may have an initiating role in the intestinal inflammation we see in IBD. Furthermore, as time passes and the IL-36 induced inflammation goes unchecked by pTregs, and perhaps by immune checkpoint inhibitors such as CTLA-4, this results in a significantly loss in body weight that coincides with destruction of the intestinal epithelium at 28 days (Fig.5.2.1.). Mice that are deficient in IL-36R signalling appear protected from disease onset, displaying a weightloss pattern similar to CTRL mice (Fig.5.2.1.A), and exhibiting a reduced T_H1 population in the periphery and colon (5.2.2.1-3A), alongside an enhanced Treg population in the periphery (Fig.5.2.2.1-2C).

Therefore, IL-36 signalling appears to initiate and maintain intestinal inflammation via enhanced T_H1 and diminished Treg responses. Whether this occurs independently, or both occurrences are mechanistically linked, still needs to be elucidated. As postulated earlier, IL-36 may be eliciting these pro-inflammatory effects via an alternate activation pathway that allows unregulated activation, proliferation, and enhanced T_H1 generation. The failure of Abatacept, a CTLA4-Ig, to demonstrate any therapeutic benefit in moderate to severe CD and UC patients in phase III trials provides evidence supporting this hypothesis[532]. If IL-36 activated T_H cells don't need CD28 mediated activation, blockade of CD80/86 ligands by preferential binding to the CTLA4-Ig would have little effect in inhibiting the T_H cell response.

The incidence of splenomegaly and lymphadenopathy we observed *Il-36r*$^{-/-}$ T_H effector cell recipient mice led us to believe that it is not just effects on T cell activation and polarisation that are mediating IL-36 induced colitis. When harvesting tissues at the termination of T cell transfer experiments, we noticed that there was a significant difference in not just the length of the colon between groups, but also in the size of the spleens and mesenteric LNs. The *Il-36r*$^{-/-}$ T_H effector cell recipients displayed substantially larger spleens and mLNs compared to their *wt* counterparts or CTRL mice (Fig.5.2.4.A&C), and these tissues contained significantly more CD4$^+$ T_H cells (Fig 5.2.5.1.C-F). Furthermore, although these *Il-36r*$^{-/-}$ T_H effector cell recipient mice exhibited larger colons than *wt* recipients (Fig.5.2.4.C), there was significantly less CD4$^+$ T_H cell infiltration observed (Fig.5.2.5.1A&B). Collectively, these results indicate that IL-36R deficiency does not impede the ability of T_H effector cells to activate and proliferate in the periphery of *Rag1*$^{-/-}$ hosts, but does regulate their egress from the spleen and mLNs and/or migration to the colon.

As previously described, for CD4$^+$ T_H cells to initiate and maintain the inflammation we see in IBD two things must happen, 1. The cells must exit the lymphoid tissue, and 2. The cells must receive and respond to a chemoattractant stimulus to initiate trafficking to the mucosa[490]. As *Il36r*$^{-/-}$ cells appear to be maintained in the periphery, this suggests there may be multiple mechanisms through which IL-36 promotes the pathogenesis of IBD. Firstly IL-36 cytokines can promote the generation of proinflammatory T cells under suboptimal activation conditions, secondly, they can instruct the generation of pathogenic CD4$^+$ T_H cell responses while inhibiting the generation of iTregs. Thirdly, IL-36 allows T_H effector cells to evade suppression by Tregs, and lastly, IL-36 also appears to play a role in either or both T_H cell egress from the lymphoid tissue and T_H cell gut homing to the intestines.

Enhanced expression of the gut homing integrin $\alpha4\beta7$ on the surface of *wt* T_H cells post adoptive transfer compared to transferred *Il36r*$^{-/-}$ $T_H$cells confirmed that IL-36 may be modulating T_H cell trafficking to the intestines. Significantly less $\alpha4\beta7^+$ and CCR9$^+$ T_H cells

infiltrating the colonic tissue of *Il36r*$^{-/-}$ $T_н$cell recipients (Fig.5.2.5.2) further endorsed this idea.

α4β7 and CCR9 expression is imprinted on lymphocytes by tolerogenic Retinoic Acid (RA/ATRA)[493,512], and this metabolite is reported to have opposing roles to IL-36 in the intestines, for instance RA promotes gut homeostasis via enhanced regulatory $T_н$ cell population expansion and immunosuppressive functioning[499,500], and simultaneously inhibits the generation of pathogenic $T_н$ cell lineages[501-507]. Interestingly, mechanistic studies revealed that IL-36α can induce both α4β7 and CCR9 expression on activated T cells, and work in synergy with ATRA to enhance their expression (Fig.5.2.6), further indicating a role for IL-36 in $T_н$ cell gut homing. Intriguingly, we demonstrated that IL-36 allows $T_н1$ cells to evade ATRA mediated suppression, and report a novel mechanism whereby ATRA/IL-36α costimulation results in the generation of pathogenic $T_н1$ cells with a gut homing phenotype (Fig.5.2.7.1 A & Fig.5.2.7.2 A). Furthermore, IL-36α induces this pathogenic gut homing phenotype more potently than other IL-1 family members, although IL-18 can also facilitate the generation of these colitogenic cells to a lesser extent (Fig.5.2.8.C). Whatsmore, this IL-36/ATRA costimulation redirects iTreg cells from adopting a tolerogenic FOXP3$^+$ positive phenotype and towards a α4β7$^+$CCR9$^+$FOXP3$^-$ phenotype (Fig.5.2.7.1 B & Fig.5.2.7.2 B).

As ATRA is continuously produced by CD103$^+$ DCs in the gut in the steady state to promote GI tolerance, these data suggest that when IL-36α is present in the microenvironment, there is an impressive potential for any residing CD4$^+$ $T_н$ cells to adopt a proinflammatory $T_н$ cell phenotype, and generate a population of proinflammatory $T_н$ cells capable of homing to the intestines and likely induce inflammation once there.

Interestingly, Jain *et al*., have reported a dual mechanism for CTLA-4 in maintaining $T_н$ cell homeostasis. Expression of this checkpoint inhibitor in Tregs allows the cells to inhibit inappropriate naïve $T_н$ cell activation, and expression in $T_н$ cells prevents them infiltrating and damaging non-lymphoid tissue[533]. Therefore, if IL-36 is circumventing CD28 mediated $T_н$ cell activation, and the ensuing CTLA-4 upregulation, this would also allow the $T_н$ cells to bypass CTLA-4 mediated prevention of non- lymphoid tissue infiltration.

Another question worthy of investigation is, does IL-36 interfere with ATRA signalling or are both effects occurring independently? ATRA signals through the Retinoic Acid Receptor α (RARα) on $T_н$ cells[492]. When RARα is blocked this inhibits the formation of Tregs, but synergistically enhances the generation of $T_н1$ cells[534]. This is the same functional effect we see IL-36 having on $T_н$ cells, therefore could IL-36 be causing $T_н$ cells to downregulate expression or activity of RARα, therefore eliciting its pro-inflammatory effect on $T_н$ cells? (Fig.6.2). Furthermore, DC produced ATRA facilitates the *de novo* generation of Foxp3$^+$ Treg cells from naive CD4$^+$CD25$^-$ $T_н$ cell populations in mice[496,535], therefore in our

Il36r$^{-/-}$ T_H effector cell recipients could enhanced/unopposed ATRA signalling be the cause of the enhanced population of FOXP3$^+$ Treg observed? Additionally, ATRA has been shown to transrepress AP-1, thereby inhibiting MAPK signalling[536]. As IL-36 signals through this pathway, perhaps IL-36 mediated induction of MAPK signalling is stronger than ATRA repression?

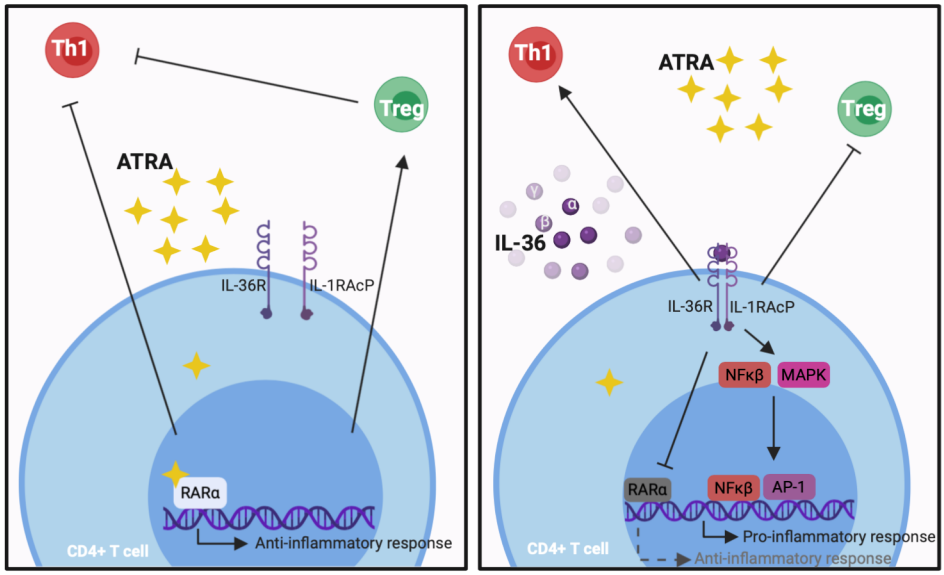


FIGURE 6.2.: Proposed interaction of IL-36 and ATRA in the generation of proinflammatory responses. ATRA interacts with the nuclear transcription factor RAR α in the nucleus to inhibit proinflammatory T_h cell generation and promote the polarisation of tolerogenic Treg cells. These Treg cells can then regulate homeostasis in the microenvironment and supress proinflammatory T_h cell responses. However, when IL-36 is present, this activates proinflammatory NFKB and MAPK pathways, promoting the polarisation and expansion of proinflammatory T_h1 cells, and inhibiting the generation of Tregs. Even though ATRA is present, the activation of IL-36r signalling is somehow interfering with RAR α /ATRA interactions, or ATRA/MAPK interactions, preventing negative regulation of pro-inflammatory t_h cell generation in the intestines. *Created with Biorender.com*.

Furthermore, genetic deletion of RARα inhibits the polarisation of T_H17 cells[492]. Once again showing an opposing role to IL-36 in T cell generation. However, several other reports have suggested that ATRA actually inhibits T_H17 differentiation, so the precise role ATRA/RARα plays on the modulation of T_H17 cells is still unclear[492].

It is important to mention here that although we describe T_H17 cells as a pro-inflammatory cell type, of which there are many reports to confirm this in the case of IBD and GI homeostasis[421], there has been emerging evidence demonstrating the existence of an anti-inflammatory T_H17 phenotype, named non-pathogenic T_H17 cells (npT$_H$17)[537]. Classical "pro-inflammatory" Th17 cells differentiate when stimulated with TGFβ, IL-6, IL-1β and IL-23, and secrete their signature IL-17a and IL-17f cytokines, which are associated with the

pathology in numerous autoimmune diseases, including CD. npT$_H$17 cells are polarised when stimulated with TGFβ and IL-6, secrete IL-17a as well as tolerogenic IL-10, and serve to inhibit autoimmune associated inflammation[537]. As our T$_H$17 culture conditions did not include IL-23, we could therefore speculate that these may be npT$_H$17 cells. This would fit in with our data showing that IL-36 enhances the proinflammatory response (T$_H$1, T$_H$2, T$_H$9), and inhibits anti-inflammatory T cell generation (Treg and possibly npT$_H$17) (Fig.4.2.3). As the IL-23R is not expressed on naïve T cells[537], it would be worth investigating the effects of IL-36 on "pathogenic" Th17 cells when the IL-23R is expressed. To achieve this the naïve CD4$^+$ T$_H$ cells would be first activated with αCD3/CD28 for 24hrs and then supplement the media with our optimised T$_H$17 cocktail + IL-23 and IL-36. The fact that our CD colitis model does not seem to be T$_H$17 mediated does little to tell us of the effect of IL-36 on the generation of T$_H$17 cells in IBD. However, there are emerging reports of anti-IL-17 therapy exacerbating disease in CD patients, and leading to the induction of IBD in psoriasis and psoriatic arthritis patients[270, 271, 538]. Therefore, IL-36 mediated inhibition of T$_H$17 generation may be intensifying colitis by impairing T$_H$17 homeostatic functioning.

Returning to the potential role for IL-36 in regulation T$_H$ cell gut homing, a key feature in the induction of GI inflammation is the trafficking of pathogenic lymphocytes from the periphery to the intestines. While expression of gut homing molecules mediates their travel to the site of inflammation, the cells must first be "released" from the lymphoid tissue[490]. CD69, which is commonly known as an early marker of T cell activation, also serves to regulate T cell egress from the lymphoid tissue[509]. CD69 negatively regulates S1P$_1$, down-regulating its expression and inhibiting its chemotactic function[509]. Interestingly, transferred *Il36r*$^{-/-}$ T$_H$ effector cells displayed an enhanced population of CD69 expressing cells compared to their *wt* T$_H$ effector cell counterparts following T cell transfer (Fig.5.2.9). Therefore, another mechanism by which IL-36 may promote T$_H$ cell trafficking to the intestines is by influencing the CD69/S1P$_1$ axis in the mLNs. Interestingly, CD69 deficiency in murine models of colitis results in opposing effects to IL-36R deficiency. For instance, there is increased T cell accumulation in colonic lamina propia *CD69*$^{-/-}$ mice[508], whereas there is an increased accumulation of T$_H$ cells in the mLNs and spleen in *Il36r*$^{-/-}$ T$_H$ effector recipient mice (Fig.5.2.4 & 5.2.5.1). Furthermore, *Il36r*$^{-/-}$ T$_H$ effector recipient mice exhibit protection from colitis (Fig.5.2.1.), whereas *CD69*$^{-/-}$ T effector cell recipients exhibit a more severe disease[515]. Collectively, these results suggest 2 possibilities. Firstly, IL-36 may be inhibiting CD69 signalling, thereby allowing S1P$_1$ to promote lymphocyte chemotaxis, or alternatively IL-36 may be directly enhancing S1P$_1$ chemotactic functioning, or indeed playing a role in both effects

It is also worthy to note here, that IL-36 may be regulating Treg generation via CD69. There are numerous reports in the literature that have demonstrated that CD69 expression enhanced FOXP3$^+$ Treg differentiation, expansion, and suppressive functioning[515-517]. As we have shown that IL-36 inhibits all of these mechanisms in Tregs (Fig.4.2.3. C, Fig 5.2.2.1 C & Fig. 5.2.2.2 C), this further supports a potential role for IL-36 promoting the pathogenesis of IBD via inhibition of CD69 signalling.

Importantly, we report enhanced expression of α4β7, CCR9, and IFNγ in human CD4$^+$ T_N cells stimulated with IL-36α (Fig.5.2.10. A, C & E), indicating that these mechanisms we are proposing have the potential to be functional in humans, and further validates the targeting of IL-36 in the clinic. Interestingly, for the past few years Vedolizumab, a monoclonal antibody directed against α4β7 has become a mainstay in the clinic for the treatment of both CD and UC. And due to α4β7s prominent role in lymphocyte gut homing, it was believed that its mechanism of action was in inhibiting T cell migration to the intestines, of which early preclinical studies reported[539-541]. However, Zeissig *et al*., recently reported that Vedolizumab only slightly effects T cell abundance and the mucosal TCR repertoire in IBD patients[542]. Moreover, the drug largely affects the innate immune response, particularly intestinal macrophage populations, and the clinical efficacy of Vedolizumab closely correlates with effects on the innate immune system[542]. In conclusion, the authors suggest that α4β7 plays a limited role in the modulation of intestinal T_N cell trafficking in humans and/or that inhibition of its function can be compensated by other mechanisms. In this regard, it is tempting to speculate that IL-36 is one of these compensatory mechanisms.

Considering the results we have presented in this study highlighting a prominent role for IL-36 in the regulation of T_N cell responses and GI homeostasis, in addition to the accumulating evidence in the literature implicating IL-36 activity in the pathogenesis of IBD, it is perhaps unsurprising that efforts to target the IL-36 axis using Spesolimab (BI655130), have entered the clinic for both CD and UC patients (Fig. 6.3). Spesolimab is a monoclonal antibody that is directed against the IL-36R, thereby inhibiting all IL-36 agonist and antagonist signalling. In an ongoing study, the efficacy of Spesolimab in promoting healing of perianal fistulas is being evaluated in patients with fistulising Crohn's Disease (NCT03752970)[422]. The clinical activity of the drug is also being assessed in a cohort of UC patients who have previously failed other biological therapy (NCT03482635)[423], and the potential benefits of introducing Spesolimab as an add on to TNFα therapy is also under investigation in UC patients (NCT03123120)[424]. Furthermore, the safety and efficacy of Spesolimab use long term, is being evaluated in an open-label phase II trial in UC patients who have effectively completed previous trials (NCT03648541)[425].

In addition to Spesolimab, several other methods are being explored to functionally target IL-36 signalling therapeutically. One of these which is showing promising pre-clinical results is the use of peptide-based inhibitors for cathepsin G and elastase in skin inflammation[543]. Henry *et al*. recently reported that IL-36 family agonists are proteolytically processed and activated by these two neutrophil granule-derived proteases[366]. Following on from these findings, the group identified peptide-based pseudosubstrates, which can antagonize the activation of IL-36 agonists via inhibited cathepsin G and elastase signalling. Using tape stripped psoriatic skin lesions from patients, the authors report elevated levels of IL-36β processing, and this is inhibited with the administration of the peptide pseudosubstrates specific for cathepsin G[543]. As elevated levels of neutrophil infiltration is characteristic of psoriasis, and enhanced neutrophil protease activity has been linked to the disease pathogenesis[544, 545], as well as the success of targeting neutrophil proteases using small molecule inhibitors in lung inflammation[546, 547], this validates the potential for successful translation for these peptide therapies in the skin. It is noteworthy that elevated levels of neutrophil elastase activity have been described in the colon of patients with IBD, but not healthy controls[548], and have been implicated in the pathogenesis of the disease[549]. Therefore, targeting neutrophil protease dependent activation of IL-36 cytokines may also represent a potential approach for novel therapeutics for IBD (Fig.6.3).

Another approach under investigation involves simultaneously blocking multiple IL-1 family cytokines associated with the pathogenesis of IBD. The aim of this is to prevent compensatory inflammation when just one cytokine is targeted. Højen *et al*. have recently developed a monoclonal antibody against the human IL-1RAcP (IL-1R3), and shown it can attenuate the functions of 6 IL-1 family members, including IL-1β and IL-36[550]. Functional studies revealed an inhibition in IL-36 driven T_H1 responses in response to anti-IL-1R3, and that blocking IL-1R3 had a broader anti-inflammatory effect than blocking IL-36 signalling alone. Furthermore, treatment with this antibody attenuated disease in murine models of peritonitis, allergic airway inflammation and psoriasis, conditions known to have multiple cytokines involved in their pathogensis[550]. As described in Chapter 1, IBD has pathogenic associations with numerous IL-1 family members, thus rendering IL-1R3 blockade an attractive potential option for therapeutic intervention.

Furthermore, preliminary data indicates that IL-36s effect on T_H1 responses is negatively regulated by SIGIRR (Fig.4.2.9), the negative regulator of IL-1 signalling. As absence of SIGIRR signalling results in a more severe form of colitis *in vivo*, associated with enhanced T_H1 and T_H17 responses[434], this may be indicative of an alternative way to indirectly target IL-36 in the clinic. By enhancing SIGIRRs pro-homeostatic function, it may be possible to suppress IL-36 induced type 1 responses, without interfering with the cytokine

family's homeostatic roles. There are reports in the literature demonstrating that IL-37 is beneficial in intestinal inflammation[358, 551], and that the cytokine mediates colitis via SIGIRR signalling[552]. Therefore, the advent of novel IL-37 biologics, may have the potential to indirectly inhibit IL-36 induced colitis.

A further consideration for therapeutically targeting IL-36 is the confounding factors that may arise due to IL-36's involvement in the microbiome. Gut microbial dysbiosis is a common occurrence in IBD, where outgrowth of certain "harmful" bacteria, fungi and virus's may be associated with disease pathogenesis. Giannoudaki *et al*. recently described a role for IL-36 in the modulation of this system, where *Il-36ra*$^{-/-}$ mice exhibited reduced weight gain and metabolic dysfunction in a model of obesity, by promoting the growth of *Akkermansia muciniphila* bacteria in the gut, which is known to have a protective role by enhancing colonic mucus secretion[396], and whose abundance has been reported to be reduced in UC[553]. Furthermore, Medina-Contreras *et al*. observed reduced levels of IL-36γ, and pro-resolving IL-36γ induced IL-22, in germ free mice during DSS colitis[397] while later studies, by the same group, revealed that IL-36 receptor signalling can modulate the composition of the intestinal microbiome, possibly through activation of the IL-23/IL-22/AMP pathway during intestinal injury and barrier repair[399]. Interestingly Scheibe *et al*., also reported lower levels of expression of Lipocalin 2 (LCN2) in *Il-36r*$^{-/-}$ mice compared to controls[377]. LCN2 is an antimicrobial peptide which plays a role in the innate response to colonic bacterial infection through iron sequestration[554, 555]. Diminished levels of LCN2, observed in the absence of IL-36R signalling, suggests an additional mechanism by which IL-36 may influence the intestinal microbiome. More recently, Sequiera *et al*. proposed a role for IL-36 in host protection and clearance of intestinal *Klebsiella pneumoniae*[426]. This group found that the presence of a mature microbiota, dominated by the phyla Bacteroidetes, was necessary to prevent *K. pneumoniae* colonisation of the intestines in mice. Interestingly, antibody mediated blockade of IL-36R signalling resulted in loss of protection from *K. pneumoniae* colonisation, while treatment with recombinant IL-36γ promoted clearance of the bacteria. These data indicate that Bacteroidetes can prime intestinal IL-36 production, thereby preventing *K. pneumoniae* colonisation[426] and further demonstrate the interplay between the IL-36 family and commensal bacteria in the gut. As other studies have found *K. pneumoniae* colonisation increases the risk of GI inflammation and the onset of colitis *in vivo*[556], as well as overrepresentation of *Klebsiella* species reported in the gut microbiota of IBD patients[557], this further highlights the importance of IL-36 cytokines regulation of the microbiome in the maintenance of GI homeostasis. Therefore, global inhibition of IL-36R signalling may interfere with mucosal barrier integrity and tissue repair processes, as well as host protection,

thereby offsetting the beneficial effects of blocking the receptor. As such it may be worthwhile to evaluate drugs that neutralize individual IL-36 family ligands.

This may be of relevance when targeting GI fibrosis, as mechanistic studies have highlighted the dichotomy of IL-36R signalling in early vs late disease. GI fibrosis is a common and often severe complication in IBD, as surgery is often the only treatment available, this frequently results in the significant and irreversible loss of intestinal tissue[558]. Early in disease, IL-36 ligands have been reported to activate intestinal fibroblasts, that then promote wound healing in acute colitis[377]. However, as chronicity develops, this IL-36 mediated fibroblast activation switches from intestinal wound healing, and instead induces intestinal fibrosis, resulting in exacerbated disease severity in both DSS and TNBS induced models of chronic colitis[404]. However, in these models, neutralization of IL-36 signalling using an anti-IL36R antibody reduced disease severity, further promoting a possible pathogenic role for IL-36 signalling in chronic intestinal inflammation and fibrotic responses[404].

A further consideration when targeting the IL-36R is the effect of IL-36 ligand blockade on those patients who exhibit both IBD and cancer. Chronic gastro inflammation is known to give rise to CRC, small bowel adenocarcinoma, intestinal lymphoma, and cholangiocarcinoma, with standardized incidence ratios of, 5.7, 27.1, 17.51, and 916.63 in IBD patients respectively[559]. While the majority of studies have highlighted a pathogenic role for the cytokine family in the pathogenesis of IBD, the presence of these cytokines, particularly IL-36α and IL-36γ, may also be extremely important in eliciting an anti-tumour response[530, 531]. Therefore, global IL-36R blockade, while having the potential to be extremely harmful in IBD patients who have already developed secondary malignancies, may also downregulate the anti-tumour response in IBD patients and increase their risk of developing one of these cancers.

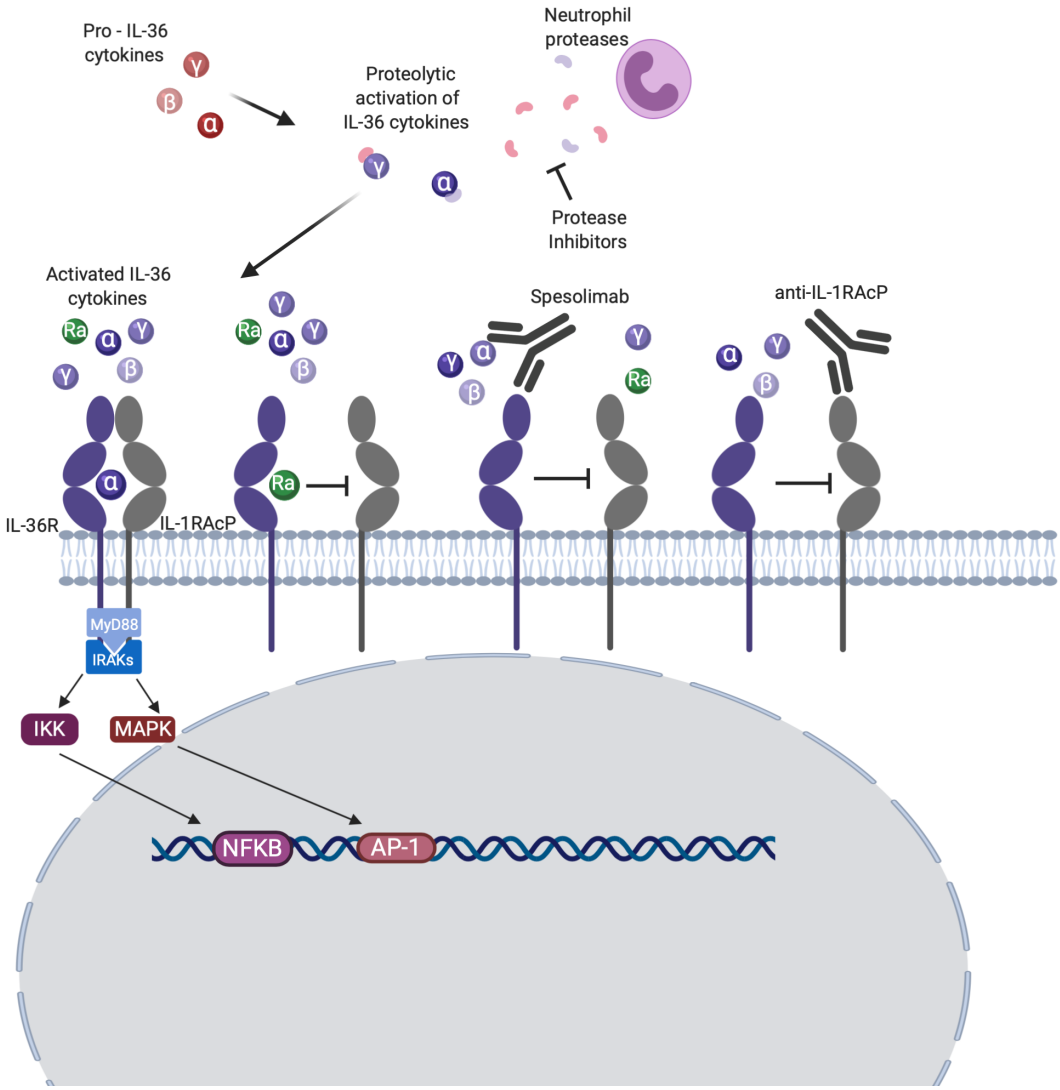


Figure 6.3.: Therapeutically targeting IL-36 family signalling. The IL-36 family members mediate inflammation through interaction with the specific IL-36r. The IL-36 agonists (IL-36α, IL-36β and IL-36γ) require proteolytic processing by neutrophil-derived proteases to elicit full activity. Once in the active form, they bind to the IL-36r, thereby recruiting the IL-1RAcP to form the IL-36r/IL-1RAcP heterodimer and initiating downstream NFKB and MAPK pro-inflammatory signalling cascades. Natural inhibition of this signalling occurs when the IL-36Ra binds to the IL-36R; this prevents the recruitment of the IL-1RAcP and so stops activation of NFKB and MAPK. Similarly, Spesolimab, a monoclonal antibody against the IL-36R, preferentially binds the IL-36R, blocking IL-36 ligand/receptor interactions, inhibiting IL-1RAcP induction and downstream signalling. Other potential modes of therapeutic intervention currently under investigation include a monoclonal antibody that binds to IL-1RAcP and specific inhibitors of neutrophil- derived proteases. *Figure created with biorender.com*. Figure taken from Leon, G., Hussey, S., & Walsh, P. T. (2020). The diverse roles of the il-36 family in gastrointestinal inflammation and resolution. *Inflammatory bowel diseases*.

6.2 CONCLUSION

While remarkable progress has been made in elucidating the central cytokines involved in the pathogenesis of IBD and targeting these in the clinic, these novel biologics seem to only have a beneficial clinical effect in certain subgroups of IBD patients. This reflects the complexity of cytokine networks at work in the inflamed intestinal tissue, which are constantly influenced by environmental and genetic factors, in addition to immune cell and microbiome plasticity.

This study has characterised the effect of IL-36 on the T_H cell responses that are associated with the pathology observed in inflammatory bowel disease, and strongly implicates the cytokine family in the pathogenesis of IBD. IL-36 has a prominent role in $CD4^+$ T_H cell activation and polarisation, with *in vivo* and *ex vivo* studies demonstrating that IL-36 potently enhances T_H1 responses, and synergistically inhibits the generation and suppressive functioning of tolerogenic regulatory T cells. In preclinical studies, it appears that IL-36 utilizes these effects on $CD4^+$ T_H cells to promote the development of colitis, and also enhances the capacity of these pro-inflammatory cells to migrate to the gut, despite the presence of tolerogenic ATRA. IL-36 can also imprint this proinflammatory gut homing phenotype on human $CD4^+$ T_H cells, indicating that these mechanisms of pathology are functional in humans. Accordingly, we report elevated levels of IL-36α in the colonic tissue and serum of paediatric IBD patients, and this occurs in an environment where IL-36Ra regulation is low. Furthermore, we identify the presence of the IL-36R on $CD3^+$ T cells in the colon of paediatric IBD patients, indicating that the elevated levels of IL-36 present have the potential to act on these cells, and thereby may be promoting IBD in these patients by the mechanisms we have just specified (Figure 6.4.).

The IL-36 family have emerged as potent orchestrators of the intestinal inflammatory response in IBD, regulating both pro- and anti-inflammatory mechanisms, and targeting this family is already being trialled in the clinic for IBD. Yet the dichotomy of this cytokine family's effects on the mucosal immune response brings doubt to the efficacy of IL-36R targeted treatment. Elucidating each of the cytokines family member's biological functions and molecular pathways, in a tissue and stage specific manner, in the inflamed intestines will be key in determining the precise patient cohort that will benefit most from IL-36R targeted therapy in IBD, and may shed light on the necessity to develop novel IL-36 ligand specific neutralizing biologics.

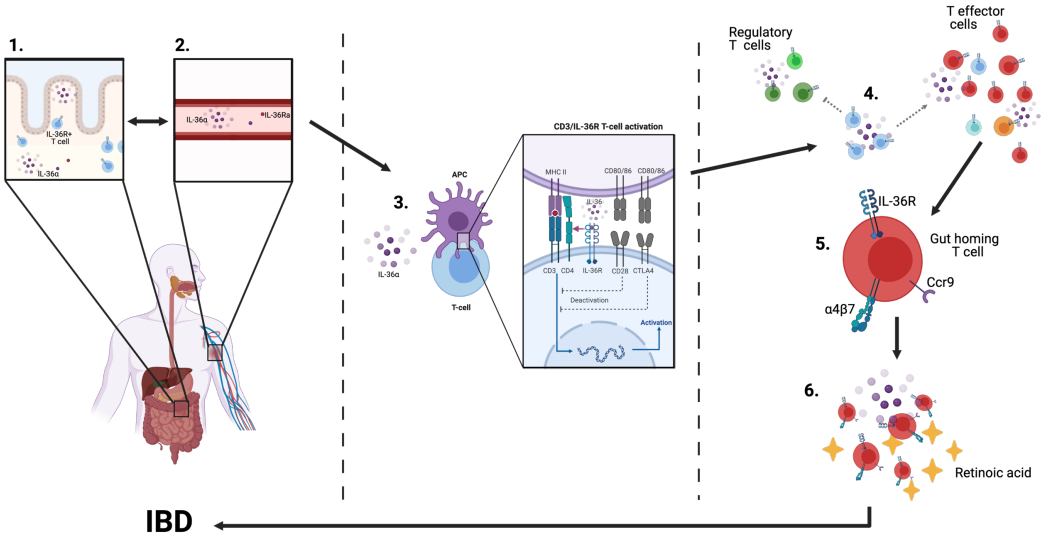


Figure 6.4: Proposed mechanism for IL-36 mediated CD4+ T cell pathology in IBD. In the intestines of paediatric IBD patients there are elevated levels of IL-36α in the same location as IL-36R+ T cells. (2) These elevated levels of IL-36α are also present in the systemic circulation, in negative correlation with the IL-36Ra, indicating there is an environment permissive to enhanced IL-36α signalling in IBD patients. In this study, we defined a mechanism whereby this elevated IL-36 in the intestines and systemic circulation may be influencing CD4+ T cells in IBD patients to amplify the inflammatory response. (3) Firstly, IL-36α can activate CD4+ T cells in the absence of classical CD28 co-stimulation. This indicates that IL-36 may act as an alternate activation pathway for CD4+ T cells, and if so, may bypass the existing regulation of CD28 mediated co-activation. (4) Once activated, IL-36α then promotes the induction of a proinflammatory phenotype in CD4+ T cells, enhancing the generation and pathogenicity of T effector cells, whist simultaneously inhibiting the expansion and suppressive capacity of Regulatory T cells. While this mechanism alone is enough to induce and maintain intestinal inflammation, IL-36 signalling also upregulates expression of integrins (α4β7) and chemokines (Ccr9) key in CD4+ T cell gut homing (5). In the intestines, retinoic acid is the main mediator of CD4+ T cell gut homing; it imprints expression of α4β7 on T cells, and promotes tolerance and maintains T cell homeostasis by promoting Treg generation, and inhibiting the polarisation proinflammatory T effector cells. Interestingly, IL-36 evades these suppressive actions of retinoic acid, and instead works in synergy with it to promote an enhanced population of pro-inflammatory T effector cells with a gut homing phenotype (6). Therefore, IL-36 evades multiple levels of homeostatic suppression to promote the generation of colitogenic CD4+ T cells that may contribute to the pathogenesis of IBD. *Figure created with biorender.com*.

CHAPTER 7.

FUTURE DIRECTIONS

Chapter 7

7.1 FUTURE DIRECTIONS

Throughout Chapter 6 I have detailed various potential mechanisms that IL-36 may be utilizing to achieve its profound pro-inflammatory effects on CD4+ T_H cell responses associated with IBD. To that end, future work on IL-36 in the context on IBD would be to further explore more deeply the specific mechanisms involved:

7.1.1 CONTINUE GENE AND PROTEIN EXPRESSION ANALYSIS ON OUR PAEDIATRIC COHORT OF IBD PATIENTS

Results from our gene expression analysis in a paediatric IBD cohort has revealed a significant increase in expression of *IL36A* in UC patients compared to CD patients controls (Fig.3.2.1.A). This is indicates that there is a dichotomy in expression of *IL36A* in the inflamed intestines between IBD subsets. However, in our protein expression analysis from the same patient cohort, while IL36α expression is still significantly elevated in our UC group compared to controls, expression of IL36α in CD group is markedly elevated, and nearing significance (p=0.0572) (Fig.3.2.2.A). Therefore, there may not actually be a disease subset dichotomy in IL36α expression, it may just be due to N numbers used. Increasing the size of the study may then elucidate if this is indeed the case. This seems likely, as Scheibe *et al*. have report elevated levels of IL36α in both CD and UC patient cohorts[377, 404]. Although, if this is not the case, this dichotomy in IL36α expression may constitute a difference between paediatric and adult inflammatory profiles in IBD.

7.1.2 MEASURE IL-38 IN THE SERUM AND COLONIC MUCOSA OF OUR PAEDIATRIC IBD COHORT.

Fonseca-Camarillo *et al*. reported temporal and opposing expression of IL-36Ra and IL-38 in the inflamed mucosa of UC patients. They report enhanced *IL-36RN* expression in the colonic mucosa of patients with active disease[395], whereas *IL-38* expression levels were enhanced in the inactive disease cohort[395]. Their finding alludes to a potential mechanism where IL-38 promotes resolution and homeostasis, and IL-36Ra modulates active inflammation in IBD. We observe a negative correlation

between IL-36α and IL-36Ra in the serum of paediatric IBD patients, indicating there is an environment permissive to unregulated IL-36α signalling. However, as IL-38 is also known to negatively regulate IL-36 signalling, perhaps there is enhanced expression of this cytokine in our paediatric cohort when IL-36Ra expression is low, and like Fonseca-Camarillo *et al*. observed, the two negative regulators of IL-36 signalling are being differentially temporally expressed. Therefore, it would be of interest to measure levels of IL-38 in the serum of the patients we have already analysed. This may expand our knowledge of natural IL-36 regulation in a paediatric IBD cohort.

7.1.3 DETERMINE IF IL-36 ENHANCES CHEMOTAXIS IN MURINE AND HUMAN CD4+ T_N CELLS.

We have reported a role for IL-36 in enhancing the gut homing capacity of CD4+ T_N cells in both mice and humans. Therefore, it would be worth investigating if this upregulation in gut homing receptors and integrins results in a functional effect, by performing transwell chemotaxis experiments. As we observed enhanced expression of α4β7 and CCR9 on these cells, these experiments should explore the migration of IL-36 stimulated T_N cells through a transwell coated with MAdCAM and CCL25.

7.1.4 DETERMINE THE EFFECT OF IL-36 ON RARA EXPRESSION.

Retinoic acid (ATRA) is present in the steady state in the intestines and serves as a homeostatic molecule, inhibiting proinflammatory T_N1 cell responses and enhancing tolerogenic Treg responses. However, IL-36 allows T_N1 cells to evade ATRA suppression and inhibits ATRA mediated Treg generation (Fig.5.2.7.1-2). As ATRA signals through RARα in T cells, does IL-36 somehow interfere with RARα signalling? Therefore, it would be interesting to determine if RARα expression and activation *in vitro* from T_N1 and iTreg cells +/- IL-36 stimulation is effected, this may give us some indication if this is a potential mechanism IL-36 is utilizing to promote inflammation in the intestines.

7.1.5 FIND THE CELL TYPE RESPONSIBLE FOR IL-36 MEDIATED ESCAPE FROM TREG SUPPRESSION.

We observed that when T_H effector cells are co-cultured with Tregs and stimulated with endogenous IL-36α, Treg mediated suppression is broken, T_H effector cell proliferation is no longer impeded (Fig.4.2.5.C & D), and IFNγ output is significantly enhanced (Fig.4.2.5.B & E). IL-36 may be eliciting these effects through 3 mechanisms:

1. IL-36 is acting directly on the Tregs, inhibiting their expansion and suppressive effects
2. IL-36 is stimulating the T_H effector cells so strongly that they overcome suppression.
3. IL-36 is stimulating the T cell depleted APCs and these in turn are causing the escape from Treg expression we have observed.

Alternatively, IL-36 may be acting through all of these mechanisms, resulting in the amplified inflammatory response we observed. Therefore, it would be worth exploring if this is the case, and substituting in *il36r* deficient cell T_H effector, Treg and APC subsets to our system will address which cell type(s) are the key responders to IL-36 cytokines in breaking Treg suppression in this assay.

7.1.6 DETERMINE IF IL-36 ALLOWS T_H EFFECTOR CELLS TO EVADE TREG SUPPRESSION *IN VIVO*.

We have demonstrated that IL-36R signalling promotes the development of colitis in the T cell transfer model of colitis. This model disease is commonly attenuated by the co transfer of Tregs alongside T effector cells. However, *in vitro* we have determined that IL-36 can mediate evasion from Treg suppression (Fig.4.2.5). Therefore, it would be worth determining if IL-36 can induce evasion from Treg mediated suppression *in vivo*, to achieve this we would induce $CD4^+CD45R\beta^{HI}CD25^{low}$ colitis, introduce a $CD4^+CD45R\beta^{low}CD25^{hi}$ population to attenuate this colitis, and then commence treatment with exogenous IL-36α.

Furthermore, substitution of *wt* Tregs with *Il36r*$^{-/-}$ Tregs would tell us if IL-36s potential evasion of Tregs *in vivo* is dependent on IL-36s effects on the T_H effector or Treg population.

7.1.7 DETERMINE THE ROLE OF IL-12 IN IL-36 MEDIATED T_N EFFECTOR CELL EVASION FROM SUPPRESSION.

We have demonstrated that IL-36 is a potent inducer of T_N1 generation and responses (Fig.4.2.3), and allows T_N effector cells to evade suppression by Tregs (Fig.4.2.5), and causes these Tregs to secrete IFNγ. IL-12 is an important cytokine in the polarisation of T_N1 cells, and is reported to reduce Tregs suppressive capacity *ex vivo*, and induce the IFNγ from these cells[460, 461]. Therefore, IL-36 could be mediating these effects on Tregs by inducing IL-12 from APCs and or/enhancing T cell sensitivity to this cytokine and is worth exploring in more detail. One way to determine if this is the case, would be to perform a Treg suppression assay, stimulate the cells with IL-36α. Following culture, it would be of interest to measure IL-12R expression on T cells by FACs, analyse the amount of IL-12 producing APCs by FACs, and measure the amount of IL-12 induced in the supernatants by ELISA. This may tell us if IL-36 is inducing IL-12 from APCs in this system. To see if IL-36 is mediating its effects via IL-12, we will then need to repeat this Treg suppression assay, and this time stimulate the cells with IL-36 and an IL-12 neutralizing antibody.

7.1.8 DETERMINE IF IL-36 IS AN ALTERNATE PATHWAY OF $CD4^+$ T_N CELL ACTIVATION AND EVASION OF TREG SUPPRESSION.

We have demonstrated that IL-36 can activate $CD4^+$ T_N cells in conditions of sub-optimal stimulus (4.2.2). T_N cells activated under IL-36 stimulus, only require TCR stimulation, circumventing the necessity for CD28 co-stimulation. However, upon CD28 activation, the checkpoint inhibitor CTLA-4 begins to be expressed on $CD4^+$ T_N cells, and serves to dampen the inflammatory response and compete with CD28 for ligation. If IL-36 is allowing $CD4^+$ T_N cells to bypass CD28 activation and subsequent CTLA-4 expression and regulation, this may explain the amplified inflammatory response we see in T cells following IL-36 stimulation.

Therefore, it would be of interest to perform a Treg suppression assay, and stimulate the cells with IL-36 +/- a CD28 antagonist or include *CD28*$^{-/-}$ T cells. This may then determine if IL-36 can evade Tregs in absence of CD28 co-stimulation. Additionally, it may be worthwhile to measure the expression of CTLA-4, and see if IL-36 activation truly is circumventing CD28 mediated CTLA-4 upregulation on T_N cells.

This assay could potentially be performed *in vivo* also, comparing the disease severity, T_N cell profile, and CTLA-4 expression in *Rag1*$^{-/-}$ mice that receive *wt* T_N

effector cells +/- exogenous IL-36α, to those that receive *Il36r*$^{-/-}$ T_H effector cells or *CD28*$^{-/-}$ T_H effector cells +/- exogenous IL-36α.

7.1.9 DETERMINE IF IL-36 MEDIATES T CELL GUT HOMING VIA CD69/S1P$_1$.

IL-36 has opposing roles to CD69 in GI homeostasis. CD69 expression is linked to lymphocyte retention in the periphery via inhibition of $S1P_1$ signalling. Expression of CD69 is upregulated on *Il36r*$^{-/-}$ T cells following T cell transfer (Fig.5.2.9), and coincide with an accumulation of lymphocytes in the spleen and mLNs (Fig.5.2.4.A&C & Fig.5.2.5.1.C-F). Therefore, it may prove valuable to measure expression of $S1P_1$ in these cells, and determine if expression of IL-36 regulates expression of CD69 and $S1P_1$. To further expand on this, CD4 T_H cells could be cultured and stimulated with exogenous IL-36, and expression of CD69/$S1P_1$ analysed.

7.1.10 DETERMINE THE ROLE OF SIGIRR IN IL-36 RESPONSES.

Preliminary data indicates that IL-36s effect on T_H1 responses is negatively regulated by SIGIRR (Fig.4.2.9.). To expand our understanding of these effects it would be of interest to compare the severity of colitis in *Rag1*$^{-/-}$ mice injected with *wt*, *Il36r*$^{-/-}$ and *Sigirr*$^{-/-}$ T_H effector cells. Analysing expression of IL-36 in these mice may reveal enhanced IL-36 signalling in *Sigirr*$^{-/-}$ recipients. If this were the case, it would be appealing to go on to treat *Sigirr*$^{-/-}$ recipients with an IL-36R neutralising antibody. Alternatively, it may prove beneficial to treat *wt* recipients with a SIGIRR agonist, perhaps IL-37, to see if this promotes homeostasis.

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APPENDIX I

9.1 PATIENT DEMOGRAPHIC TABLES

Patient Information		**Numbers**
ELISA Cohort		
Disease Groups	UC	31
	CD	42
	Ctrl	24
Sex	Female	34
	Male	63
Age	0-5 years	12
	5-10 years	12
	10-15 years	61
	15+	12
CD Phenotype	L1	1
	L2	9
	L3	18
	L4a	7
	L4b	2
	PA disease only	0
	Oral Disease Only	0
	Undefined	5
UC Phenotype	E1	3
	E2	4
	E3	0
	E4	24
CD Disease Severity	Remission	4
	Mild	9
	Moderate	12
	Moderate - Severe	4
	Severe	13
UC Disease Severity	Remission	0
	Mild	13
	Moderate	14
	Moderate - Severe	0
	Severe	4

Patient Information		Numbers
Gene Expression Cohort		
Disease Groups	UC	15
	CD	18
	Ctrl	22
Sex	Female	28
	Male	27
Age	0-5 years	4
	5-10 years	16
	10-15 years	24
	15+	11
CD Phenotype	L1	1
	L2	3
	L3	10
	L4a	1
	L4b	1
	PA disease only	2
	Oral Disease Only	0
	Undefined	0
UC Phenotype	E1	2
	E2	0
	E3	0
	E4	13
CD Disease Severity	Remission	0
	Mild	4
	Moderate	0
	Moderate - Severe	12
	Severe	2
UC Disease Severity	Remission	0
	Mild	7
	Moderate	5
	Moderate - Severe	0
	Severe	3

CD Classification:

L1: limited terminal ileal disease

L2: isolated colonic disease

L3: ileal-colonic disease

L4a: disease proximal to the ligament of treitz

L4b: disease distal to the ligament of treitz

PA: perianal disease

UC Classification:

E1: proctitis only

E2: left-sided disease

E3 extensive disease

E4: pancolitis

APPENDIX II

10.1 Papers published during PhD:

10.1.1. The Diverse Roles of the IL-36 Family in Gastrointestinal Inflammation and Resolution.

Leon, G., Hussey, S., & Walsh, P. T. (2020). *Inflammatory Bowel Diseases*.

Basic Science Review Article

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The Diverse Roles of the IL-36 Family in Gastrointestinal Inflammation and Resolution

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The interleukin (IL)-36 family is a member of the IL-1 superfamily of cytokines and, in common with other IL-1 family members, has been shown to exhibit pleiotropic effects in homeostasis and inflammation. Although the important role these cytokines play in the skin has been widely reported, recent evidence suggests that IL-36 family members are expressed and can also exert significant influence at the intestinal mucosa. In this review, we summarize current knowledge surrounding the role of the IL-36 in the intestines. In particular, we examine its likely dichotomous role as a mediator of both inflammation and resolution, highlighting its overlapping roles in innate and adaptive inflammation at the mucosa and its contribution to pathophysiology of inflammatory bowel disease. We also summarize the complexities of targeting this cytokine family in a clinical setting.

Key Words: IBD, Crohn's, colitis, IL-36, IL-36R

INTRODUCTION

The gastrointestinal (GI) system represents the largest and most sophisticated immune organ of the entire body. Dynamic layers of mucosal immunoregulation maintain the delicate balance between vigilance and tolerance that is central to GI homeostasis. Cytokines are integral mediators in this system, with the interleukin (IL)-1 cytokine family in particular playing key and often dichotomous roles. The IL-1 family contains 11 immunomodulatory cytokines, comprising 8 agonistic ligands (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, and IL-37) and 3 natural antagonists (IL-1Ra, IL-36Ra, and IL-38).[1] In humans, the genes encoding these cytokines are located on chromosome 2, with the exception of IL-18 and IL-33, which are mapped on chromosome 11 and 9, respectively.[2]

These cytokines elicit their effects through 4 specific heterodimeric receptor complexes, with each receptor consisting of an intracellular TIR domain, a transmembrane helix, and an extracellular ligand-specific binding domain (IL-1R1, IL-33R [ST2], IL-18Rα, and IL-36R). Recruitment of an accessory protein is necessary to initiate signal transduction. Upon appropriate ligand interaction with either the IL-1R1, the IL-33R, or the IL-36R, the IL-1R accessory protein (IL-1RAcP) is recruited, whereas in the case of IL-18, the IL-18Rβ chain is recruited to form the IL-18R. Dimerization of these receptor complexes initiates nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pro-inflammatory signaling cascades.[1]

Due to the central role of IL-1 member signaling in inflammation, a number of distinct mechanisms have evolved to regulate their activity. These include receptor antagonists, decoy receptors, and neutralizing binding proteins.[1, 3] The IL-1Ra and IL-36Ra, alongside IL-38, preferentially bind the IL-1R and IL-36R, respectively, and this action disrupts the recruitment of the IL-1RAcP, thereby preventing receptor dimerization and downstream signal transduction.[3] The decoy receptor, IL-1R2, lacks the intracellular TIR domain required for signal transduction, and so it acts as a molecular sink for both IL-1α and IL-1β cytokines. Interestingly, IL-1R2 also exists in a soluble form and can exert these neutralizing effects in a cell extrinsic manner, similar to the mechanisms through which soluble IL-33R (ST2) and IL-18 binding protein (IL-18bp) have been described to neutralize their respective cytokines signaling.[1, 3, 4]

The Complex Roles of the Interleukin-1 Family in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), typically subcategorized as either Crohn's disease (CD) or ulcerative colitis (UC), is a chronic inflammatory disorder of the GI tract whose incidence is on the rise globally.[5] Its etiology is unknown, but there is accumulating evidence demonstrating

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that interactions between environmental, genetic, and immunological factors promote the development and pathogenesis of these chronic inflammatory disorders. Prevalence of IBD is highest in developed western countries, with approximately 1.6 million people in United States and up to 2 million people in Europe affected.[5, 6] More recent epidemiological studies have also shown an increase of disease incidence in developing countries, such as in Eastern Europe, South America, Africa, and Asia.[5, 6] Although the precise causes of IBD remain relatively unknown, such differences in epidemiological patterns highlight the likely importance of environmental and genetic factors in the development of the disease.

Though both CD and UC share the common characteristic of GI inflammation, their disease presentation and histological and immunological profiles are quite distinct. Crohn's disease can affect anywhere along the GI tract from mouth to anus, most frequently in the ileum and colon, and commonly causes inflammation in a discontinuous transmural pattern, resulting in a thickened submucosa, granulomas, fissuring ulceration, and the development of fistulas and strictures in the affected areas.[7, 8] In contrast in UC, inflammation is continuous, presenting from the rectum to colon and only involves the mucosal and submucosal layers.[7] In both disorders, disease course in highly variable; some patients experience chronic abdominal pain, rectal bleeding, fever, and diarrhea, whereas others experience long periods of remission from active disease.[7, 8] This heterogeneity in disease presentation and severity highlights the complexity of differential immunological factors and inflammatory pathways that are involved in the pathogenesis of IBD. For instance, CD is frequently associated with a dominant type 1 inflammatory response, with the involvement of T helper (Th)-1 CD4+ T cells, whereas UC is characterized by an atypical type 2 response, mediated by Th2-like CD4+ T cells.[7] The Th17 responses have been implicated in the pathogenesis of both CD and UC. Interestingly, these cells show functional plasticity and can be converted into interfern (IFN)-γ producing Th1 cells or anti-inflammatory Tregs, suggesting that they may serve as a homeostatic mediator in the balance between inflammation and resolution in disease.[9] Given the potent ability of IL-1 family members to drive inflammation, it is not surprising that many members of this family have been implicated as being involved in gastrointestinal inflammation and, in particular, with the pathogenesis of IBD. However for many IL-1 family members, a more complex picture has emerged, with often conflicting mechanistic roles as mediators of inflammation and homeostasis and resolution described.

The prototypical family members, IL-1α and IL-1β, have been described to play a pathogenic role in the context of IBD.[10, 11] Accordingly, elevated levels of monocyte-derived IL-1β have been reported in IBD patients,[10, 12–14] with disease severity correlating with the increased IL-1 levels found in inflamed tissue biopsies.[15] Furthermore, deletion of *IL-1β* was shown to confer protection in the 2,4,6-trinitrobenzene sulfonic acid (TNBS) model of colitis.[16] Although such reports indicate a pathogenic role for IL-1 in the intestine, a more complex picture is beginning to emerge that demonstrates IL-1 can also play critical, context-dependent,roles in GI homeostasis. For example, a recent study by Zhou et al described the necessity for constitutive expression of IL-1β in the steady state to facilitate Treg generation via innate lymphoid cell 3 (ILC3) mediated IL-2 production.[17] In addition, specific deletion of IL-1 signaling in IL-C3 cells causes disruption in GI homeostasis, oral tolerance to dietary antigens, and Treg maintenance, whereas ILC3 mediated IL-2 production was found to be significantly reduced in Crohn's disease patients.[17] These studies highlight the importance of a balanced and tightly regulated IL-1 signaling axis in the intestinal mucosa to facilitate gut homeostasis and allude to the intricacies involved in targeting cytokines as a potential therapy. Though inhibition of IL-1 signaling has proven effective in other inflammatory conditions,[18] there has been little success therapeutically targeting IL-1 in IBD.

Similar to IL-1α, IL-18 is also expressed constitutively in the gut mucosa, indicating a potential role in GI homeostasis.[19] Interleukin-18 is generally regarded as a pro-inflammatory mediator, enhancing the production of IFNγ, stimulating the differentiation of Th1 cells,[20] and priming natural killer cell cytotoxicity.[21] Interestingly, IL-18 activity can be regulated through cleavage of pro-IL-18, mediated by the NLRP6 and NLRP3 inflammasomes, which are known regulators of colonic homeostasis.[22, 23] NLRP6 is predominantly expressed in intestinal epithelial cells (IECs) and promotes mucosal renewal, mucus secretion, and proliferation.[23] And IEC-derived IL-18, mediated by the NLRP6-inflammasome, has been shown to prevent gut colonization by colitogenic microbiota.[24] Recent studies have shown that deficiency in *NLRP3* exacerbated chemically induced colitis. This increased severity in disease occurred in association with insufficient amounts of IL-18 in the mucosa, and administration of recombinant IL-18 (rIL-18)-induced protection in this model via enhanced barrier repair and epithelial regeneration.[22] In addition, IL-18 has also been reported to regulate the adaptive inflammatory response in the GI tract by orchestrating the balance between pro-inflammatory Th17 and anti-inflammatory Treg differentiation in the colon.[25] In this context, IEC-derived IL-18 promotes the generation of Tregs while synergistically inhibiting the generation of Th17 cells, thus maintaining colonic homeostasis in the steady state.[25]

On the other hand, increased levels of IL-18 have been observed in patients with CD,[26, 27] correlating with studies highlighting the role of IL-18 in the breakdown of the mucosal barrier and amplifying epithelial inflammation during disease. Specific deletion of *IL-18* or its receptor, *IL-18R*, in IECs has conferred protection in dextran sodium sulfate (DSS) colitis,[28] and this protection is further enhanced in a *IL-18*/*IL-1β* double knockout model.[16] Furthermore, IL-18bp$^{-/-}$ mice exhibit significantly exacerbated disease, alongside a reduction in mature goblet cells. This phenotype is reversed in the absence of IL-18R

expression on IECs, suggesting a pathogenic role for IL-18R signaling in the colonic epithelium. Further mechanistic studies demonstrated a role for IL-18 in inhibiting goblet cell development and maturation, thereby promoting mucosal barrier dysfunction, which is a common characteristic of UC.[28] It is also noteworthy that genome-wide association studies (GWASs) have linked mutations in genes involved in the IL-18 signaling pathway with increased IBD susceptibility,[29, 30] enhancing the rationale for targeting the IL-18 pathway therapeutically.

Interleukin-33 is generally regarded as a mucosal alarmin, typically involved in type 2 immune responses, which can play important roles in innate immunity in the intestine.[31] Although IL-33 is constitutively expressed in the epithelium and endothelium, its receptor ST2 (IL-33R) is primarily expressed on immune cells, including T cell and innate lymphoid cell subsets. This pattern of expression is indicative of the key role IL-33 plays in the orchestration of immune responses at mucosal surfaces.[4] Elevated levels of IL-33 have been observed in the colon and serum of ulcerative colitis (UC) patients in which a Th2-like inflammatory profile is commonly observed.[32–35] Furthermore, when mesenteric lymph node CD4+ T cells from piroxicam-accelerated colitis (PAC) *IL-10*$^{-/-}$ mice were challenged with rIL-33, Th2-associated cytokines were produced.[33] Though these data indicate a pro-inflammatory role for IL-33 in the pathogenesis of UC, preclinical studies have also revealed a complex and dichotomous role for this cytokine often with conflicting results. First, various studies have shown that *IL-33*$^{-/-}$ mice are highly susceptible to the development of TNBS- and DSS-induced colitis[36] and administration of rIL-33 attenuates disease.[37–39] In contrast, using the same model, other groups have observed opposing results.[35, 40, 41] Sedholm et al observed elevated levels of IL-33 in both DSS- and TNBS-induced colitis, and in this case, administration of rIL-33 increased disease severity, whereas *St2*$^{-/-}$ mice were somewhat protected.[35] Furthermore, investigations using senescence-accelerated (SAMP) mice, which develop an enteric UC-like disease, revealed that IEC-derived IL-33 correlates with disease severity and that blockade of this pathway alleviates disease.[39, 42] A recent study by Groβ et al provided an interesting insight into complexity of IL-33 signaling in IBD and offered a potential explanation for conflicting data described previously. In these studies, an exacerbation of disease was evident when rIL-33 is administered during the acute phase of DSS-induced colitis, whereas prolonged rIL-33 administration was found to lead to enhanced recovery from disease and ameliorated intestinal inflammation in the chronic phase.[43] These results suggest distinct temporal roles for IL-33 signaling in disease. With such complex and dichotomous roles, the precise impact of IL-33 on the development and progression of IBD is still unclear and requires further study before it can begin to be assessed therapeutically.

Interleukin-37 is a unique anti-inflammatory member of the IL-1 family. Expression of this cytokine has been found to be elevated in the sera of both UC and CD patients, and higher levels of IL-37-producing cells have also been found in inflamed colon tissue biopsies in CD.[44, 45] In pediatric patients, elevated levels of this cytokine correlate with disease severity,[46] and humanized transgenic mouse studies have demonstrated that IL-37 can act to dampen mucosal inflammation and protect against DSS-induced colitis.[47]

As described thus far, IL-1 family members evoke a sophisticated dichotomous role in the GI tract, both as mediators of inflammation and in the maintenance of homeostasis. Here, we will review current knowledge surrounding the role of the most recently described members of this family, the IL-36 subfamily, in the intricate cytokine networks that regulate intestinal homeostasis; how alterations in their activity may drive the pathogenesis of IBD; and current efforts to target these cytokines therapeutically.

THE INTERLEUKIN-36 FAMILY OF CYTOKINES

Interleukin-36 is a relatively novel member of the IL-1 cytokine superfamily. It was initially discovered 2 decades ago when DNA database screens identified 4 new members of the IL-1 family based on their homology at both gene and protein levels.[48, 49] They were originally designated names based on their order of discovery, from IL-1F5 to IL-1F9, but were later reclassified as IL-36Ra (IL-1F5), IL-36α (IL-1F6), IL-36β (IL-1F8) and IL-36γ (IL-1F9) and are collectively known as the IL-36 subfamily of cytokines. As mentioned, the IL-36 family is composed of 3 agonistic ligands (IL-36α, IL-36β, and IL-36γ) and one specific receptor antagonist (IL-36Ra).[48, 49]

Like other IL-1 family members, such as IL-1β,[50] IL-36 agonists are generated in a biologically inactive "pro" form that requires proteolytic processing to enable their pro-inflammatory activity.[51] Truncation of their n-termini results in over a 1000-fold increase in their activity,[51] and Henry et al recently reported that this cleavage is due to neutrophil granule-derived proteases. Interleukin-36α, IL-36β, and IL-36γ are activated differentially by cathepsin G, elastase, and proteinase-3.[52] Elastase has also been shown to activate IL-36Ra, facilitating its anti-inflammatory effects.[53] Interleukin-36 ligands signal by first binding to the IL-36R; this induces the IL-1RAcP to act as a co-receptor, and the dimerization of this IL-36R/IL-1RAcP complex facilitates downstream pro-inflammatory signal transduction. Regulation of IL-36 family signaling is mediated by both the IL-36Ra, and possibly IL-38, which preferentially bind the IL-36R and inhibit IL-1RAcP recruitment, thereby preventing activation of IL-36 mediated signaling cascades.[51]

All IL-36 cytokines have been identified in both humans and mice. Interestingly, their general chromosomal location and gene organization is quite similar, sharing a considerable sequence homology between the species of 91% for *IL-36Ra*,

62% for *IL-36β*, 56% for *IL-36γ*, and 54% *IL-36α*. This genomic conservation serves to highlight the importance of these cytokines between species.[54]

Interleukin-36 Expression in the Intestines

In recent years, IL-36 family members have gained significant attention due to their dysregulation in inflammatory conditions, such as psoriasis,[55] rheumatoid arthritis (RA),[56] osteoarthritis (OA),[57] systemic lupus erythematosus (SLE),[58] and inflammatory bowel disease (IBD),[59] and have been shown to regulate both parenchymal and immune cell responses.[59–61] In the GI tract, IL-36 cytokines are derived from various sources such as the intestinal epithelium and parenchyma cells and immune cells such as CD14+ macrophages, CD123+ plasmacytoid DCs, and CD8+ T cells.[62] Expression levels of IL-36α and IL-36γ in particular have been shown to be elevated in the inflamed intestinal mucosa of both mice and IBD patients, and such increases seem to be driven by tissue damage and the intestinal microbiota (Fig. 1).[59, 60, 63, 64]

These data contrast with other tissue sites where IL-36 cytokines have also been reported to be mediators of inflammation. For example in psoriatic skin, IL-36β is produced by and can act on keratinocytes, which express the IL-36R.[65] In addition, elevated levels of expression of IL-36γ are found in the serum of psoriasis patients and may represent a biomarker for disease severity.[55, 56] Interleukin-36 family members are also highly expressed in the synovial tissues of patients with RA, OA, and psoriatic arthritis (PsA).[56, 57] In these tissues, $CD138^+$ plasma cells are thought to be the main cellular source of IL-36α.

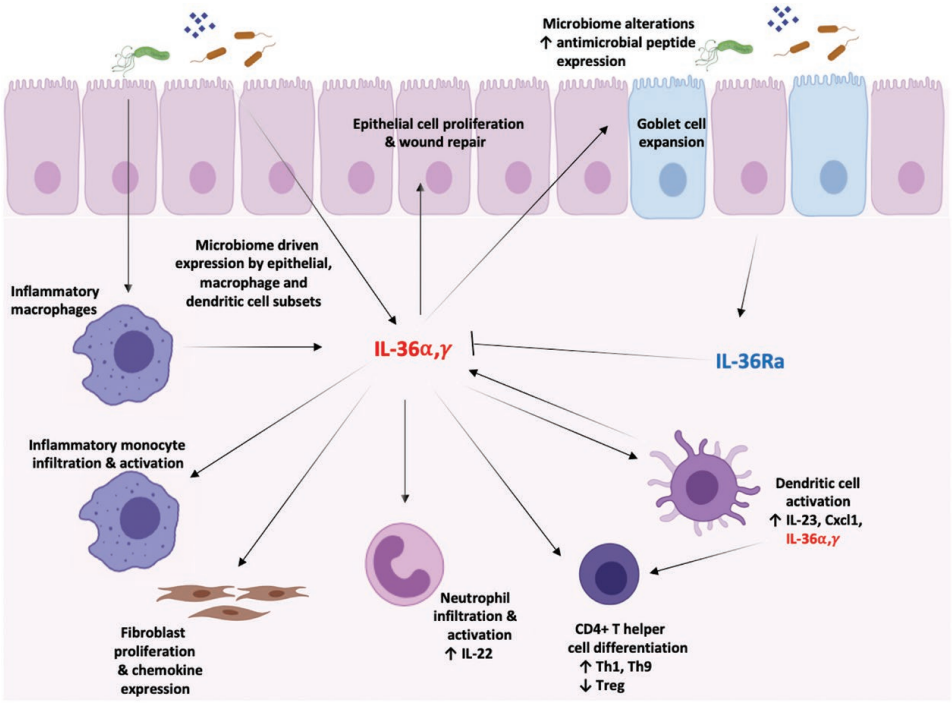

FIGURE 1. The effects of IL-36 family members at the intestinal mucosa. The IL-36 family of cytokines are mediators of inflammation and homeostasis at the intestinal mucosa where they can stimulate diverse responses from both immune and parenchymal cell subsets. Their effects can be dichotomous, simultaneously promoting mucosal homeostasis and barrier integrity while also having the potential to recruit and activate inflammatory cells to the mucosa, expand pathogenic cell populations associated with IBD, and induce fibrosis and microbiotic dysbiosis. Figure created with biorender.com.

INTERLEUKIN-36 AND IBD

Historically, research on immune involvement in IBD was starkly divided into groups focusing either on the innate arm or the adaptive arm of the immune response, and crosstalk between the 2 was generally ignored.[66] However, recent GWAS confirmed the relevance of already characterized pathways and also revealed strong evidence for an association between innate and adaptive immunity in mucosal homeostasis.[29] As IL-36 cytokines have been described to mediate both innate and adaptive inflammation, they may play critical roles in this crosstalk and the sensitive balance of mucosal immunity in the intestine (Fig. 1).

Interleukin-36 as a Mediator of Innate Inflammation in the Intestine

Similar to other IL-1 family members, IL-36 cytokines have been shown to have a dichotomous role in regulating the innate immune response in mouse models of colitis. Recently, Russell et al observed a significant increase in expression of IL-36α in the colonic mucosa of UC patients and enhanced expression of both IL-36α and IL-36γ in the colons of mice undergoing active DSS-induced colitis.[59] To determine the pathophysiological effect of the elevated levels of IL-36 observed in colitis, Russell et al compared acute DSS onset and severity between wild type (WT) mice and *IL-36r*$^{-/-}$ mice. This analysis demonstrated that in the absence of IL-36R signaling, a less severe form of colitis was induced, alongside reduced inflammatory cell infiltrate into the colonic lamina propria, with decreased numbers of neutrophils and macrophages detected when compared with WT mice.[59]

Similarly, Medina-Contreras et al also observed elevated expression of IL-36γ mRNA in both IBD patients and in the colons of WT mice undergoing acute DSS colitis.[64] This study reported enhanced expression of IL-36 by M1 macrophages and IECs in the colonic lamina propria (Fig. 1), in addition to a decreased disease activity index (DAI) in *IL-36r*$^{-/-}$ mice compared with their WT counterparts. Notably, they also uncovered a significant defect in the ability of *IL-36r*$^{-/-}$ mice to resolve DSS-induced inflammation. After 5 days of DSS exposure, both WT and *IL-36r*$^{-/-}$ mice were returned to normal drinking water, at which point WT mice began to spontaneously recover from colitis as expected. In contrast, *IL-36r*$^{-/-}$ mice continued to exhibit an elevated DAI, and the failure to appropriately resolve disease was observed upon study termination, where *IL-36r*$^{-/-}$ mice were found to exhibit a significantly higher grade of colonic inflammation.[64] The failure to recover from DSS-induced damage was associated with a marked reduction in expression of IL-36γ induced IL-22 by colonic neutrophils.[64] In follow-up studies, the same group demonstrated that IL-36R signaling acts as an upstream driver of the IL-23/IL-22/AMP pathway during intestinal injury and barrier repair.[67]

In agreement with Medina-Contreras et al, Scheibe et al also found that *IL-36r*$^{-/-}$ mice exhibited increased DSS disease severity, along with diminished survival rates and an increased bacterial burden in the colonic wall.[61] Consistent with other studies, they also reported elevated levels of IL-36 cytokines in the colons of patients with IBD[59, 61, 64]; IL-36α was found to be expressed by colonic inflammatory macrophages and DCs, whereas IL-36γ expression was found in IECs.[61] Scheibe et al further reported that stimulation of murine mucosal IECs with IL-36R ligands resulted in marked proliferation, both in vitro and in vivo, via Myd88 pathway signaling,[61] indicating this response is a direct mechanism of intestinal inflammation resolution (Fig. 1).[61]

Collectively, these studies indicate that IL-36 plays a pathogenic role in the "damage phase" of innate colitis by amplifying the inflammatory response in the mucosa; however, as the disease resolves, IL-36R signaling is necessary to modulate the innate responses of IECs and neutrophils to promote intestinal barrier repair and wound healing. Although these findings bear striking similarities with related IL-1 family members, as described previously, it remains to be determined whether IL-36 cytokines play a more prominent role in either intestinal inflammation or homeostasis and resolution in such settings. The development of tissue/cell specific knockout mice will play an important role in segregating the importance of these apparently opposing effects.[68, 69]

The Regulation of the Adaptive Response by IL-36

As well as playing a significant role in modulating innate inflammatory responses and homeostasis in the intestine, there is significant evidence that IL-36 cytokines can also influence the activation of adaptive immune cell subsets and thereby play key overlapping instructive roles in immune crosstalk. T cells represent the key cell population from the adaptive immune response involved in the pathogenesis of IBD, and in recent years, numerous groups have reported a role for IL-36 in directly regulating T cell responses.[59, 60, 70, 71] Interleukin-36β and IL-36γ have been shown to promote $CD8^+$ T cell activation,[70] Additionally, Th1 polarization is enhanced by IL-36α[59] and IL-36β,[71] whereas IL-36γ promotes Th9 polarization and inhibits iTreg differentiation via MyD88 and NFκBp50-dependent signaling pathways.[60] As dysregulated T cell responses in the gut mucosa are a defining characteristic of IBD, these studies highlight the potential impact IL-36 may have on disease development and severity.

Accordingly, Russell et al reported altered colonic T cell responses in *IL-36r*$^{-/-}$ mice using the *Citrobacter rodentium* model of colitis, demonstrating reduced mucosal Th1 responses in the absence of IL-36R signaling.[59] Subsequently, Harusato et al found elevated levels of IL-36γ expression in WT mice in both the *Helicobacter hepaticus* and T cell transfer models of colitis.[60] Using the oxazolone induced model of disease, which elicits a Th2/Th9-dependent colitis comparable to human UC, they further demonstrated that IL-36r$^{-/-}$ mice also exhibited a milder form of disease.[60]

A significant increase in IL-36γ mRNA was also observed in the inflamed colonic tissue of WT mice during active disease, and *IL-36γ*$^{-/-}$ mice also displayed attenuated disease.[60] Significantly, a diminished population of IL-9 producing CD4^{+} T cells and increased numbers of CD4^{+} Treg cells were found in the colonic tissue of *IL-36r*$^{-/-}$ mice after oxazolone treatment.[60] Mechanistic in vitro studies further demonstrated that IL-36-dependent regulation of the Th9/Treg balance occurs through modulation of IL-2/STAT5 and IL-4/STAT6 signaling. In addition, it was also demonstrated using the T cell transfer model of colitis that recipients of IL-36r$^{-/-}$ effector CD4+ T cells exhibited a significantly milder form of disease compared with recipients of WT CD4+ T cells.[60] As the T cell transfer model is characterized by Th1/Th17 induced mucosal inflammation, the reduction in disease severity could be hypothesized to be due to a reduced ability to mount an effective Th1 response in the absence of IL-36 signaling. In agreement with these findings, Scheibe et al recently reported a pathogenic role for IL-36 in the Th1 dependant TNBS model of colitis. Interleukin-36r$^{-/-}$mice exhibit a significantly less severe form of disease, and neutralization of IL-36 signaling using an anti-IL-36R antibody ameliorates disease in WT mice undergoing chronic TNBS[72] colitis.

These data indicate that IL-36 cytokine-dependent direct and indirect modulation of mucosal T cell responses may represent an important mechanism through which these cytokines influence inflammation in the GI tract (Fig. 1). Furthermore, the potent stimulatory effects of IL-36 family members across a wide range of mucosal inflammatory cell responses indicate an important role as mediators at the interface between innate and adaptive inflammation in the intestine.

Interleukin-38 as a Potential Regulator of Interleukin-36R Signaling-mediated IBD

Interleukin-38 serves to negatively regulate IL-1 family mediated inflammation, possibly acting as a specific antagonist of IL-36 subfamily signaling.[73] Enhanced levels of IL-38 have been observed in colon samples from UC and CD patients,[62, 74] along with a significant increase of IL-38-expressing B cells infiltrating the lamina propria when compared with healthy controls.[75] The increase in IL-38 observed in CD patients is specific to inflamed biopsies and correlates with enhanced IL-1β, IL-17A, and IL-6 expression in these tissues,[74] indicating that IL-38 expression may be increased as a consequence of mucosal inflammation during active disease. In UC, enhanced levels of IL-36 family member mRNA, including IL-36Ra, were found in the colonic mucosa of patients with active disease.[62] However in this instance, IL-38 expression levels were found to be elevated in the inactive disease cohort,[62] perhaps suggesting a resolving role for this cytokine in UC, where IL-36Ra serves to regulate active inflammation and IL-38 promotes resolution and homeostasis. These data potentially present a unique IL-36Ra/IL-38 signaling axis that serves to modulate IL-36 agonist signaling in a disease subtype, stage specific manner, and suggest an innovative target for the therapeutic treatment of IBD and GI tract inflammation. Furthermore, a number of groups have reported increased IL-38 expression in the colon of DSS-exposed mice,[74, 75] and treatment with recombinant IL-38 protein alleviates disease, in association with a downregulation of macrophage-derived IL-1β and TNF-α expression in the colon.[75]

Interleukin-36 Cytokines as Mediators of Intestinal Fibrosis

Gastrointestinal fibrosis is a common complication in IBD, particularly CD and chronic progressive UC. Surgery is often the only option for treatment and results in significant and irreversible loss of intestinal tissue.[76] Recently Scheibe et al[72] reported significantly elevated amounts of IL-36α^{+} inflammatory macrophages adjacent to α-smooth muscle actin–positive myofibroblasts and smooth muscle cells in stenotic CD regions when compared with controls.

Previously, the group had identified IL-36 ligands as potent activators of intestinal fibroblasts that promote wound healing in acute colitis.[61] In this study, IL-36 stimulation of colonic fibroblasts resulted in enhanced expression of gut-homing chemokines Cxcl1, Cxcl2, and Ccl20 and promoted neutrophil migration in vitro. Mice deficient in IL-36R signaling displayed reduced numbers of leukocytes in the colon after 10 days of DSS,[61] indicating that IL-36 signaling has a role in fibroblast-mediated leukocyte recruitment in the mucosa (Fig. 1).

Subsequently, when Scheibe et al used a chronic model of colitis, IL-36 mediated fibroblast activation was reported to induce intestinal fibrosis and exacerbate disease severity in both DSS and TNBS induced models.[72] Functional studies implicated the ability of IL-36 to induce expression of genes involved in the regulation of fibrosis and tissue remodeling in mediating these effects. Furthermore, increased levels of fibrotic markers were found in the colonic tissue from CD and UC patients, and these correlated with enhanced levels of IL-36α expression. Interestingly, neutralization of IL-36 signaling using an anti-IL36R antibody was also reported to reduce disease severity in chronic DSS and TNBS models, further highlighting a possible pathogenic role for IL-36 signaling in chronic intestinal inflammation and fibrotic responses.[72]

This dichotomy in IL-36's effect in disease pathology may be due to different types of inflammation associated with early vs late disease stages. For instance, acute colitis has been associated with a Th1 type inflammatory response; however as chronicity develops, this changes to an innate and adaptive mediated inflammation, exhibiting a predominately more Th2 type of cytokine profile that is typically associated with fibrosis.[77–80]

It is also noteworthy that Sommerfeld et al recently reported a role for IL-36^{+} macrophages mediating fibrosis via IL-17.[81] As type 17 responses contribute to autoimmunity in psoriasis and inflammatory arthritis and are implicated

in the pathogenesis of fibrotic diseases of the heart, lung, and liver, these data suggest that IL-36 family cytokines may play an important role in fibrotic responses across several tissue sites.[81]

The Emerging Role for Interleukin-36 in Regulating the Microbiome

Gut microbial dysbiosis is a common occurrence in IBD, where outgrowth of certain "harmful" bacteria, fungi, and viruses may be associated with disease pathogenesis. Giannoudaki et al recently described a role for IL-36 in the modulation of this system, in which *IL-36ra*$^{-/-}$ mice exhibited reduced weight gain and metabolic dysfunction in a model of obesity by promoting the growth of *Akkermansia muciniphila* bacteria in the gut, which is known to have a protective role by enhancing colonic mucus secretion[63] and whose abundance has been reported to be reduced in UC.[82] Furthermore, Medina-Contreras et al observed reduced levels of IL-36γ and proresolving IL-36γ induced IL-22 in germ-free mice during DSS colitis;[64] however, later studies by the same group revealed that IL-36 receptor signaling can modulate the composition of the intestinal microbiome, possibly through activation of the IL-23/IL-22/AMP pathway during intestinal injury and barrier repair[67] (Fig. 1).

Interestingly, Scheibe et al also reported lower levels of expression of Lipocalin 2 (LCN2) in *IL-36r*$^{-/-}$ mice compared with controls.[61] Lipocalin 2 is an antimicrobial peptide that plays a role in the innate response to colonic bacterial infection through iron sequestration.[83, 84] Diminished levels of LCN2 observed in the absence of IL-36R signaling suggests an additional mechanism by which IL-36 may influence the intestinal microbiome.

More recently, Sequiera et al proposed a role for IL-36 in host protection and clearance of intestinal *Klebsiella pneumoniae*.[85] This group found that the presence of a mature microbiota dominated by the phyla Bacteroidetes was necessary to prevent *K. pneumoniae* colonization of the intestines in mice. Interestingly, antibody-mediated blockade of IL-36R signaling resulted in loss of protection from *K. pneumoniae* colonization, whereas treatment with recombinant IL-36γ promoted clearance of the bacteria. These data indicate that Bacteroidetes can prime intestinal IL-36 production, thereby preventing *K. pneumoniae* colonization,[85] and further demonstrate the interplay between the IL-36 family and commensal bacteria in the gut. As other studies have found *K. pneumoniae* colonization increases the risk of GI inflammation and the onset of colitis in vivo,[86] in addition to the overrepresentation of *Klebsiella* species reported in the gut microbiota of IBD patients,[87] this further highlights the importance of IL-36 cytokine regulation of the microbiome in the maintenance of GI homeostasis.

TARGETING INTERLEUKIN-36 THERAPEUTICALLY

Cytokines are considered key constituents in the immunopathogenesis of IBD and play an important role in defining the persistence vs the resolution of intestinal inflammation. Gastrointestinal homeostasis is achieved by complex orchestration of pro- and anti-inflammatory effector cytokines at the mucosal surface, and consequently, targeting these inflammatory modulators has offered an attractive potential therapy for patients. There have already been some notable successes using this approach, with the use of anti-TNFα biologics, infliximab, adalimumab, and certolizumab.[88, 89] It is well established that the use of these biologics, in particular infliximab and adalimumab, can induce and maintain clinical response and remission, reduce systemic inflammation, and induce mucosal healing in patients with IBD.[90] Treatment with these biologics has shown efficacy in around 80% of patients; however, approximately 30% of patients lose this response, and 20% of patients are resistant to TNFα inhibitors.[91]

This leaves a significant cohort of patients which may benefit from the use of alternative cytokine-based therapeutic approaches. One such therapeutic, ustekinumab, is gaining popularity in the clinic for treating CD. This biologic is a monoclonal antibody that targets the common p40 subunit of IL-12 and IL-23, effectively inhibiting the generation of Th1/Th17 cells.[92] The efficacy of this therapy has been well documented in the CERTIFI[93] and UNITI[94] trials, in which patients who had previously failed anti-TNFα therapy showed an induction and maintenance in clinical response.

This illustrates how effective cytokine therapies can be in intestinal inflammation and highlights the complexity of the cytokine networks active in the inflamed tissue. Blockade of a singular cytokine is unlikely to be effective in all cases of IBD, whereas precision inhibition on a case by case basis and employing strategies modulating multiple inflammatory mediators may yield more effective results and clinical benefit to the patients.

In this regard, IL-36 may represent an attractive candidate for therapeutic intervention in IBD. As described previously, accumulating evidence implicates IL-36 activity in the pathogenesis of IBD, with increased levels of expression observed in the inflamed mucosa of both CD and UC patient cohorts, and several studies have validated its functional role as a mediator of mucosal inflammation. In addition, directly targeting IL-36R activity can improve outcomes in preclinical models of colitis, and blockade of this receptor has proven effective in the treatment of human generalized pustular psoriasis (GPP).[95] In light of these findings, it is perhaps unsurprising that efforts to target the IL-36 axis using spesolimab (BI655130), a monoclonal antibody that is directed against the IL-36R, have entered the clinic for both CD and UC patients (Fig. 2). In an ongoing study, the efficacy of spesolimab

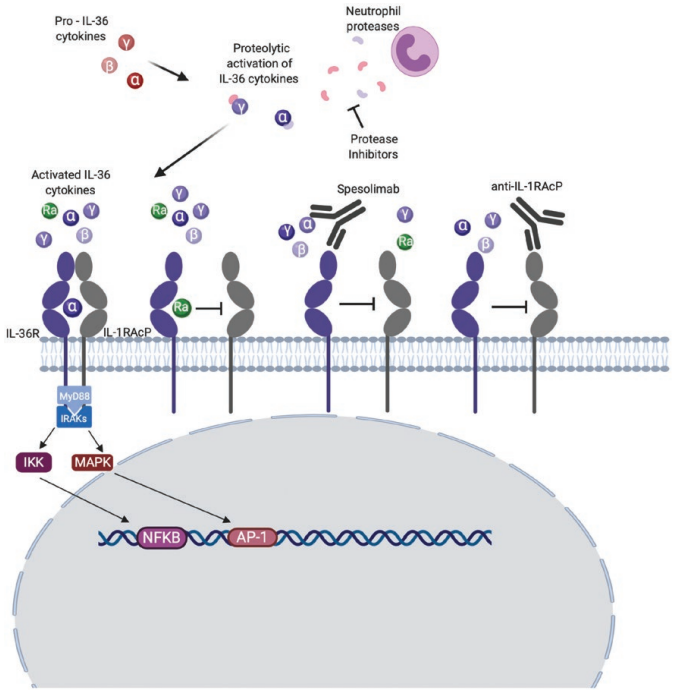

FIGURE 2. Therapeutically targeting IL-36 family signaling. The IL-36 family members mediate inflammation through interaction with the specific IL-36R. The IL-36 agonists (IL-36α, IL-36β and IL-36γ) require proteolytic processing by neutrophil-derived proteases to elicit full activity. Once in the active form, they bind to the IL-36R, thereby recruiting the IL-1RAcP to form the IL-36R/IL-1RAcP heterodimer and initiating downstream NFκB and MAPK pro-inflammatory signaling cascades. Natural inhibition of this signaling occurs when the IL-36Ra binds to the IL-36R; this prevents the recruitment of the IL-1RAcP and so stops activation of NFκB and MAPK. Similarly, spesolimab, a monoclonal antibody against the IL-36R, preferentially binds the IL-36R, blocking IL-36 ligand/receptor interactions, inhibiting IL-1RAcP induction and downstream signaling. Other potential modes of therapeutic intervention currently under investigation include a monoclonal antibody that binds to IL-1RAcP and specific inhibitors of neutrophil-derived proteases. Figure created with Biorender.com.

in promoting healing of perianal fistulas is being analyzed in patients with fistulizing Crohn's disease (NCT03752970).[96] The clinical activity of the drug is also being assessed in UC patients who have previously failed other biological therapy (NCT03482635),[97] and the potential use of spesolimab as an add on to TNFα therapy is also under investigation in UC patients (NCT03123120).[98] Furthermore, the safety and efficacy of long-term spesolimab use is being analyzed in an open-label phase 2 trial in UC patients who have effectively completed previous trials (NCT03648541).[99]

In addition to spesolimab, several other methods are being explored to functionally target IL-36 signaling therapeutically. One of these that is showing promising preclinical results is the use of peptide-based inhibitors for cathepsin G and elastase in skin inflammation.[100] Henry et al recently reported that IL-36 family agonists are proteolytically processed and activated by these 2 neutrophil granule-derived proteases.[52] Taking another step further, the group identified peptide-based pseudosubstrates, which can antagonize the activation of IL-36 agonists via inhibited cathepsin G and elastase signaling. Using tape-stripped psoriatic skin lesions from patients, the authors report elevated levels of IL-36β processing, and this is inhibited with the administration of the peptide pseudosubstrates specific for cathepsin G.[100] Because elevated levels of neutrophil infiltration is characteristic of psoriasis and enhanced neutrophil protease activity has been linked to the disease pathogenesis,[101,

[102] in addition to the success of targeting neutrophil proteases using small molecule inhibitors in lung inflammation,[103, 104] this validates the potential of a success for these peptide therapies in the skin. Furthermore, elevated levels of neutrophil elastase activity have been described in the colon of patients with IBD but not healthy controls[105] and have been implicated in the pathogenesis of the disease.[106] Therefore, targeting neutrophil proteases activation of IL-36 pro-inflammatory agonists represents a potential target for novel therapeutics (Fig. 2).

Another approach under investigation involves simultaneously blocking multiple IL-1 family cytokines associated with IBD pathogenesis to prevent compensatory inflammation when one cytokine is targeted. Højen et al recently developed a monoclonal antibody against the human IL-1RAcP (IL-1R3) and shown it can attenuate the functions of 6 IL-1 family members, including IL-1β and IL-36.[107] Functional studies revealed an inhibition in IL-36-driven Th1 responses in response to anti-IL-1R3 and that blocking IL-1R3 had a broader anti-inflammatory effect than blocking IL-36 signaling alone. Furthermore, treatment with this antibody attenuated disease in murine models of peritonitis, allergic airway inflammation, and psoriasis, conditions known to have multiple cytokines involved in their pathogensis.[107] As described earlier, IBD has pathogenic associations with numerous IL-1 family members, thus rendering IL-1R3 blockade an attractive potential option for therapeutic intervention.

A further consideration for therapeutically targeting IL-36 is the confounding factors that may arise due to IL-36's involvement in the microbiome as described previously. Global inhibition of IL-36R signaling may interfere with mucosal barrier integrity, tissue repair processes, and host protection, thereby offsetting the beneficial effects of blocking the receptor. Therefore, it may be worthwhile to evaluate drugs that neutralize individual IL-36 ligands. This may be of particular relevance when targeting GI fibrosis, as mechanistic studies have highlighted the dichotomy of IL-36R signaling in early vs late disease. Todorović et al recently reported the efficacy of using a small molecule antagonist against IL-36γ in targeting plaque psoriasis.[108] Using a small molecule, high throughput screen, the authors identified A-552 as a potent inhibitor of IL-36γ signaling, and this was confirmed in human preclinical models using punch biopsies from the skin on psoriasis patients. Interestingly, this molecule does not exert an antagonist effect on IL-36α,[108] highlighting the importance of targeted therapeutics for individual patient requirements.

CONCLUSIONS

Remarkable progress has been made in elucidating the main cytokines involved in the pathogenesis of IBD and targeting these in the clinic. Yet, these novel biologics seem to only have a beneficial clinical effect in certain subgroups of IBD patients. This reflects the complexity of cytokine networks at work in the inflamed intestinal tissue, which is constantly influenced by genetic and environmental factors, in addition to immune cell and microbiome plasticity. The IL-36 family has emerged as potent modulators of the intestinal inflammatory response in IBD, regulating both pro- and anti-inflammatory mechanisms. Interleukin-36 ligands are released by various gut resident cells and elicit effects on both immune and nonimmune cells in the mucosa and possibly influence gut resident microbiota, offering multiple targets for therapeutic development. However, due to the complex and often dichotomous roles of IL-36 cytokines reported to date, further studies are required to define the specific mechanisms through which IL-36 signaling may impact GI inflammation across different stages of disease to determine whether it represents a viable target for therapeutic intervention in IBD.

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1O.1.2 KERATINOCYTE INTERLEUKIN-36 RECEPTOR EXPRESSION ORCHESTRATES PSORIASIFORM INFLAMMATION IN MICE.

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Research Article

Keratinocyte interleukin-36 receptor expression orchestrates psoriasiform inflammation in mice

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The IL-36 family cytokines have emerged as important mediators of dermal inflammation in psoriasis and have been reported to provide a proinflammatory stimulus to a variety of immune and stromal cell subsets in the inflamed skin. However, it remains to be determined which cell type, if any, in the skin plays a predominant role in mediating IL-36 cytokines instructive role in disease. Here, we demonstrate that targeted deletion of *Il36r* in keratinocytes results in similar levels of protection from psoriasiform inflammation observed in "global" *Il36r*-deficient mice. Mice with deficiency in IL-36 receptor expression on keratinocytes had significantly decreased expression, comparable with *Il36r*-deficient mice, of established mediators of psoriatic inflammation, including, IL-17a, IL-23, IL-22, and a loss of chemokine-induced neutrophil and IL-17A–expressing γδ T-cell subset infiltration to the inflamed skin. These data demonstrate that keratinocytes are the primary orchestrating cell in mediating the effects of IL-36–driven dermal inflammation in the imiquimod model of psoriasiform inflammation and shed new light on the cell-specific roles of IL-36 cytokines during psoriatic disease.

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Introduction

Psoriasis is a common debilitating autoinflammatory disease of the skin which represents a massive socioeconomic burden, affecting up to 3% of the population, with profound impacts on patient's physical and psychological well-being (Gelfand et al, 2004; Mrowietz et al, 2014). The IL-36 family of cytokines is emerging as central orchestrating mediators of psoriatic disease. The family consists of three separate agonistic ligands, designated IL-36α, IL-36β, and IL-36γ and a specific IL-36 receptor antagonist all of which act through a specific IL-36 receptor (Walsh & Fallon, 2018). Similar to the more extensively characterised "classical" IL-1 cytokines, IL-1α and IL-1β, IL-36 cytokines are also thought to act as important drivers of inflammation but function in a more tissue restricted manner (Gabay & Towne, 2015). A particular focus on the role of IL-36 family members as orchestrators of inflammatory skin disease has recently emerged. This focus stems from the identification of specific mutations in the gene encoding the IL-36 receptor antagonist (*IL36RN*), which reduce its activity leading to the development of an autoinflammatory condition characterised by a severe form of generalized pustular psoriasis (Marrakchi et al, 2011; Onoufriadis et al, 2011). Numerous studies, using both murine models of psoriasis, as well as patient tissues, have expanded and effectively translated these discoveries, further implicating IL-36 family members as central orchestrators of dermal inflammation, even in more prevalent forms of disease such as psoriasis vulgaris (Blumberg et al, 2007, 2010; Carrier et al, 2011; Tortola et al, 2012; Bachelez et al, 2019). This has prompted a significant effort among pharmaceutical companies to develop monoclonal antibody strategies aimed at targeting the IL-36R signaling axis for the treatment of psoriasis, which have very recently been validated in early clinical trials (Ganesan et al, 2017; Mahil et al, 2017). Although the central role of IL-36 cytokines in psoriatic inflammation has been established, many questions surrounding their precise mechanism of action in this setting remain unanswered. IL-36 receptor stimulation has been reported to promote proinflammatory responses in various skin cell subsets, including keratinocytes, fibroblasts, macrophages, dendritic cells, and various T cell subsets (Towne & Sims, 2012; Walsh & Fallon, 2018). However, the question of which responding cell types, if any, are critical in driving IL-36–dependent dermal inflammation is unclear.

We have addressed this question using a newly generated transgenic mouse (*Il36rΔK*), demonstrating that keratinocyte-specific expression of Il36r is required to orchestrate psoriasiform disease. Critically, Il36r deficiency in keratinocytes only mirrored the protection from psoriasiform inflammation, induced through the topical administration of Aldara cream, observed in "globally" Il36r-deficient mice. This protection from skin inflammation occurred in association with a failure to induce the expression of established mediators of psoriatic inflammation including IL-17a, IL-23, and IL-22. In addition, although the heightened expression of IL-36 family cytokines themselves was only marginally altered in the inflamed skin of *Il36rΔK* mice, the induced expression of known IL-36–responsive genes encoding Il17c, and the antimicrobial peptides S100a8 and S100a9 were also diminished. Significantly, loss of Il36r expression in keratinocytes also resulted in a loss of infiltration of neutrophils and IL-17a–expressing Vγ4+ γδ T cells to the inflamed

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skin. This study demonstrates the central orchestrating role for keratinocyte-specific IL-36 responses in driving psoriasiform inflammation.

Results and Discussion

Loss of expression of Il36r in keratinocytes results in similar levels of protection from psoriasiform inflammation to those observed in *Il36r*$^{-/-}$ mice

In an effort to identify which cell types play an instructive role in mediating IL-36–driven dermal inflammation, we generated a novel *Il36r* floxed (*Il36r*$^{fl/fl}$) mouse to facilitate cell-specific deletion of *Il36r* gene, its expression, and responses (Fig S1). Although IL-36 family cytokines have been reported to stimulate various cell subsets of immune and stromal origin in the skin, we sought to specifically examine the role of keratinocytes given their reported expression of the IL-36 receptor among human patients, responses to IL-36 stimulation ex vivo, and established role in the pathogenesis of psoriatic disease (Blumberg et al, 2007, 2010; Carrier et al, 2011; Tortola et al, 2012; Mahil et al, 2016; Madonna et al, 2019). To address this question, we crossed the *Il36r*$^{fl/fl}$ mouse with *K14Cre*$^{+}$ transgenic mice, in which the Cre recombinase is expressed in keratinocytes under the control of the *keratin-14* gene promoter, to generate mice in which Il36r expression was specifically deleted among keratinocytes in the skin (*Il36rΔK* mice) (Wang et al, 1997; Dassule et al, 2000). *Il36rΔK* mice were overtly normal and showed no evidence of baseline-altered skin homeostasis or inflammation, which was comparable with that observed in *Il36r*$^{fl/fl}$ littermates (Fig S2). Specific *Il36r* deletion was confirmed through analysis of IL-36r protein expression in both uninflamed and inflamed skin induced through daily topical administration of 5% Aldara cream, which contains the TLR7 agonist imiquimod, for 6 d, by immunohistochemistry (Fig 1A). These data demonstrate that epidermal keratinocytes represent the major cell type expressing the IL-36r in the skin of wild-type mice and confirm that this expression is lost in *Il36rΔK* mice. We also examined the levels of gene expression of the Il36r in the inflamed skin of these mice, demonstrating that overall Il36r expression is significantly decreased in *Il36rΔK* skin (Fig 1B). Together, these data demonstrate that the Il36r is predominantly expressed in keratinocytes in inflamed skin, and this expression is lost in the *Il36rΔK* mice.

To confirm a specific loss of responsiveness to IL-36 ligands in keratinocytes, we next examined the induction of Il17c gene expression in isolated primary keratinocytes stimulated for 24 h with IL-36α. Expression of Il17c has previously been found to be up-regulated in keratinocytes in response to IL-36 stimulation (Hashiguchi et al, 2018). As predicted, IL-36 responsiveness is lost in keratinocytes isolated from *Il36rΔK* mice (Fig 1C). Importantly, and in contrast to keratinocytes, IL-36α–induced expression of Cxcl1 in *Il36rΔK* dermal fibroblasts is maintained, indicating that functional expression of the IL-36 receptor in these skin-resident stromal cells is maintained in *Il36rΔK* mice (Fig 1D). Similarly, BMDCs from *Il36rΔK* respond to IL-36α to a similar degree as BMDCs from *Il36r*$^{fl/fl}$ mice (Fig S3), demonstrating that functional IL-36r responses are preserved in non-keratinocytes in *Il36rΔK* mice.

To investigate the functional importance of keratinocyte-specific expression of IL-36r in the context of psoriatic inflammation, we analysed disease pathogenesis among *Il36rΔK* mice using the Aldara-induced model of psoriasiform inflammation. This model has previously been reported to be sensitive to treatment with frontline current therapeutic approaches currently in use in psoriasis patients (Pantelyushin et al, 2012; Mitsui et al, 2015; Shibata et al, 2015; Shimizu et al, 2019). Strikingly, deficiency of the IL-36r in keratinocytes alone resulted in a similar level of protection from disease to that observed in *Il36r*$^{-/-}$ mice, whereas wild-type *Il36r*$^{fl/fl}$ littermate controls developed marked disease. Furthermore, this protection was evident in terms of ear thickening (Fig 1E), as well as overall histological scoring incorporating levels of acanthosis, desquamation, parakeratosis, and infiltration, evaluated through hematoxylin and eosin staining of treated skin (Fig 1F and G).

Aldara-induced psoriasiform inflammation in mice has previously been shown to be profoundly regulated by IL-36 family cytokine signaling. In agreement with our study, mice deficient in the *Il36r* across all tissues and cell types have been shown to be largely protected from disease pathogenesis, whereas mice deficient in the *Il36rn* gene (*Il36rn*$^{-/-}$), encoding the IL-36 receptor antagonist, exhibited an exacerbated disease phenotype (Tortola et al, 2012). Similarly, treatment with anti-IL-36 receptor blocking antibodies can effectively suppress psoriasiform inflammation in this model (Ganesan et al, 2017; Mahil et al, 2017). Although no studies to date have investigated which specific responding cell type, if any, plays an important role in mediating these effects, Tortola et al (2012) have reported, through bone marrow chimera studies, that radioresistant cells mediate the pathogenic effects of IL-36r signaling in the Aldara model (Tortola et al, 2012). Furthermore, relative expression levels of the IL-36 receptor were found to be higher in keratinocytes, fibroblasts, and neutrophils compared with dendritic cells and T-cell subsets (Mahil et al, 2017). Such observations are consistent with our findings that IL-36R expression on keratinocytes is required to mediate skin disease.

Expression of Il36r on keratinocytes is required for the activation of key pathogenic pathways in psoriasiform inflammation

As a first step in evaluating the mechanism through which IL-36–dependent keratinocyte responses mediate dermal inflammation, we examined the levels of expression of several genes previously characterised as playing important roles in driving psoriatic skin inflammation. As expected, topical Aldara treatment of wild-type *Il36r*$^{fl/fl}$ skin induced the expression of key proinflammatory mediators such as Il17a, Il23, and Il22. However, these effects were lost in *Il36r*$^{-/-}$ mice and also in mice lacking expression of *Il36r* only in keratinocytes (*Il36rΔK*) (Fig 2A). In addition, the expression levels of Il17c and genes encoding the antimicrobial peptides, S100a8 and S100a9, which are induced by IL-36 cytokines specifically in keratinocytes and implicated in disease progression (Mahil et al, 2017), were also reduced in the skin of *Il36rΔK* mice (Fig 2B).

It has been previously reported that IL-36 family cytokines can promote their own expression in inflamed skin and possibly act in a feed forward fashion, in tandem with other pathogenic cytokines, to perpetuate inflammation and disease pathogenesis (Milora et al, 2015; Hernandez-Santana et al, 2019; Madonna et al, 2019). Therefore, we

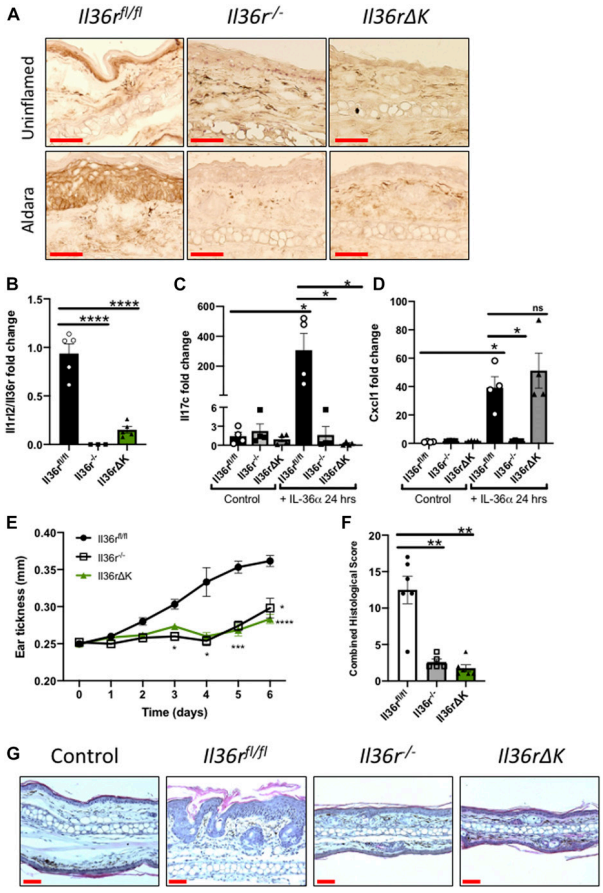

Figure 1. Deletion of *Il36r* gene in keratinocytes results in similar levels of protection from psoriatic inflammation to those observed in *Il36r*$^{-/-}$ mice.
(A) Representative micrographs obtained after immunohistological detection of IL-36R staining using DAB (brown) in fresh frozen sections from mice ears of control, *Il36r*$^{fl/fl}$, *Il36r*$^{-/-}$, and *Il36rΔK* mice after vehicle (uninflamed) or Aldara cream (5% Imiquimod [IMQ]) topical administration for 6 d. Scale bar = 1 μm. **(B)** Relative Il36r gene expression levels in the skin of *Il36r*$^{fl/fl}$ (n = 5), *Il36r*$^{-/-}$ (n = 3), and *Il36rΔK* (n = 5) mice after 7-d Aldara treatment. **(C, D)** Il17c gene expression levels in keratinocytes and (D) Cxcl1 gene expression levels in fibroblasts, untreated, and treated with recombinant mouse IL-36 α for 24 h. **(E, F)** Ear thickness and (F) combined histological scoring of *Il36r*$^{fl/fl}$ (n = 6), *Il36r*$^{-/-}$ (n = 5), and *Il36rΔK* (n = 6) mice after six consecutive days of Aldara cream (5% IMQ) topical administration. **(G)** Representative micrographs obtained after hematoxylin and eosin staining of ear sections of control (vehicle-treated Il36r$^{fl/fl}$) and *Il36r*$^{fl/fl}$, *Il36r*$^{-/-}$, and *Il36rΔK* mice after 6-d Aldara cream topical administration. Scale bar = 1 μm. Data shown in (E) are representative of three independent experiments with similar results. **(B, C, D)** Data show means ± SEM. Statistical analyses were performed using one-way ANOVA multiple comparisons test with Tukey's correction in Fig 1B–D, two-way ANOVA multiple comparisons test with Bonferroni correction in Fig 1E, and Mann–Whitney test in Fig 1F. Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001.
Source data are available for this figure.

investigated whether loss of IL-36r activity among keratinocytes might alter the induction of IL-36 family gene expression in inflamed skin. Interestingly, the induction of Il36a, Il36b, and Il36g gene expression upon Aldara treatment were largely maintained, albeit at slightly reduced levels, irrespective of *Il36r* expression (Fig 2C), indicating that factors, other than the IL-36 cytokines themselves, can drive their own expression in inflamed skin.

These data demonstrate that keratinocyte responses to IL-36 signaling are sufficient to orchestrate the activation of key pathogenic pathways in psoriasis-like dermal inflammation. Although it has previously been established that IL-23 and IL-17a expression and activity are positively regulated by IL-36 cytokines, in the context of dermal inflammation, our findings provide an important advance in identifying keratinocytes as the key cells in mediating these effects (Tortola et al, 2012; Pfaff et al, 2017). In agreement with several previous studies, we have also found that IL-36 cytokines can induce the expression of genes expressed by keratinocytes such as Il17c and S1008a and S1009a. Our analysis of *Il36* family gene expression has revealed that IL-36 cytokines themselves play a relatively minor role in positively regulating their own expression in inflamed skin. In this regard, it is noteworthy that dendritic cells have been shown to play a critical role in mediating IL-36–dependent psoriasiform inflammation in this model. It is tempting to speculate that these cells, upon activation by mediators other than IL-36, can

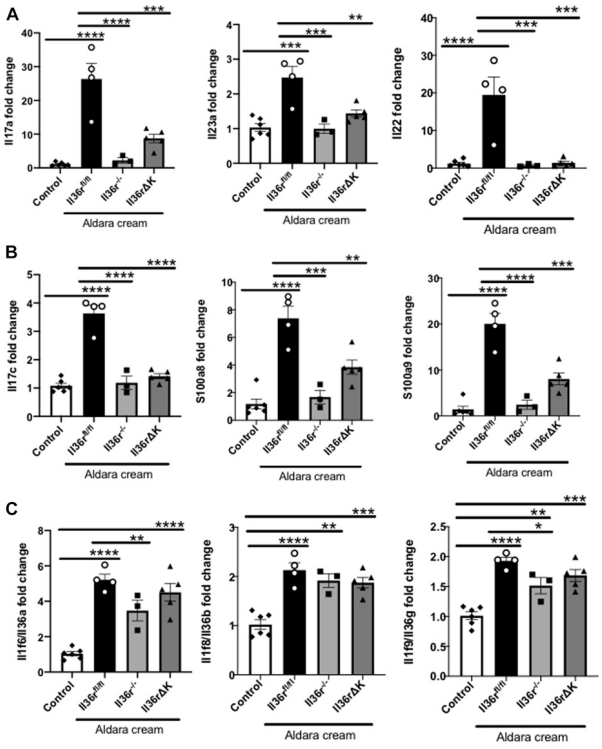

Figure 2. Levels of induced inflammatory gene expression in the skin of *Il36r*$^{fl/fl}$, *Il36r*$^{-/-}$, and *Il36rΔK* mice after 4-d Aldara treatment as determined by qRT-PCR.
(A) Relative gene expression levels of inflammatory mediators associated with psoriatic inflammation Il17a, Il23, and Il22 in cDNA from ear extracts of control uninflamed (n = 6) and *Il36r*$^{fl/fl}$ (n = 4), *Il36r*$^{-/-}$ (n = 3), and *Il36rΔK* (n = 5) mice after 4 d Aldara treatment. **(B, C)** Relative gene expression levels of keratinocyte-associated genes Il17c, S100a8, and S100a9 and, (C) relative gene expression of IL-36 family ligands, Il36a, Il36b, and Il36g. Data show means ± SEM. Statistical analysis was performed of changes in expression relative to control uninflamed skin using one-way ANOVA Multiple comparisons with Tukey's correction. Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001.
Source data are available for this figure.

provide an important source of IL-36 family expression in this setting (Tortola et al, 2012; Numata et al, 2018; Madonna et al, 2019). Together, these findings also have direct relevance to the "feed-forward" model of psoriatic inflammation, wherein T-cell–derived IL-17 family cytokines can act in synergy with IL-36 family members, as well as other inflammatory mediators, to promote keratinocyte hyperplasia characteristic of disease (Milora et al, 2015; Campbell et al, 2017; Pfaff et al, 2017). Our results indicate that IL-36–derived signaling in keratinocytes also plays an earlier role in the initiation of this amplified response through directly regulating IL-17a expression, possibly through the induction of IL-23.

Expression of Il36r on keratinocytes promotes the recruitment of neutrophils and IL-17a–expressing γδ T cells to the inflamed dermis

Psoriasiform inflammation as a result of topical Aldara administration results in the activation and recruitment of inflammatory immune cells which contribute directly to epidermal hyperplasia. Key cell subsets involved in this process include neutrophils and γδ T cells, which provide a major source of pathogenic IL-17a expression (Cai et al, 2011). It has previously been demonstrated that targeting IL-36r signaling in this model can suppress these responses (Tortola et al, 2012). Therefore, we next sought to examine what influence, if any, Il36r expression on keratinocytes plays in the recruitment and activation of these cells. As previously reported, Aldara treatment for 4 d led to a significant infiltration of cells of hemopoietic origin to the skin of wild-type mice when compared with baseline numbers of CD45$^+$ cells present in (control) treated wild-type *Il36r*$^{fl/fl}$ skin (Fig 3A) (Tortola et al, 2012). In contrast, Aldara treatment of *Il36rΔK*, as well as *Il36r*$^{-/-}$, mice did not induce significant infiltration of CD45$^+$ cells, demonstrating the IL-36 cytokine activity in keratinocytes is required to regulate the inflammatory cell infiltration in psoriatic skin (Fig 3A).

In terms of specific pathogenic immune cell subsets, the Vγ4$^+$ subset of γδ T cells are a prominent source of IL-17a in mouse skin and are thought to clonally expand and play a central pathogenic

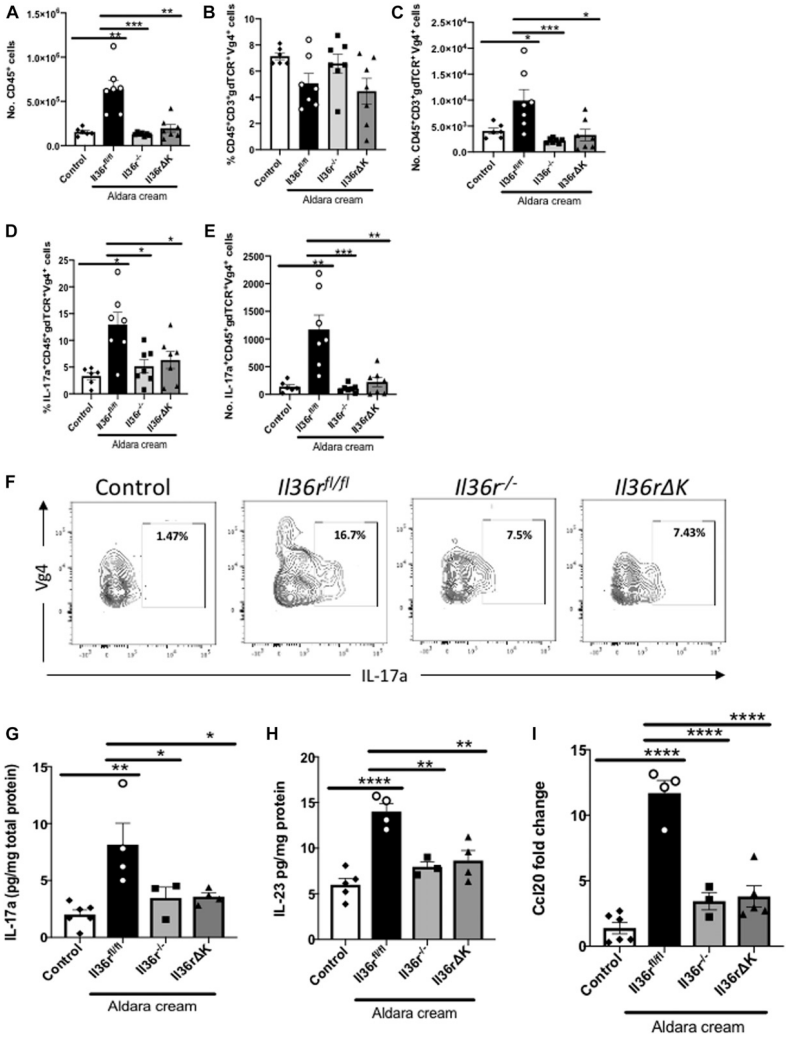

Figure 3. Keratinocyte expression of Il36r directs IL-17a–producing Vγ4$^+$ γδ T cells to the inflamed dermis.
(A) Total numbers of CD45$^+$ cells after 4-d Aldara treatment. **(B, C)** Percentage and (C) total cell counts of Vγ4$^+$ γδ T cells in the ear skin of vehicle-treated (control) (n = 6) and Aldara-treated *Il36r*$^{fl/fl}$ (n = 7), *Il36r*$^{-/-}$ (n = 7), and *Il36rΔK* (n = 7) mice as determined by flow cytometry. **(D, E)** Percentage and (E) total cell counts of IL-17a–producing Vγ4$^+$ γδ T cells in the same mice. **(F)** Representative plots of Il-17a expression levels in Vγ4$^+$ γδ T cells as determined by flow cytometry. **(G, H)** Expression levels of (G) IL-17a and (H) IL-23 protein expressed as pg/mg of total protein in the skin of littermate control (n = 6), and *Il36r*$^{fl/fl}$ (n = 5), *Il36r*$^{-/-}$ (n = 3), and *Il36rΔK* (n = 4) mice. **(I)** Relative Ccl20 gene expression levels in the ear skin of control (n = 6), and Aldara-treated *Il36r*$^{fl/fl}$ (n = 4), *Il36r*$^{-/-}$ (n = 3) and *Il36rΔK* (n = 5) mice. Data show means ± SEM. Statistical

role in the inflamed dermis in the Aldara model (Hartwig et al, 2015; Prinz & Sandrock, 2015; Ramírez-Valle et al, 2015). Therefore, we examined the relative levels of Vγ4$^+$ γδ T cells, as well as their expression of IL-17a, in the inflamed skin of *Il36rΔK*, *Il36r*$^{-/-}$, and *Il36r*$^{fl/fl}$ mice, compared with levels found in uninflamed skin. Although the percentage of Vγ4$^+$γδTCR$^+$ T cells within the CD45$^+$ compartment did not change between groups, the overall numbers of infiltrating Vγ4$^+$γδTCR$^+$ T cells were significantly elevated in the inflamed skin of *Il36r*$^{fl/fl}$ control mice. In contrast, numbers of this subset were significantly, and similarly, reduced in *Il36rΔK* and *Il36r*$^{-/-}$ inflamed skin (Fig 3B and C). As expected, the percentage and number of Vγ4$^+$γδTCR$^+$ T cells expressing IL-17A were also significantly increased in the inflamed skin of *Il36r*$^{fl/fl}$ mice. This increase, both in terms of expression on a per cell basis, and overall cell numbers, was lost in the inflamed skin of both *Il36rΔK* and *Il36r*$^{-/-}$ mice, demonstrating that keratinocyte-specific IL-36r signaling is required for the activation, recruitment, and/or expansion of IL-17$^+$Vγ4$^+$ γδ T cells in the inflamed dermis (Fig 3D–F). Importantly, this overall decrease in IL-17a–expressing cells was reflected in the amount of total IL-17a protein detected in the inflamed skin at this time point (Fig 3G), as well as a failure to induce expression of both IL-23 protein and the Ccr6 ligand, Ccl20, which is a key signal in recruiting IL-17a–expressing cells to inflamed skin (Mabuchi et al, 2011; Campbell et al, 2017) (Fig 3H and I).

In addition to IL-17a$^+$ γδ T cells, we also examined the levels of infiltration of neutrophils as a key pathogenic cell subset, previously reported to be recruited to the inflamed skin by IL-36 cytokines in this model (Tortola et al, 2012). This analysis demonstrated that the recruitment of CD45$^+$CD11b$^+$Ly6G$^+$ neutrophils was significantly diminished, as determined by percentage and overall cell number, in the inflamed skin of both *Il36rΔK* and *Il36r*$^{-/-}$ mice (Fig 4A–C). This lack of neutrophil infiltration occurred in association with a failure to induce protein expression of the neutrophil chemo attractant Cxcl1 in the inflamed skin. Cxcl2 levels were also examined and found to be somewhat reduced in *Il36rΔK* skin, albeit not to significant levels (Fig 4D and E). The numbers of other inflammatory cell subsets, including CD11b$^+$F4/80$^+$ macrophages, CD11b$^+$CD11c$^+$ dendritic cells, and αβ TCR$^+$ T cells were not found to be significantly altered in *Il36r*$^{fl/fl}$ skin in response to Aldara treatment at this time point (Fig S4).

Collectively, these data demonstrate the key instructive role that IL-36 signaling in keratinocytes plays in driving the recruitment to the skin of key pathogenic cell subsets required for the pathogenesis of psoriasiform inflammation. These observations add to the established importance of the IL-36 family in driving the pathogenesis of disease with implications for the future development of therapeutic strategies for patients. In particular, these data indicate that targeting of the IL-36 family specifically in keratinocytes, such as through the topical administration of specific inhibitors may provide a suitable approach. These observations also raise the possibility that IL-36 cytokines may play an important bridging mechanism between environmental factors, such as the skin microbiome and the pathogenesis of psoriatic inflammation. Along these lines, we, and others, have recently shown that these cytokines can alter the composition of the intestinal microbiome (Ngo et al, 2018; Giannoudaki et al, 2019). As this study demonstrates the significance of IL-36 activity on the outermost cells of the skin barrier in driving psoriatic inflammation, it will be of interest to determine whether IL-36 family members can play a similar instructive role on the skin microbiome.

Materials and Methods

Mice

All experiments were performed with 8–16-wk-old male and female mice. All animal experiments were performed with ethical approval by Trinity College Dublin Animal Research Ethics Committee and under license by the Irish Health Products Regulatory Authority (project authorization no: AE19136/P036).

Generation of *Il36rΔK* mice

Il1rl2floxed (*Il36r*^{fl}) transgenic mice were generated on a C57Bl/6 background by Cyagen Biosciences (see Fig S1A for outline of strategy). Exon 4 was selected as the conditional KO region and deletion of this exon was predicted to result in loss of function of the mouse *Il1rl2* gene. Mouse genomic fragments containing homology arms and conditional KO region were amplified from the BAC clone by using high-fidelity Taq and were sequentially assembled into a targeting vector together with recombination sites and selection markers (Fig S1A). After confirming correctly targeted ES clones via Southern blotting, the clones were selected for blastocyst microinjection, followed by chimera production. Founders were confirmed as germ line–transmitted via crossbreeding with wild-type C57Bl/6 mice. Mutant mice received were heterozygous for the transgene. Heterozygous mice were mated to obtain homozygous in house (Fig S1B). Transgenic mice were identified by DNA extraction of ear tissue and amplification by PCR of the transgene. The K14^{Cre} mice (B6N.Cg-Tg(KRT14-cre)1Amc/J) were obtained from Jackson Laboratories (Strain 004782). In these mice, the expression of Cre recombinase in keratinocytes is controlled by a human keratin 14 promoter. Il36rflox mice were mated with K14^{Cre} mice to generate K14^{Cre+}Il36r$^{fl/fl}$ (*Il36rΔK*).

Aldara model of psoriasiform inflammation

Il36r$^{fl/fl}$, *Il36rΔK*, and *Il36r*$^{-/-}$ mice on C57BL/6 background were bred in house. *Il36r*$^{-/-}$ mice were obtained from Amgen under Material Transfer Agreement that is described previously (Russell et al, 2016). Aldara cream (5% imiquimod; MEDA Pharmaceuticals) or Vaseline was applied to adjacent mice ears daily for up to 7 d. Ear thickness

analyses performed were one-way ANOVA multiple comparisons with Tukey's correction in Fig 3G–I, Mann–Whitney test used in Fig 3 A–C and E, and *t* test in Fig 3D. Significant differences are indicated as follows: *P < 0.05; ***P < 0.001; ****P < 0.0001.
Source data are available for this figure.

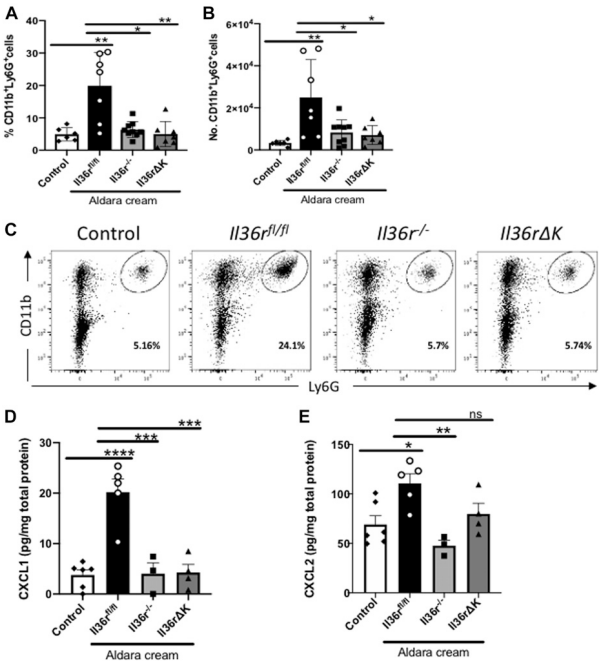

Figure 4. Keratinocyte expression of *Il36r* directs neutrophil infiltration to the inflamed dermis.
(A, B) Percentage and (B) total cell numbers of $CD11b^+Ly6G^+$ cells in the $CD45^+$ cell compartment, from vehicle-treated littermate control (n = 6) as well as $Il36r^{fl/fl}$ (n = 7), $Il36r^{-/-}$ (n = 9), and *Il36rΔK* (n = 7) mice after 4-d Aldara treatment. **(C)** Representative plots for staining of $CD11b^+Ly6G^+$ neutrophils in vehicle-treated and the inflamed dermis of $Il36r^{fl/fl}$, $Il36r^{-/-}$, and *Il36rΔK* mice by flow cytometry. **(D, E)** Protein levels of Cxcl1 and Cxcl2 expressed as pg/mg of total protein from littermate control (n = 6), $Il36r^{fl/fl}$ (n = 5), $Il36r^{-/-}$ (n = 3), and *Il36rΔK* (n = 4) mice after 4-d Aldara treatment. Data show means ± SEM. Statistical analyses were performed using one-way ANOVA multiple comparisons with Tukey's correction except in Fig 4A and B in which Mann–Whitney test was used. Significant differences are indicated as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.
Source data are available for this figure.

was measured before the initiation of the experiment (day 0) and every day subsequently on both Vaseline and Aldara-treated ears using a thickness gauge (Hitec).

Histopathological analysis of tissue

Ear tissues were recovered and fixed overnight in 10% neutral buffered formalin (Medical Supply) before dehydration and embedding into paraffin blocks. Sections of 5 μm thickness were cut, stained with hematoxylin and eosin and scored blindly for pathological manifestations of psoriatic inflammation on a scale up to four (0, no differences over control; 1, mild; 2, moderate; 3, marked; 4, severe) for acanthosis, desquamation, parakeratosis, and infiltration. The scores for each parameter were combined into a total histological severity score.

DAB staining

Ear tissue was recovered and frozen in OCT. 5-μm-thickness sections were obtained using a cryostat (Leica) and 3,3-DAB immunohistochemical staining was performed on the sections using ImmPRESS Anti-Rag Ig Kit and ImmPACT DAB (Brown) peroxidase substrate (Vector Laboratories) following the manufacturer's instructions. The primary antibody used was mouse anti-IL-36R (M616; Amgen) at a dilution of 1/900. The sections were counterstained with Mayer's Hematoxylin for 5 min.

ELISA

Ear tissues from $Il36r^{fl/fl}$, *Il36rΔK*, and $Il36r^{-/-}$ mice were lysed and homogenized with RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Sigma-Aldrich) using Bead-Bug prefilled tubes with 1.5-mm Zirconium beads (Sigma-Aldrich) in a FastPrep-24 5G system (MP Biomedicals). Protein content of the lysates was quantified using Bicinchoninic Acid kit (Sigma-Aldrich) following the manufacturer's instructions. Levels of mouse cytokines Il-17A (homodimer), Il-23 (eBioscience), Cxcl1, and Cxcl2 (DuoSet ELISA; R&D Systems) were measured in protein lysates by ELISA following the manufacturer's instructions. Ears from $Il36r^{fl/fl}$ and *Il36rΔK* littermates were used as non-inflamed control tissue to determine relative levels of induced protein expression.

qRT-PCR

Ear tissues from mice were stored in RNAlater (Sigma-Aldrich) at −80°C. Isolate II RNA Mini kit (Bioline) was used to obtain total RNA following the manufacturer's instructions. The tissues were covered by lysis buffer and disrupted using BeadBug-prefilled tubes with 1.5-mm Zirconium beads (Sigma-Aldrich) in a FastPrep-24 5G system (MP Biomedicals). High-Capacity cDNA kit (Applied Biosystems) was used to perform reverse transcription. Real-time PCR was performed in triplicate using specific TaqMan Gene Expression Assays (Table 1) and TaqMan Fast Universal PCR Master Mix in a QuantStudio 3 System (Applied Biosystems). Normalization was performed using 18S ribosomal RNA, and relative gene expression levels were obtained by the ΔΔCt method. Levels of induced gene expression were determined by comparison with baseline levels found in the ear skin from *Il36r*$^{fl/fl}$ and *Il36rΔK* littermates used as non-inflamed control tissues.

Keratinocytes and fibroblasts isolation

Keratinocytes and dermal fibroblast cells from *Il36r*$^{fl/fl}$, *Il36rΔK*, and *Il36r*$^{-/-}$ adult mouse skin were isolated using protocols described previously (Khan & Gasser, 2016; Li et al, 2017).

To assess the effects of IL-36 on both keratinocytes and fibroblasts, the cells were stimulated with recombinant mouse IL-36α (aa8-160) (R&D Systems) for 24 h, followed by analysis of gene expression for indicated genes by qRT-PCR.

BMDC differentiation and stimulation

Bone marrow was extracted from the femur and tibia of *Il36r*$^{fl/fl}$, *Il36r*$^{-/-}$, and *Il36r*^{ΔK} mice and cultured in 10 ml of complete RPMI (10% FCS + 1% penicillin streptomycin) (cRPMI) supplemented with 20 ng/ml recombinant mouse GM-CSF protein (Sigma-Aldrich) at 37°C. On days 3 and 6 of culture, the medium was refreshed with 10 ml of cRPMI + 20 ng/ml GM-CSF. On day 7, the cells were examined for CD11b/CD11c coexpression by flow cytometry and were then placed in culture at 3 × 10^5 cells/well with or without 100 ng/ml rmIL-36α (R&D Systems) for 24 h. After 24 h, the supernatants were harvested for analysis of Cxcl1 expression by ELISA (R&D Systems).

Table 1. TaqMan assays used in qRT-PCR to obtain gene expression data.

Gen	TaqMan assay code
Ccl20	Mm01268754_m1
Il17c	Mm00521397
Il1f6/Il36a	Mm00457645_m1
Il1f8/Il36b	Mm01337546_g1
Il1f9/Il36g	Mm00463327_m1
Il1rl2/Il36r	Mm00519245_m1
Il22	Mm01226722_g1
Il23	Mm01160011_g1
Il17a	Mm00439618_m1
S100a8	Mm00496696_g1
S100a9	Mm00656925_m1
Cxcl1	Mm04207460_m1

Cell isolation for flow cytometry analysis

Ears were cut into small pieces that were digested with 3 mg/ml Dispase II in HBSS (Sigma-Aldrich) solution for 90 min at 37°C with agitation. After removing Dispase II solution, the tissues were digested with 1.5 mg/ml Collagenase D (Roche) in PBS for additional 90 min at 37°C with agitation. After digestion, the cell extracts were washed with RPMI with 10% FCS and 5 mM EDTA and disaggregated into single-cell suspension by dissociation of tissues and passing through 100- and 40-μm cell strainers consecutively.

Flow cytometry

Surface and intracellular protein expression of cells was analysed using a BD LSRFortessa Cell Analyzer (BD Biosciences) with further analysis carried out using FlowJo software (BD Biosciences). Harvested cells from individual mice were first counted by trypan blue exclusion and an aliquot taken for analysis of CD45 expression levels to evaluate infiltrating cell numbers. For intracellular staining of IL-17a, the remaining cells were stimulated with PMA (10 ng/ml), ionomycin (1 μg/ml), and brefeldin A (5 μg/ml) during 4 h, then surface staining was performed and cells were fixed and permeabilized using eBioscience Foxp3/Transcription Factor Staining Buffer Set (eBiosciences) following the manufacturer's instructions. Alternatively, for analysis of neutrophils, the cells were stained immediately with the indicated surface markers, without restimulation and subsequently analysed. The following mouse antibodies were used for surface staining CD11c (N418), Ly6G (1A8), F4/80 (BM8), TCRβ chain (H57-597), CD45 (30-F11), CD11b, Vγ4 (UC3-10A6) (BioLegend), TCR gamma/delta (eBioGL3), and CD3e (145/2C11). Intracellular staining was performed using the mouse antibody Il-17a (eBio17B7). Dead cells were excluded from all analyses using Aqua Live/Dead stain (Invitrogen). All antibodies were purchased from eBioscience unless otherwise stated. The gating strategy used to identify and quantify immune cell subsets is shown in Fig S5.

Statistical data analysis

Data were assessed for normal distribution and homoscedasticity. One-way ANOVA or Mann–Whitney U-test was applied as indicated in figure legends to compare the differences among the groups using Prism 8 software (GraphPad). Statistical significance details for each graph are indicated in the respective figure legends.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.201900586.

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Author Contributions

YE Hernandez-Santana: conceptualization, data curation, formal analysis, investigation, and writing—original draft.
G Leon: data curation, formal analysis, investigation, and writing—original draft.
D St Leger: data curation and investigation.
PG Fallon: resources.
PT Walsh: conceptualization, data curation, formal analysis, investigation, and writing—original draft.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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10.1.3 CURRENT PERSPECTIVES ON THE INTERLEUKIN-1 FAMILY AS TARGETS FOR INFLAMMATORY DISEASE.

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Clinical

European Journal of Immunology HIGHLIGHTS

REVIEW

Current perspectives on the interleukin-1 family as targets for inflammatory disease

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Since the first description of interleukin-1 (IL-1) and the genesis of the field of cytokine biology, the understanding of how IL-1 and related cytokines play central orchestrating roles in the inflammatory response has been an area of intense investigation. As a consequence of these endeavours, specific strategies have been developed to target the function of the IL-1 family in human disease realizing significant impacts for patients. While the most significant advances to date have been associated with inhibition of the prototypical family members IL-1α/β, approaches to target more recently identified family members such as IL-18, IL-33 and the IL-36 subfamily are now beginning to come to fruition. This review summarizes current knowledge surrounding the roles of the IL-1 family in human disease and describes the rationale and strategies which have been developed to target these cytokines to inhibit the pathogenesis of a wide range of diseases in which inflammation plays a centrally important role.

Keywords: Canakinumab · Inflammation · Inflammatory disease · Interleukin-1 family · Therapy

Introduction

Since the earliest descriptions of the prototypical cytokine interleukin-1 (IL-1) as a soluble endogenous pyrogen, the investigation of how this cytokine, along with subsequently identified related family members, can play centrally important roles in inflammation continues to expand [1]. While these early discoveries can justifiably be described as having contributed to the genesis of the field of cytokine biology, the IL-1 family of cytokines quickly became recognised as critical regulators of inflammatory processes across a range of human diseases [2]. As a result, there has been significant interest in targeting these cytokines for therapeutic intervention in patients where inflammation contributes to the mechanistic basis of disease [3].

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The IL-1 family consists of seven agonistic ligands (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-37), and three specific receptor antagonists (IL-1Ra, IL-36Ra and IL-38) which mediate their effects through four distinct heterodimeric receptor complexes. These receptors include specific ligand binding chains (IL-1R1, IL-33R (ST2), IL-18Rα and IL-36R), which upon ligand interaction recruit either the IL-1R accessory protein (IL-1RAcP), in the case of IL-1R1, IL-33R and IL-36R, or the IL-18Rβ chain in the case of the IL-18R (Fig. 1) [4]. Once activated, these receptor complexes initiate pro-inflammatory intracellular signalling cascades driven by NF-KB and MAPK dependent pathways and can play instructive roles in driving both innate and adaptive inflammatory responses both systemically and in a tissue specific manner.

The basic mechanisms which define the integral role of the IL-1 family of cytokines in the regulation of inflammation have been recently described in extensive detail elsewhere [5, 6]. In this review, we provide an overview of the rationale and strategies employed to target the activity of individual IL-1 family members

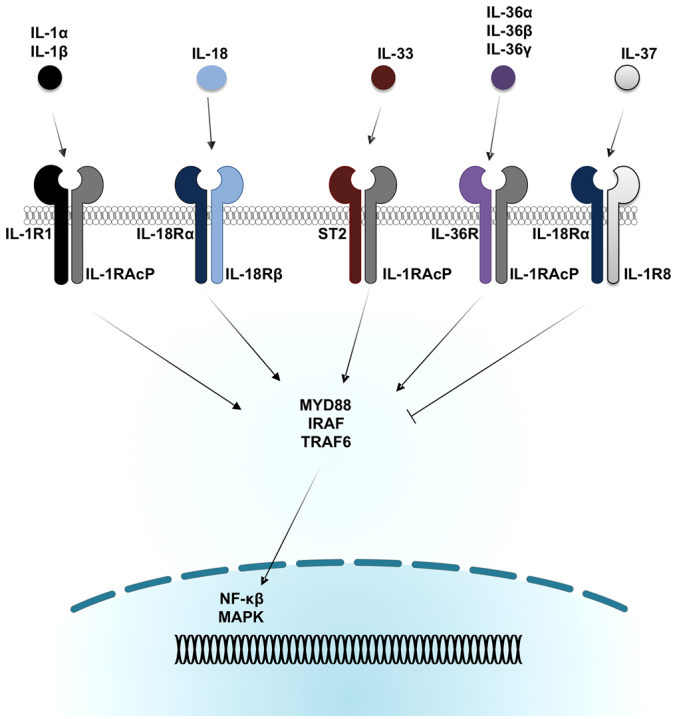

Figure 1. Members of the IL-1 family of cytokines and their cognate receptors. IL-1, IL-33 and IL-36 cytokines initiate signalling by binding to the IL-1R, ST2 and IL-36R respectively, which, upon ligand binding, form heterodimeric receptors with the IL-1RAcP. This results in the activation of MAPK and NFKB signalling, and subsequent pro-inflammatory gene expression, via MyD88, IRAF and TRAF6 dependent signalling mechanisms (as denoted by the arrows). Similarly, IL-18 activates these same pathways through the formation of an IL-18Rα and IL-18Rβ heterodimeric receptor. In contrast, IL-37 initiates an immunosuppressive signalling programme through interacting with IL-18Rα in association with IL-1R8 (SIGIRR).

across a range of inflammatory disease conditions. Furthermore, we describe how such efforts have also confirmed the previously underappreciated role for inflammation as a contributory mechanism in the pathogenesis of diseases such as atherosclerosis, which had been considered to develop through largely noninflammatory mechanisms. While these approaches have already realized hugely significant impacts in terms of patient care, these benefits will almost certainly expand as we learn more about the mechanisms through which the activity of these cytokines are regulated, as well as their specific roles in mediating the inflammatory response.

Regulation of IL-1 family cytokine activity in homeostasis and inflammation

As IL-1 cytokines play a central role in inflammation a number of distinct mechanisms have evolved to restrict their activity and maintain homeostasis [2]. In providing an endogenous balance against unregulated activity, such mechanisms are a centrally important consideration in efforts to modulate IL-1 family activity to treat disease. In addition, discoveries revealing that several autoinflammatory disease conditions result from monogenic mutations in genes which critically regulate IL-1 family member activity, has added hugely to our understanding of how these cytokines direct inflammation and homeostasis. Such observations have underscored significant, and ongoing, efforts aimed at targeting IL-1 family activity for therapeutic benefit among patients.

As well as the distinct proinflammatory ligands described above, the IL-1 family also consists of endogenous specific receptor antagonists, such as IL-1Ra and IL-36Ra. When these antagonists are present in excess, they preferentially bind to IL-1R1 and IL-36R respectively, thereby inhibiting ligand interaction and appropriate receptor assembly. As well as IL-1Ra, a decoy receptor IL-1R2 has been identified which inhibits the activity of IL-1α

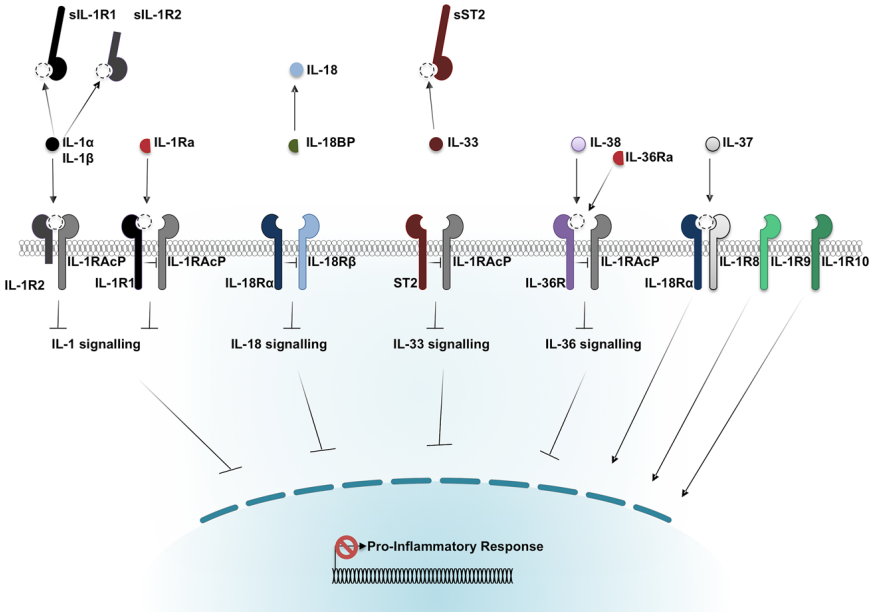

Figure 2. Negative Regulation of IL-1 family signalling. (From left to right) Specific mechanisms have evolved to regulate proinflammatory signalling by IL-1 family cytokines and their receptors. These include inhibition of IL-1α/β through neutralisation with soluble forms of the IL-1R (sIL-1R1/2), specific inhibition of the IL-1R complex through association with cell surface expressed IL-1R2, or direct antagonism through excess IL-1Ra expression. IL-18 activity is regulated through activity of the IL-18 binding protein (IL-18BP) which preferentially binds free IL-18 restricting its ability to interact with the IL-18R. Similarly, IL-33 bioavailability and activity is limited by the expression of the soluble form of its receptor ST2 (sST2). Initiation of IL-36 dependent signalling is regulated through competitive binding to the IL-36R of the specific IL-36Ra and also through the activity of IL-38. Uniquely, IL-37 acts to restrict proinflammatory responses through engagement with the IL-18Rα chain, utilizing IL-1R8 as a coreceptor. IL-1R9 and IL-1R10 are more recently described IL-1 family receptors which are also thought to exert a negative regulatory/ inhibitory function.

and IL-1β through preferential sequestration of these ligands both intra, as well as extracellularly. As IL-1R2 lacks an intracellular TIR domain, required to initiate signalling pathways, it can act as a molecular sink for IL-1 cytokines. Interestingly, IL-1R2 (and IL-1R1) can also be found in a soluble form where it may exert these neutralizing effects in a cell extrinsic fashion. In a similar way, IL-18 binding protein (IL-18BP) and a soluble form of the IL-33R (sST2) can act to neutralize the activity of their respective cytokines in the extracellular microenvironment (Fig. 2) [7]. Most recently, IL-38 has been shown act in a similar fashion to IL-36Ra, as an inhibitor of IL-36R dependent inflammation [8]. Indeed, upon its initial discovery the IL-38 protein was found to share 41% homology to IL-1Ra and 43% homology to IL-36Ra, which strongly indicated a similar antagonist function. However, unlike IL-36Ra, IL-38 appears to act as a 'non classical' antagonist, with inhibitory effects only observed at lower concentrations, indicating that it acts in a mechanistically distinct fashion from IL-36Ra.

Further negative regulation of IL-1 family signalling is provided by negative regulatory receptors which act to restrict proinflammatory signalling at the cell surface. These include IL-1R8 (also known as TIR8, SIGIRR), IL-1R9 and 10 (also known as TIGIRR2 and TIGIRR1) [4]. IL-1R8 is unique among IL-1 family receptors in that it contains a single extracellular Ig domain as well as specific amino acid substitutions in its intracellular TIR domain. IL-1R8 expression has been found to suppress IL-1 family receptor signalling through blocking the assembly of receptor complexes, and preferential sequestering of intracellular adaptor molecules, such as MyD88, which are required for the initiation of pro-inflammatory signalling [9]. More recently, it has been proposed that IL-1R8, in conjunction with the IL-18Rα chain, can act as a receptor for the immunosuppressive IL-37 and may play a role in mediating its anti-inflammatory activity (Fig. 2) [10]. The activity of IL-1R9 and IL-1R10 are far less extensively characterized, although homology of both receptors with IL-18BP may indicate similar regulatory roles, particularly in tissues of the central

nervous system [4]. Similar to the role of IL-1R8 in mediating IL-37 immunosuppression, it has also been proposed that IL-1R9 may act as a coreceptor for IL-38 and play an important role in mediating its anti-inflammatory effects [11]. Indeed IL-38 has recently been found to ameliorate skin inflammation, possibly through interacting with IL-R9 [12].

Interleukin-1α/β

Unsurprisingly, most progress has been made in our understanding of the respective roles of the initially discovered family members IL-1α and IL-1β. Both cytokines stimulate inflammatory responses through binding to the type 1 IL-1 receptor (IL-1R1) and this interaction can be blocked through preferential binding of the endogenous interleukin-1 receptor antagonist (IL-1Ra). Despite exerting similar pro-inflammatory activity in this way, there are several important distinguishing features between both ligands. IL-1α is largely membrane bound and plays a predominantly local rather than systemic role. By contrast, IL-1β is the primary circulating form of IL-1, involved in a broad spectrum of inflammatory disorders. Although full length IL-1α can exhibit functional activity, it can be further processed by the Ca^{2+} dependent protease calpain, as well as other inflammatory proteases, to potentially enhance its pro-inflammatory activity [13, 14]. Low levels of IL-1α are constitutively expressed in numerous cell types, in particular epithelial cells, vascular endothelium, keratinocytes and platelets, and its expression is increased upon exposure to stress/inflammatory signals. As well as in its membrane-associated form, IL-1α can also be expressed intracellularly in cytosolic and nuclear fractions [15]. While cell surface IL-1α acts locally by activating IL-1R1 signalling in an intra- and paracrine manner, nuclear localized IL-1α is linked to pro-inflammatory activation of transcription [16]. Cytosolic and nuclear IL-1α are also recognised as "alarmins" which can act to initiate sterile inflammation upon release into the microenvironment during the course of cell injury or necrotic cell death [17].

In contrast, the precursor of IL-1β (ProIL-1β) is biologically inactive, with its expression driven by microbe-associated molecular patterns (MAMPs), danger-associated molecular patterns (DAMPs), as well as cytokines such as tumour necrosis factor-α (TNFα), IL-18, IL-1α or IL-1β itself. ProIL-1β cleavage, to its active form, is mediated by inflammasomes, such as NOD-like receptor protein 3 (NLRP3), that activate the cysteine protease caspase-1 [18]. Caspase-1–independent activation of ProIL-1β, mediated by neutrophil-derived serine proteases, has also been described [19–21]. The expression, activation and secretion of IL-1β is mainly associated with innate immune cell subsets including monocytes, macrophages, and dendritic cells and IL-1β is generally considered to be a major pro-inflammatory mediator of the systemic inflammatory response. Ligand binding to IL-1R1 leads to the recruitment of the IL-1 receptor accessory protein (IL-1RAcP), enabling the initiation of pro-inflammatory signalling in a process which is under tight regulation through the distinct mechanisms described above.

Disruption of such regulatory mechanisms has been associated with several rare autoinflammatory disease conditions. These diseases are driven by dysregulated IL-1β activity, and arise due to inherited mutations in genes encoding proteins which regulate IL-1 production and secretion. Such conditions, described to date, include Familial Mediterranean Fever (FMR), Cryopyrin-Associated Periodic Syndrome (CAPS) which includes Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells Syndrome (MWS) and Neonatal-Onset Multisystem Inflammatory Disease (NOMID), as well as others with no known genetic association. These conditions typically present as periodic fever, neutropenia, fatigue, myalgia, elevated CRP levels, and in severe cases with joint deformation and developmental disability [22, 23]. Unsurprisingly, therapeutic intervention with specific therapies targeting IL-1 have been found to dramatically improve patient outcomes in these conditions (Table 1) [24–26].

Targeting IL-1 in human disease

Currently three different therapeutic strategies have been approved to target the activity of IL-1 in an expanding number of systemic inflammatory diseases, with many more in clinical development [27]. Anakinra, a recombinant, non-glycosylated human IL-1Ra, which acts to broadly inhibit inflammation mediated by both IL-1α and IL-1β, was FDA approved in 2001 for the treatment of adult rheumatoid arthritis (RA) and NOMID. Rilonacept, also referred to as IL-1 Trap, is a dimeric fusion protein consisting of portions of IL-1R1 and the IL-1RAcP linked to the Fc portion of immunoglobulin G1. Rilonacept functions as an inhibitor with affinity for IL-1α, IL-1β and IL-1Ra, and is in clinical use for the treatment of CAPS, in adults and children. Canakinumab is an anti-IL-1β monoclonal antibody that binds to human IL-1β and neutralizes its inflammatory activity by blocking its interaction with IL-1 receptors while sparing effects from IL-1α or IL-1Ra. Canakinumab was originally approved in 2009 and indicated to treat FCAS, MWS, as well as systemic juvenile idiopathic arthritis (sJIA) [28]. Administration of canakinumab every 2 weeks offers an advantage over the human IL-1Ra (anakinra) which due to rapid clearance must be injected daily, and is often poorly tolerated by patients for the above indicated conditions [29]. In addition to notable success in treating patients with rare autoinflammatory diseases as described above, targeting IL-1 activity has shown considerable promise in more common disease states considered to have inflammatory IL-1 activity as a key pathogenic mechanism.

Common inflammatory diseases responsive to anti IL-1 therapies

While investigations into inhibiting IL-1 activity across a broad spectrum of human inflammatory disease conditions continue, in certain settings, clear efficacy has been reported. These include rheumatological diseases such as gout and RA, metabolic disorders

Table 1. IL-1 family targeted therapies

IL-1 targeted therapy	Therapeutic strategy	Target	IL-1 associated disease
Anakinra	Recombinant IL-1Ra	IL-1R1	**CAPS** (17), **Rheumatoid Arthritis** (24), **Gout** (21), **Osteoarthritis** (24), **Type 2 Diabetes** (27), **Colorectal Cancer** (41), **Cardiovascular Disease** (35,36)
Rilanocept	IL-1R1 and IL-1RAcp fusion protein	IL-1α IL-1β IL-1Ra	**CAPS** (18), **Gout** (22)
Canakinumab	IL-1β neutralizing antibody	IL-1β	**CAPS** (19), **Stills Disease**, **Gout** (23), **Type 2 Diabetes** (28), **Cardiovascular Disease** (37), **Lung Cancer** (37)
Tadekinig Alfa	Recombinant IL-18 Binding Protein	IL-18	**AOSD** (63, 64)
GSK3772847/ CNTO-7160	ST2 blocking antibody	IL-33R/ST2	**Asthma** (NCT03393806, NCT03207243*), **Atopic Dermatitis** (NCT02345928*)
AMG282/ MSTT1041A/ RG6149	ST2 blocking antibody	IL-33R/ST2	**Asthma** (NCT01928368, NCT02918019*), **Atopic Dermatitis** (NCT03747575*), **COPD** (NCT03615040*), **Chronic rhinosinusitis with nasal polyps** (NCT02170337)
ANB020/ Etokimab	IL-33 neutralizing antibody	IL-33	**Asthma** (NCT03469934*), **Atopic Dermatitis** (NCT03533751*), **peanut allergy** (NCT02920021), **Chronic rhinosinusitis with nasal polyps** (NCT03614923*)
SAR440340/ REGN3500	IL-33 neutralizing antibody	IL-33	**COPD** (NCT03546907*), **Asthma** (NCT02999711, NCT03112577, NCT03387852*), **Atopic Dermatitis** (NCT03738423, NCT03736967*)
BI655130	IL-36R blocking antibody	IL-36R	**Ulcerative Colitis** (NCT0364854*), **Crohns Disease** (NCT03752970*), **Generalized Pustular Psoriasis** (NCT02978690*)
ANBO19	IL-36R blocking antibody	IL-36R	**Generalized Pustular Psoriasis** (NCT03619902*), **Palmoplantar Pustulosis** (NCT03633396*)

*Clinical trial details can be accessed at www.clinicaltrials.gov.

including Type 2 diabetes (T2D) and atherosclerosis and recent developments in the treatment of malignancy (Table 1).

In the instance of gout, swollen and painful joints, resulting from the formation and deposition of pro-inflammatory uric acid crystals, are symptoms of disease pathogenesis. Uric acid crystals, along with TLR stimulation through fatty acid DAMPS, can induce IL-1β secretion in vitro, mimicking the inflammation in the joints of gout patients and prompting the investigation of anti-IL-1 therapies in this setting [30]. Gout patients respond well to anakinra [31], rilonacept [32] and canakinumab [33] with a reduction in clinical severity and prolonged periods without flares compared to conventional steroid treatments. Anakinra has also been effective in many other joint and muscular diseases including osteoarthritis and RA with a reduction in joint destruction observed during the course of disease progression [34]. Despite such observations, a question remains as to whether, particularly among RA patients, anti-IL-1 agents offer any significant improvement over current dominant therapeutics in use such as anti-TNF biologics.

In T2D, obesity can lead to insulin resistance, with a gradual loss of insulin producing beta cells, resulting in hyperglycaemia. Glucose has been shown to directly drive IL-1β expression from beta cells [35] and can increase deposition of amyloid polypeptide which enhances IL-1β expression and contributes to the beta cell loss in diabetic disease [36]. Treatment of T2D with Anakinra or canakinumab have been found to improve insulin production and glycaemic control, while leading to decreased levels of C-reactive protein (CRP) and IL-6 [37, 38]. These studies point to a beneficial effect of anti-IL-1 therapy in T2D, likely through specific blockade of IL-1β. However, a small scale preliminary trial in patients with

T2D using an IL-1α neutralising antibody (MABp1), reduced levels of glycosylated haemoglobin (HbA1c), an indicator of the average blood glucose levels in the previous 2–3 months, suggesting IL-1α blockade may also be beneficial [39]. A possible protective role for canakinumab and anakinra has also been assessed in recent-onset Type 1 diabetes. However, while both agents were found to be safe, they were not effective as single immunomodulatory drugs in improving β-cell function among these patients [40].

IL-1 has also been at the centre of hypotheses linking inflammation with the pathogenesis of atherosclerosis. Cholesterol crystals, an established pathogenic stimulus in cardiovascular disease, can serve as an endogenous danger signal when engulfed by inflammatory monocytes to directly trigger the NLRP3 inflammasome and IL-1β production [41]. Furthermore, in preclinical animal models of atherosclerosis, NLRP3 deficient mice displayed reduced plaque severity and protection from disease, providing a mechanistic link between cholesterol deposition and a systemic pro-inflammatory state in atherosclerosis disease [42]. More recent studies have reported that cholesterol crystals interact with neutrophils to trigger the release of neutrophil extracellular traps (NETs) which prime macrophages to produce the precursor ProIL-1β [43]. IL-1 has also been implicated as mediating pathological events that can occur in the heart following a myocardial infarction, including inflammation and remodelling which act to weaken viable heart muscle tissue contributing to subsequent heart failure [44]. Pilot studies, examining the effects of administration of anakinra for 14 days following myocardial infarction, led to lowering of CRP levels, as well as reduced progression to heart failure [45]. Similarly, anakinra has also been reported to improve clinical scores associated with poor outcomes among ischaemic stroke patients [46]. These studies, alongside the reported beneficial effects of targeting IL-1 in Type 2 diabetic patients with increased risk of atherosclerotic disease, led to the design of The Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS). The CANTOS trial was the first large scale human trial initiated to test the inflammation hypothesis of atherothrombosis, through specifically targeting IL-1β. The primary outcome from the CANTOS trial assessed the incidence of cardiovascular death, myocardial infarction, or stroke and demonstrated that canakinumab was superior to placebo at preventing these adverse cardiac events [47]. This confirmation that targeting IL-1 can benefit patients in an array of cardiovascular disease settings, will likely prompt further investigations, as more information concerning the role of IL-1 in cardiovascular pathology is uncovered.

The significance of IL-1 in tumour development is also currently under investigation. IL-1α and IL-1β have been found to be abundantly expressed in advanced tumours and associated with higher tumour grade and invasiveness [48]. In addition, preclinical studies in mice have demonstrated that blocking IL-1β decreases tumour invasion, growth, and metastases indicating that specifically targeting IL-1β may be beneficial [49]. Anakinra, which neutralizes both IL-1 ligands, has been reported to inhibit colon tumour growth in preclinical studies [50] and has been investigated as a therapy across various malignancies. In metastatic colorectal cancer patients, anakinra administration was reported to provide significant survival benefit and improved quality of life scores, when added to standard-of-care chemotherapy for colorectal cancer [51]. However, in such settings the relative short half-life of anakinra and requirement for continuous administration is problematic and can hinder patient adherence, prompting investigation of anti-IL-1 neutralising antibodies [52, 53]. Along these lines, an IL-1α neutralizing antibody (MABp1) has been demonstrated to improve symptoms in patients with metastatic or unresectable colorectal cancer in a phase 3 study, revealing its potential to be used along with standard of care in the advanced treatment stages of colorectal cancer [54]. As canakinumab had been hypothesized to reduce metastatic disease in part through alteration of adhesion molecule function, incident cancers were tracked in the CANTOS trial. Here canakinumab significantly reduced the incidence of lung cancer and deaths from all cancers [47]. This has subsequently led to the design of a combination trial looking at canakinumab with a programmed cell death protein 1 (PD-1) inhibitor in patients with non-small cell lung cancer [55]. As more trial results are reported greater evidence supporting a role for targeting IL-1 in cancer therapy programs can be assessed more fully.

Interleukin-18

Similar to IL-1β, IL-18 is expressed as an intracellular precursor protein (ProIL-18) which undergoes caspase-1 mediated cleavage into its active form [56]. However, unlike IL-1β, the IL-18 precursor protein is expressed constitutively across a range of different cell types and tissues and exhibits a distinct immunostimulatory profile [57]. Most notably, IL-18 has been described as a potent stimulator of IFNγ expression and was originally identified as IFNγ inducing factor [58]. In this capacity, IL-18 is recognized as a Th1 response inducing factor alongside IL-12, and indeed requires the presence of IL-12 to elicit this function. As well as enhancing IFNγ expression from CD^{4+} T cells, IL-18 can also work in concert with IL-12 to drive NK cell effector function and expression of IFNγ, and induce NK cell expansion through enhanced IL-2 sensitivity [59–61]. IL-18 induces such responses through stimulating a unique heterodimeric receptor consisting of IL-18Rα ligand binding and IL-18Rβ accessory chains. Similar to the IL-1RAcP and other IL-1 family receptors, while the IL-18Rα chain is widely expressed, the IL-18Rβ chain, required to induce intracellular signalling, is usually absent from the cell surface unless its expression is induced by other pro-inflammatory factors such as IL-12 [57].

ProIL-18 may also be released from dying cells whereupon it can be activated extracellularly, in a caspase-1 independent fashion, by neutrophil or cytotoxic cell derived proteases [62, 63]. In its secreted form, active IL-18 is tightly regulated by the IL-18BP, which exhibits a high affinity for the cytokine and is present in the serum of healthy individuals in significant molar excess over active IL-18 [64, 65]. Disruption of this balanced regulation leads to enhanced IL-18 driven inflammatory responses which can, if unchecked, play important roles in disease.

Targeting IL-18 in human disease

While efforts to target IL-18 in human disease are less advanced compared to IL-1, there has been significant progress in this regard. Similar to IL-1, distinct autoinflammatory conditions have recently been described in which elevated IL-18 activity has been implicated in disease pathogenesis [66]. These include NLRC4 associated autoinflammatory disorders, in which patients with gain of function mutations in the *NLRC4* inflammasome gene, exhibit systemic inflammation, characterised by a macrophage activation syndrome(MAS)-like presentation and severe enterocolitis early in life [67, 68]. Notably, IL-1 blockade (anakinra) as a therapeutic strategy for these patients was found to have mixed results, indicating that alternative mediators may play a more prominent role. Indeed, it was noted that very high levels of 'free' IL-18 were present in patients' serum, offering a potential mechanistic basis for disease and, following these observations, it has recently been shown that treatment with recombinant IL-18BP offers an effective therapeutic approach [69, 70].

MAS is also known as secondary haemophagocytic lymphohistiocytosis (sHLH), and is a life-threatening condition which can arise as a complication of infection, malignancy and rheumatic diseases such as systemic juvenile idiopathic arthritis (sJIA) and adult-onset Still's disease (AOSD). Several reports have demonstrated that levels of 'free' and total IL-18 are elevated in many sHLH patients, while it has recently been reported that levels of 'free' IL-18 are also significantly elevated among patients with sJIA and AOSD [71–75]. These observations provided a sound rationale for an investigation of recombinant IL-18BP as a treatment for AOSD patients in a phase II study, which demonstrated that targeting IL-18 represents an effective therapeutic strategy [76, 77]. Although the studies described above have clearly established a therapeutic benefit of targeting IL-18 activity in human disease, it is as yet unclear whether these effects can completely be ascribed to the inhibition of its IFNγ inducing activity. In this regard, it is notable that preclinical studies indicate that IL-18 can elicit a wide range of both pro-inflammatory, and indeed pro-resolving effects, independent of downstream IFNγ activity, across a range of different tissues [57].

IL-18 activity has also been extensively characterised in the pathogenesis of several other chronic inflammatory disease conditions including Crohn's disease, rheumatoid arthritis, psoriasis, cardiovascular disease and respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma [57]. As such, it seems likely that inhibition of IL-18 may represent a suitable strategy for disease intervention. In particular, targeting IL-18 activity for conditions associated with intestinal inflammation/colitis such as inflammatory bowel diseases may offer significant potential. The rationale for this approach is underscored by the clear pathogenic function of IL-18 in the colon demonstrated in elegant preclinical studies by several groups, as well as the severe colitis evident among NLRC4 associated autoinflammatory disease patients described above [67, 68, 78, 79]. Indeed, both recombinant IL-18BP and an anti-IL-18 neutralising antibody have been investigated as potential therapeutics for Crohn's disease, although to date it remains unclear as to whether this approach has been successful. As our understanding of the role of IL-18 as a mediator of inflammation in the context of human disease continues to expand, its potential as a novel therapeutic target for further conditions is also likely to grow.

Interleukin-33

IL-33 was identified in 2005 through computational analysis of sequence databases, while searching for a ligand for the, until then, orphan receptor ST2, which was originally identified in 1989 [80, 81]. IL-33 is mainly expressed by epithelial cells, endothelial cells and fibroblasts, across various tissues including the lung, skin, stomach and central nervous system [80, 82]. In homeostasis, IL-33 is normally localized in the cell nucleus, and in common with IL-1α, the full-length cytokine is biologically active [83, 84]. Moreover, it has been demonstrated that cleavage of full-length IL-33 by apoptotic caspase-7 or 3 leads to an inactive form of the cytokine. Apoptotic cells release the inactive truncated form of IL-33, while necrotic cells can release the full-length IL-33 which is biologically functional [84, 85]. Thus, IL-33 also acts as an alarmin or DAMP and is secreted in response to necrosis and tissue damage, leading to the recruitment and activation of immune cells. After release, IL-33 can further mature into more bioactive isoforms through processing by neutrophil and mast cell proteases [86, 87].

IL-33 signals through a heterodimeric receptor complex comprised of ST2 and IL-1RAcP, while a soluble form of ST2 (sST2) acts as a decoy receptor, negatively regulating IL-33 signalling. ST2 is expressed in the cell surface of various immune cells, and binding of IL-33 predominantly induces type 2 immune responses, underlining its important role in helminth infection, allergy and asthma. IL-33 signalling promotes cytokine and chemokine production and degranulation of mast cells, basophils, and eosinophils. It is a major activator of type 2 innate lymphoid cells (ILC2) and Th2 cells, and also acts on Treg cells, alternatively activated macrophages, dendritic cells and NK cells. IL-33 promotes the differentiation of macrophages towards an alternatively activated phenotype, and induces eosinophilia and goblet cell hyperplasia by activating ILC2 cells to produce IL-5 and IL-13 [88–90].

Targeting IL-33 in human disease

To date, much of the development of strategies to target IL-33 has focused on its role in allergic inflammation. In mouse models of asthma and allergic airway inflammation, blockade of the IL-33/ST2 axis by anti-IL-33 neutralising antibodies or administration of sST2 has been effective in attenuating airway inflammation [91, 92]. Anti-IL-33 treatment has also been effective in allergic rhinitis models, as well as other forms of airway inflammation including cigarette smoke-induced lung inflammation and a fungal induced asthma model in mice [93–95]. IL-33 is also implicated in chronic obstructive pulmonary disease (COPD), where

elevated levels of IL-33 were found in the lungs of patients, and in a mouse model of chronic obstructive lung disease induced by parainfluenza virus infection [96]. In chronic rhinosinusitis with nasal polyps, a type 2 mediated inflammatory disease, epithelial cell derived IL-33 and IL-33-responsive ILC2s producing IL-13, appear to play an important role in disease pathogenesis [97]. Other allergic diseases in which IL-33 appears to play an important role include food allergy and anaphylaxis [98–100], and atopic dermatitis (AD) [101]. In a mouse model of food anaphylaxis, blockade of ST2 or deficiency of ST2 gene expression attenuated disease severity [100]. In both humans and mouse models of AD, IL-33 was elevated in skin keratinocytes [101]. Overexpression of IL-33 in the skin of mice causes an AD-like pathology [102], whereas IL-33 blockade or deficiency in IL-33 or ST2 genes can reduce disease severity in other models [103, 104].

IL-33 has also been implicated in the pathogenesis of other non-allergic inflammatory conditions. Expression of both IL-33 and ST2 were found to be elevated in the synovium and serum of patients with rheumatoid arthritis [105–108], and disease activity was attenuated in a mouse model of collagen induced arthritis, by blocking IL-33 signalling [105, 106]. A possible role for IL-33 in central nervous system (CNS) inflammation was also indicated by increased levels of IL-33 in the serum, peripheral leukocytes and CNS of multiple sclerosis patients [109]. However, studies in the experimental autoimmune encephalomyelitis (EAE) mouse model have shown conflicting results as to whether IL-33 exerts positive or negative effects in disease development. [110–112]. Similarly, IL-33 appears to play dichotomous roles in the gastrointestinal system. IL-33 is elevated in colonic epithelial and lamina propria cells in ulcerative colitis (UC), and its expression correlates with disease activity [113, 114]. However, preclinical studies in mice have resulted in conflicting outcomes, with various studies describing both pathogenic and pro-resolving roles for IL-33 in intestinal inflammation [115–120].

Three distinct strategies for therapeutic targeting of the IL-33/ST2 axis are currently in development: soluble decoy receptors, IL-33 neutralizing antibodies, and anti-ST2 blocking antibodies [121]. Anti-IL-33 and anti-ST2 antibodies are currently under evaluation in clinical trials for a range of allergic conditions including asthma, atopic dermatitis, peanut allergy, chronic rhinosinusitis with nasal polyps, as well as in COPD (Table 1). Decoy receptors, including 'IL-33 Trap', which is a fusion of sST2 and the accessory protein IL-1RAcP, are also under development but have not yet been fully investigated in a clinical setting [122]. Before therapeutic strategies targeting IL-33 can be further explored in non-allergic inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis, further investigation is required to determine a definitive role of IL-33 in these settings.

Interleukin-36

The IL-36 cytokine subfamily was reclassified from earlier designations in 2010 [123], having also been identified previously based upon sequence homology with other IL-1 family members. The IL-36 subfamily consists of three agonistic ligands, IL-36α, β, γ, and one endogenous antagonist, IL-36Ra [124], all of which bind specifically to the IL-36 receptor, which is a heterodimer of the IL-36R (IL-1Rrp2) subunit and IL-1RAcP. When present in excess, IL-36Ra can bind to the IL-36R and inhibit recruitment of IL-1RAcP and the subsequent activation of downstream signalling pathways [125]. IL-38 has also been described as a possible IL-36 subfamily member, which can act as an antagonist of IL-36R [8]. Similar to other IL-1 family members, IL-36 cytokines also appear to require proteolytic processing to achieve optimal activity, in a process which is thought to be caspase-1 independent, and occurs in the presence of neutrophil derived proteases [126]. Although different cell types have been found to express IL-36 family members, including macrophages, dendritic cells (DCs), neural cells, T cells, keratinocytes, fibroblasts and epithelial cells in various tissues [124], their function is largely associated with epithelial barrier surfaces. Accordingly, much of the focus on investigating the role of the IL-36 cytokines in driving inflammatory diseases has thus far focused on such tissue sites as the skin and gastrointestinal tract [127].

Targeting IL-36 in human disease

In the skin, IL-36 cytokines are of particular relevance in the pathogenesis of a severe form of psoriatic inflammation known as generalized pustular psoriasis (GPP). GPP is a common manifestation among patients with loss-of-function mutations in the *IL36RN* gene, which encodes IL-36Ra, leading to a rare autoinflammatory condition known as Deficiency of Interleukin-36 Receptor Antagonist (DITRA) [128–131]. Several preclinical studies have shed further light on the role of IL-36 regulation of dermal inflammation during psoriasis pathogenesis. Blumberg et al. first demonstrated an important role for these cytokines in promoting dermal inflammation in mice, which shared many characteristics with human psoriasis [132]. These observations were followed by detailed preclinical studies by Tortola et al., demonstrating that IL-36 cytokines can orchestrate psoriasis from inflammation [133]. Using the imiquimod induced model of psoriasis, it was observed that deletion of the *Il1rl2* (IL36R) gene significantly diminished psoriatic inflammation in mice, while disease was severely exacerbated in the absence of *Il36rn,* the IL-36Ra gene. In this setting, IL-36 cytokines were found to direct the infiltration and activation of macrophages, neutrophils and IL-17A expressing γδ T cells to the inflamed skin [133]. Interestingly, bone marrow chimera studies indicated that radioresistant cells are implicated in directing psoriasis-like inflammation in this model, suggesting that non-hematopoietic cell expression of IL-36R is required for psoriasis onset. In support of these observations, it has also been demonstrated that keratinocytes can both express, and respond to, IL-36 family cytokines, which act in concert with IL-17A in an amplification cycle to propagate dermal inflammation [134]. The identification of a monogenic association of the IL-36Ra gene among DITRA

patients has sparked significant interest in targeting the IL-36 family to treat psoriatic inflammation. While it seems intuitive that such a strategy is likely to have significant impact among DITRA patients, a recent clinical study has indicated that this approach will also benefit GPP patients in which the *IL36RN* gene is not mutated, including those with gain of function mutations in the *CARD14* gene, or indeed, no identified genetic association[135]. IL-36 blockade may be a suitable approach, not only for these relatively rare autoinflammatory conditions, but also for more common forms of psoriasis, particularly among patients who are unresponsive to current frontline therapies. Indeed, the use of anti-IL-36R blocking antibodies has been shown extensively to inhibit psoriasis like dermal inflammation in mice providing validation for this approach [136, 137]. In addition, individuals with loss of function mutations in the *IL1RL2* gene, encoding the IL-36R, appear to have normal overall immune function, indicating that IL36R blockade represents a safe therapeutic option in humans [137].

As well as important drivers of dermal inflammation, several studies have also investigated the role of the IL-36 family in intestinal inflammation. In colon tissues, elevated levels of IL-36α and IL-36γ have been detected in patients with Crohn's disease and ulcerative colitis [138–141]. Similar to related IL-1 family members, the role of IL-36 in the gut is complex. While some studies have demonstrated that IL-36 has a pro-inflammatory role [138, 142], other studies have described its role in promoting the resolution of intestinal inflammation [140, 141]. On the one hand, IL-36 signalling facilitates neutrophil and inflammatory monocyte infiltration to intestinal tissues and can regulate the balance of pro-inflammatory mucosal CD^{4+} T cell subsets [138, 143, 144]. In contrast, IL-36R signalling can also promote the recovery of intestinal damage and accelerate mucosal healing [140, 141]. In addition, a more recent study has demonstrated that IL-36R signalling may promote intestinal fibrosis in both mice and patients with inflammatory bowel disease [145]. Although these data have underscored a current clinical evaluation of anti-IL-36R blocking antibodies among IBD patients, given such contrasting outcomes, further studies are required to determine precisely how IL-36 cytokines can mediate such apparent opposing effects in the gut.

While most studies to date have focused on IL-36 as mediator of inflammatory diseases in the skin and intestine, these cytokines have also been implicated in diseases of other tissues. Patients affected by rheumatoid arthritis, psoriatic arthritis and osteoarthritis all have elevated levels of IL-36 family members [146]. However, *Il1rl2* deficient mice were not found to exhibit any alterations in the pathogenesis of arthritic disease in preclinical models examined to date [147]. IL-36 cytokines may also play a role in the lung, as IL-36γ is highly expressed in asthma patients [148]. However, it is currently unclear as to what the mechanistic significance of these observations are.

Interleukin 37

Uniquely among the IL-1 family, IL-37 appears to act to restrict inflammatory responses in a variety of cells and tissues. Originally identified in 2000, its specific role as a natural immune suppressive cytokine has only recently come to light, sparking significant interest [149, 150]. Requiring proteolytic processing, possibly through caspase-1, for full activity IL-37, can act in both an extracellular and intracellular capacity to suppress inflammation. In its secreted form, IL-37 appears to bind the IL-18Rα chain leading to the subsequent recruitment of IL-1R8 as a coreceptor, resulting in the activation of anti-inflammatory intracellular signalling pathways [10]. The human *IL37* gene encodes 5 transcripts, of which the IL-37b isoform has been the most studied. However, unlike other IL-1 family members, no homologue of IL-37 has been found to exist in mice, arguably hindering the investigation of its unique function. Humanised IL-37 transgenic mice have been helpful in this regard and have revealed that IL-37 can exhibit broad immunosuppressive functions inhibiting both innate and adaptive immune responses [149]. Most notably, these mice exhibit reduced severity across a range of inflammatory disease models including experimental colitis, obesity driven metabolic disease and endotoxic shock among others [10, 151, 152].

A possible role for IL-37 in human disease is also beginning to be uncovered. Perhaps most significantly, several mutations in the *IL37* gene been described to be associated with the severity of rheumatoid arthritis, ankylosing spondylitis and coronary artery disease [153–155]. Expression levels of IL-37 have also been described to be altered in many disease settings with both increased and decreased levels observed [149]. Together these observations raise significant implications for the possibility of harnessing IL-37 activity to treat inflammatory disease in humans. In this regard it is particularly noteworthy that administration of recombinant IL-37 has also proven effective in treating preclinical models of inflammatory diseases including obesity dependent metabolic disease, asthma, endotoxaemia and rheumatoid arthritis [156–159]. Such observations hold considerable promise for the future translation of these effects to patients.

Conclusion

While unquestionable progress has been made in advancing therapeutic targeting of the IL-1 family in inflammatory disease over recent decades (Table 1), new discoveries continue to expand possibilities and reveal novel indications where such approaches may be of benefit [160]. Recent discoveries surrounding the mechanisms which regulate the expression and activation of IL-1 family members are also opening possibilities for novel approaches to target these pathways e.g. through NLRP3, protease and gasdermin D/pyroptosis inhibitors [161–163]. While a greater understanding of the specific, and often unique, roles each family member plays in driving systemic and tissue specific inflammatory responses will expedite these endeavours, some significant gaps in our knowledge still remain. In particular, the importance of more recently identified anti-inflammatory family members such as IL-37 and IL-38 in human disease have yet to be clarified. Similarly, uncovering the respective roles of intrinsic negative regulatory receptors such as IL-1R8, in homeostasis and disease, will likely represent critical

steps forward. As the field develops, it is probable that the number of strategies to target the IL-1 family will continue to expand across a broader range of inflammatory disease indications.

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Abbreviations: **CAP**: Cryopyrin-associated periodic syndrome · **DAMPs**: Danger-associated molecular patterns · **DITRA**: Deficiency of interleukin-36 receptor antagonist · **FCAS**: familial cold autoinflammatory syndrome · **FMR**: familial mediterranean fever · **IL-1**: Interleukin-1 · **IL-1Ra**: Interleukin-1 receptor antagonist · **IL-1RAcP**: IL-1 receptor accessory protein · **ILC2**: innate lymphoid cells · **MAMPs**: Microbe-associated molecular patterns · **MWS**: Muckle-Wells syndrome · **NOMID**: neonatal-onset multisystem inflammatory disease

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10.2 IN PRESS:

10.2.1. UCP3 RECIPROCALLY CONTROLS CD4+ TH17 AND TREG CELL DIFFERENTIATION.

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