The role of IL-18 in intestinal immune regulation



Oliver James Harrison

Lincoln College, University of Oxford

A thesis submitted for the degree of

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Abstract

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Elevated levels of the cytokine interleukin-18 (IL-18) are found in many chronic inflammatory disorders, including inflammatory bowel disease (IBD). However, the role of IL-18 in mucosal immunity and inflammation is not well understood. At mucosal and environmental interfaces, Th17 cells have been shown to contribute to protection from pathogenic infection. In contrast, regulatory T (Treg) cells maintain intestinal homeostasis by preventing aberrant inflammatory responses to the resident microbiota. We demonstrate that under homeostatic conditions, colonic Th17 cells highly express IL-18 receptor (IL-18R1) and that intestinal epithelial cell production of IL-18 acts directly on $CD4^+$ T cells to limit colonic Th17 differentiation. Furthermore, whilst IL-18R1-signalling is dispensable for induction of colitis, we observed a critical role for IL-18R1-signalling in $Foxp3^+$ Treg mediated control of colitis. Together, these studies demonstrate that the intestinal epithelium regulates colonic $CD4^+$ T cell responses through production of the cytokine IL-18.

Education is an admirable thing, but it is well to remember from time to time that nothing that is worth knowing can be taught – Oscar Wilde

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Abbreviations

AIRE	Autoimmune regulator
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Antigen presenting cell
APECED	Autoimmune polyendocrine candidiasis ectodermal dystrophy
APRIL	A proliferation inducing ligand
ASF	Altered Schaedler flora
Bcl11b	B-cell lymphoma/leukemia 11b
Blimp-1	B lymphocyte induced maturation protein 1
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cell
CCR	Chemokine (CC) motif receptor
CD	Crohn's disease
CFU	Colony forming unit
cLPL	Colon lamina propria leukocytes
CSR	Class switch recombination
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCR	Chemokine (CXC) motif receptor
CX_3CR	Chemokine (CXXXC) motif receptor
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
DTR	Diphtheria toxin receptor
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
Foxp3	Forkhead box P3
FSC	Forward scatter
Gadd45β	Growth arrest and DNA damage 45 β
GALT	Gut-associated lymphoid tissue
GATA3	GATA binding protein 3
GF	Germ-free
GFP	Green fluorescent protein
GI	Gastrointestinal
GITR	Glucocorticoid-induced tumour necrosis factor receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome-wide association study
H&E	Haematoxylin and eosin
H.h.	*Helicobacter hepaticus*
HPRT	Hypoxanthine phosphoribosyl transferase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
IMDM	Iscove's Modified Dulbecco's Media
i.p.	Intraperitoneal
i.v.	Intravenous
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
IRF4	Interferon regulatory factor 4
iTreg	Induced regulatory T cell

JAK	Janus kinase
LIP	Lymphopenia induced proliferation
LP	Lamina propria
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAMP	Microbe-associated molecular pattern
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MyD88	Myeloid differentiation primary response gene (88)
NFκB	Nuclear factor κB
NK cell	Natural killer cell
NKT cell	Natural Killer T cell
NLR	NOD-like receptor
NLRC	NLR family CARD domain-containing protein
NLRP	Nucleotide-binding domain and leucine rich repeat containing protein
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
OX40	Tumour necrosis factor superfamily member 4
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
PP	Peyer's patch
PRR	Pathogen recognition receptor
RA	Retinoic acid
Rag	Recombination activating gene
RaP	Receptor accessory protein
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RORγt	Retinoic acid receptor-like orphan receptor γt
RPMI	Roswell Park Memorial Institute Medium
S.D.	Standard deviation
S.E.M	Standard error of the mean
SFB	Segmented filamentous bacteria
sIgA	Secretory immunoglobulin A
Smad	Sma and Mad Related Family
SPF	Specified pathogen free
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor expressing in T cells
TCR	T cell antigen receptor
T_{FH}	T follicular helper cell
TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
Tr1 cell	T regulatory type 1 cell
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRIF	Tir-domain-containing-adaptor protein inducing IFN-β
tTreg	Thymic Treg cell
Tyk2	Tyrosine kinase 2
UC	Ulcerative colitis

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Chapter 1 Introduction

1.1 General Introduction

The mammalian immune system is charged with the formidable task of detecting and eliminating threats to the host, including microbial pathogens and tumours, whilst maintaining tolerance to beneficial or innocuous foreign and self-antigens. Two branches of immunity have co-evolved in this regard, with the innate immune system providing immediate defence against pathogens and instructing initiation of antigen-specific adaptive immune responses that provide immunity upon subsequent exposure or infection. The balance between tolerance and immunity is key to host survival, and this is especially true at mucosal surfaces, particularly the intestine. The gastrointestinal tract is a site of preferential colonization by commensal organisms, consisting of fungal, viral and a multitude of bacterial species. Initial microbial colonization of the host occurs during birth and continues until a stable commensal microbiota is established during childhood (Tannock 2007). Microbial colonization of the gastrointestinal tract acts as a vital triggering stimuli for maturation of the mucosal immune system and the presence of a commensal microbiota further benefits the host by providing resistance to invading pathogens and metabolism of dietary components (Macpherson et al. 2005, Hooper et al. 2012). A dynamic molecular dialogue between microbiota and host ensures this colonization occurs as a state of mutualism, the breakdown of which can result in chronic pathologies of the gastrointestinal tract such as inflammatory bowel diseases (IBD) (Kaser et al. 2010, Maloy and Powrie 2011).

1.2 Multiple mechanisms enforce intestinal immune homeostasis

1.2.1 Intestinal epithelial cells (IEC)

The intestine constitutes a vast network of non-lymphoid and secondary lymphoid tissues, and as such, is home to numerous populations of leukocytes. Reflecting the unique challenge of maintaining intestinal immune tolerance, many of these cellular populations are found to be enriched in, or exclusive to, the intestine. In addition to

the array of hematopoietic cell populations critical for immune tolerance within the intestine, the intestinal mucosa is anatomically specialized to promote homeostasis (Hill and Artis 2010, Harrison and Maloy 2011). The intestinal epithelium consists of a single layer of specialized intestinal epithelial cell (IEC) subsets derived from multi-potent and highly proliferative $Lgr5^{+}$ stem cells located within the intestinal crypts (van der Flier and Clevers 2009). Whilst the cellular composition of the intestinal epithelium varies with anatomical location, both the colon and small intestine possess populations of absorptive and secretory cells, including enterocytes, colonocytes, goblet, endocrine and Paneth cells (Wright 2000, Simons and Clevers 2011). Goblet cell-derived mucins form a layer of membrane tethered and soluble mucus components that create not only a viscous protective barrier but a matrix loaded with secretory IgA and IEC-derived antimicrobial peptides (AMP) forging a layer impermeable to the majority of intestinal bacteria (Hill and Artis 2010). The finding that mice deficient in the major mucin, Muc2, develop spontaneous colitis highlights the importance of mucus production in immune homeostasis within the intestine (Heazlewood et al. 2008). However, in addition to compartmentalising commensal bacteria, IEC engage in an active dialogue with the luminal contents. Pattern-recognition receptors (PRR), including Toll-like receptors (TLR) and Nod-like receptors (NLR), are germ line encoded sensors of microbial and host-derived danger signals (Schroder and Tschopp 2010). Tonic PRR-signalling within intestinal epithelial cells (IEC) drives cell-intrinsic proliferation, survival, AMP production and fortification of intercellular tight junctions, limiting bacterial translocation to the lamina propria (Maloy and Powrie 2011). Furthermore, homeostatic PRR-signalling within the intestinal epithelium serves to regulate not only localization but also composition of the microbiota. For example, deficiency in the cytosolic PRR, NLRP6, results in an altered intestinal microbiota with elevated abundance of the bacterial phyla *Bacteroidetes* (*Prevotellaceae*) and TM7 (Elinav et al. 2011). NLRP6-deficient mice also exhibited increased susceptibility to experimental DSS (dextran sulphate sodium) colitis and elegant cross-fostering and cohousing experiments revealed the

colitogenic microbiota to be both vertically and horizontally transmissible (Elinav et al. 2011). Thus, the intestinal epithelium utilises multiple pathways to influence the localization and composition of the microbiota to promote intestinal tolerance. Dysbiosis, an imbalance in the composition of microbial communities, occurs in patients with IBD, though whether this is cause or a consequence of the inflammatory environment remains unclear (Kaser et al. 2010).

1.2.2 Antigen presenting cells (APC)

In line with their important roles in host defense and tolerance, the intestine contains abundant and heterogeneous populations of myeloid antigen presenting cells (APC). Subsets from distinct developmental origins vary in anatomical localization, phenotype and function (Coombes and Powrie 2008, Varol et al. 2010). Recent studies of intestinal APC have utilised the differential expression of CD103 (αE integrin) and CX_3CR1 (receptor for Fractalkine (CX3CL1)) to identify two major tissue resident populations, which appear to contribute to intestinal tolerance in distinct ways (Varol et al. 2010). $CD103^+$ DC can take up intestinal antigens and following CCR7-dependent migration to the mesenteric lymph node (MLN), initiate T cell responses with an intestinal tropism through induction of intestinal homing receptors CCR9 and $\alpha4\beta7$ (Iwata et al. 2004, Johansson-Lindbom et al. 2005, Jaensson et al. 2008). Under homeostatic conditions, $CD103^+$ DC preferentially promote regulatory T cell responses (Coombes et al. 2007, Sun et al. 2007, Schulz et al. 2009). However, this function is not hard-wired and is controlled by factors in the intestinal microenvironment providing a mechanism through which the balance between tolerance and immunity can be controlled (Laffont et al. 2010, Kinnebrew et al. 2012). In addition to effects on T cell mediated immunity, $CD103^+$ DC also promote T cell independent immunoglobulin A (IgA) class switch recombination by intestinal B cells contributing to host defense and intestinal barrier function (Uematsu et al. 2008). By contrast, intestinal CX_3CR1^+ APC (consisting of dendritic cells and macrophages) are thought to be non-migratory and are poor primers of naïve T cells (Schulz et al.

2009). CX_3CR1^+ APC accumulate within the lamina propria following microbial colonization where they promote intestinal tolerance through a variety of mechanisms (Niess and Adler 2010). CX_3CR1^+ APC lie adjacent to the epithelial barrier, and project transepithelial processes into the intestine to sample luminal antigens (Rescigno et al. 2001, Niess et al. 2005). Additionally, whilst largely hypo-responsive to PRR agonists, colonic macrophages remain highly phagocytic and aid intestinal tolerance through clearance of apoptotic cells and debris, whilst also acting to expand populations of regulatory T cells (Murai et al. 2009, Hadis et al. 2011, Smith et al. 2011). Together these data indicate that lymphoid and tissue-resident populations of APC collaborate to instigate and maintain a tolerogenic intestinal microenvironment.

1.2.3 B cells and IgA

B cells are abundant within the gut associated lymphoid tissue and an intimate relationship exists between the commensal microbiota and intestinal B cell responses. Originating within the bone marrow, B cells are dedicated to the production of antibodies, which is initiated through V(D)J recombination to generate IgM and IgD expressing immature B cells (Stavnezer et al. 2008). Antigen recognition elicits somatic hyper-mutation (SHM) and class switch recombination (CSR) to increase antibody affinity and generate distinct effector responses through utilisation of different constant antibody regions, including, IgE, IgG and IgA. Secretory IgA (sIgA) is a major class of antibody production at mucosal surfaces, including within the intestine where the Peyer's patches represent a key site for induction of IgA responses in a mechanism dependent upon transforming growth factor β_1 (TGF-β_1) (Cerutti 2008). sIgA is a dimeric antibody that binds to the polymeric Ig receptor on the basolateral surface of IEC and is subsequently translocated across the epithelium and released into the mucus layer. sIgA inhibits binding of commensal bacteria and soluble antigens to the intestinal epithelium and their progression to the lamina propria. Commensal microbiota trigger IEC to produce

B cell activating factor (BAFF)(Katsenelson et al. 2007) and a proliferation-inducing ligand (APRIL)(He et al. 2007), both factors that promote IgA$^+$ B cell induction. Induction of IgA is highly specific to the invoking commensal bacteria where it acts to limit the composition and function of the commensal microbiota (Hapfelmeier et al. 2010) and alterations in relative abundance of commensal bacteria occur as a result of IgA-deficiency (Fagarasan et al. 2002, Wei et al. 2011).

1.2.4 Innate Lymphoid Cells (ILC)

Innate Lymphoid Cells (ILC) are a recently recognised leukocyte lineage, which arise from a common lymphoid precursor and share functional characteristics with CD4$^+$ T cells, with the key exception of rearranged antigen receptors (Spits and Cupedo 2012). ILC are currently categorised into 3 distinct lineages characterised by expression of lineage specifying transcription factors, ILC1 (T-bet$^+$), ILC2 (GATA3$^+$) and ILC3 (Rorγt$^+$) resembling Th1, Th2 and Th17 cells respectively (Pearson et al. 2012, Spits et al. 2013). ILC are emerging as key regulators of the commensal microbiota and are essential for early innate immune protection from mucosal pathogens (Monticelli et al. 2011, Sonnenberg et al. 2012). Commensal microbiota derived signals drive IL-22 production from ILC3 (Satoh-Takayama et al. 2008, Sanos et al. 2009, Sawa et al. 2011) which is essential to limit dissemination of potentially pathogenic *Alcaligenes xylosoxidans* (Sonnenberg et al. 2012) and for protection from the murine attaching/effacing pathogen, *Citrobacter rodentium* (Sonnenberg et al. 2011). Conversely, dysregulated ILC responses can promote chronic tissue pathology within the intestine (Buonocore et al. 2010), and similar pro-inflammatory populations of ILC3 have been reported in patients with IBD (Geremia et al. 2011).

1.3 CD4$^+$ T cells

1.3.1 Thymic selection of CD4$^+$ T cells

Within adults, continuous production of T cells requires the trafficking of bone marrow derived common haematopoietic stem cell progenitors to the thymus (Bhandoola and

Sambandam 2006). Multi-potent precursors develop within a supportive environment of stromal and haematopoietic APC, where passage through a $CD4^-$ $CD8^-$ double negative (DN) stage limits potential for development to other lymphoid and myeloid lineages (Chi et al. 2009). Rag-dependent rearrangement of the T cell antigen receptor (TCR)β locus and assembly of a surface preTCR complex allows DN thymocytes to proceed through β-selection, halt somatic recombination of TCRβ gene loci and up-regulate expression of both CD4 and CD8 co-receptors to become $CD4^+$ $CD8^+$ double positive (DP) thymocytes (Schatz and Ji 2011). DP thymocytes undergo active rearrangement of the TCRα gene locus, resulting in production of a functional TCRαβ complex on the thymocyte cell surface. Somatic recombination of T cell antigen receptor (TCR) gene loci in immature thymocytes results in production of many T cells with potentially useful antigen specificity. By contrast, many thymocytes develop with irrelevant antigen specificity, or furthermore, with potentially self-reactive antigen receptors. However, thymic selection mechanisms function to restrict and sculpt the TCR repertoire. Thymocytes bearing a TCR with affinity for self-peptides are positively selected and receive survival signals from Major histocompatibility complex class-II (MHC-II) expressing cortical thymic epithelial cells (cTEC) (Stritesky et al. 2012). Thymocytes failing to proceed beyond positive selection die by neglect. Thymocytes having undergone positive selection are further subject to negative selection. DP thymocytes cease somatic recombination of the TCR gene loci, down regulate either CD4 or CD8 expression and migrate to the thymic medulla where they interact with medullary thymic epithelial cells (mTEC) presenting tissue specific antigens in an Aire-dependent manner (Mathis and Benoist 2009). Thymocytes bearing rearranged TCR with intermediate affinity for self-peptides proceed through the negative selection checkpoint, whilst those with high affinity (self-reactive clones) undergo apoptosis to remove them from the mature T cell pool (clonal deletion). At the threshold between intermediate and high affinity TCR, clonal diversion of $Foxp3^+$ regulatory T (Treg) cells occurs, allowing emergence of peripheral $CD4^+$ T cells with high self-reactivity able to enforce dominant peripheral

tolerance (Xing and Hogquist 2012). The role for Aire and Foxp3 in central and peripheral tolerance respectively, is highlighted by human patients bearing mutations in these genes that develop fatal autoimmune disorders early in life (Bennett et al. 2001, Wildin et al. 2001, Anderson et al. 2002, Anderson et al. 2005, Villasenor et al. 2005, Ziegler 2006, Mathis and Benoist 2009). Finally, mature T cells that have undergone thymic selection leave the thymus to re-circulate through the bloodstream and secondary lymphoid tissues (Gowans 1959).

1.3.2 Differentiation of $CD4^+$ T cell subsets.

The functional specialisation of $CD4^+$ T cells allows effective tailoring of the immune response to a diverse array of pathogens and immunogenic insults. A combination of environmentally derived factors are key to $CD4^+$ T cell differentiation during the initiation and maintenance of an immune response, including TCR signalling, co-stimulation, metabolic signals and the surrounding cytokine milieu (Zhu et al. 2010). In the time since the original description of Th1 and Th2 subsets by Mosmann and Coffman (Mosmann et al. 1986, Mosmann and Coffman 1989), additional states of $CD4^+$ T cell lineage specification have been described, including regulatory T (Treg) cells and Th17 cells. These are individually described below in greater detail.

1.3.3 Th1 cells

Differentiated $CD4^+$ T cells are characterised by distinct expression of cell surface molecules, transcription factors and production of inflammatory cytokines. Th1 cells are identifiable by their production of IFN-γ, although they may also secrete IL-2, TNF-α, GM-CSF and lymphotoxin and by their expression of the lineage-specifying transcription factor T-box expressed in T cells (T-bet) (Mosmann et al. 1986, Szabo et al. 2000, Zhu et al. 2010). Activation of naïve $CD4^+$ T cells in the presence of IFN-γ drives signal transducers and activators of transcription 1 (STAT1) dependent induction of T-bet and IFN-γ-inducible transcription factor 1 (IRF1) (Lighvani et al. 2001, Kano et al. 2008). The lineage-specifying transcription factor, T-bet, up-

regulates IL-12Rβ2 expression in conjunction with IRF-1-dependent expression of IL-12Rβ1, which together form a functional receptor for IL-12 (Mullen et al. 2001, Afkarian et al. 2002, Kano et al. 2008). Activation of STAT4 downstream of IL-12 stimulation further augments IFN-γ production and T-bet expression and orchestrates full commitment to, and heritability of, the Th1 lineage (Mullen et al. 2002, Yang et al. 2007, Schulz et al. 2009). IL-12 stimulation also triggers STAT4-activation and DNA-binding at the promoter and conserved non-coding sequences (CNS) of the *Il18r1* locus where it promotes transient histone H3 and H4 acetylation (Yu et al. 2007). Transient histone acetylation by STAT4 prevents recruitment of DNA methyltransferase Dnmt3a and cofactor Dnmt3L to the *Il18r1* locus, resulting in elevated *Il18r1* expression in differentiating Th1 cells (Yu et al. 2007). Similar findings have been reported for the *Ifng* locus in differentiating Th1 cells, where T-bet and STAT4 promote increased transcription, histone acetylation and reduced DNA methylation to sustain IFN-γ production (Agarwal and Rao 1998, Mullen et al. 2002, Zhang and Boothby 2006, Lewis et al. 2007). Stimulation with IL-18 synergises with IL-12 signalling to promote IFN-γ production and cellular proliferation independently of TCR-signalling in a mechanism dependent upon Growth arrest and DNA damage 45 β (Gadd45β) (Robinson et al. 1997, Yoshimoto et al. 1998, Nakahira et al. 2001, Smeltz et al. 2001, Yang et al. 2001, Nakahira et al. 2002). Cross-regulation of distinct $CD4^+$ T cell lineages is exemplified by the demonstration that T-bet-deficient $CD4^+$ T cells have elevated expression of GATA3, a Th2 cell lineage specifying transcription factor (Zheng and Flavell 1997, Usui et al. 2006). T-bet represses expression of GATA3 and direct interactions between T-bet and GATA3 inhibit GATA3 target gene binding (Szabo et al. 2000, Hwang et al. 2005). *In vivo*, this cross-regulation presents as a spontaneous Th2 cell mediated asthma in T-bet-deficient mice (Finotto et al. 2002, Finotto et al. 2005, Thieu et al. 2008) and as such, mice deficient in either T-bet or STAT4 have largely diminished Th1 cell differentiation, and exhibit increased susceptibility when challenged with a variety of intracellular pathogens (Suzuki et al. 1988, Scott et al. 1989, Suzuki and Remington

1990, Kaplan et al. 1996, Thierfelder et al. 1996). By contrast, exacerbated and aberrant Th1 cell responses have been linked to pathology in models of chronic inflammatory disorders, including multiple sclerosis (Bettelli et al. 2004), diabetes (Trembleau et al. 1995) and IBD (Powrie et al. 1994).

1.3.4 Th2 cells

Th2 cells are essential for host defence against a variety of extracellular parasites, through production of a range of effector cytokines including IL-4, IL-5, IL-13 and IL-25 (Paul and Zhu 2010, Zhu et al. 2010). However, aberrant Th2 cells responses can result in inflammatory and allergic disorders, including asthma (Robinson et al. 1992). Indeed, Th2 cell associated cytokines trigger B cell class switching to IgE production, promote eosinophilia and mast cell accumulation at mucosal surfaces, and regulate mucus secretion by epithelial cells (Paul and Zhu 2010). Extensive *in vitro* characterisation has identified a key role for IL-4 stimulation in differentiation of Th2 cells. Th2 cell lineage specification is dependent upon the transcription factor GATA3, which is induced in response to IL-4 driven activation of STAT6 (Zheng and Flavell 1997) and GATA3-deficient $CD4^+$ T cells do not produce Th2 cytokines (Pai et al. 2004, Zhu et al. 2004). Furthermore, regulation of Th2 locus chromatin accessibility, in addition to induction and maintenance of IL-4Rα expression, highlight a key role for IL-2/STAT5-signalling in Th2 cell lineage specification (Liao et al. 2008). Th2 cell development is also bolstered by a variety of transcription factors induced following TCR stimulation, including IRF4 (Lohoff et al. 2002), c-MAF (Ho et al. 1996), TCF-1 (Yu et al. 2009), Gfi-1 (Shinnakasu et al. 2008) and several NFAT family members (Ranger et al. 1998). As with T-bet in Th1 cells, Th2 cell associated transcription factors act to restrain development of alternative effector lineages. GATA3 directly interacts with Runx3 to limit IFN-γ production (Yagi et al. 2010) and suppresses expression of IL-12Rβ2 (Ouyang et al. 1998) and STAT4 (Usui et al. 2003). Furthermore, expression of *Il18r1* is repressed in differentiated Th2 cells, where STAT6 and GATA3 binding at intronic CNS of *Il18r1* mediate chromatin

remodelling and epigenetic silencing of the locus (Yu et al. 2008). Whilst much is known about *in vitro* requirements for Th2 cell lineage induction and stability, differentiation of Th2 cells *in vivo* is less well understood, and the development of Th2 cells can occur independently of IL-4 (Finkelman et al. 2000, Jankovic et al. 2000). As such, allergen or parasite induced TSLP, IL-33 and IL-25 can contribute to induction and maintenance of Th2 cell responses *in vivo* (Paul and Zhu 2010). Until recently, production of IL-9 was considered a hallmark of Th2 cells, due to its contribution to allergic inflammation through activation of mast cells. However, more recent findings demonstrate that naïve $CD4^+$ T cells cultured in the presence of IL-4 and TGF-β_1 produce high levels of IL-9 and IL-10, in the absence of other Th2 cell cytokines, and have been termed Th9 cells (Dardalhon et al. 2008, Veldhoen et al. 2008). Whilst multiple transcription factors including IRF4, PU.1 and STAT6 promote Th9 cell differentiation, a lineage defining transcription factor has yet to be identified (Jabeen and Kaplan 2012).

1.3.5 Th17 cells

Th17 cells are readily identifiable by their production of signature cytokines IL-17A, IL-17F and IL-22 (Aggarwal et al. 2003, Murphy et al. 2003, Liang et al. 2006) and expression of cell surface receptors CCR6 and IL-23R (Hirota et al. 2007, Awasthi et al. 2009). Early differentiation of Th17 cells is driven by TGF-β_1 in the presence of pro-inflammatory cytokines, including IL-1β, and STAT3-activating cytokines, such as IL-6 or IL-21 (Bettelli et al. 2006, Mangan et al. 2006, Sutton et al. 2006, Veldhoen et al. 2006, Korn et al. 2007, Nurieva et al. 2007, Zhou et al. 2007, Chung et al. 2009). The transcriptional network underlying Th17 cell lineage specification is dependent upon the transcription factor Retinoic acid receptor-like orphan receptor γt (Rorγt), encoded by *Rorc* (Ivanov et al. 2006). Mice deficient in Rorγt are almost devoid of Th17 cells, with the few remaining cells dependent upon compensation from the transcription factor family member, Rorα (Ivanov et al. 2006, Yang et al. 2008). Of importance clinically is the continued reliance of Th17 cells on transcriptional

regulation by Rorγt, as small molecule inhibitors of Rorγt inhibit expression of Th17 cell signature genes in differentiated Th17 cells and limit Th17 cell-mediated immunopathology *in vivo* (Huh et al. 2011). While Rorγt is necessary for Th17 differentiation, it is not sufficient, and multiple other transcription factors are involved, either by promoting Rorγt expression, or in cooperation with Rorγt to regulate modules of Th17 cell signature genes (Littman and Rudensky 2010, Ciofani et al. 2012, Yosef et al. 2013). IL-6 signalling in naïve $CD4^+$ T cells elicits production of the common-γ chain cytokine family member IL-21, and both IL-6 and IL-21 trigger STAT3-activation, which in turn promotes expression of Rorγt (Korn et al. 2007, Nurieva et al. 2007, Zhou et al. 2007). Additional transcription factors help shape the Th17 lineage, including BATF (Schraml et al. 2009), IRF4 (Brustle et al. 2007), Runx1 (Zhang et al. 2008), Aiolos (Quintana et al. 2012) and Aryl-hydrocarbon receptor (Ahr) (Quintana et al. 2008), as well as metabolic sensors mTORC1 (Delgoffe et al. 2011, Kim et al. 2013) and HIF-1α (Dang et al. 2011, Shi et al. 2011) and select components of the NFκB family, including IKKα (Li et al. 2011), IκBζ (Okamoto et al. 2010) and Rel (Ruan et al. 2011).

Investigations into the cellular sources of, and requirement for, TGF-β_1 in Th17 cell lineage specification *in vivo* have been extensive, providing evidence for both direct and indirect modulation of Th17 cell fate. TGF-β_1 acts indirectly to promote Th17 cell differentiation through inhibition of Th1 and Th2 cell differentiation and curtail production of effector cytokines known to inhibit Th17 cell differentiation, including IFN-γ, IL-4 and IL-2 (Takimoto et al. 2010). In line with these findings, TGF-β_1 directly represses expression of Eomesodermin, a Th1 associated transcription factor, which itself negatively regulates transcription of *Il17a* and *Rorc* (Ichiyama et al. 2011). Furthermore, both T-bet- and STAT6-deficient $CD4^+$ T cells differentiate to a Th17 cell phenotype in the absence of TGF-β_1, demonstrating that inhibition of Th1 and Th2 cell differentiation is an indirect mechanism by which this cytokine promotes Th17 cell differentiation (Das et al. 2009). In contrast, direct contribution to Th17 cell

differentiation by TGF-β_1 occurs in the presence of IL-6 through activation of ROCK2, a serine threonine kinase whose targets include IRF4, a factor essential for induction of Rorγt, and thus, Th17 cell differentiation (Huber et al. 2008, Chung et al. 2009, Biswas et al. 2010, Mudter et al. 2011). However, the strict requirement for TGF-β_1 in differentiation of naïve $CD4^+$ T cells to Th17 cells is controversial and has been challenged by investigators demonstrating that a combination of IL-1β, IL-6 and IL-23 is sufficient to drive Th17 cell differentiation, in the absence of TGF-βR-signalling (Ghoreschi et al. 2010). Interestingly, genetic and experimental comparison of Th17 cells polarised with either TGF-β_1 and IL-6 and those polarised with IL-1β, IL-6 and IL-23 demonstrated marked differences in their phenotype, and function, in line with previous observations that whilst sufficient to permit Th17 cell polarisation, TGF-β_1 and IL-6 required additional IL-23 mediated signals to drive tissue destruction in models of autoimmunity (McGeachy et al. 2007). Culture of Th17 cells in the presence of IL-1β, IL-6 and IL-23 leads to high expression of classically Th1 cell associated effector molecules, including transcription factors T-bet and Hlx, and Th1 cell surface antigens, such as CXCR3, and IL-18R1 (Ghoreschi et al. 2010). The dispensability of TGF-β_1 for Th17 cell differentiation in this study is in contrast to a number of early reports that $CD4^+$ T cell responsiveness to TGF-β_1, and indeed T-cell derived TGF-β_1 were essential for Th17 cell differentiation *in vivo* (Li et al. 2007). Furthermore, recent studies suggest autocrine production of TGF-β_1 promotes Th17 cell fate *in vivo* (Gutcher et al. 2011). Whilst the data are currently controversial and these conflicting results are not easily reconciled, it is worth noting that the concentration of TGF-β_1 in distinct inflammatory environments and tissues likely differs, and Th17 cell differentiation is sensitive to the concentration of TGF-β_1 (Zhou et al. 2008).

STAT3-activation in response to IL-6 or IL-21 induces the expression of IL-23R in a manner dependent upon Rorγt (Zhou et al. 2007, Zhou et al. 2008). Additionally, TGF-β_1 augments STAT3-mediated expression of IL-23R in a dose dependent

manner, where low concentrations of TGF-β_1 bolster expression and high concentrations inhibit IL-23R expression (Zhou et al. 2008). IL-23 stimulation of differentiating Th17 cells promotes maintenance of lineage differentiation and acquisition of pathogenic effector potential (Murphy et al. 2003, Langrish et al. 2005, Mangan et al. 2006, Sutton et al. 2006, McGeachy et al. 2009). IL-23R-signalling can also promote expression of pro-inflammatory cytokines not classically associated with Th17 cells, including IFN-γ (Lee et al. 2009) and GM-CSF (Codarri et al. 2011, El-Behi et al. 2011). However, the factors required for production of IL-22 from distinct effector T cell subsets remain ambiguous. Early reports detail the production of IL-22 by Th17 cells, though differential requirements for exogenous TGF-β_1 in the presence of IL-6 have been reported, where the absence of TGF-β_1 resulted in high IL-22 and minimal IL-17A production, and conversely, exogenous TGF-β_1 led to low levels of IL-22 compared to high frequencies of IL-17A production (Zheng et al. 2007, Basu et al. 2012). By contrast, activation of the ligand-dependent transcription factor aryl hydrocarbon receptor (AHR) is sufficient to induce production of IL-22 from conventional Th17 cells (Veldhoen et al. 2008), though a lineage-specifying transcription factor for Th22 cells is also yet to be identified.

1.3.6 T follicular helper (T_{FH}) cells

The discovery that the transcriptional repressor B cell leukaemia/lymphoma 6 (Bcl-6) was essential for lineage specification to a T_{FH} phenotype confirmed the identity of this $CD4^+$ T cell subset specialised in provision of help to B cell responses (Johnston et al. 2009, Nurieva et al. 2009, Yu et al. 2009). T_{FH} are characterised by high expression of CXCR5, ICOS, PD-1, CD40L and production of IL-21, through which they elicit formation of germinal centres (GC) to promote B cell survival and class switch recombination (Crotty 2011). Similarly to Th17 cells, STAT3-activating cytokines IL-6 and IL-21 play a key role in T_{FH} lineage specification (Vinuesa et al. 2005, Fazilleau et al. 2009), although this has recently been complemented by reports highlighting STAT3-independent pathways can also promote differentiation of T_{FH} cells *in vivo* (Poholek et al. 2010, Eto et al. 2011). Whilst the lineage relationship

between T_{FH} and other $CD4^+$ effector T cell subsets is not clear, the transcriptional repressor Bcl-6 has been demonstrated to be essential for lineage specification to T_{FH} cells by antagonising differentiation of Th1, Th2 and Th17 cells (Fazilleau et al. 2009). Emerging evidence demonstrates that T_{FH} cells may represent a transitional phenotype under polarising conditions associated with other effector populations. Early during Th1 cell differentiation, a Th1/T_{FH} intermediary stage occurs, where IL-12 dependent STAT4-signalling promotes Bcl-6 and IL-21 expression. Within committed Th1 cells, T-bet represses expression of Bcl-6 and IL-21, though accessibility of the Bcl-6 locus remains permissive (Oestreich et al. 2011, Oestreich et al. 2012). Furthermore, elegant fate mapping studies recently demonstrated that Peyer's patch T_{FH} cells could arise from "ex-Th17" cells, where they promote the development of IgA-producing B cells (Hirota et al. 2013). Thus, whether T_{FH} cells represent a diversionary fate of other developing $CD4^+$ T cell lineages or a truly distinct lineage remains unclear.

1.3.7 Plasticity of effector $CD4^+$ T cells

The theory of "master transcriptional regulators" states that each $CD4^+$ T cell subset is dependent upon a distinct transcription factor. As such, Th1, Th2, Th17 and T_{FH} cells require T-bet, GATA3, Rorγt and Bcl-6 respectively, whereas Treg cell differentiation requires Foxp3. Th9 cells and IL-10 producing $Foxp3^-$ Tr1 cells represent two additional cellular subsets for which key transcription factors are yet to be determined, thus whether they represent distinct lineages remains to be seen. However, recent data suggest that this paradigm is likely too simplistic, and *in vivo* a far more complicated interaction between "master" transcription factors results in diversity and flexibility of $CD4^+$ T cell polarisation (Oestreich and Weinmann 2012). For example, during lymphocytic choriomeningitis virus (LCMV) infection, IL-12 and IFN-dependent mechanisms reprogram committed $GATA3^+$ Th2 cells to a $GATA3^+$ T-bet^+ phenotype expressing a hybrid transcriptome (Hegazy et al. 2010). In this setting, T-bet expression by committed $GATA3^+$ cells acts to limit Th2 cell mediated

immunopathology. By contrast, epigenetic analysis of Th17 cells demonstrates the presence of permissive histone marks at the *Ifng* and *Tbx21* loci within Th17 cells, indicating that Th17 cells are poised to express T-bet and produce IFN-γ (Wei et al. 2009, Mukasa et al. 2010). Additionally, the conversion of established Th17 cells to Th1-like cells in response to stimulation with IL-23 and IL-12 has been demonstrated *in vitro* (Lee et al. 2009), and IL-17A$^+$IFN-γ$^+$ effector CD4$^+$ T cells are found at sites of tissue pathology in both mice and humans (Hue et al. 2006, Kullberg et al. 2006, Cosmi et al. 2008, Kebir et al. 2009). IL-23 mediated Th17 cell expression of T-bet, and associated production of IFN-γ, appears key to the emergence of "pathogenic" Th17 cells, including those within the intestine (McGeachy et al. 2007, Bending et al. 2009, Yang et al. 2009, Ahern et al. 2010, Ghoreschi et al. 2010, Hirota et al. 2011, Lee et al. 2012, Morrison et al. 2013). As such, the ability of T-bet to limit or exacerbate immunopathology mediated by "distinct" CD4$^+$ T helper subsets represents a key role for co-expression of "master transcription factors" in determining the outcome of a variety of immune responses. Therefore, determining the factors underlying CD4$^+$ T cell plasticity will be key to understanding the requirement for this process in immune responses during both health and disease.

1.3.8 Regulatory T (Treg) cells

The intestine contains a high frequency of regulatory T cell populations and early studies revealed their importance in preventing intestinal inflammation (Izcue et al. 2009, Harrison and Powrie 2013). More recent studies have emphasized the importance of Foxp3 (Forkhead box P3)-expressing populations in particular. Foxp3 is a transcription factor vital for the induction and maintenance of a regulatory T cell phenotype (Josefowicz et al. 2012). Mutations in the X-linked gene encoding Foxp3 illustrate the essential role for $Foxp3^+$ Treg cells in immunological tolerance in both mice and humans (Ziegler 2006). The majority of patients with Immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome bear loss of function mutations within the FOXP3 gene and suffer an early onset fatal autoimmunity (Chatila et al. 2000, Bennett et al. 2001, Wildin et al. 2001). Although IPEX syndrome affects many organs and tissues, the intestine is most frequently affected, emphasizing the key role of $Foxp3^+$ Treg cells in establishing and maintaining tolerance within the intestine (Gambineri et al. 2003).

Induction of Foxp3 expression and acquisition of the regulatory T cell program occurs at both thymic and extra-thymic locations. Thymus-derived $Foxp3^+$ Treg cells (tTreg) differentiate in response to high-affinity TCR interactions with MHC-II presented self-antigens (Stritesky et al. 2012, Xing and Hogquist 2012). Extra-thymic acquisition of Foxp3 expression and Treg function is also dependent upon TCR-signalling but also strongly influenced by environmental factors (Bilate and Lafaille 2012). Indeed there is convincing evidence that the intestinal microenvironment is a preferential site of antigen-induced Treg (iTreg) development, most likely reflecting high immune challenge and the continuous need for active immune suppression to maintain intestinal homeostasis (Izcue et al. 2009).

1.3.9 Characteristics of thymus-derived Foxp3$^+$ Treg cells.

Of the peripheral Foxp3$^+$ Treg cell pool, the majority probably originate within the thymus where a balance between NFκB signalling and PI3K/Akt/mTOR pathways within developing thymocytes is key to their differentiation (Feuerer et al. 2009, Hori 2010). TCR-signalling represents a major source of these instructive signals, and synergistic responses to TCR ligation and co-stimulatory signals via CD28 (Salomon et al. 2000, Tai et al. 2005) link TCR signalling to NFκB by promoting activation of PKCθ (Gupta et al. 2008), CARMA-1 (Barnes et al. 2009, Molinero et al. 2009), TAK1 (Wan et al. 2006) and IKK (Schmidt-Supprian et al. 2003, Long et al. 2009). The NFκB family member c-Rel and atypical inhibitor of NFκB ($I\kappa B_{NS}$) both appear to heavily influence the development of thymic Foxp3$^+$ Treg cells, where they act at the pioneer element CNS3 of Foxp3 to promote gene expression (Isomura et al. 2009, Ruan et al. 2009, Schuster et al. 2012). Additionally, STAT5-activating survival signals via IL-2 (and to lesser extents IL-7 and IL-15) are essential for induction of Foxp3 expression in Treg cell precursors (Burchill et al. 2008, Lio and Hsieh 2008, Hsieh et al. 2012). Additional factors also contribute to induction and maintenance of Foxp3 expression within thymic Foxp3$^+$ Treg cells, including Foxo1 and Foxo3 (Harada et al. 2010, Kerdiles et al. 2010, Ouyang et al. 2010, Ouyang et al. 2012, Samstein et al. 2012), Creb-ATF-1 (Kim and Leonard 2007) and NFAT (Tone et al. 2008).

Foxp3$^+$ Treg cells utilise a broad αβ-TCR repertoire distinct from conventional effector CD4$^+$ T cells (Hsieh et al. 2006, Pacholczyk et al. 2006), likely reflecting their elevated affinity for self-peptides and the higher threshold of signalling required to promote clonal diversion (Stritesky et al. 2012, Xing and Hogquist 2012). However, the interaction affinity between peptide/MHC-II complex and TCR is not the sole determinant for expression of Foxp3 by developing thymocytes. Generation of transgenic mice bearing naturally occurring Treg cell TCRs demonstrated a small thymic niche for each self-peptide specific clone (Bautista et al. 2009). Intra-clonal

competition limits thymic Foxp3$^+$ Treg cell emergence, ensuring development of a diverse repertoire of Foxp3$^+$ Treg cells (Bautista et al. 2009). Both thymic DC and epithelial cells present antigens involved in Foxp3 induction and establishment of the regulatory T cell program (Wirnsberger et al. 2009). Upon emergence from the thymus, Foxp3$^+$ Treg cells migrate to peripheral lymphoid and non-lymphoid tissues, where they act to maintain tissue homeostasis through regulation of inappropriate immune activation.

1.3.10 Characteristics of extra-thymically induced Foxp3$^+$ Treg cells.

Extra-thymic induction of Foxp3$^+$ iTreg cells from mature CD4$^+$ T cells is thought to primarily occur under two physiological states, during either sub-immunogenic or chronic inflammatory conditions (Bilate and Lafaille 2012). CD4$^+$ T cell activation in the absence of inflammation (sub-immunogenic conditions) results in extra-thymic Foxp3 induction in a variety of settings, including blockade of CD4 co-receptor engagement using non-depleting monoclonal antibody (mAb) (Cobbold et al. 2004), administration of continuous low dose antigen under sterile settings (i.v. injection or osmotic pumps) (Apostolou and von Boehmer 2004), antigen presentation in the absence of inflammation (Kretschmer et al. 2005) or in tolerogenic microenvironments, like the mesenteric lymph nodes and intestinal lamina propria (Mucida et al. 2005, Hadis et al. 2011).

The presence of tTreg and iTreg populations in the intestine raises the question of the functional roles of these populations. This has been difficult to decipher, as few cell surface markers distinguish between these two populations. *In vitro* activation of naive CD4$^+$ T cells in the presence of TGF-β_1 and IL-2 is a commonly employed method of generating Foxp3$^+$ iTreg cells. Whilst showing some facets of Treg cell function, as assessed *in vitro*, the genetic composition of these cells differs greatly to those generated in different tissue contexts *in vivo* (Haribhai et al. 2009, Feuerer et al. 2010). Helios is a putative tTreg marker, an Ikaros transcription factor family

member encoded by *Ikzf2*, a gene enriched in the Foxp3$^+$ Treg cell signature (Hill et al. 2007, Thornton et al. 2010). However, it is now apparent that a substantial proportion of experimentally induced Foxp3$^+$ iTreg can express Helios, and as such, its use as a marker of Foxp3$^+$ Treg cell origin should be employed with caution (Verhagen and Wraith 2010, Gottschalk et al. 2012). In the absence of inflammation, the use of neuropilin-1 as a selective marker of tTreg appears promising; mucosa-derived iTreg are Nrp-1^{lo} compared to their tTreg Nrp-1^{Hi} counterparts (Weiss et al. 2012, Yadav et al. 2012). Interestingly, recent data suggest a role for neuropilin-1 in the maintenance of Foxp3$^+$ Treg cell stability and survival (Delgoffe et al. 2013).

Despite these caveats, sophisticated genetic models have provided strong support for collaboration between iTreg and tTreg populations in control of intestinal inflammation. Efficient prevention of T cell transfer colitis by Foxp3$^+$ tTreg cells requires the emergence of Foxp3$^+$ iTreg cells from the colitogenic transferred naïve CD4$^+$ T cells (Haribhai et al. 2009). A non-redundant role for iTreg in maintenance of intestinal tolerance under homeostatic conditions has been demonstrated through a series of elegant genetic manipulations of the Foxp3 locus. Within the Foxp3 locus, three conserved non-coding sequences (CNS1-3) promote size, stability and composition of the Foxp3$^+$ Treg cell population (Zheng et al. 2010). *In vivo*, CNS3-deficiency leads to drastically decreased thymic output of Foxp3$^+$ tTreg and CNS2-dependent mechanisms appear responsible for heritable maintenance of Foxp3 expression in proliferating Treg cells (Zheng et al. 2010). Foxp3 CNS1 contains a TGF-β/NFAT response element, and synergistic binding of Smad3 and NFAT at CNS1 are suggested to be required for Foxp3 induction (Tone et al. 2008). CNS1-deficient mice have unaffected tTreg populations, but defective induction of Foxp3$^+$ iTreg, particularly within the GALT (gut-associated lymphoid tissue) (Zheng et al. 2010, Josefowicz et al. 2012). This highly selective blockade in extra-thymic Foxp3$^+$ Treg induction led to neither fatal autoimmunity nor exacerbated colonic Th1 or Th17 responses, as may have been expected upon loss of intestinal immune tolerance. Instead, complete loss of intronic Foxp3 CNS1 manifested as pronounced Th2

responses, primarily at mucosal sites (Josefowicz et al. 2012). These findings are consistent with earlier studies showing a key role for iTreg cell development in oral tolerance towards nominal antigens and for control of allergic responses, and illustrate the importance of immune deviation towards a Foxp3$^+$ Treg program for mucosal tolerance (Curotto de Lafaille et al. 2008).

Whether shared responsibilities or specific functional roles exist for these developmentally distinct populations is not clear. One obvious possibility is that tTreg and iTreg populations contribute different antigen specificities, increasing the frequency of potentially responsive Foxp3$^+$ Treg cell populations that can be activated. It is known that Foxp3$^+$ Treg cells utilise a broad αβ-TCR repertoire distinct from that of Foxp3$^-$ effector T cells (Hsieh et al. 2006, Pacholczyk et al. 2006). Recent studies indicate that colonic Foxp3$^+$ Treg utilise a TCR repertoire distinct from Foxp3$^+$ Treg found in systemic tissues, suggesting that the local antigenic milieu shapes the colonic Foxp3$^+$ Treg pool (Lathrop et al. 2011). As may be expected, microbial specificities are increased within colonic Foxp3$^+$ Treg populations and transgenic mice bearing a TCR repertoire derived from colonic Foxp3$^+$ Treg did not select thymic Foxp3$^+$ Treg, suggesting they are specific for exogenous antigens (Lathrop et al. 2011). Furthermore, when T cells bearing these TCR were rendered unable to adopt a Foxp3$^+$ Treg cell fate they developed colitogenic potential. These data are compatible with the notion that the intestinal microenvironment supports local differentiation of microbe-reactive iTreg as a means of preventing development of inflammatory effector responses. Further understanding of the specificity of intestinal Foxp3 expressing cell populations is required to fully understand the role of these cells in antigen-specific tolerance induction.

1.3.11 Foxp3$^+$ Treg cell mediated suppression in the intestine.

Although a source of early debate, it is now widely accepted that Foxp3$^+$ Treg cells utilise multiple regulatory mechanisms to control host immune responses. Production of regulatory cytokines (TGF-β, IL-10 and IL-35), modulation of APC function (LAG-3,

CTLA-4, Granzyme/Perforin) and alteration of cellular metabolism (CD25, CD39/CD73, IDO induction in DC) have all been implicated in Treg cell function in distinct inflammatory settings (Vignali et al. 2008, Shevach 2009). Production of IL-10 and TGF-β_1 are key cytokine pathways necessary for immune tolerance within the intestine and here their roles are highlighted in more detail.

1.3.11.1 TGF-β_1

The Transforming Growth Factor family of cytokines have developmental and physiological roles throughout the mammalian lifespan. Encoded by individual genes, the TGF-β family consists of 3 isoforms, TGF-β_1, TGF-β_2 and TGF-β_3, which signal via the TGF-βR complex (TGF-βRI and TGF-βRII). TGF-βR-activation triggers intrinsic kinase activity and phosphorylation of downstream signalling components, for example, transcription factors Sma and Mad family (Smad)2/3 (Derynck and Zhang 2003). Inhibitory Smads, such as Smad7, act to negatively regulate TGF-βR signalling, making this a finely tuned system. Further control occurs at the post-transcriptional level as TGF-β_1 is secreted to the cell surface in a latent complex, requiring further post-translational modification to become biologically active. Activation of the latent TGF-β_1 complex requires enzymatic cleavage of latency-associated peptides, or integrin mediated conformational change (Khalil 1999). The dominant role for TGF-β_1 in immunological tolerance is highlighted by the T cell dependent, early onset, fatal autoimmune disease of *Tgfb1*$^{-/-}$ mice that occurs within a few weeks of birth (Shull et al. 1992, Diebold et al. 1995, Letterio et al. 1996, Kobayashi et al. 1999). Similar to Foxp3-deficiency, complete ablation of TGF-β_1 production, or of TGF-βR expression in T cells, leads to catastrophic autoimmunity (Shull et al. 1992, Li et al. 2006). By contrast, quantitative reductions in mice with impaired but not abolished TGF-β regulatory pathways leads to intestinal inflammation, indicating the intestine is particularly sensitive to TGF-β signalling (Izcue et al. 2009). Genetic perturbation of Smad signalling, through loss of Smad3/4, or forced expression of Smad7 results in intestinal inflammation,

implicating active TGF-β_1 in intestinal tolerance. Likewise, inability to activate latent TGF-β_1 in either T cells or myeloid cells results in loss of intestinal homeostasis. Mice with APC-specific ablation of integrin sub-units, αv or β8, or T cell specific deletion of the convertase furin are prone to development of intestinal inflammation (Lacy-Hulbert et al. 2007, Travis et al. 2007, Yang et al. 2007, Pesu et al. 2008). In addition to the importance of T cell produced TGF-β_1, it is also crucial that T cells can respond to this cytokine (Li et al. 2006) and naïve T cells unable to respond to TGF-β cannot be controlled by Treg cells (Fahlen et al. 2005). However, whether TGF-β_1 mediates T cell suppression by inhibition of proliferation, acquisition of colitogenic potential, or generation of iTreg cell populations warrants further investigation. Furthermore, the distinct signalling pathways initiated by different TGF-β isoforms remain to be examined in this and multiple other contexts.

1.3.11.2 IL-10

IL-10, a regulatory cytokine, is produced by multiple leukocyte populations and protects the host by limiting inflammatory responses to microbial challenge (Saraiva and O'Garra 2010). IL-10 signals via a receptor complex consisting of IL-10Rα and IL-10Rβ sub-units, promoting STAT3 activation via Jak1 and Tyk2 (Glocker et al. 2011). By contrast with the fatal consequences of Foxp3-deficiency, genetic ablation of IL-10 or IL-10R leads to severe colitis that is dependent upon on the presence of a triggering microbiota (Kuhn et al. 1993, Davidson et al. 1996, Sellon et al. 1998).

IL-10 production is enriched within mucosal tissues and $CD4^+$ T cells at these sites are an abundant source (Uhlig et al. 2006). Furthermore, both $Foxp3^+$ and $Foxp3^-$ populations contribute to the production of IL-10 within the gastrointestinal tract. However, strikingly there is compartmentalization of this response, with high levels of IL-10 production in the colon primarily restricted to $Foxp3^+$ cells and the reverse in the small intestine where $Foxp3^-$ cells represent the major source (Uhlig et al. 2006, Maynard et al. 2007). Functional studies support a key role for $CD4^+$ T cell-derived

IL-10 in intestinal homeostasis, as adoptive transfer of IL-10$^{-/-}$ CD4^{+} T cells to lymphopenic hosts results in severe intestinal inflammation and specific deletion of IL-10 from CD4^{+} T cells led to spontaneous colitis (Davidson et al. 1996, Asseman et al. 1999, Roers et al. 2004). Early studies showed that IL-10-deficient Treg cells could prevent, but not cure, colitis in the T cell transfer model (Asseman et al. 1999, Uhlig et al. 2006). In a lymphocyte-replete model, deletion of IL-10 from Foxp3^{+} Treg also leads to intestinal inflammation, albeit with less severity than deletion in total CD4^{+} T cells, implicating CD4^{+} Foxp3^{-} cells as a functional source of IL-10 that contributes to intestinal homeostasis (Rubtsov et al. 2008).

The IL-10R complex is expressed upon innate and adaptive immune cell populations within the intestine and acts to control intestinal CD4^{+} T cells through direct and indirect mechanisms. Myeloid-specific ablation of STAT3 leads to spontaneous colitis driven by dysregulated CD4^{+} T cell responses. In this setting, defective IL-10R signalling in colonic macrophages resulted in elevated levels of co-stimulatory molecules CD80/86 and hyper-activation of colitogenic CD4^{+} T cells (Takeda et al. 1999, Kayama et al. 2012). Direct IL-10R signalling into CD4^{+} T cells also serves to limit colitogenic responses within the intestine. Intestinal Th17 and Foxp3^{+} Treg express IL-10Rα, and CD4-specific expression of a dominant-negative IL-10Rα results in selective expansion of Th17 and IFN-γ/IL-17A co-producing populations during intestinal inflammation (Huber et al. 2011).

In addition to effects on effector CD4^{+} T cells, there is also accumulating evidence that IL-10R signalling into Foxp3^{+} Treg cells is important for their function. Thus, IL-10Rβ$^{-/-}$ Foxp3^{+} Treg cells lost Foxp3 expression and failed to protect mice from intestinal inflammation following naïve CD4^{+} T cell transfer (Murai et al. 2009). In a genetic model, specific deletion of IL-10Rα in Foxp3^{+} Treg cells resulted in spontaneous intestinal inflammation characterized by selective dysregulation of Th17 cells (Chaudhry et al. 2011). However, in that and other lymphocyte replete settings,

Foxp3$^+$ Treg cells demonstrated remarkable stability and were not observed to lose expression of Foxp3 (Rubtsov et al. 2010, Chaudhry et al. 2011). Whether loss of Foxp3 expression in lymphopenia driven colitis represents a failure to maintain the Treg program or is a specific consequence of the cell transfer system requires further study, especially given the interest in Treg cell therapy in the clinical setting. Finally, it should not be overlooked that many other cell types produce IL-10 including B cells and macrophages and that non-T cell derived IL-10 can also play a functional role in intestinal homeostasis (Mizoguchi et al. 2002, Murai et al. 2009, Hadis et al. 2011).

1.3.12 Functionally specialised Foxp3$^+$ Treg cells

An emerging concept in T cell biology is that of adaptation to the tissue environment and context dependent specialization of the adaptive immune response is well documented (Littman and Rudensky 2010). Differentiation of CD4$^+$ T cells to distinct effector populations is dependent upon appropriate TCR-signalling, co-stimulation and surrounding cytokine milieu. Expression of lineage-specifying transcription factors T-bet, GATA3 and Rorγt is required for Th1, Th2 and Th17 responses respectively (Zheng and Flavell 1997, Szabo et al. 2000, Ivanov et al. 2006). However, expression of these differentiation programs is not mutually exclusive and it is increasingly appreciated that interplay between these factors contributes to the fate and flexibility of effector T cell responses (Oestreich and Weinmann 2012). As has been described for effector CD4$^+$ T cell populations, it is now evident that Foxp3$^+$ Treg cells can acquire expression of transcription factor modules that allow context-dependent immune suppression (Barnes and Powrie 2009). For example, in response to IFN-γ or IL-27, Foxp3$^+$ Treg cells upregulate expression of the Th1 cell transcription factor, T-bet (Koch et al. 2009, Hall et al. 2012). T-bet expression in Foxp3$^+$ Treg promotes CXCR3 expression and migration to Th1-inflamed sites facilitating control of the Th1 response (Koch et al. 2009). Equally, the reliance of Th17 cells upon STAT3-dependent cytokines for their differentiation is mirrored by the STAT3-dependency of Foxp3$^+$ Treg-mediated control. Thus, targeted deletion of

STAT3 (or IL-10Rα, which signals via STAT3) in Foxp3$^+$ Treg led to severe intestinal inflammation dominated by accumulation of Th17 cells (Chaudhry et al. 2009, Chaudhry et al. 2011).

The majority of peripheral Foxp3$^+$ Treg express transcription factors IRF4 and/or GATA3, suggesting these transcription factors may play more general roles in Foxp3$^+$ Treg cell function (Zheng et al. 2009, Wang et al. 2011, Wohlfert et al. 2011, Rudra et al. 2012). Indeed, specific loss of IRF4 in Foxp3$^+$ Treg results in an autoimmune syndrome characterized by mucosal Th2 responses, similar to the phenotype reported in mice deficient in Foxp3 CNS1 (Zheng et al. 2009, Josefowicz et al. 2012). IRF4 seems to be an important pioneer transcription factor involved in multiple CD4$^+$ T cell effector phenotypes including Th2, Th17 and T_{FH} differentiation (Lohoff et al. 2002, Rengarajan et al. 2002, Huber et al. 2008, Bollig et al. 2012, Glasmacher et al. 2012). Based on these findings IRF4 may play multiple roles in Foxp3$^+$ Treg cells that promote specific modules of suppression. In support of this, B lymphocyte-induced maturation protein-1 (Blimp-1) is a regulator of IL-10 production and its expression in Foxp3$^+$ Treg cells is dependent upon IRF4 (Cretney et al. 2011). Furthermore, IRF4-deficient Foxp3$^+$ Treg cells are unable to control Th1 and Th17 responses in a T cell transfer model of colitis (Zheng et al. 2009). Further understanding of the various molecular mechanisms through which IRF4 expression in Foxp3$^+$ Treg cells contributes to mucosal immune tolerance is required.

Beyond its essential role in embryogenesis, within the hematopoietic system, the transcription factor GATA3 is required for thymic T cell development and mature CD4$^+$ T cell differentiation to a Th2 lineage (Zheng and Flavell 1997). In addition, this lineage-specifying factor promotes immune tolerance through transcriptional enhancer activity at Foxp3 CNS2 to maintain Foxp3 expression in regulatory T cells (Wang et al. 2011). Genetic ablation of GATA3 in Foxp3$^+$ Treg results in a systemic autoimmune syndrome, dermatitis and intestinal inflammation (dependent upon

experimental system and facility), that was due to reduced expression of Foxp3 mRNA and protein on a per-cell basis and an associated decrease in regulatory capability (Wang et al. 2011, Wohlfert et al. 2011, Rudra et al. 2012). Alongside decreased Foxp3 expression, $Foxp3^+$ Treg specific ablation of GATA3 results in mildly elevated intestinal Th2 responses, with increased frequencies of $GATA3^+$, IL-4, IL-5 and IL-13 producing populations in the intestinal lamina propria (Rudra et al. 2012), although whether the two phenotypes are mutually exclusive remains unclear.

Adaptation of $Foxp3^+$ Treg to the tissue microenvironment and the ability to utilise multiple regulatory mechanisms is key to instigating and maintaining active immunological tolerance. Distinct antigen specificities, enrichment in regulatory cytokine production and utilization of effector transcription factor networks demonstrate that adaptation of $Foxp3^+$ Treg to the tissue microenvironment is critical in maintaining intestinal immune tolerance. The role of the host immune system in continuous tolerance to the microbiota is evident; yet, the commensal microbiota itself is intimately involved in the dynamic molecular dialogue required for immune tolerance at both mucosal and peripheral sites.

1.3.13 Modulation of $CD4^+$ T cells by intestinal microbiota and diet

Colonization of the host by the commensal microbiota and the presence of multiple dietary components is key to proper mucosal immune cell function. Indeed, individual bacterial species promote differentiation of distinct intestinal T cell populations. Reared in a sterile environment, and on a diet free of live microbes, germ-free (GF) mice provide a useful experimental tool to explore the relationship between the host immune system and commensal microbiota. Controlled colonization of germ-free mice allows analysis of the role for individual or multiple bacterial species in mutualistic interactions with the host immune system.

Segmented Filamentous Bacteria (SFB) are gram-positive members of the *Clostridiales* genus able to penetrate the small intestinal mucus layer and interact with the epithelium to colonize the host. Colonization with SFB results in accumulation of effector T cells within the small intestine, primarily Th17, and to a lesser extent Th1 cells (Gaboriau-Routhiau et al. 2009, Ivanov et al. 2009). The mechanisms through which SFB promotes effector T cell polarization and accumulation within the intestine remain largely unknown. Whether direct interactions between SFB and the intestinal epithelium, host immune cells, or production of unique metabolic modulators is required for induction of effector T cells is not understood. Equally, whether the immune response to SFB colonization is antigen-specific remains to be established. Identifying the genetic attributes of SFB that drive this highly immunostimulatory response will be crucial to understanding how distinct bacterial species modulate the mucosal immune system.

Regulatory T cell populations are also affected by microbial colonization, which can lead to accumulation of intestinal Foxp3$^+$ Treg populations and induction of IL-10 production (Honda and Littman 2012). For example, *Bacteroides fragilis* is a human commensal able to induce IL-10 production from intestinal T cells and limit Th17 responses during intestinal inflammation (Round and Mazmanian 2010). Colonization of germ-free mice with a cocktail of 46 spore-forming murine *Clostridia* strains promotes accumulation of colonic Foxp3$^+$ Treg (Atarashi et al. 2011). The nature of the exact strains, microbial attributes and associated host molecular responses to this complex gnotobiotic mixture are as yet unknown. However, accumulation of Foxp3$^+$ Treg and induction of IL-10 production are associated with increased colonic levels of active TGF-β_1 in these gnotobiotic mice. As discussed earlier, whilst the reliability of Helios expression to determine tTreg from iTreg is still controversial, *Clostridium*-colonized gnotobiotic mice showed a shift from Helios$^+$ Foxp3$^+$ to Helios$^-$ Foxp3$^+$ Treg following colonization, suggestive of iTreg generation (Atarashi et al. 2011). The antigen-specificity of *Clostridia*-induced colonic Foxp3$^+$ Treg was not

assessed, but in the context of TCR repertoire of colonic Foxp3$^+$ Treg, it is possible to hypothesize that these iTreg would bear TCR-specificity for *Clostridia*-derived epitopes. Furthermore, analysis of TCR repertoire in this setting may aid identification of individual *Clostridia*-strains responsible for Foxp3$^+$ Treg accumulation and/or IL-10 induction. In addition, colonization of GF mice with a benign altered Schaedler flora (ASF), comprised of eight commensal species, promoted accumulation of colonic Helios$^-$ Foxp3$^+$ Treg that served to promote intestinal tolerance during experimental DSS colitis (Geuking et al. 2011). Similarly, infection with the mouse pathogen *Helicobacter hepaticus* induces an IL-10-producing Treg response that prevents bacteria-driven chronic intestinal inflammation (Kullberg et al. 2002, Kullberg et al. 2006). Together these data suggest that induction of a Treg/IL-10 producing response may be an important part of host-commensal mutualism allowing persistence of microbes in the absence of a damaging inflammatory response. It is clearly of interest to further understand the molecular pathways and metabolites that control microbe-induced Treg function as these may provide novel immune modulators and underlie the poorly characterized properties of probiotic bacteria. Clostridia are fermenting bacteria and it is notable that fermentation products such as short chain fatty acids promote gut health (Maslowski et al. 2009). Whether iTreg induction and IL-10 production following bacterial colonization requires constant microbial exposure remains to be examined and the use of reversible colonization systems will be a useful tool to examine this question.

Retinoic acid (RA) is a metabolite of diet-derived vitamin-A, a nutrient essential for multiple physiological processes, including an array of immunological functions (Hall et al. 2011). Within the GALT, RA promotes both regulatory and effector immune responses through imprinting of intestinal homing receptors α4β7 and CCR9 on activated T cells, and acts as a co-factor in the *de novo* generation of Foxp3$^+$ iTreg by CD103$^+$ DC (Coombes et al. 2007, Sun et al. 2007). Induction of Foxp3 expression by CD103$^+$ DC is dependent on TGF-β_1 and is enhanced by RA,

accordingly $CD103^+$ DC highly express mRNA for *aldh1a1* and *aldh1a2*, the retinal metabolizing enzymes required for RA synthesis (Coombes et al. 2007). *In vivo* corroboration of the role for RA in iTreg induction stems from the observation that mice reared on a vitamin-A deficient diet are defective in iTreg generation upon oral antigen exposure (Hall et al. 2011). As such, during intestinal homeostasis, RA promotes regulatory responses to bolster immunological tolerance to dietary antigens. However, recent studies demonstrate context dependent roles for this metabolite in intestinal immune tolerance. Thus, RA acts to promote effector responses in settings of intestinal inflammation. Oral infection with *Toxoplasma gondii* is controlled by a strong intestinal Th1 response, which is impaired in vitamin-A deficient mice (Suzuki et al. 1988, Hall et al. 2011). Furthermore, normal tolerance to dietary antigens is disturbed in patients with coeliac disease, whereby aberrant immune responses to gluten, a component of wheat, result in intestinal inflammation. Contrary to its recognized role in oral tolerance, RA, in combination with IL-15, a cytokine highly abundant in the mucosa of patients with coeliac disease, promotes pro-inflammatory cytokine production from intestinal APC to drive intestinal inflammation (DePaolo et al. 2011). As such, RA can act as a pro-inflammatory co-adjuvant in settings of intestinal stress, whilst promoting intestinal tolerance during homeostasis.

1.4 Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) represents an umbrella-term covering a range of chronic inflammatory disorders primarily of the gastrointestinal tract, but often with extra-intestinal manifestations (Xavier and Podolsky 2007). The two major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). A disease of unknown aetiology, data from experimental and clinical studies demonstrate that IBD results as a loss of intestinal homeostasis with aberrant immune responses to components of the resident intestinal microbiota. As described above, complex interactions between the microbiota, mucosal immune system, and intestinal tissue cells govern

multiple layers of regulation that control intestinal immunity. Experimental models of IBD have been key to the demonstration that chronic inflammation in IBD results due to either over-production of pro-inflammatory pathways, or to the loss of key negative regulatory mediators (Maloy and Powrie 2011).

Diagnosis of IBD is dependent upon clinical examination using endoscopic, radiological and histological techniques, and patients often present with clinical symptoms varying from abdominal pain to bloody diarrhoea, weight loss and fever (Baumgart and Sandborn 2007, Kaser et al. 2010). Suggestive of distinct aetiologies, the tissue pathologies apparent within patients with CD and UC differ greatly. In CD, lesions can occur throughout the gastrointestinal (GI) tract, are characterised by transmural inflammation comprising dense cellular infiltrates and granulomas, and occur most frequently in the terminal ileum and large intestine. Meanwhile, tissue pathology in UC is restricted to the colon, affects only the mucosal surface, and pathology is often characterised by formation of crypt abscesses, changes in crypt architecture and goblet cell depletion (Kaser et al. 2010). The development of strictures and fistulas also represent severe complications resulting from chronic inflammation. Although these aberrant inflammatory responses primarily affect the gastrointestinal tract, patients with IBD frequently exhibit extra-intestinal inflammatory responses affecting the skin, liver, eyes and joints, and patients also have significantly elevated risk of developing colon and small bowel cancers (Ullman and Itzkowitz 2011). Current frontline therapy for UC is 5-aminosalicylic acid (5-ASA), a bowel-targeted salicylic acid derivative, and for CD, combinations of antibiotics, 5-ASA and corticosteroids (Baumgart and Sandborn 2007). Furthermore, recently the use of biological agents targeting TNF-α, including Infliximab, have seen some success, though patients often become refractory to treatment, mount neutralising antibody responses and are also at greater risk of re-activation of latent infections, including Tuberculosis (Kaser et al. 2010). Ultimately, many patients go on to require surgical intervention.

CD and UC are both chronic inflammatory diseases of the gastrointestinal tract, but are considered distinct disorders, and this is reflected in their aetiology. UC is characterised by Th2 type responses, with elevated concentrations of pro-inflammatory cytokines IL-5 and IL-13 (Heller et al. 2005). Furthermore, Natural Killer T (NKT) cell production of IL-13 is thought to drive pathology in this disease (Fuss et al. 2004). By contrast, CD is associated with a strong Th1 and Th17 cell responses, highlighted by the increased expression of IL-12 (Monteleone et al. 1997, Fuss et al. 2006), IL-12Rβ2 (Parrello et al. 2000), IL-18 (Monteleone et al. 1999, Pizarro et al. 1999), T-bet and IFN-γ (Fuss et al. 1996), IL-17A (Annunziato et al. 2007) and IL-22 (Brand et al. 2006).

1.4.1 Genetic susceptibility to Inflammatory Bowel Disease

Genome wide association studies (GWAS) and successive meta-analyses have identified multiple genetic loci associated with susceptibility to either Crohn's disease and/or ulcerative colitis. A considerable proportion of these genes are involved in immune cell function, and many are involved in promoting epithelial barrier integrity, bacterial handling and innate and adaptive immune cell responses (Khor et al. 2011, Jostins et al. 2012). Polymorphisms amongst genetic loci encoding NOD2, ATG16L1 and IL-23R are all strongly associated with susceptibility to IBD, though they are also present amongst clinically healthy members of the population, suggesting that breakdown in intestinal immune homeostasis occurs as the result of multiple genetic deficiencies and triggering environmental factors, including those from the intestinal microbiota (Khor et al. 2011). By contrast, mutations in genes encoding IL-10 or IL-10R subunits are found in patients with very severe early-onset IBD (eo-IBD) and are associated with immune hyper-activation within the intestine (Glocker et al. 2009). Many of these patients are unresponsive to immunosuppressive therapies and require bone marrow transplantation. Recent studies suggest up to 15% of eo-IBD cases are attributable to mutations in the IL-10/IL-10R axis, clearly illustrating the

important role of this pathway in intestinal homeostasis (Kotlarz et al. 2012). Single nucleotide polymorphisms in *IL10* have also been associated with susceptibility to adult UC and CD although in this setting the genetic contribution to disease is modest (Franke et al. 2008, Franke et al. 2010, Jostins et al. 2012).

1.4.2 Intestinal microbiota and IBD

It is widely accepted that dysregulated immune responses to components of the resident intestinal microbiota underlie the chronic gastrointestinal inflammation of patients with IBD. This is highlighted by observations that therapeutic treatment with antibiotics, or faecal stream diversion, can ameliorate disease in some instances (Baumgart and Sandborn 2007). Though no single identifiable infectious agent has been associated with IBD, recent studies highlight global changes in the intestinal microbiota of patients with IBD (termed dysbiosis), with a reduced overall diversity of their intestinal microbiota and decreased frequencies of bacteria with reported anti-inflammatory properties including the *Bacteroidetes* order and *F.prausnitzii,* in addition to elevated frequencies of adherent/invasive *E.coli* (AIEC) species (Sartor 2008, Hill and Artis 2010, Frank et al. 2011). Whether the dysbiosis observed in IBD patients underlies this aberrant immune response to commensal components, or is secondary to on-going intestinal inflammation remains unknown.

1.5 Animal Models of Inflammatory Bowel Disease

The advent of animal models of colitis was key to delineating multiple pro-inflammatory and regulatory circuits underlying inflammation within the intestine. Animal models of IBD can be broadly classified as either acute, or chronic. Administration of chemicals, either via drinking water (DSS; dextran sulphate sodium) or rectally (TNBS; 2,4,6-trinitrobenzenesulfonic acid), damages the intestinal epithelium allowing translocation of commensal bacteria and triggers acute inflammation followed by epithelial repair. Additionally, acute infectious models of colitis, including *Citrobacter rodentium*, also promote acute intestinal inflammation,

and also permit dissection of protective immune responses within the intestine and detailed analysis of host/pathogen interactions. The chronic models of IBD used within this thesis better portray the aetiology and pathology of clinical IBD, though these models are by no means perfect, and lack many attributes of IBD, including the relapsing/remitting disease progression observed in patients. The models of IBD used in this thesis are outlined below.

1.5.1 T cell transfer colitis model

The T cell transfer colitis model has been key in revealing an important role for $CD4^+$ T cells in promoting intestinal inflammation. Despite efficient elimination of self-reactive T cells during thymic negative selection, the peripheral naïve $CD4^+$ T cell population contains clones reactive to both self-antigens along with the commensal microbiota. Accordingly, transfer of naïve $CD4^+$ T cells into immune deficient and lymphopenic *Rag1*$^{-/-}$ or *Scid* mice results in cellular proliferation, differentiation and elicits systemic and intestinal inflammation (Powrie et al. 1993). In this model, mice develop severe colitis, which recapitulates some key features of the intestinal tissue pathology observed in patients with IBD, including lamina propria leukocyte infiltration, epithelial hyperplasia and goblet cell depletion. Accumulation of activated and differentiated T cells in the colonic lamina propria tissue drives intestinal inflammation in recipient mice. Production of IL-23 from innate immune cell populations is essential for intestinal inflammation in the T cell transfer colitis model, where it acts directly on $CD4^+$ T cells to promote emergence of colitogenic effector T cell populations, whilst acting to limit differentiation of peripherally induced $Foxp3^+$ Treg cells (Hue et al. 2006, Izcue et al. 2008, Ahern et al. 2010). Additionally, T cell transfer colitis can be prevented, or established inflammation resolved, by adoptive transfer of $Foxp3^+$ Treg cells (originally $CD45RB^{lo}$ and then $CD25^+$), with differential requirements for regulatory mechanisms dependent upon experimental setting (Izcue et al. 2009, Harrison and Powrie 2013). For example, production of IL-10 by $Foxp3^+$ Treg cells is required for cure, but not prevention, of T cell transfer colitis, whilst DC

expression of the integrin CD103 and OX40 expression by Foxp3^{+} Treg cells are required for prevention of T cell transfer colitis (Asseman et al. 1999, Annacker et al. 2005, Griseri et al. 2010).

As a reductionist system to interrogate the role of single genes and their products to induction or regulation of intestinal inflammation, the T cell transfer colitis model provides an extremely useful tool. Genetic deficiencies of key factors can be selectively deleted in either the recipient *Rag1*$^{-/-}$ mouse, or in donor CD4^{+} T cells populations, allowing analysis of the contribution of distinct immune cell compartments. Furthermore, this model is dependent upon the presence of the commensal microbiota, and antigen-specificity towards components of the intestinal microbiota is required for induction of intestinal inflammation (Feng et al. 2010). However, caveats of the T cell transfer colitis model include the lack of numerous adaptive immune cell populations, including B cells, CD8 T cells and NKT cells, all of which may potentially contribute to intestinal inflammation. Furthermore, adoptive transfer of CD4^{+} T cells to a lymphopenic environment results in vigorous lymphopenia driven proliferation, expansion and intra-clonal competition, which may polarise the overall antigen-specificity of the T cell repertoire towards only highly reactive clones (Kieper et al. 2005). Nonetheless, the T cell transfer model of colitis has been supremely useful for elucidating pathways fundamental to induction and regulation of intestinal inflammation.

1.5.2 *Helicobacter hepaticus* induced IBD in immune competent mice.

Helicobacter hepaticus (*H. hepaticus*) is one of a small number of culturable bacteria that have been directly linked to development of chronic intestinal inflammation in susceptible mouse strains. *H. hepaticus* is a gram-negative, micro-aerophilic, helical bacteria with bipolar flagella, that is extremely motile and is able to persistently colonise the murine caecum, colon and liver, where it was first isolated from A/JCr mice exhibiting chronic hepatitis (Fox et al. 1994). Oral infection of susceptible

mouse strains, such as *Il10*$^{-/-}$ mice or 129*SvEv Rag2*$^{-/-}$ mice, triggers marked inflammation throughout the large intestine, including the colon (colitis) and caecum (typhlitis), collectively termed typhlocolitis (Burich et al. 2001, Maloy et al. 2003, Tomczak et al. 2003, Maggio-Price et al. 2006). Upon infection, *H. hepaticus* localises to the mucus layer on the apical surface of the intestinal epithelium, in addition to successfully penetrating the intestinal crypts, where it emerges as a dominant component of the mucosal microbiota (Young et al. 2004). However, WT C57BL/6 mice are highly resistant to *H. hepaticus* triggered intestinal inflammation despite life-long colonisation and induction of robust adaptive immune responses, in part due to induction of *H. hepaticus*-specific Treg cells that actively prevent pathogenic effector responses to *H. hepaticus* through secretion of IL-10 (Kullberg et al. 2002). Thus, the pathogenic potential of *H. hepaticus* infection in WT mice can be revealed following perturbation of IL-10 dependent regulatory pathways (Burich et al. 2001, Kullberg et al. 2006). In this model, IL-23-dependent $CD4^+$ T cell responses drive tissue pathology in both the colon and caecum, where the disease is particularly severe (Kullberg et al. 2006). The $CD4^+$ T cell effector response is characterised by potent Th1 and Th17 cell responses, similar to that described in the T cell transfer model of colitis, and antibody-mediated blockade of IFN-γ significantly attenuates intestinal inflammation (Kullberg et al. 2006).

Together, the use of these models of chronic inflammatory intestinal inflammation allow detailed mechanistic insight to the regulatory and pro-inflammatory responses occurring during intestinal immune homeostasis and inflammation and provide well controlled experimental conditions for assessment of the suitability of immune candidates for future therapies.

1.6 IL-1 family cytokines and the inflammasome

The IL-1 cytokine family contains 11 members, which mediate a wide array of immune responses, and consists of IL-1α, IL-1β, IL-1Ra, IL-18, IL-33 and IL-1F5-10 (Sims and Smith 2010). All genes encoding IL-1 family cytokines possess a highly

conserved structure, identical location of certain introns and conserved usage of key amino acid sequences, likely arising from duplication of a common gene (Sims and Smith 2010). All IL-1 family cytokines exert their biological effects through ligation and activation of extracellular cytokine receptors, however; only *Il1rn* (encoding IL-1Ra) demonstrates a classical signal peptide for secretion by the endoplasmic reticulum and Golgi apparatus (Sims and Smith 2010). How other IL-1 family members are secreted remains to be determined, yet it is clear that both IL-1β and IL-18 are translated and stored within the cytoplasm as inactive precursors, which require further enzymatic cleavage of pro-domains to render them biologically active.

NLRs are cytosolic sensors of exogenous and endogenous danger signals, which when triggered promote formation of a multi-molecular complex termed an inflammasome, which acts as a platform for activation of caspase-1, leading to maturation and release of IL-1 family cytokines, IL-1β and IL-18 (Schroder and Tschopp 2010). Caspases are cysteine proteases synthesised as inactive zymogens, which require activation to mediate their key roles in inflammation and cell death. Activation of caspase-1 occurs downstream of many inflammasome assemblies, which are often dependent upon the adaptor molecule Asc, and are identified by the contributing NLR family member. The NLRP3- and NLRC4-dependent inflammasomes are the best characterised to date, though others dependent upon NLRP6 (Elinav et al. 2011), NLRP1 (Boyden and Dietrich 2006, Bruey et al. 2007, Faustin et al. 2007) and AIM2 (Burckstummer et al. 2009, Fernandes-Alnemri et al. 2009, Hornung et al. 2009, Roberts et al. 2009) have been described. A similar mode of activation occurs for NLRP3 and NLRC4, though the initiating stimuli differ. NLRP3 activation results in conformational change, oligomerisation and clustering, promoting recruitment of the central adaptor molecule, Asc, itself essential for binding and recruitment of pro-caspase-1. Clustering of multiple pro-caspase-1 molecules triggers autocatalysis and activation of caspase-1, which mediates subsequent cleavage of pro-IL-1β and pro-IL-18 that is required for the release of the active

forms of these pro-inflammatory cytokines. The remaining IL-1 family members are not processed by the inflammasome (Davis et al. 2011).

Within the last decade, inflammasomes have emerged as central regulators of the innate immune response to a variety of infectious agents and inflammatory insults (Schroder and Tschopp 2010). However, genetic mutations in *NLRP3*, resulting in chronic activation of this complex present as severe autoinflammatory disorders, including Familial cold autoinflammatory syndrome (FCAS), Muckle Wells syndrome (MWS) and Neonatal onset multisystem inflammatory disease (NOMID) (Masters et al. 2009). Patients with these autoinflammatory disorders respond remarkably well to treatment with biological therapies designed to block IL-1/IL-1R1-interactions (Lachmann et al. 2009, Kuemmerle-Deschner et al. 2013, Moll and Kuemmerle-Deschner 2013).

IL-1 family cytokine receptors also share significant structural homology, characterised by intracellular IL-1/TLR (TIR) domains and extracellular immunoglobulin superfamily domains. Consistent with their expression of homologous TIR domains, signalling downstream of IL-1 family receptors is akin to that of TLR (Sims and Smith 2010). In the instance of IL-18, initial binding at low affinity to IL-18R1 allows recruitment of the high affinity IL-18 Receptor accessory protein (RaP) resulting in co-localisation of two TIR domains (Parnet et al. 1996). Upon receptor assembly and the juxtaposition of the TIR domains, recruitment of various key adaptor molecules, including MyD88, IL-1R-associated kinase 4 (IRAK4) and TNFR-associated factor 6 (TRAF6) promotes biological activity of IL-18, which is typically associated with activation of NFκB and MAPK-signalling pathways (Sims and Smith 2010).

1.7 IL-18 and intestinal inflammation

IL-18 was originally discovered in mice infected with *Propionibacterium acnes* and subsequently challenged with lipopolysaccharide (LPS), as a novel factor able to induce IFN-γ production from T cells, and thus was termed IFN-γ-inducing factor

(IGIF) (Okamura et al. 1995). The ability of IL-18 to elicit IFN-γ production from T cells was dependent upon the presence of IL-2 (Kohno et al. 1997). A variety of haematopoietic and non-haematopoietic cells can express IL-18, including Kupffer cells, macrophages, dendritic cells, keratinocytes, osteoblasts, adrenal cortex cells, epithelial cells and microglia (Conti et al. 1997, Matsui et al. 1997, Stoll et al. 1997, Udagawa et al. 1997, Stoll et al. 1998, Prinz and Hanisch 1999, Gracie et al. 2003). Within the intestinal mucosa, IL-18 production has been reported from epithelial cells, dendritic cells and macrophages (Monteleone et al. 1999, Pizarro et al. 1999, Kanai et al. 2000). Early reports indicated that IL-18 was elevated in patients with IBD, especially those with CD. IL-18 was detectable in the serum of patients with CD, levels of IL-18 were elevated in active lesions compared to non-involved tissue, and the level of IL-18 correlated with severity of inflammation (Monteleone et al. 1999, Pizarro et al. 1999, Kanai et al. 2000). Interestingly, these studies also highlighted a distinct shift in production of IL-18 within CD patients. Using immunohistochemistry (IHC) analysis, IL-18 localised to the epithelium within non-involved regions, whereas in inflamed lesions, production of IL-18 was from lamina propria cells morphologically described as macrophages (Pizarro et al. 1999). This altered distribution of IL-18 under inflammatory conditions appears specific to CD, as IL-18 localised to epithelial cells in patients with UC, regardless of inflammatory severity (Pizarro et al. 1999). Furthermore, the bioactivity of mature IL-18 is regulated by production of an endogenous IL-18 binding protein (IL-18BP), levels of which are also elevated under inflammatory conditions, including CD, thus the contribution of IL-18 to clinical intestinal inflammation remains unclear (Corbaz et al. 2002, Naftali et al. 2007, Leach et al. 2008).

Genetic studies have identified a number of polymorphisms within the inflammasome/IL-18 immune axis that are associated with susceptibility to IBD. Single nucleotide polymorphisms in *Nlrp3*, leading to decreased expression, have been identified as associated with susceptibility to IBD (Schoultz et al. 2009, Villani et

al. 2009, Cummings et al. 2010), although this association was not observed in all patient cohorts (Lewis et al. 2011). Furthermore, ubiquitin-mediated trafficking of sensory components tempers NLRP3 inflammasome activation following degradation within the autophagosome (Shi et al. 2012). Mutations in components of the autophagy pathway, including the key adaptor ATG16L1, are also associated with IBD susceptibility (Hampe et al. 2007), and ATG16L1-deficient mice demonstrate enhanced susceptibility to acute DSS colitis, a phenotype rectified through antibody-mediated blockade of IL-1β and IL-18 (Saitoh et al. 2008). Additionally, GWAS studies have directly identified polymorphisms in the IL-18R1-IL-18RaP locus as being associated with susceptibility to both early-onset and adult IBD (Wellcome Trust Case Control 2007, Barrett et al. 2008, Zhernakova et al. 2008, Imielinski et al. 2009). The effects of the polymorphisms on IL-18 receptor complex expression and function remain unknown.

A number of studies have directly assessed the role of IL-18 in induction of intestinal inflammation, most often employing the acute DSS model of colitis. Early studies using monoclonal antibody mediated blockade of IL-18 demonstrated a pathogenic role for this cytokine, and blockade of IL-18 was associated with decreased tissue levels of TNF-α and IFN-γ (Kanai et al. 2001, Siegmund et al. 2001). In these initial studies, depletion of macrophages or IL-18 blockade limited tissue pathology, suggesting that macrophage-derived IL-18 may promote intestinal inflammation (Kanai et al. 2001). Later studies using transgenic mice overexpressing IL-18 suggested that IL-18 may in fact promote monocyte/macrophage accumulation within the lamina propria, though whether this was a direct result of IL-18R-signalling or a reflection of the elevated inflammation was not investigated (Ishikura et al. 2003). Together, these investigations suggested that IL-18 derived from lamina propria mononuclear cells promotes intestinal inflammation during acute challenge with DSS. However, more recent studies focusing on the role of distinct inflammasome components in intestinal immune regulation have challenged this conclusion. Recent reports documented that *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and caspase-1$^{-/-}$ mice were hyper-susceptible

to DSS colitis, and that this susceptibility phenotype could be partially rescued by administration of exogenous IL-18 (Dupaul-Chicoine et al. 2010, Zaki et al. 2010). Thus, conflicting results demonstrate IL-18 may play both protective and pathogenic roles in this model of acute intestinal inflammation. The role of IL-18 in chronic models of intestinal inflammation has not been extensively investigated. However, consistent with the hypothesis that IL-18 drives pathogenic responses within the colonic lamina propria, local administration of an adenovirus expressing antisense IL-18 mRNA was sufficient to ameliorate established colitis in SCID mice transferred with naïve $CD4^+$ T cells (Wirtz et al. 2002). The precise mechanisms through which IL-18 may regulate intestinal immune homeostasis remain unclear, and the identification of such pathways was a key aim of this thesis.

1.8 Thesis aims

Accumulating evidence from animal models and genetic studies in humans has identified a potential involvement for the pro-inflammatory cytokine IL-18 in intestinal inflammation. Despite this, little research has been conducted on the requirement for IL-18 in murine models of chronic intestinal inflammation, with most investigators focussing on acute models of chemically driven colitis, which have produced conflicting results. We sought to analyse the role of IL-18 within the intestine during immune homeostasis and inflammation, and to do so, we took advantage of the models of chronic intestinal inflammation developed within our laboratory.

Chapter 2 Materials and Methods

2.1 Mice

C57BL/6J (B6) WT, B6 *Il18r1*$^{-/-}$, B6 *Il18*$^{-/-}$, B6 *SJL* CD45.1, B6 IL-23R^{gfp}, B6 IL-10^{gfp}, B6 Foxp3^{hCD2}, B6 *Rag1*$^{-/-}$, B6 *Rag1*$^{-/-}$ *Il18r1*$^{-/-}$ and B6 *Rag1*$^{-/-}$ *p19*$^{-/-}$ mice were maintained in isolator cages in specific pathogen-free (SPF) facilities at the University of Oxford. B6 *Il18r1*$^{-/-}$ and B6 *Il18*$^{-/-}$ mice were originally purchased from The Jackson Laboratory (Maine, USA) and B6 *Il18r1*$^{-/-}$ mice were crossed to B6 *Rag1*$^{-/-}$ or B6 Foxp3^{hCD2} IL-10^{gfp} mice to generate B6 *Rag1*$^{-/-}$ *Il18r1*$^{-/-}$ and *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice, respectively. B6 Foxp3^{hCD2} mice were a generous gift from Professor Herman Waldmann, and Professor Richard Flavell kindly provided B6 IL-10^{gfp} mice. In addition to routine SPF screening, mice were regularly screened for *Helicobacter* species. All experiments were conducted in accordance with the UK Scientific Procedures Act (1986) under a Project License (PPL) authorized by the UK Home Office Animal Procedures Committee and approved by the Sir William Dunn School of Pathology Local Ethical Review Committee.

2.2 Antibodies

Antibodies used are detailed in Table 2.1. All antibodies are specific for murine targets unless stated otherwise. Antibodies used *in vivo* for cytokine blockade or cell depletion were shown to contain <0.1 EU endotoxin/ml assessed by Limulus Amebocyte Lysate (LAL) assay (Lonza, Belgium).

Table 2.1 Antibodies used for FACS and immunofluorescence staining, *in vitro* and *in vivo* stimulation/blockade

Antigen	Clone	Source	Fluorochrome	Concentration
Immunofluorescence				
IL-18	Rabbit pAb	Abcam	-	1:200
E-cadherin	Mouse pAb	BD Bioscience	-	1:400
Cell surface staining				
CD16/32	93	eBioscience	-	1:200
CD4	GK1.5	eBioscience	PeCy7, APC	1:400
CD4	RM4-5	eBioscience	PE, FITC	1:400
TCRβ	H57-597	eBioscience	PerCP-Cy5.5	1:400
CD45.1	A20	eBioscience	eFluor450	1:200
CD45.2	104	eBioscience	FITC	1:200
CD25	7D4	eBioscience	PE, Bio	1:400
CD45RB	16A	eBioscience	FITC	1:200
B220	RA36B2	eBioscience	PerCP-Cy5.5	1:400
hCD2	RPA-2.10	eBioscience	APC, PE	1:200
IL-18R1	112614	R&D Systems	FITC, APC	1:25
GITR	DTA-1	eBioscience	PE	1:100
CTLA4	UC10-4B9	eBioscience	PE	1:100
ICAM-1	KAT-1	eBioscience	PE	1:100
CD103	2E7	eBioscience	PE	1:100
CXCR3	CXCR3-173	eBioscience	APC	1:200
ICOS	15F9	eBioscience	APC	1:200
OX40	OX-86	eBioscience	PE	1:100
PD-1	J43	eBioscience	eFluor450	1:200
CD62L	MEL-14	eBioscience	PE	1:400
Intracellular staining				
Foxp3	FJK-16s	eBioscience	APC, PE	1:100
IL-17A	eBio17B7	eBioscience	APC, PE	1:100
IFN-γ	XMG1.2	eBioscience	eFluor450, PE	1:100
Isotype controls				
IgG1	R3-34	eBioscience	FITC/PE/APC	1:100
IgG1	A110-1	BD Bioscience	APC	1:100
IgG2a	R35-95	eBioscience	FITC/APC/PE	1:100
IgG2b	A95.1	BD Bioscience	PerCP-Cy5.5	1:100
In vitro				
CD3 (agonistic)	145-2C11	eBioscience	-	1μg/ml
CD28 (agonistic)	37.51	eBioscience	-	1μg/ml
IFN-γ	XMG1.2	eBioscience	-	10μg/ml
IL-4	11B11	eBioscience	-	10μg/ml
In vivo				
IL-10R	1B1.2 1C4	In house	-	1mg/week

2.3 Bacteria

2.3.1 Culture of *Helicobacter hepaticus*

Helicobacter hepaticus NCI-Frederick Isolate 1A (American Type Culture Collection, Hh1A, strain 51449) was grown on blood agar plates containing selection antibiotics, Trimethoprim (5µg/ml), Vancomycin (10µg/ml) and Polymixin-B (25U/ml) (TVP; Oxoid, UK) at 37°C under a microaerophilic atmosphere (10% H_2, 10% CO_2, N_2 balance) in CampyPak jars (Oxoid). Once established, bacteria were expanded in liquid cultures of Tryptone Soy Broth (TSB) with TVP and 10% Foetal Calf Serum (FCS) (PAA Solutions, UK) in vented Erlenmeyer flasks under a microaerophilic atmosphere, shaking at 170rpm at 37°C. Liquid cultures were split daily and inoculated at OD_{600} 0.05. Bacterial viability was assessed prior to infection with a Live/Dead assay kit (Molecular Probes, UK), following the manufacturers recommended protocol.

2.3.2 Assessment of *Helicobacter hepaticus* colonisation

At sacrifice, caecal contents were isolated and total genomic DNA purified using a Stool DNA Isolation Kit (Qiagen, UK). Isolated stool DNA was subjected to qRT-PCR analysis using *H. hepaticus cdt*B gene specific primers using a Chromo4 detection cycler (MJ Research, UK). Quantitation of *H. hepaticus* colonisation was performed by generating a standard curve using serial dilutions of pure *H. hepaticus* DNA. Whole caecal DNA (20ng in 4µl) was added to reaction mixtures of final volume 25µl and *H. hepaticus* DNA concentration expressed as [*H. hepaticus* DNA]/20ng total caecal DNA.

2.3.3 Assessment of Segmented Filamentous Bacteria colonisation

At sacrifice, caecal contents were isolated and total genomic DNA purified using a Stool DNA Isolation Kit (Qiagen, UK) following the manufacturers recommended protocol. Isolated stool DNA was subjected to qRT-PCR analysis using SFB 16s rRNA gene specific primers, as described (Barman et al. 2008) using a Chromo4 detection cycler (MJ Research).

2.4 Flow cytometry

2.4.1 Antibodies

Flow cytometry antibodies are described in Table 2.1. Stained cells were acquired on a Dako Cyan (Beckman Coulter, Oxford, UK), LSRII or Fortessa (BD Bioscience, Oxford, UK) and sorted on a FACSAriaIII (BD Bioscience, Oxford, UK), MoFlo, or Astrios (Beckman Coulter, UK). Flow cytometry data was analysed using FlowJo software (TreeStar Inc. Oregon, USA).

2.4.2 Surface staining

Single cells suspensions (~$5x10^5$ - $1x10^6$ cells/well) were stained with the antibodies detailed in Table 2.1. All staining incubations were performed in 50µl PBS/0.1% BSA in round bottom 96-well plates for 20 min at 4°C. Cells were first stained with Fc-block (anti-CD16/32) (eBioscience, UK) to prevent subsequent non-specific antibody binding. In addition, cells were stained with Fixable Viability Dye eFluor® 780 (eBioscience, UK) to distinguish live cells according to manufacturers recommendations. Cells were washed with PBS/0.1% BSA then resuspended in PBS/0.1% BSA containing a cocktail of fluorescently labelled antibodies. Cells were again washed in PBS/0.1% BSA and either analysed/sorted immediately or resuspended in Fixation/Permeabilisation buffer (eBioscience, UK) and kept at 4°C overnight, protected from direct light, for future analysis or intracellular staining.

2.4.3 Restimulation of cells for intracellular cytokine staining

Single cell suspensions were isolated from organs as described in section 2.5. Cells were restimulated for 4 hours in 96 well round bottom plates (Corning) at 37°C in IMDM media supplemented with 10% FCS, 2mM L-glutamine, 100U/ml Penicillin/Streptomycin (all PAA solutions) with 0.1µg/ml Phorbol 12-myristate 13-acetate (PMA), 1µg/ml Ionomycin and 10µg/ml Brefeldin A (all Sigma Aldrich, UK), or 10µg/ml Monensin (BioLegend, UK). Following restimulation, cells were centrifuged and the supernatant discarded. Cells were twice washed in PBS/0.1% BSA and cell surface stained and fixed as in section 2.4.2.

2.4.4 Intracellular staining

Fixed cells were washed twice in PBS/0.1% BSA with centrifugation at 1600rpm before being resuspended in 100µl Fix/Perm Buffer (eBioscience, UK) and for 60 minutes incubated at 4°C protected from light. Cells were washed twice with 200µl Fix/Perm Buffer before being resuspended in 50µl Fix/Perm Buffer containing a cocktail of fluorescently labelled antibodies and incubated at 4°C for 30 minutes protected from light. Cells were washed twice in Fix/Perm Buffer and then twice in PBS/0.1% BSA with centrifugations at 1600rpm before being resuspended in 400µl PBS/0.1% BSA for analysis.

2.5 Cell isolation, enrichment and sorting

2.5.1 Isolation of spleen and lymph node cells

Suspensions of single cells were prepared from individual lymphoid organs by passage through 70 - 100µM cell strainers (Falcon, BD Biosciences, Oxford, UK), or 70µM pore nylon mesh (Plastok Associates, UK). Cells were washed in PBS/0.1% BSA followed by centrifugation at 1500 rpm for 5 min. Red blood cell lysis was performed by addition of ACK lysis buffer (10mM $KHCO_3$, 150mM NH_4Cl, 0.1mM Na_2.EDTA, pH 7.2-7.4) for 3 min at room temperature. Cells were washed, filtered through 70µM cell strainers and resuspended in ice-cold PBS/0.1% BSA.

2.5.2 Isolation of bone marrow cells

Bone marrow cell were isolated for *in vitro* and *in vivo* experiments in an identical manner. Briefly, at sacrifice, hind limbs were carefully removed to ensure sterility and integrity of the whole bone structure. Muscle and connective tissue was dissected away and whole bones briefly washed in 70% EtOH. Femurs and tibias were flushed with ice-cold PBS, cells filtered through 70µM cell strainers and red blood cell lysis performed for *in vitro* experiments. Cells were resuspended in ice-cold PBS for *in vivo* use, or appropriate cell culture media for *in vitro* experiments.

2.5.3 Isolation of colonic lamina propria leukocytes (cLPL)

Large intestines (colon and caecum) were cut open longitudinally and the luminal contents removed. Whole colons were cut into multiple small pieces (~2cm) and

placed in ice-cold PBS/0.1% BSA in a 50ml tube (Falcon, BD Biosciences, Oxford, UK). Tubes were shaken by hand to remove any remaining luminal contents, supernatants aspirated and replaced with ~10ml pre-warmed (37°C) RPMI/5% FCS supplemented with 5mM EDTA (VWR International, UK). Tubes were placed in a shaking incubator (15 min, 37°C, 200rpm) to remove intestinal epithelial cells. Supernatant containing the epithelial layer was aspirated, washed and resuspended in ice-cold PBS/0.1% BSA for analysis. This washing process was repeated a total of 3 times. Next, in order to neutralise the EDTA activity, ~10ml of RPMI/5% FCS containing 15mM Hepes (PAA, UK) was added to each sample for 10 min at room temperature. Supernatant was discarded and replaced with pre-warmed RPMI 1640/10% FCS containing 0.4mg/ml type VIII collagenase (Sigma Aldrich, UK) and 40µg/ml DNase I (Roche, UK). Tubes were shaken (60 min, 37°C, 200rpm) in order to aid tissue digestion. Supernatants were collected and filtered through 70µM nylon mesh, washed in PBS/0.1% BSA and resuspended in ~10ml ice-cold RPMI/5% FCS/EDTA to quench collagenase activity. Cells were centrifuged and resuspended in 3ml P30 solution (30% Percoll (GE, Amersham, UK)). Overlaying 3ml P75 with 4ml P40 and then 3ml P30 containing colonic lamina propria leukocytes created a three-layered discontinuous gradient. Gradients were centrifuged (1800 rpm, 10°C, minimal acceleration, no brake) and enriched leukocytes isolated from the P40/P75 interface. Cells were then washed, centrifuged (1700 rpm) and resuspended in ice-cold PBS/0.1% BSA.

2.5.4 Enrichment of $CD4^+$ T cells

Single cell suspensions prepared from the spleen (as in section 2.5.1) were incubated with a panel of rat anti-mouse mAb against cell surface markers CD8, MHC-II, CD11b and B220, for 20 min on ice. Cells were washed and resuspended in ice-cold PBS/0.1% BSA at $1x10^8$ cells/ml. Sheep anti-rat IgG-coated beads (Dynal, Invitrogen) were washed twice in PBS/0.1% BSA and mixed with the cells at a 1:1 ratio. The cell/bead mixture was incubated with constant rotation for 30 min at 4°C. The suspension was then placed on a magnet (Dynal, Invitrogen) and the enriched

cell suspension, depleted of beads, was aspirated to a fresh 14ml tube (Falcon, BD Biosciences, UK). This process was repeated twice more for a total of 3 enrichments on the magnet. Cell suspensions were washed with PBS/0.1% BSA and resuspended in ice-cold PBS/0.1% BSA. Harvested cells were consistently ~80% $CD4^+$ T cells.

2.5.5 Fluorescence-activated cell sorting (FACS)

Isolated spleen cells were enriched for $CD4^+$ cells as described in section 2.5. Enriched $CD4^+$ T cells were surface stained for expression of CD4, CD25, CD62L, CD44, CD45RB and hCD2 using antibodies (described in section 2.2) at a concentration of $1x10^8$/ml. Stained cells were washed in ice-cold PBS/0.1% BSA, filtered through 70μM cell strainers and resuspended at $5x10^7$ cells/ml and purified using a FACSAriaIII (BD Bioscience, UK), MoFlo or Astrios (Beckman Coulter, UK) cell sorter. Naive cells were sorted as $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ (*in vivo)* or $CD4^+$ $CD62L^+$ $CD44^-$ $CD25^-$ (*in* vitro) and regulatory cells as either $CD4^+$ $CD25^+$ $CD45RB^{lo}$ or $CD4^+$ $hCD2^+$ $CD45RB^{lo}$. Routine re-analysis was utilised to assess purity which was typically >97%.

2.6 Induction of intestinal inflammation

2.6.1 T cell transfer colitis

Naïve $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ T cells were isolated following the steps described above (section 2.5) and injected intraperitoneally (i.p.) into sex matched recipient mice ($4x10^5$ cells/mouse) in 200μl ice-cold sterile PBS. Mice were monitored weekly to assess clinical signs of disease and cachexia (weight loss). Mice were euthanized at the development of severe weight loss (>20% of initial mass) or when clinical symptoms of colitis were observed. In some experiments, mice were euthanized at earlier time points.

2.6.2 Regulatory T cell mediated prevention of intestinal inflammation

Naïve $CD4^+$ T cells and $CD4^+$ $Foxp3^+$ T cells were isolated following the steps described above (section 2.5). Naïve $CD4^+$ T cells were mixed at a 2:1 ratio with $CD4^+$ $Foxp3^+$ Treg cells and injected i.p. into sex matched recipient mice ($6x10^5$

cell/mouse total) in 200µl ice-cold sterile PBS. Mice were monitored weekly to assess clinical signs of disease and cachexia. Mice were euthanized at the development of severe weight loss (>20% of initial mass) or when clinical symptoms of colitis were observed. In some experiments, where indicated, mice were euthanized at earlier time points.

2.6.3 Induction of T cell mediated colitis in lymphocyte replete mice by infection with *H. hepaticus* and concomitant IL-10R blockade

H. hepaticus was cultured as described in section 2.3.1. Mice were administered $1x10^8$ CFU *H. hepaticus* by oral gavage with a curved 22G feeding needle (Popper and Sons, UK), 3 times on consecutive days. On the first day of *H. hepaticus* infection, and every 7 days following, mice were administered 1mg of αIL-10R mAb by i.p. injection. On day 28, mice were euthanized for histological and further analysis.

2.7 Generation of bone marrow chimeric mice

Recipient mice were exposed to 2 doses of 550 Rad γ-irradiation from a ^{137}Cs source given ~180 minutes apart. Bone marrow cells were isolated as described in section 2.5.2, and $1x10^7$ total bone marrow cells were injected intravenously (i.v.) into irradiated, sex matched, recipient mice in 200µl sterile ice-cold PBS. Mice were monitored daily for 7 days following irradiation and then allowed to rest for >6 weeks to allow full reconstitution of the haematopoietic compartment.

2.8 Generation of samples for cytokine analysis

2.8.1 Preparation of whole colon tissue

At sacrifice, ~5mm pieces of the distal, mid and proximal colon, terminal ileum and liver were dissected, snap frozen on liquid nitrogen and stored at -80°C. Samples were thawed at room temperature and subjected to 2 rounds of homogenisation using Lysing Matrix D Beads (MP Biomedicals, Cambridge, UK) using a FastPrep 24 Homogeniser set at 6.0m/s for 40 seconds. Samples destined for protein analysis were homogenised in 600µl PBS containing protease inhibitor (Roche, Burgess Hill, UK) according to the manufacturers protocol. Samples destined for RNA analysis

were homogenised in 600µl Buffer RLT (Qiagen, Crawley, UK) containing 2-mercaptoethanol (Sigma Aldrich, UK). Following homogenisation, samples were centrifuged at 13,000 rpm for 5 minutes and supernatants removed. For analysis of protein, homogenate supernatants were aliquoted and stored at -20°C. In order to extract RNA, homogenate was loaded onto an appropriate RNAeasy Spin column (Qiagen, UK) and RNA isolated as described in section 2.9.1.

2.8.2 Preparation of colonic tissue explants

Upon sacrifice, ~1cm pieces of distal colon were dissected and weighed using an analytical balance. Tissue samples were rested for 30 minutes in PBS/0.1% BSA supplemented with 100U/ml Penicillin/Streptomycin, then cultured overnight in 400µl RPMI/10% FCS/100U/ml Pen/Strep/ 2mM L-glutamine/ 0.05mM 2-mercaptoethanol in 48 well plates at 37°C and 5% CO_2. Supernatants were harvested and stored at -20°C for future cytokine analysis.

2.8.3 Preparation of *ex vivo* cell culture supernatants

At sacrifice, colonic lamina propria leukocytes were isolated as described in section 2.5.3, and $5x10^4$ – $1x10^6$ cells were cultured overnight in 100µl RPMI/10% FCS/100U/ml Pen/Strep/ 2mM L-glutamine/ 0.05mM 2-mercaptoethanol in round bottom 96-well plates at 37°C and 5% CO_2. Following culture, cells were centrifuged in the plate in order to pellet cells and supernatants collected and stored at -20°C for future analysis. Cells were then lysed in Buffer RLT containing 2-mercaptoethanol and stored at -20°C for future RNA isolation and analysis.

2.8.4 Preparation of *in vitro* cell culture supernatants

Following appropriate experimental stimulations, for example, Th17 differentiation, cells were pelleted by centrifugation and supernatants stored for future analysis as in 2.8.7.

2.8.5 Quantification of protein concentration

Protein concentration from isolated tissues was determined by means of Bradford Assay. Protein Assay solution (Bio-Rad, UK) was diluted 1/5 in H_20 to create a working solution as recommended in the manufacturers protocol. Diluted Protein

Assay solution (180µl) was mixed with protein sample (20µl) in a flat bottom plate. In order to quantitate protein concentration, a standard curve was created by 2-fold dilution of a standard solution of BSA (1mg/ml) with PBS as a blank. Protein concentration was analysed by measuring OD_{600}.

2.8.6 Cell supernatant protein precipitation

In order to concentrate cell supernatants for Western blot analysis, proteins were precipitated with methanol/chloroform. Harvested cell supernatants were mixed with 0.25 volumes of ice-cold chloroform and 1 volume of ice-cold methanol, and centrifuged (13,000 rpm, 10 min, 4°C). The chloroform layer forming above the interface was aspirated and discarded, and the protein interface washed by addition of 1 volume of ice-cold methanol. Following further centrifugation (13,000 rpm, 20 min, 4°C), the supernatant was discarded and protein pellet dried with the aid of a desk lamp. Protein samples resuspended in Lane Marker Reducing Buffer (Thermo Scientific, UK) were boiled for 5 min at 95°C, cooled on ice and loaded onto SDS-PAGE gels for electrophoresis.

2.8.7 Quantification of cytokine concentration

Tissue explants or cell culture supernatants were harvested as described in sections 2.8.2, 2.8.3 and 2.8.4, Cytokine concentration was analysed using a FlowCytomix Bead Assay (eBioscience, UK) according the manufacturers recommendations. Sample data was acquired on a Dako Cyan flow cytometer and analysed with FlowCytomix Pro software. Cytokine concentration were normalised to account for cell number or tissue explant weight.

2.9 Preparation and analysis of RNA, cRNA and cDNA

2.9.1 RNA Isolation (Routine analysis)

RNeasy Mini Plus Kit, RNeasy Mini Kit or RNeasy Micro Kit (all Qiagen, UK) were routinely used for isolation of total RNA. RNA was isolated following the manufacturers protocol including optional DNase I digestion of genomic DNA. Isolated RNA was eluted in RNase-free water and RNA concentration determined using with a NanoDrop machine (Thermo Scientific, UK) using RNase-free water as

a blanking value. Isolated RNA was stored at -80°C or converted to cDNA by reverse transcription using Superscript III Reverse Transcriptase and Oligo d(T) primers (Invitrogen, UK) in line with the manufacturers instructions.

2.9.2 Quantitative PCR

Quantitative PCR (qPCR) analysis of gene expression utilised either of 2 systems, Taqman or SyBR Green. Where primers/probes were designed "in-house", care was taken to ensure PCR products were intron-spanning to avoid amplifying genomic DNA. In all cases, both control samples containing water, or RNA not subject to reverse transcription, were utilised. For SyBR Green reactions, melting point analysis was utilised to identify a single PCR product to ensure primer specificity. For Taqman-based analysis, primer/probe sets were either purchased from Applied Biosystems, or designed and purchased from Invitrogen. Taqman primers (300nmol) were used in combination with Taqman probes (100nmol) labelled with the FAM/TAMRA fluorophore/quencher pair. All qPCR mixtures, 4µl of cDNA was added to create a final volume of 20µl, with reactions run in triplicate and fluorescence detected using a Chromo4 detection cycler (MJ Research, UK). Quantification of gene expression was normalised to that of "house-keeping" genes, GAPDH, HPRT or CD3. Relative mean gene expression differences were calculated through the use of the $2^{-\Delta C(t)}$ method.

2.9.3 RNA Isolation (for microarray analysis)

RNA was isolated from sorted cell populations using the RiboPure Kit (Ambion) according to the manufacturers instructions. RNA concentration and integrity were analysed with a BioAnalyser 2100 (Agilent, UK) according to the manufacturers instructions. RNA was immediately snap frozen on dry ice and stored at -80°C prior to amplification.

2.9.4 RNA Amplification, Hybridisation and Microarray analysis

RNA was prepared as described in section 2.9.3 above. Only samples with RNA integrity number (RIN) values above 7 were used for future analysis. Total isolated RNA was subjected to 2-rounds of amplification before being converted to

biotinylated cRNA using TargetAmp™ 2-Round Biotin-aRNA Amplification Kit (Cambio, UK) according to the manufacturers protocol. Biotinylated cRNA was hybridised to MouseWG-6 v2.0 Expression BeadChip (Illumina). cRNA hybridisation and sample acquisition were performed by the microarray core facility at the Wellcome Trust Centre for Human Genetics, Oxford. Raw intensity data was analysed and exported from Bead Studio and subsequent analysis was performed "in house" with help from Dr. Chris Schiering. Microarray analysis was performed using GeneSpring GX12 software (Agilent). Data was normalised using 75% percentile shift normalisation algorithm and baseline transformed to the median of all samples. Statistical significance was determined using an unpaired t-test followed by Benjamini Hochberg false discovery rate multiple testing correction. P value cut-off was set to 0.05.

2.10 Assessment of systemic and intestinal inflammation

2.10.1 Assessment of cachexia

Prior to induction of colitis, mice were weighed on an analytical balance. Mice were then subsequently weighed to monitor systemic inflammation at intervals dependent upon the experimental model. Following adoptive transfer of naïve T cells, mice were monitored and weighed weekly. Weight loss was represented as % of initial mass and statistical significance determined by repeated-measures ANOVA. Mice showing clinical signs of disease or losing >20% initial body weight were sacrificed regardless of experimental duration.

2.10.2 Assessment of splenic inflammation

At the termination of an experiment, excised spleens were weighed on an analytical balance and total cell count determined by manual counting of single cell suspensions as described in section 2.5.1.

2.10.3 Assessment of intestinal inflammation

At sacrifice, mice were dissected and ~1cm sections of distal, mid and proximal colon were isolated and rinsed in PBS/0.1% BSA. Tissue sections were fixed in 4% formyl saline (Sigma Aldrich, UK) for >24 hours before being paraffin wax embedded.

Transverse sections of 4μm thickness were cut using a microtome (Leica Biosystems) and mounted onto microscope slides with or without Haematoxylin and Eosin staining (Leica Biosystems). Histological analysis was performed as described previously (Izcue et al. 2008). Briefly, intestinal inflammation was scored using a semi-quantitative scale (0-12) in a blinded fashion according to parameters identified in Table 2.2 (below). Mean inflammation scores of 3 colon sections were pooled to provide a representative reflection of total intestinal inflammation. Representative histological micrographs were recorded using a CoolScope (Nikon, Japan).

Table 2.2 – Criteria used for histological assessment of intestinal inflammation

1. Epithelium	**Hyperplasia and/or**	**Goblet Cell Depletion**
0	None	None
1	Mild (1.5x)	Mild (25%)
2	Moderate (2-3x)	Marked (25-50%)
3	Severe (>3x)	Substantial (>50%)
2. Lamina Propria		
0	None – few leukocytes	
1	Mild – some leukocytes at tips of crypts	or – many lymphoid follicles
2	Moderate – marked infiltrate	
3	Severe – dense infiltrate throughout	
3. Area affected	**(% of section)**	
0	None	
1	< 25%	
2	25 – 50%	
3	> 50%	
4. Severe Inflammation		
0	None	
1	Sub-mucosal inflammation	or few crypt abscesses (< 5)
2	Sub-mucosal inflammation	and few crypt abscesses (< 5)
2	Many crypt abscesses (> 5)	or Extensive sub-mucosal inflammation
3	Many crypt abscesses (> 5)	and Extensive sub-mucosal inflammation

3	Ulceration	or Extensive fibrosis

2.11 Cellular immunological assays

2.11.1 Generation of bone marrow derived dendritic cells

Bone marrow was isolated as in 2.5.2. including ACK lysis of red blood cells. Bone marrow cells were cultured in complete RPMI containing 5% cell supernatant from the X63 cell line, which constitutively produces GM-CSF. Cells were cultured in 3ml of media in 6 well plates at $1x10^6$/ml for 3 days, before addition of an additional 3ml of culture media. On day 6, 5ml media was exchanged for fresh culture media, cells we used between day 7 and day 9 post-isolation. Cells were > 80% $CD11c^+$ when tested.

2.11.2 $CD4^+$ cell differentiation

Naïve $CD4^+$ $CD62L^+$ $CD44^-$ $CD25^-$ T cells were FACS sorted as in section 2.5. Cells were cultured in complete IMDM media at 37°C and 5% CO_2. Cells were cultured in 2 different manners.

2.11.3 Naïve $CD4^+$ T cell differentiation on agonistic mAb.

Cells ($2.5x10^5$ cells/well) were cultured in 96 well flat bottom tissue culture grade plates coated with agonistic anti-CD3 mAb (5µg/ml) and soluble agonistic anti-CD28 mAb (1µg/ml). Where indicated, cytokines were added at the following concentrations, unless stated otherwise; IL-1β (10ng/ml), IL-2 (100U/ml), IL-4 (10ng/ml), IL-6 (10ng/ml), IL-12 (10ng/ml), IL-18 (10ng/ml), IL-23 (10ng/ml), TGF-β_1 (250pg/ml or 2.5ng/ml) (All from R&D Systems). For differentiation, cells were cultured in the presence of IL-12 (Th1), IL-4 and IL-2 (Th2), TGF-β_1 (250pg/ml), IL-1β, IL-6 and IL-23 (Th17) or IL-2 and TGF-β_1 (2.5ng/ml) (iTreg). All differentiation experiments were performed in the presence of neutralising antibodies against IFN-γ ± IL-4 ("In house" - 10µg/ml) where appropriate.

2.11.4 Naïve $CD4^+$ T cell differentiation on BMDC

Cells were cultured in 1ml volume in 48 well flat bottom tissue culture grade plates containing $1x10^5$ BMDC and $2.5x10^5$ naïve $CD4^+$ T cells. Cells were stimulated with soluble anti-CD3 (1µg/ml), LPS (100ng/ml; *E. coli* Serotype R515, Alexis

Biochemicals) and TGF-β_1 (1ng/ml). All differentiation experiments were performed in the presence of neutralising antibodies against IFN-γ ± IL-4 ("In house" - 10μg/ml).

For naïve $CD4^+$ T cell differentiation to Th17 cells, following 4-5 days of culture, cells were restimulated as in section 2.4.3, and both surface and intracellular stains performed as in section 2.4.2 and 2.4.4. For iTreg induction assays, following 3 days of culture, both surface and intracellular stains were performed as in section 2.4.2 and 2.4.4 without restimulation.

2.12 Statistics

Statistical analysis of weight loss data utilised a repeated measure ANOVA followed by Bonferroni post-hoc test. Non-parametric tests were used for all other statistical analyses. The Mann-Whitney U test and Wilcoxon matched pairs signed rank test were utilised for comparison of 2 unpaired, or paired, groups respectively. Statistical analysis of more than 2 groups utilised a Kruskal-Wallis one-way ANOVA followed by Dunn's post-hoc test. Divergence of experimental data from a hypothetical value was statistically analysed using a 1-column t test. Statistical analysis was performed in Prism 6.0 (GraphPad Software, Inc.). Data is represented as mean ± standard error of the mean (S.E.M.) Experimental differences were considered statistically significant only when $p \leq 0.05$.

Chapter 3 Direct regulation of colonic Th17 cell differentiation by intestinal epithelial cell derived IL-18

3.1 Introduction

Th17 cells are enriched at mucosal sites (Weaver et al. 2007), where they produce high levels of IL-17A, IL-17F and IL-22 (Langrish et al. 2005, Liang et al. 2006) by which means they play a key role in protective immunity from a variety of extracellular pathogens (Ye et al. 2001, Happel et al. 2003, Huang et al. 2004, Kelly et al. 2005, Ishigame et al. 2009). By contrast, Th17 cells have also been implicated in numerous chronic inflammatory and autoimmune disorders, including intestinal inflammation (Cua et al. 2003, Hue et al. 2006, Kullberg et al. 2006, Uhlig et al. 2006, Korn et al. 2009, Ahern et al. 2010). The factors required for Th17 cell lineage specification under non-inflammatory conditions *in vivo* are beginning to be elucidated. Whilst IL-6 is critical for acquisition of pathogenic effector Th17 cells under inflammatory conditions, the requirement for this cytokine in homeostatic Th17 cell differentiation within the intestine remains controversial (Ivanov et al. 2006, Shaw et al. 2012). By contrast, IL-1β is essential for naïve cell differentiation to a Th17 cell phenotype under both homeostatic and inflammatory conditions at intestinal and extra-intestinal sites (Sutton et al. 2006, Chung et al. 2009, Shaw et al. 2012). Indeed, *Il1r1*$^{-/-}$ mice are resistant to EAE, where Th17 cells mediate pathology, but are hyper-susceptible to enteric infection with *Citrobacter rodentium*, where Th17 cells mediate protective immunity (Sutton et al. 2006, Chung et al. 2009, Lebeis et al. 2009, Torchinsky et al. 2009). Furthermore, we have previously demonstrated that IL-1R1-signalling on CD4^{+} T cells is required for Th17 cell survival and colitogenic potential during T cell transfer colitis (Coccia et al. 2012). IL-1 family cytokines are key co-regulators of CD4^{+} T cell lineage specification, and the role of IL-1β in Th17 cell differentiation is mirrored by the contribution of IL-33 and IL-18 to the differentiation of Th2 and Th1 cells, respectively (Guo et al. 2009, Sims and Smith 2010). How IL-1 family cytokines cross-regulate the differentiation of distinct CD4^{+} T

cell effector subsets is still incompletely understood. Whilst IL-18 is not essential for Th1 cell differentiation, activation of STAT4 in response to IL-12 stimulation promotes the expression of IL-18R1, whereupon IL-18 stimulation can promote proliferation and production of IFN-γ (Okamura et al. 1995, Robinson et al. 1997, Smeltz et al. 2001). Aberrant Th1 cell responses within the gastrointestinal tract are thought to be key contributors to tissue pathology in IBD (Zenewicz et al. 2009), but the role of IL-18 in exacerbating or perpetuating pathogenic Th1 cell responses in the gut is not well characterised.

The potent ability of IL-18 to elicit pro-inflammatory responses is reflected by the array of regulatory mechanisms controlling its secretion and continued bioavailability. Whilst pro-IL-18 (24kDa) is translated as an inactive form, enzymatic cleavage by caspase-1 is required to release mature IL-18 (18 kDa) into the extracellular space. Caspase-1 activation is dependent upon inflammasome assembly, which occurs in response to detection of endogenous or exogenous danger signals (Schroder and Tschopp 2010, Franchi et al. 2012). Thus, IL-18 maturation and secretion requires cellular stress or damage. Once secreted from the cell, inhibitory factors compete with the IL-18R complex to limit the actions of IL-18. In addition to the presence of a soluble neutralising IL-18Rβ (Andre et al. 2003, Fiszer et al. 2007), IFN-γ stimulation elicits production of a soluble IL-18 binding protein (IL-18BP), creating a negative feedback loop to limit the actions of mature IL-18 (Novick et al. 1999). Together, these observations highlight the potent ability of IL-18 to promote Th1 cell responses, and the checkpoints in place to controls its bioactivity.

The role of IL-18R1-signalling in Th1 cell differentiation has been investigated and recent studies elucidating the different cytokine requirements for Th17 differentiation identified *Il18r1* as an IL-23 regulated gene in differentiating Th17 cells, (Ghoreschi et al. 2010), however, the impact of IL-18 on distinct $CD4^+$ T cell subsets within the intestine is yet to be determined. However, before we assessed the role of IL-18 in

intestinal inflammation, we undertook to determine if IL-18 was regulating intestinal immunity in the steady state. In this chapter we aimed to characterise the source of IL-18 within the intestine, and determine the role of this cytokine in promoting distinct $CD4^+$ T cell subsets under homeostatic conditions.

3.2 Results

3.2.1 IL-18R1 is highly expressed by colonic Th17 cells

We initially sought to confirm published observations of *Il18r1* expression by distinct $CD4^+$ T cell subsets differentiated *in vitro*. Naïve $CD4^+$ T cells were either activated in the absence of exogenous differentiation factors (Th0), or activated and polarised under Th1, Th2, Th17 and iTreg skewing conditions. Efficient polarisation of effector and regulatory populations was confirmed by high expression of lineage-associated factors *Ifng, Il4, Il17a* and *Foxp3* (Figure 3.1). We found that high levels of both *Il18r1* and *Il18rap* were expressed under Th1, Th17 and also iTreg polarising conditions (Figure 3.1). In contrast, and in line with reported findings (Smeltz et al. 2001), expression of both *Il18r1* and *Il18rap* was low under Th0 and Th2 polarising conditions (Figure 3.1).

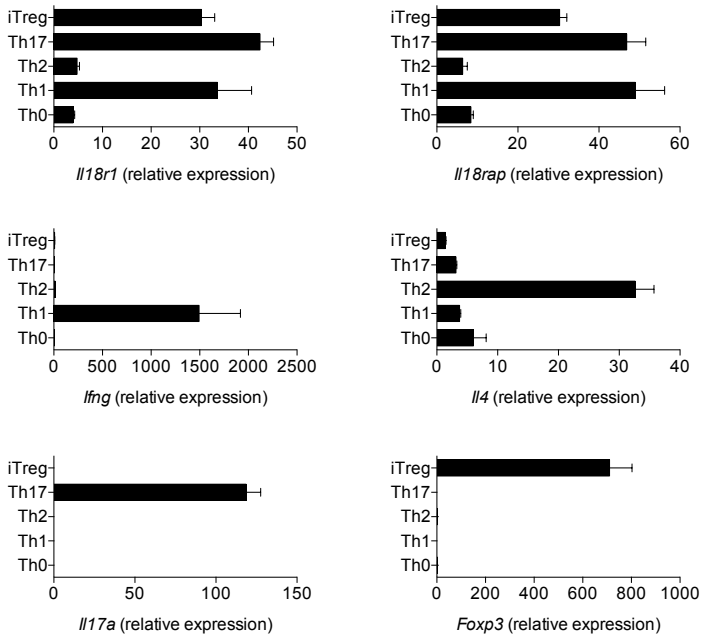


Figure 3.1 *In vitro* polarised Th1, Th17 and Foxp3$^+$ Treg cells express IL-18R components.
FACS-purified naïve WT CD4$^+$ CD25$^-$ CD44$^-$ CD62L$^+$ T cells were activated with plate-bound anti-CD3 (1µg/ml) and soluble anti-CD28 (1µg/ml) and cultured for 4 days under either Th0 (anti-IFN-γ [10µg/ml], anti-IL-4 [10µg/ml]), Th1 (IL-12 [10ng/ml], anti-IL4 [10µg/ml]), Th2 (IL-4 [10ng/ml], anti-IFN-γ [10µg/ml], Th17 (TGF-β_1 [250pg/ml], IL-6 [20ng/ml], IL-1β [10ng/ml], IL-23 [10ng/ml]) or iTreg (TGF-β_1 [2.5ng/ml], IL-2 [100U/ml]) polarising conditions. qRT-PCR analysis of gene expression was calculated relative to *Hprt1*. Data shown is pooled from 3 individual experiments, where bars represent the mean and error bars represent S.E.M.

We next sought to confirm the expression of IL-18R1 by distinct $CD4^+$ T cell subsets *in vivo.* A balance between effector and regulatory $CD4^+$ T cell responses to the microbiota ensures intestinal homeostasis, and Th1, Th17 and $Foxp3^+$ Treg cell populations are present under homeostatic conditions within the intestine. We utilised flow cytometric analysis of isolated leukocytes from steady state WT mice to investigate expression of IL-18R1 by distinct $CD4^+$ T cell populations. In agreement with the gene expression data, IL-18R1 expression was evident on intestinal Th1, Th17 and $Foxp3^+$ Treg cell subsets under homeostatic conditions, however, the proportion of IL-18R1-expressing cells varied between cellular subsets and tissue of origin. Analyses of colonic lamina propria leukocytes (LPL) revealed that IL-18R1 was almost ubiquitously expressed by intestinal Th17 cells (>90%), was highly expressed by Th1 cells (70%), and was expressed by around 40% of $Foxp3^+$ Treg cells (Figure 3.2). Furthermore, IL-18R1 expression was also detectable on Th1, Th17 and $Foxp3^+$ Treg cell populations from peripheral lymphoid organs (MLN and spleen); though the frequency of IL-18R1 expression on peripheral $CD4^+$ T cell subsets was markedly lower than the equivalent population derived from the colonic lamina propria (Figure 3.2). Nevertheless, the hierarchy of IL-18R1 expression was maintained throughout mucosal and peripheral tissue sites (Th17>Th1>$Foxp3^+$) (Figure 3.2). In order to confirm that IL-18R1 expression on distinct $CD4^+$ T cells within the intestine was not an artefact of *ex vivo* restimulation or intracellular staining, we utilised two transgenic reporter mouse strains to assess IL-18R1 expression by isolated $CD4^+$ T cells *ex vivo*. Previous studies within our laboratory used IL-$23R^{gfp/+}$ mice to indicate that $CD4^+$ T cell production of IL-17A within the intestine is limited to the IL-$23R^+$ population (Chris Schiering, D.Phil thesis). In accordance with this cytokine staining, and consistent with our earlier findings with *ex vivo* re-stimulated intestinal Th17 cells, we observed that >90% of intestinal IL-$23R^+$ $CD4^+$ T cells expressed IL-18R1 under homeostatic conditions (Figure 3.3A,B). In a similar vein, we utilised dual-reporter $Foxp3^{hCD2}$ IL-10^{gfp} mice (Kamanaka et al. 2006, Komatsu et al. 2009) to assess IL-18R1 expression by populations of intestinal

Foxp3$^+$ Treg cells. FACS analyses of hCD2$^+$ cells confirmed that roughly 40% of Foxp3$^+$ Treg cells within colonic LPL expressed IL-18R1 under homeostatic conditions (Figure 3.3C,D) in line with data gained through intracellular staining. In addition, GFP$^+$ (IL-10$^+$) and GFP$^-$ (IL-10$^-$) hCD2$^+$ T cells exhibited comparable expression of IL-18R1, indicating that it was not enriched within the IL-10 producing Foxp3$^+$ Treg population (Figure 3.3D). Together these data indicate that IL-18R1-expressing CD4$^+$ T cells are enriched within the intestinal microenvironment, and raised the possibility that in addition to promoting Th1 cell differentiation, IL-18R1-signalling may act to regulate intestinal Th17 and/or Foxp3$^+$ Treg cell populations within the intestine.

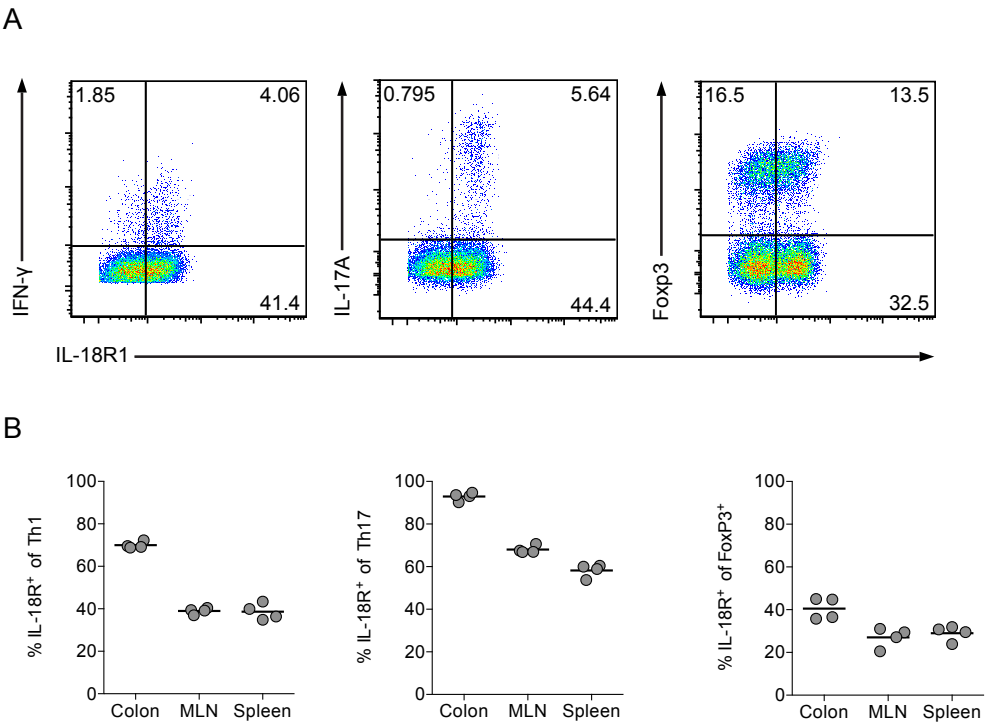


Figure 3.2 Colonic Th1, Th17 and Foxp3$^+$ Treg cells express IL-18R1.
Lymphoid and non-lymphoid tissue leukocytes were isolated from steady state WT mice and Th1, Th17 and Foxp3$^+$ Treg cell expression of IL-18R1 analysed by flow cytometry.
A) Representative FACS plots of IL-18R1 expression on colonic Th1, Th17 and Foxp3$^+$ Treg cells, gated on live CD4$^+$ TCRβ$^+$ cells.
B) Frequencies of IL-18R1$^+$ Th1, Th17 and Foxp3$^+$ Treg cells isolated from colon, MLN and spleen of steady state WT mice.
Data shown represents 1 of 3 independent experiments with similar results. Horizontal bars represent the mean and each dot represents an individual mouse. n=4 mice/individual experiment.

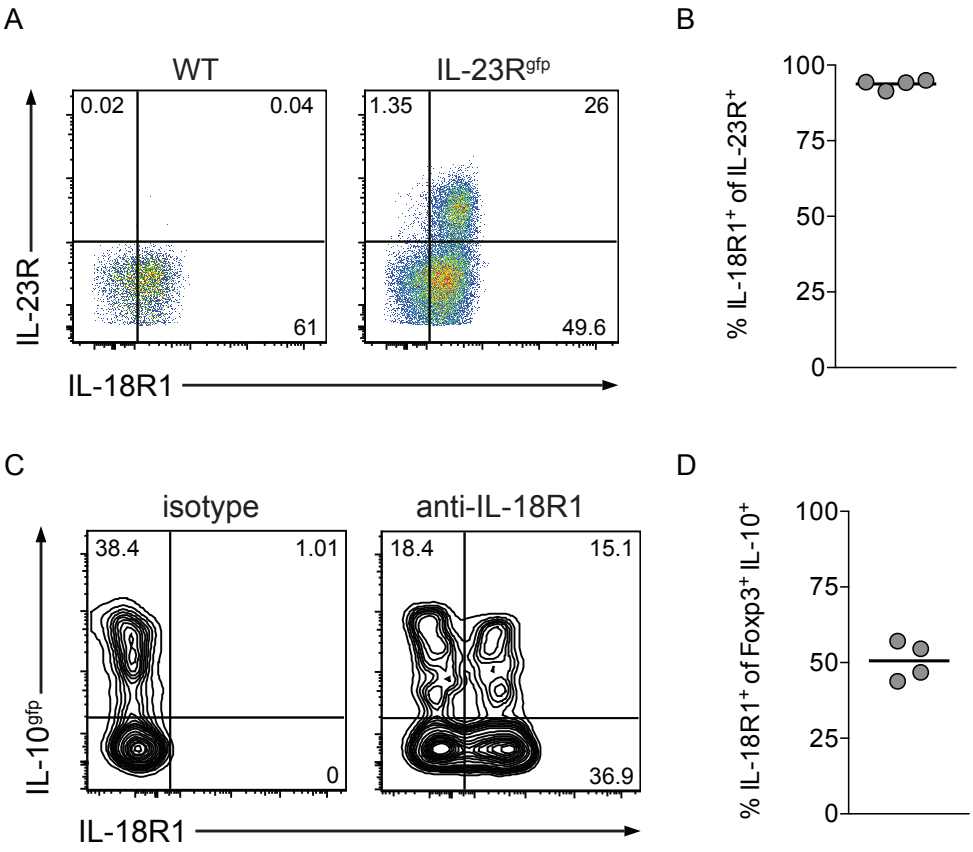


Figure 3.3 Expression of IL-18R1 on colonic IL-23R$^+$ CD4$^+$ T cells and Foxp3$^+$ Treg cells.
Colonic LPL were isolated from IL-23R$^{gfp/+}$ and Foxp3^{hCD2} IL-10^{gfp} reporter strains and live CD4$^+$ TCRβ$^+$ cell expression of IL-18R1 analysed by flow cytometry.
A) Representative FACS plots of IL-18R1 and IL-23R expression on colonic CD4$^+$ T cells from WT (non-gfp) and IL-23R$^{gfp/+}$ mice.
B) Quantitation of IL-18R1 expression by colonic IL-23R$^+$ CD4$^+$ T cells.
C) Representative FACS plots of IL-18R1 and IL-10(gfp) expression by colonic CD4$^+$ Foxp3$^+$ Treg cells from Foxp3^{hCD2} IL-10^{gfp} mice.
D) Quantitation of IL-18R1 expression by colonic Foxp3$^+$ IL-10$^+$ Treg cells.
Data are representative of results from 2 independent experiments with similar results, each with 4 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse.

3.2.2 Canonical IL-18R1 signalling regulates colonic Th17 cell differentiation

In order to determine the impact of IL-18 on intestinal $CD4^+$ T cells, we assessed the frequency of regulatory and effector $CD4^+$ T cells in *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice. We utilised both *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice, because in addition to canonical IL-18/IL-18R1 interactions, IL-18 independent IL-18R1-signalling has been reported (Gutcher et al. 2006, Lewis and Dinarello 2006). Indeed, Gutcher *et al*. demonstrated IL-18R1-signalling on innate immune cells, independently of IL-18, acted to promote pathogenic Th17 cell differentiation and tissue pathology in the EAE model of central nervous system autoimmunity. We observed that the frequency and total number of colonic LPL $CD4^+$ $TCR\beta^+$ cells was unaffected in *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice when compared to WT mice (Figure 3.4A,B). However, we found that genetic deficiency of either IL-18 or IL-18R1 resulted in significantly increased frequencies of intestinal Th1 and Th17 cells when compared to WT mice (Figure 3.4C,E). By contrast, Th1 and Th17 cell frequencies in peripheral lymphoid organs remained unaffected by *Il18/Il18r1*-deficiency (data not shown). The elevated intestinal Th1 and Th17 cell frequencies were not the result of decreased $Foxp3^+$ Treg cell development or accumulation as no significant differences were observed in colonic $Foxp3^+$ Treg cell frequency between WT, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice (Figure 3.4D,E). Thus, given the equivalent increases in effector $CD4^+$ T cell frequencies in both *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice, we concluded that intestinal $CD4^+$ T cell frequencies were being regulated through canonical IL-18/IL-18R1-signalling.

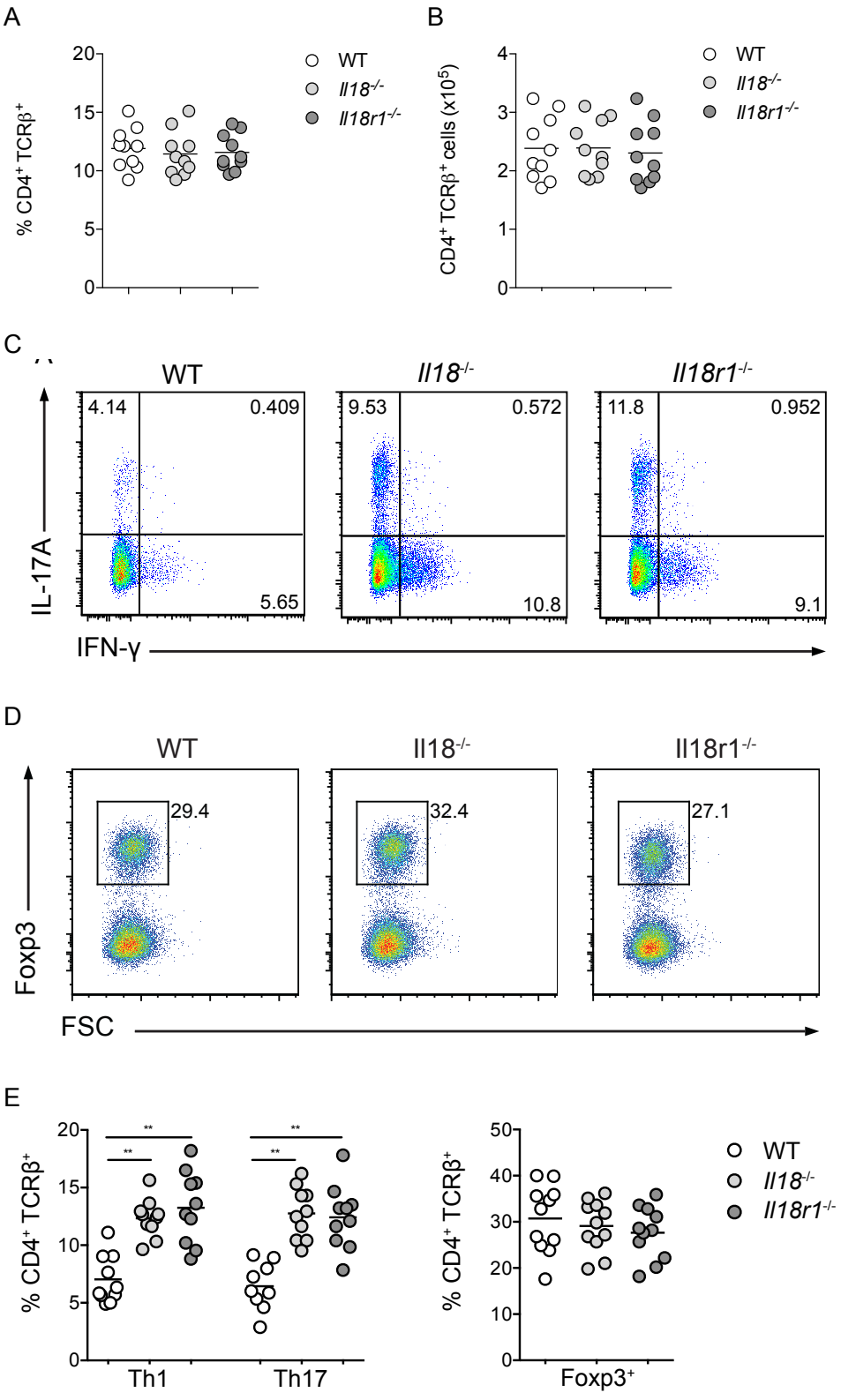


Figure 3.4 Canonical IL-18R1 signalling inhibits colonic effector CD4+ T cell differentiation.

Colonic LPL were isolated from co-housed steady state WT, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice and analysed for Th1, Th17 and Foxp3^{+} Treg cell frequencies by flow cytometry.
A) Frequencies of colonic CD4^{+} TCRβ^{+} cells in WT, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice.
B) Total numbers of colonic CD4^{+} TCRβ^{+} cells in WT, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice.
C) Representative FACS plots of colonic Th1 and Th17 cell populations.

D) Representative FACS plots of colonic Foxp3$^+$ Treg cell populations.
E) Frequencies of colonic Th1, Th17 and Foxp3$^+$ Treg cells.
All FACS plots are gated on live CD4$^+$ TCRβ$^+$ cells. Data shown represents 2 independent pooled experiments. Horizontal bars represent the mean and each dot represents an individual mouse. n=10-11 mice/group. Statistical significance determined with Mann-Whitney test. (** = $p<0.01$).

Based on intracellular cytokine staining of colonic LPL for IFN-γ and IL-17A following restimulation with 12-phorbol 13-myristate acetate (PMA) and Ionomycin, we concluded that frequencies of colonic Th1 and Th17 cells were elevated in the absence of IL-18R1-signalling. Whilst early data suggested that MyD88, through which TLR, IL-1R1 and IL-18R1 all signal, did not regulate intestinal Th17 cell differentiation (Atarashi et al. 2008, Ivanov et al. 2009), recently published data suggest that PMA/ionomycin restimulation may exaggerate the production of IL-17 from Th17 cells (Hirota et al. 2011). Indeed, a recent study using *ex vivo* restimulation with anti-CD3 revealed a role for MyD88-signalling in Th17 cell differentiation within the intestine, likely downstream of TLR and IL-1R1 signalling in APC and T cells respectively (Shaw et al. 2012).

To ensure that our observations were not an artefact of chemical restimulation with PMA and ionomycin, we isolated colonic LPL from steady state WT and *Il18r1*$^{-/-}$ mice and restimulated with anti-CD3. In accordance with an elevated frequency of intestinal Th17 cell *in vivo*, we found that, upon restimulation with anti-CD3, colonic LPL isolated from *Il18r1*$^{-/-}$ mice produced significantly more IL-17A, but not GM-CSF or IL-22, compared to WT colonic LPL (Figure 3.5A). However, whilst levels of IFN-γ production were elevated upon anti-CD3 restimulation of colonic LPL isolated from *Il18r1*$^{-/-}$ mice relative to WT colonic LPL (Figure 3.5A), this increase did not reach statistical significance, likely reflecting the contribution of other cellular subsets, including γδ T cells and CD8^{+} T cells, to the production of IFN-γ following anti-CD3 stimulation.

Furthermore, consistent with the increased IL-17A responses detectable *in vitro*, we also found that the expression levels of genes encoding S100A8 and S100A9 anti-microbial peptides (AMP), which are induced by IL-17A, were significantly elevated in total colonic tissue of *Il18r1*$^{-/-}$ mice when compared to WT controls, whereas expression of the IL-22 induced AMP, Reg3γ (Onishi and Gaffen 2010), was equivalent (Figure 3.5B).

Distinct resident bacterial species can alter intestinal $CD4^+$ T cell phenotype and frequency, with colonisation by segmented filamentous bacteria (SFB) being particularly associated with intestinal Th17 differentiation (Gaboriau-Routhiau et al. 2009, Ivanov et al. 2009, Hooper et al. 2012). However, the increased generation of intestinal Th17 cells observed in *Il18/Il18r1*$^{-/-}$ mice was not due to elevated colonisation with SFB as *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice had comparable SFB levels to WT mice, as assessed by qRT-PCR for 16S rRNA of this species (Figure 3.5C). In addition, as differences in intestinal microbiota have been reported for mice deficient in key inflammasome components and IL-18 (Elinav et al. 2011), in all the experiments described herein, WT, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice were co-housed for >3 weeks prior to analysis, to equalise differences in resident intestinal microbiota. Overall, these data show that canonical IL-18R1-signalling acts to limit differentiation of colonic Th17 cells under homeostatic conditions.

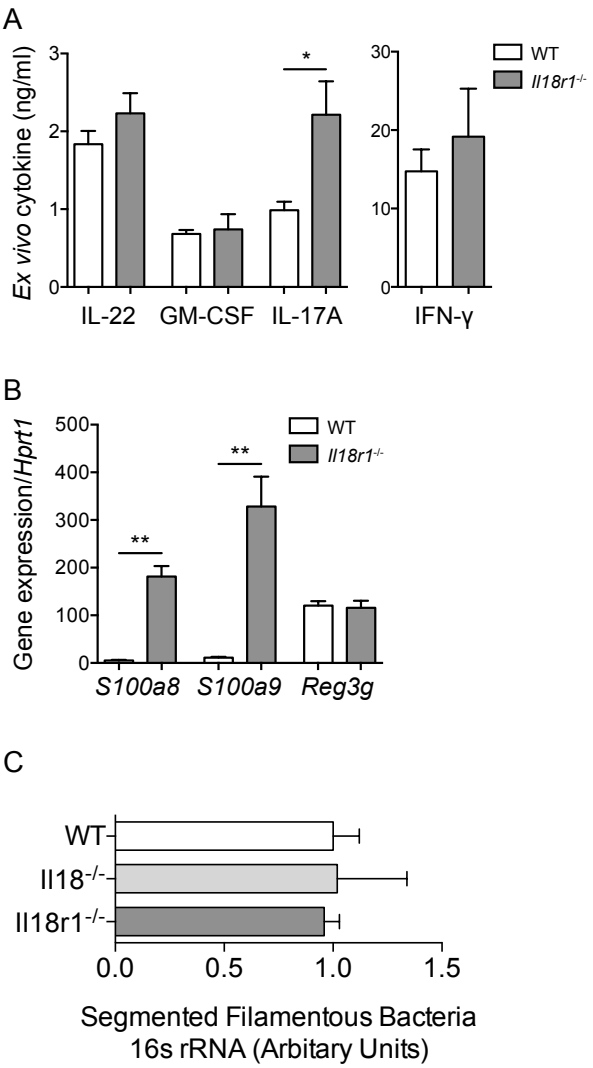


Figure 3.5 IL-18R1-deficiency is associated with elevated colonic levels of IL-17A and IL-17A-regulated genes.

A) Colonic LPL were isolated from steady state WT and *Il18r1*$^{-/-}$ mice and 1x10^6 cells were restimulated with soluble anti-CD3 (1µg/ml) for 16hrs. Quantification of cytokine production was assessed by flow-cytometry based bead array.

B) Whole distal colon tissue was isolated from WT and *Il18r1*$^{-/-}$ mice before extraction of total RNA. qRT-PCR expression of genes encoding anti-microbial peptides was calculated relative to *Hprt1*.

C) Caecal contents of steady state WT and *Il18r1*$^{-/-}$ mice were isolated and subject to DNA extraction before qRT-PCR based analysis of SFB colonisation levels.

Data represent 1 of 2 independent experiments with similar results, each with 4-5 mice/group. Bars represent the mean and error bars represent S.E.M. Statistical significance between experimental groups was determined using Students t test. (*=p<0.05, **=p<0.01).

3.2.3 Intestinal epithelial cell production of IL-18 limits colonic Th17 cell differentiation

We next sought to determine the cellular source of IL-18 within the steady state intestine. Amongst the heterogeneous populations of intestinal mononuclear antigen presenting cells (Coombes and Powrie 2008, Varol et al. 2010, Merad et al. 2013), $CD11b^{+}$ $CD11c^{lo/-}$ $F4/80^{+}$ tissue resident macrophages produce IL-1β upon microbial colonisation of the intestine to promote Th17 cell differentiation (Shaw et al. 2012). We therefore hypothesised that tissue resident macrophages could also represent a cellular source of IL-18 within the intestine, given that IL-1β and IL-18 are both inflammasome processed cytokines (Schroder and Tschopp 2010). However, recent studies reported that mice lacking key inflammasome components within the non-haematopoietic cellular compartment exhibited increased susceptibility to acute colitis triggered by administration of dextran sulphate sodium (DSS) (Dupaul-Chicoine et al. 2010, Zaki et al. 2010). This susceptibility could be partially rescued by administration of exogenous IL-18, implying a protective role for IL-18 derived from non-haematopoietic cells during acute colitis. Furthermore, *Il18* expression within intestinal epithelial cells (IEC) was elevated in the presence of an intestinal microbiota when compared to germ free controls (Pott et al. 2012). Therefore, IEC represent another potential source of IL-18 during homeostatic conditions.

We undertook two complementary approaches to determine the cellular source of IL-18 within the healthy intestine. First, we used immunofluorescence microscopy on formalin-fixed and paraffin-embedded colon sections to localise IL-18. Analysis of colonic tissue sections from WT steady state mice identified ubiquitous cytoplasmic IL-18 staining within E-cadherin^{+} cells of the intestinal epithelium, with very few IL-18^{+} cells identifiable in the underlying lamina propria tissue (Figure 3.6). Importantly, the specificity of the IL-18 staining was verified using sections from *Il18*$^{-/-}$ mice, which do not produce IL-18 in any tissue (Figure 3.6B).

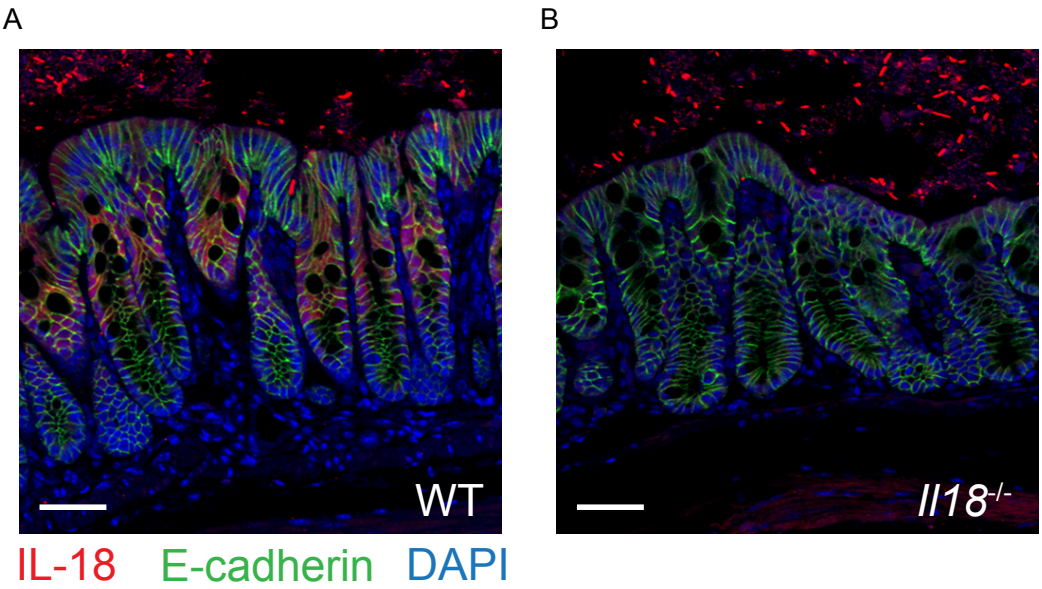


Figure 3.6 Intestinal epithelial cells express IL-18 in the healthy colon.
Distal colon tissue from steady state A) WT and B) *Il18*$^{-/-}$ mice was isolated, formalin-fixed and paraffin-embedded. Transverse sections (4µm) were cut on a microtome and mounted on glass microscopy slides. Tissue was subject to xylene-mediated wax removal, rehydration, antigen retrieval and blocking with goat serum. Samples were stained with rabbit anti-mouse IL-18 and mouse anti-mouse E-cadherin, followed by fluorescently conjugated secondary antibodies and DAPI. Images were acquired on an Olympus F1000 confocal microscope under 20x magnification and analysed using Olympus Fluoview software. Scale bar = 50µm Representative images taken from analysis of 4 individual mice.

An important caveat of the immunofluorescence staining is that the anti-IL-18 does not discriminate between pro-IL-18 and the active, cleaved form of the cytokine. Thus, to confirm our immunofluorescence staining represented IEC secreting active IL-18, and to determine a functional role for IEC-derived IL-18 on colonic $CD4^+$ T cell populations, we generated bone marrow (BM) chimeric mice by reconstituting lethally irradiated WT or *Il18*$^{-/-}$ mice with WT bone marrow cells. After BM reconstitution was complete, we used Western blotting to detect active IL-18 in colonic tissue homogenates. In accordance with the immunofluorescence staining, BM chimeric mice lacking IL-18 selectively in the non-haematopoietic compartment (WT➔*Il18*$^{-/-}$) had no detectable mature IL-18 in the colon, despite the presence of WT haematopoietic cells (Figure 3.7A). Conversely, the control BM chimeras (WT➔WT) had readily detectable mature IL-18 in the colon (Figure 3.7A). Thus, under steady state conditions, production of IL-18 within the intestine is strictly limited to IEC. Furthermore, flow cytometric analysis of colonic LPL $CD4^+$ T cells from BM chimeric mice revealed that IEC-derived IL-18 negatively regulated intestinal Th17 differentiation because WT➔*Il18*$^{-/-}$ BM chimeric mice had significantly elevated frequencies of intestinal Th17 cells when compared to WT➔WT control BM chimeric mice (Figure 3.7B,D). Conversely, in this chimeric setting, IEC-derived IL-18 promoted intestinal Th1 cell differentiation, because WT➔*Il18*$^{-/-}$ BM chimeric mice had significantly decreased frequencies of intestinal Th1 cells when compared to WT➔WT control chimeras (Figure 3.7B,D). However, in contrast to effector $CD4^+$ T cell frequencies, $Foxp3^+$ Treg cell frequency was not affected by the absence of IEC-derived IL-18 (Figure 3.7C,D). In accordance with elevated Th17 cell frequencies *in vivo*, expression levels of *S100a8* and *S100a9*, but not *Reg3g*, were elevated in the absence of IEC-derived IL-18 (Figure 3.7E). These data indicate that under steady state conditions, intestinal epithelial cells are the primary source of constitutive IL-18 production in the gut, which acts to inhibit Th17 cell differentiation.

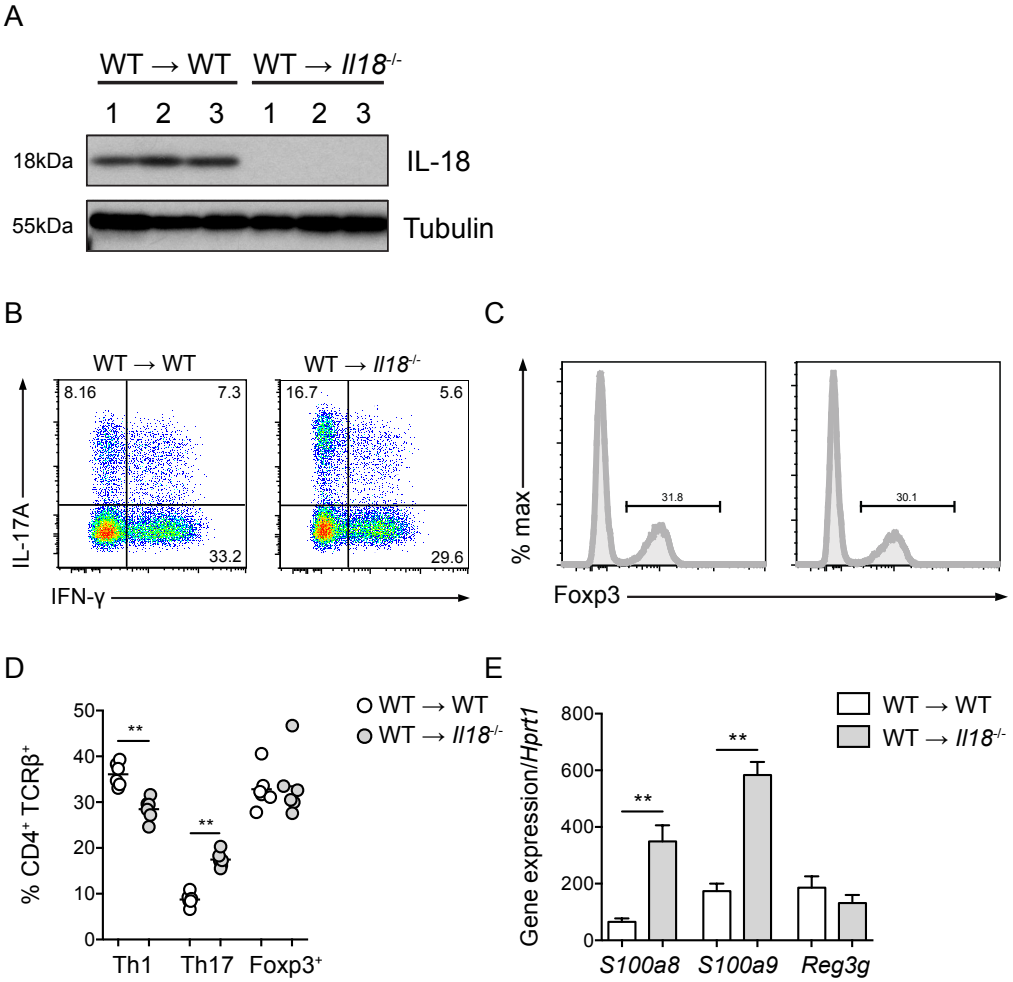


Figure 3.7 IEC-derived IL-18 inhibits colonic Th17 cell differentiation.
Lethally irradiated WT and *Il18*$^{-/-}$ recipients were transferred i.v. with 1x10^{7} bone marrow cells isolated from CD45.1 WT mice and allowed to reconstitute for 6 weeks before further analysis.
A) Western blot analysis of total colonic tissue of BM chimeric mice, probed with anti-IL-18 and anti-Tubulin. Each lane represents an individual mouse.
B) Representative FACS plots of colonic Th1 and Th17 cell frequencies from BM chimeric mice, gated on CD4^{+} TCRβ^{+} CD45.1^{+} cells.
C) Representative FACS histograms of colonic Foxp3^{+} Treg cell frequency from BM chimeric mice, gated on CD4^{+} TCRβ^{+} CD45.1^{+} cells.
D) Frequencies of colonic Th1, Th17 and Foxp3^{+} Treg cells from BM chimeric mice.
E) Antimicrobial peptide gene expression from distal colon of BM chimeric mice, assessed by qRT-PCR and relative to *Hprt1.*
Data shown represents 1 of 2 independent experiments, each with n=5-6 mice/group. Bars represent the mean, errors bars represent S.E.M, and each dot represents an individual mouse. Statistical significance determine using Mann Whitney test. (* =p<0.05, **=p<0.01).

3.2.4 Cell intrinsic IL-18R1-signalling regulates colonic Th17 cell differentiation

To formally determine whether the phenotype of elevated intestinal Th17 cell differentiation we observed in *Il18r1*$^{-/-}$ mice was the result of a cell-intrinsic role for IL-18R1-signalling in intestinal Th17 cells, we used two complementary approaches.

We first employed a previously described *in vitro* cell differentiation system whereby co-culturing naïve CD4^{+} T cells with bone marrow derived dendritic cells (BMDC) in the presence of anti-CD3, LPS and TGF-β_1 leads to efficient Th17 cell polarisation (Veldhoen et al. 2006, Chung et al. 2009). Under these conditions, we achieved efficient Th17 cell polarisation of WT CD4^{+} T cells, which was significantly inhibited by addition of exogenous IL-18 (Figure 3.8). Furthermore, when *Il18r1*$^{-/-}$ naïve CD4^{+} T cells were co-cultured under these conditions, they underwent polarisation to a Th17 lineage to a significantly higher degree than WT T cells, and this was not inhibited by addition of exogenous IL-18 (Figure 3.8). Taken together, these data indicate that IL-18R1-signalling directly inhibits Th17 cell differentiation *in vitro* in a cell-intrinsic manner.

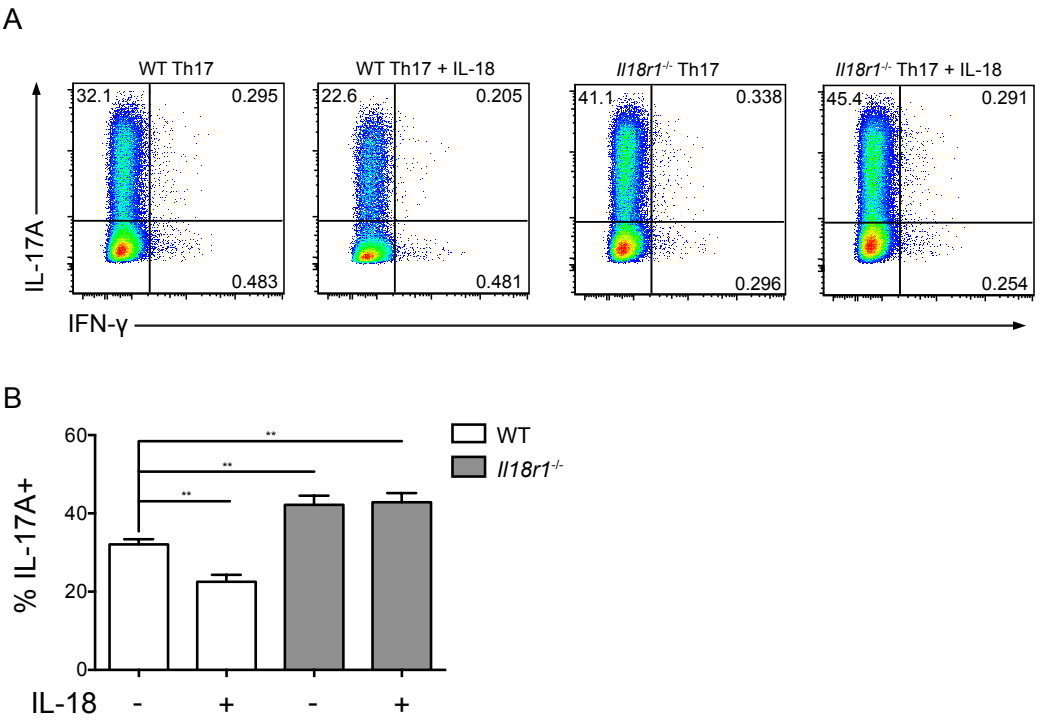


Figure 3.8 IL-18 inhibits Th17 differentiation *in vitro*.
FACS-purified naïve $CD4^+$ $CD25^-$ $CD44^-$ $CD62L^+$ T cells ($2.5x10^5$) isolated from either WT or *Il18r1*$^{-/-}$ mice were co-cultured for 5 days with WT BMDC ($1x10^5$), in the presence of soluble anti-CD3 (1µg/ml), LPS (100ng/ml), TGF-β_1 (1ng/ml), anti-IFN-γ (10µg/ml) and anti-IL-4 (10µg/ml) before analysis of cytokine production by intracellular staining and flow cytometry.
A) Representative FACS plots of Th1 and Th17 cell frequencies.
B) Quantitation of Th17 cell frequencies from different co-cultures.
Data is representative of 1 of 3 independent experiments with similar results. Each experimental group was performed in triplicate in each experiment. Bars represent the mean, error bars represent S.E.M. Statistical significance between experimental groups was determined using Students t test. (*=p<0.05, **=p<0.01)

Next, in order to formally demonstrate this cell-intrinsic IL-18R1 requirement *in vivo*, we reconstituted lethally irradiated *Rag1*$^{-/-}$ recipient mice with a 1:1 mixture of WT (CD45.1) and *Il18r1*$^{-/-}$ (CD45.2) bone marrow cells. Flow cytometric analyses revealed that reconstitution of splenic CD8^{+} T cells and B220^{+} B cells, as well as CD4^{+} T cells in the spleen, MLN and colonic LP, was equivalent from both WT and *Il18r1*$^{-/-}$ sources, indicating that *Il18r1*$^{-/-}$ deficiency did not generally affect lymphocyte reconstitution in mixed BM chimeras (Figure 3.9A,B). However, analyses of intestinal CD4^{+} T cells from mixed BM chimeric mice revealed that *Il18r1*$^{-/-}$ CD4^{+} T cells gave rise to significantly higher frequencies of Th17 cells when compared with WT CD4^{+} T cells (Figure 3.9C,D,F). Conversely, the mixed BM chimeras contained significantly lower frequencies of intestinal Th1 cells derived from *Il18r1*$^{-/-}$ CD4^{+} T cells than from WT CD4^{+} T cells (Figure 3.9C,D,F). Interestingly, cell-intrinsic regulation of Th17 cell differentiation by IL-18R1-signalling occurred primarily within the colonic LP, as WT and *Il18r1*$^{-/-}$ CD4^{+} T cells gave rise to equivalent Th17 cell frequencies in peripheral lymphoid tissues (Figure 3.9G). In contrast, Th1 cell differentiation demonstrated a more global regulatory role for IL-18R1-signalling, as significantly lower Th1 cell frequencies were generated from *Il18r1*$^{-/-}$ CD4^{+} T cells in both mucosal and peripheral lymphoid tissues (Figure 3.9G). Consistent with our previous observations, equivalent frequencies of Foxp3^{+} Treg cells were derived from WT and *Il18r1*$^{-/-}$ CD4^{+} T cells in all compartments in the mixed BM chimeric mice (Figure 3.9G). Together, these data demonstrate that IEC-derived IL-18 acts via IL-18R1 directly on CD4^{+} T cells within the intestine to regulate Th17 cell differentiation.

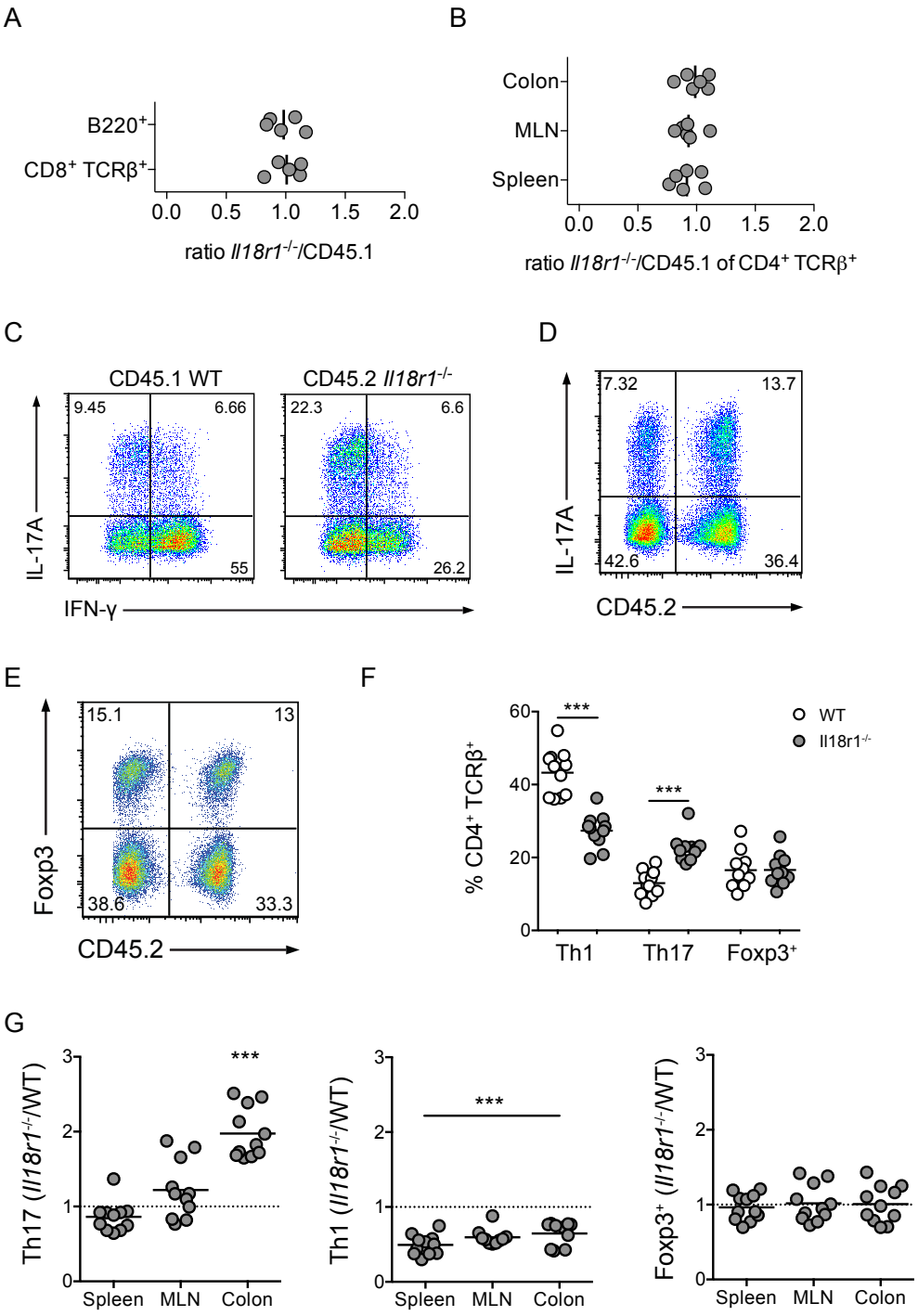


Figure 3.9 Inhibition of colonic Th17 differentiation by IL-18R1 is cell intrinsic.
Lethally irradiated *Rag1*$^{-/-}$ recipients were transferred with a 1:1 mixture of WT (CD45.1) and *Il18r1*$^{-/-}$ (CD45.2) bone marrow cells. Following 6 weeks reconstitution, colonic LPL were isolated and Th1, Th17 and Foxp3^{+} Treg cell frequencies were determine by intracellular staining and flow cytometry.
A) Relative contribution of WT and *Il18r1*$^{-/-}$ derived cells to splenic CD8^{+} T cells and B220^{+} B cells.
B) Relative contribution of WT and *Il18r1*$^{-/-}$ derived cells to splenic, MLN and colonic CD4^{+} TCRβ^{+} cell population.
C) Representative FACS plots of colonic Th1 and Th17 cell frequencies from mixed BM chimeric mice.

D) Representative FACS plots of colonic Th17 cell frequency by both WT and *Il18r1*$^{-/-}$ derived cells from mixed BM chimeric mice.
E) Representative FACS plots of colonic Foxp3^{+} Treg cell frequency by both WT and *Il18r1*$^{-/-}$ derived cells from mixed BM chimeric mice.
F) Frequencies of colonic Th1, Th17 and Foxp3^{+} Treg cells from mixed BM chimeric mice.
G) Relative contribution to Th1, Th17 or Foxp3^{+} Treg cell pool by WT and *Il18r1*$^{-/-}$ derived cells in lymphoid compartments of mixed BM chimeric mice.
Data shown is pooled from 2 independent experiments. n=10-12 chimeric mice/group. Bars represent the mean and each dot represents a single chimeric mouse. Statistical significance determined using Mann Whitney (F) or one-column T test (G). (* =$p<0.05$, **=$p<0.01$, ***=$p<0.001$).

3.3 Discussion

Colonisation by resident intestinal microbiota stimulates maturation of the mucosal immune system, including the accumulation and differentiation of effector and regulatory $CD4^+$ T cell subsets (Littman and Rudensky 2010), and also influences induction and progression of models of autoimmunity and chronic inflammation (Chervonsky 2010, Wu et al. 2010, Berer et al. 2011, Strickland et al. 2011). Microbial colonisation triggers accumulation of intestinal Th17 cells, and SFB is a particularly potent inducer of Th17 cell differentiation under homeostatic conditions (Gaboriau-Routhiau et al. 2009, Ivanov et al. 2009). Induction of serum amyloid proteins (Ivanov et al. 2009), ATP (Atarashi et al. 2008) and production of IL-1β (Shaw et al. 2012) in response to commensal bacteria have all been suggested to contribute to the generation of intestinal Th17 cells, but the host signalling pathways linking the microbiota to intestinal Th17 cell differentiation remain to be fully elucidated. Published data from other groups demonstrates that intestinal epithelial cell expression of *Il18* is induced by the commensal microbiota (Pott et al. 2012), and *Il18* expression within the intestinal epithelium is regulated by STAT3-signalling (Pickert et al. 2009). Thus, if IL-18 expression within the intestinal epithelium is regulated by the commensal microbiota, this could occur through two mechanisms, which may not be mutually exclusive. TLR-mediated detection of commensal bacterial products by IEC may directly trigger *Il18* transcription; indeed, *Il18* promoter activity upstream of non-coding exon 1 has been shown to be LPS-sensitive (Tone et al. 1997). Alternatively, indirect regulation through a haematopoietic cell intermediary could regulate *Il18* expression within the intestinal epithelium. For example, IL-22 production from $NKp46^+$ ILC populations is dependent upon the commensal microbiota, and activation of STAT3 (most likely in response to IL-22) within IEC regulates expression of *Il18* (Satoh-Takayama et al. 2008, Pickert et al. 2009, Sanos et al. 2009). The direct requirements for induction of IL-18 within intestinal epithelial cells, including the identity of triggering bacterial species and host derived signalling pathways are of great interest.

With this in mind, whilst we have not formally assessed the mechanism by which pro-IL-18 is processed and secreted from the intestinal epithelium, this is highly likely to be dependent upon activation of the inflammasome. Indeed, recent findings from our laboratory demonstrate that homeostatic production of IL-18 within the steady state intestine is dependent upon Asc, a key adaptor protein for inflammasome formation, which is highly expressed by IEC (Naren Srinivasan, D.Phil thesis).

If indeed induction of IEC-derived IL-18 occurs upon colonisation by a singular bacterial species, as for SFB and Th17 cells, a potential means by which we could identify the triggering agent would be to build upon and extrapolate from recent seminal studies by Elinav *et al*, and their demonstration that mice deficient for a novel inflammasome component, Nlrp6, and key downstream molecules Asc, caspase-1 and IL-18 are host to a dysbiotic intestinal microbiota (Elinav et al. 2011). Interestingly, absence of Nlrp6 resulted in decreased production of IL-18 within the intestine and whole microbiome-sequencing demonstrated a resultant increase in the abundance of bacterial phyla *Bacteroidetes* (*Prevotellaceae*) and TM7. Additional differences in ecological landscape of microbial communities between *Nlrp6*$^{-/-}$ and *Il18*$^{-/-}$ mice were also eluded to, but not formally demonstrated. Using the findings of Elinav *et al.* as an example to work with, if we hypothesise that elevated abundance of TM7 in the context of Nlrp6-deficiency is due to either direct recognition of TM7 by Nlrp6, or that Nlrp6-dependent pathways are required for appropriate immune responses to control TM7 “outgrowth”, then in a similar tack, identifying bacterial species emergent in the context of IL-18-deficiency would likely yield some of the species triggering its induction and/or activation. The controlled mono-colonisation of germ-free mice would then be an excellent means through which to conclusively demonstrate the ability of a single bacterial species to elicit the generation of IEC-derived IL-18.

In accordance with the known role of IL-18 in promoting Th1 cell differentiation, we demonstrated expression of IL-18R1 on Th1 cells both *in vitro* and *in vivo*. In addition, we demonstrated expression of IL-18R1 by Th17 and Foxp3$^+$ Treg cells *in vitro* and *in vivo*. Whilst naïve CD4$^+$ T cells do not express IL-18R components, the mechanism of IL-18R1 expression induction on Th1 cells has been extensively characterised, with STAT4-binding at conserved non-coding sequences of *Il18r1* required for gene expression (Yu et al. 2007, Yu et al. 2008), the transcription factors required for chromatin remodelling preceding and promoting expression of IL-18R1 on Th17 and Foxp3$^+$ cells are as yet unknown. IL-23R-signalling promotes gene expression through activation of STAT3, and to a lesser extent STAT4, to promote the effector function of Th17 cells (Parham et al. 2002). Thus, STAT4-dependent mechanisms may also control expression of IL-18R1 on Th17 cells in response to stimulation with IL-23 and induction of IL-18R1-signalling may represent a cell-intrinsic checkpoint to limit differentiation and/or acquisition of a pathogenic effector phenotype. Indeed, we demonstrate that IL-18R1-signalling directly on CD4$^+$ T cells acts to limit Th17 cell differentiation within the colonic lamina propria. The requirements for IL-18R1-expression on Foxp3$^+$ Treg cells are discussed in detail in Chapter 5.

We observed divergent effects of IL-18R1-signalling on colonic Th1 cell differentiation dependent upon the experimental setting. Using intact steady state mice, we observed that *Il18*$^{-/-}$ or *Il18r1*$^{-/-}$ mice had elevated colonic Th1 cell frequencies when compared with WT control mice. However, transplantation of bone marrow cells into irradiated lymphocyte replete or lymphopenic *Rag1*$^{-/-}$ recipients demonstrated a key role for IL-18/IL-18R1-signalling in promoting Th1 cell differentiation. Furthermore, the contribution of IL-18R1-signalling to Th1 cell differentiation was more marked in mixed BM chimera *Rag1*$^{-/-}$ recipients, where the highest frequencies of colonic Th1 cell differentiation were observed. As such, whilst IL-18R1-signalling is dispensable for colonic Th1 cell differentiation under truly

steady state conditions, generation of bone marrow chimeras represents an inflammatory context, as demonstrated by the increased frequencies of $CD4^+$ effector T cells following reconstitution. As such, in this context, IL-18R1-signalling promotes Th1 cell differentiation under inflammatory environments, as has been previously described. Additionally, competition for Th1 cell polarising cytokine IL-12 in mixed bone marrow chimeras may also exacerbate the effect of IL-18R1-deficiency in this setting. Reciprocal regulation between IL-12Rβ2 and IL-18R1 on differentiating Th1 cells (Xu et al. 1998) would also result in *Il18r1*$^{-/-}$ $CD4^+$ T cells being unable to compete with WT cells for IL-12 in mixed BM chimeric mice, which would further exacerbate the inability of IL-18R1-deficient cells to undergo Th1 cell differentiation in this setting. On-going experiments utilising adoptive transfer of isolated $CD4^+$ T cells to CD45.1 WT lymphocyte replete recipients will be key to delineating the contributions of lymphopenia, competition and IL-18R1-signalling to colonic Th1 cell differentiation. However, this still does not fully explain the elevated frequency of colonic Th1 cells observed in the absence of IL-18R1-signalling under homeostatic conditions. As IL-18R1-dependent control of colonic Th1 cell frequencies appeared cell-extrinsic, we hypothesised that IL-18R1 signalling on $Foxp3^+$ Treg cells may affect their function within the intestine, and as such, elevated Th1 cell frequencies may actually reflect elevated immune cell activation in the intestine, confusing in the light of the published role of IL-18R1-signalling in bolstering Th1 cell differentiation. The role of IL-18R1-signalling in $Foxp3^+$ Treg cell function is the subject of discussion in Chapter 5, and experiments aimed to "rescue" the elevated Th1 cell differentiation in *Il18r1*$^{-/-}$ mice by adoptive transfer of WT $Foxp3^+$ Treg cells are currently in progress. However, more advanced tools for future analysis of IL-18R1-signalling in specific $CD4^+$ T cell subsets will be required, for example, when IL-18R1$^{fl/fl}$ mice become available they could be crossed with IL-17A^{Cre} mice to more definitively address the effects of IL-18R1 signals on Th17 cells.

IL-1R1 and IL-18R1 both rely on the key adaptor molecule MyD88 for their signalling (Adachi et al. 1998). Thus exactly how different combinations of IL-1R1-, and IL-18R1-derived signals interact to regulate lineage specification of Th17 cells requires investigation. It will be important to confirm the requirement for MyD88 signals in regulation of Th17 cell differentiation by IL-18, however, beyond this, proteomics based analysis of post-translational modifications in differentiated Th17 cells following stimulation with IL-1β and/or IL-18 could be key to a more detailed understanding of the signalling interactions. With the idea of clinical applications and targeted therapies in mind, the aim of these experiments would ultimately be to either restrain or boost specific Th17 cell responses, dependent upon the clinical setting. Equally, current therapies aimed at targeting IL-18 in chronic inflammatory disorders may benefit from patient monitoring for intestinal manifestations, given the high frequency of IBD susceptibility associated polymorphisms within the healthy population.

Together, the data presented within this chapter clearly define a regulatory role for IL-18 in limiting commensal microbiota induced Th17 cells within the colonic lamina propria. Moreover, we identified intestinal epithelial cells as the major source of mature IL-18 within the intestine, from where IL-18 acts directly on IL-18R1-expressing $CD4^+$ T cells to limit Th17 differentiation in a cell-intrinsic manner.

Chapter 4 Role of IL-18R1-signalling in chronic $CD4^+$ T cell mediated intestinal inflammation

4.1 Introduction

Having established a regulatory role for IL-18R1-signalling in limiting colonic Th17 cells differentiation under homeostatic conditions, we next investigated the role of IL-18R1-signalling on the induction of chronic intestinal inflammation in $CD4^+$ T cell mediated models of IBD.

Although Th17 cells mediate tissue pathology in a variety of inflammatory contexts, the requirement for IL-17A in protective intestinal immunity and induction of intestinal inflammation remains unclear. Although loss of IL-17A renders mice and humans hyper-susceptible to certain mucosal pathogens, including *Candida albicans* (Huang et al. 2004, LeibundGut-Landmann et al. 2007, Puel et al. 2010), *Salmonella typhimurium* (Ishigame et al. 2009, Geddes et al. 2011) and *Citrobacter rodentium* (Ishigame et al. 2009), the role of IL-17A in experimental IBD models appears more complex (Korn et al. 2009). Chemically driven models of acute colitis reported a protective role for IL-17A during intestinal inflammation, with IL-17A-deficient mice developing exacerbated tissue pathology and increased cellular infiltration to the colonic lamina propria following challenge with DSS (Ogawa et al. 2004). Conversely, selective ablation of STAT3 in $Foxp3^+$ Treg cells led to a spontaneous chronic colitis dominated by emergence of Th17 cells, and disease was ameliorated by treatment with anti-IL-17A mAb (Chaudhry et al. 2009). However, whilst *Rorc*$^{-/-}$ naïve $CD4^+$ T cells, which cannot give rise to Th17 cells, were unable to mediate intestinal inflammation upon transfer to *Rag1*$^{-/-}$ mice, $CD4^+$ T cell derived IL-17A was dispensable for the induction of colitis in this setting, and this was ascribed to be due to redundancy between IL-17A and IL-17F (Izcue et al. 2008, Leppkes et al. 2009, O'Connor et al. 2009). In line with these findings, whilst the frequency of IL-17A-producing $CD4^+$ T cells and ILC are elevated in the intestine of patients with IBD

(Cosmi et al. 2008, Geremia et al. 2011), recent phase II clinical trial results demonstrate that antibody mediated blockade of IL-17A does not limit, and could exacerbate disease readouts, with some patients suffering from fungal infections, reflecting the role of IL-17A in host defence (Korn et al. 2009, Hueber et al. 2012).

The role of Th1 cells in experimental IBD is also complex. Adoptive transfer of T-bet- or IRF1- deficient naïve $CD4^+$ T cells to *Rag1*$^{-/-}$ recipient mice did not induce severe intestinal inflammation, and STAT4-deficient naïve $CD4^+$ T cells induced colitis with delayed kinetics, demonstrating a prominent role for colitogenic Th1 cell responses in the T cell transfer IBD model (Simpson et al. 1998, Neurath et al. 2002, Kano et al. 2008). However, *Rag1*$^{-/-}$ recipient mice genetically deficient for IL-12 still developed severe colitis upon adoptive transfer of WT naïve $CD4^+$ T cells that was accompanied by robust Th1 accumulation in the inflamed gut (Hue et al. 2006), suggesting that IL-12 independent, STAT4-dependent induction of T-bet expression likely drives the acquisition of pathogenic potential in this scenario. In line with these observations, early data on IL-23 focused on induction of IFN-γ production by $CD4^+$ T cells (Oppmann et al. 2000). More recently, the induction of colitogenic $CD4^+$ T cell populations has been shown to rely upon intrinsic IL-23R-signalling and to correlate with acquisition of an IL-$17A^+$/IFN-γ^+ phenotype, which is associated with co-expression of Rorγt and T-bet (Ahern et al. 2010, Hirota et al. 2011, Lee et al. 2012, Morrison et al. 2013).

We hypothesised that IL-18R1-signalling on $CD4^+$ T cells may contribute to induction of colitogenic $CD4^+$ T cells and thus induction of intestinal inflammation. Firstly, STAT4-dependent mechanisms control IL-18R1-expression on developing and committed Th1 cells (Yu et al. 2007, Yu et al. 2008), and IL-23 dependent signals promote IL-18R1 expression on differentiating Th17 cells (Ghoreschi et al. 2010). We hypothesised that IL-18R1-signalling may play a role in the transition of Th17 cells to a pathogenic IL-$17A^+$/IFN-γ^+ phenotype and/or emergence of Th1 cells associated

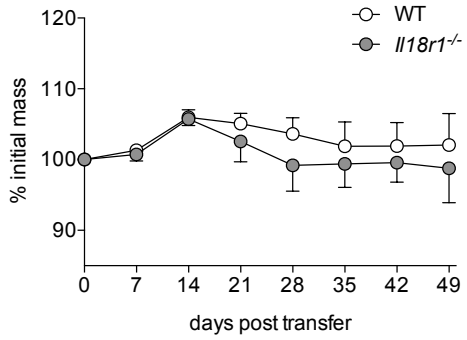
with tissue pathology in the intestine. To address this question we utilised two complementary models of $CD4^+$ T cell mediated chronic intestinal inflammation; T cell transfer colitis induced in lymphopenic *Rag*$^{-/-}$ hosts following adoptive transfer of naive $CD4^+$ T cells (Powrie et al. 1993); and severe typhlocolitis induced in WT B6 mice by infection with *H. hepaticus* and concomitant blockade of IL-10R-signalling (Kullberg et al. 2006).

4.2 Results

4.2.1 Intrinsic IL-18R1-signals are dispensable for effector $CD4^+$ T cell function *in vivo*.

To address whether IL-18R1 expression on $CD4^+$ T cells is essential for induction of pathogenic effector responses within the gastrointestinal tract, we initially utilised the well-characterised T cell transfer model of colitis (Powrie et al. 1993, Powrie et al. 1994, Powrie et al. 1994). Thus, we adoptively transferred FACS-purified $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ naïve T cells obtained from either WT or *Il18r1*$^{-/-}$ donor mice into *Rag1*$^{-/-}$ recipient mice and assessed the development of systemic and intestinal inflammation. We found that adoptive transfer of naïve $CD4^+$ T cells from WT or *Il18r1*$^{-/-}$ mice elicited similar systemic inflammation in *Rag1*$^{-/-}$ recipient mice, characterised by wasting disease (Figure 4.1A) and increases in splenic mass and cell number (Figure 4.1B). Furthermore, naïve $CD4^+$ T cells isolated from either WT or *Il18r1*$^{-/-}$ mice elicited comparable intestinal inflammation upon adoptive transfer to *Rag1*$^{-/-}$ recipient mice, with no significant differences in either disease incidence or severity (Figure 4.2A-C). In accordance with the finding that both WT and *Il18r1*$^{-/-}$ naïve $CD4^+$ T cells elicited severe intestinal inflammation upon adoptive transfer to a lymphopenic environment, we observed that accumulation of $CD4^+$ T cells within lymphoid tissues (spleen and MLN) and in the colonic lamina propria was unaffected by the absence of cell-intrinsic IL-18R1-signalling (Figure 4.3A,B).

A



B

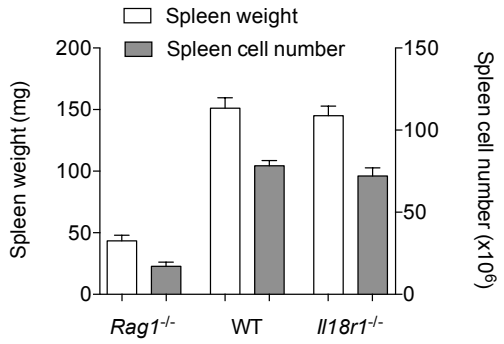


Figure 4.1 IL-18R1-signalling on CD4$^+$ T cells is dispensable for systemic inflammation induced during T cell transfer colitis.
FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ mice were transferred i.p. to *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease and assessed for systemic inflammation.
A) Representative weight loss curves, shown as percentage of initial mass.
B) Spleen weight (white bars) and spleen cell number (grey bars) at point of sacrifice. Data shown represents 1 of 3 independent experiments with similar results, each using n=5 mice/group. Data are represented as mean ± S.E.M. Statistical significance was determined using (A) 2-way ANOVA and (B) Mann-Whitney tests.

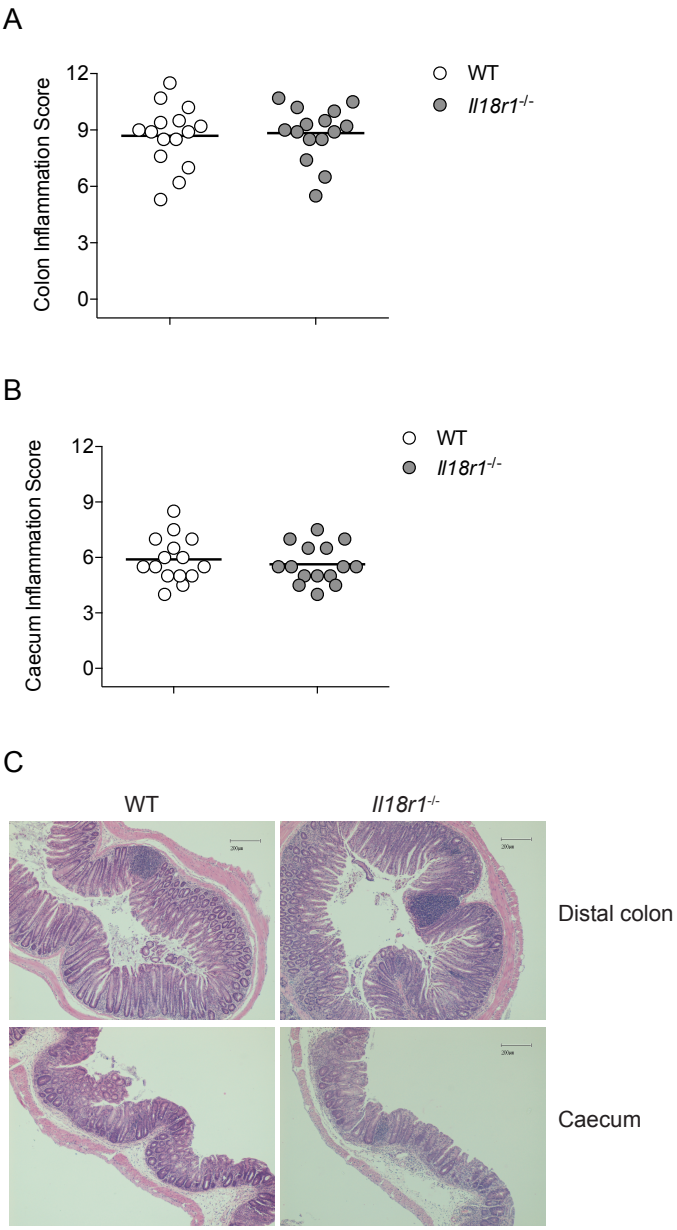
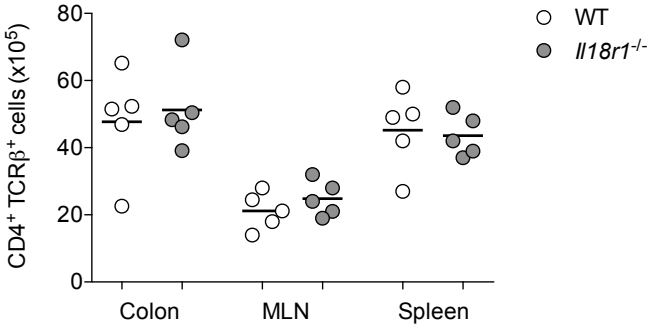


Figure 4.2 IL-18R1-signalling on $CD4^+$ T cells is dispensable for intestinal inflammation induced by naïve T cell transfer.
FACS-purified naïve $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ T cells ($4x10^5$) isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ mice were transferred i.p. to *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease and assessed for intestinal inflammation.
A) Mean inflammation scores for proximal, mid and distal colon tissue sections.
B) Inflammation scores for caecal tissue sections
C) Representative photomicrographs of colonic and caecal inflammation.
Data shown represents 3 pooled independent experiments, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

A



B

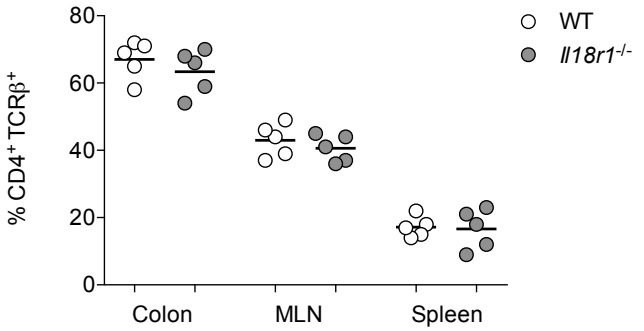


Figure 4.3 IL-18R1-signalling on $CD4^+$ T cells is dispensable for lymphoid reconstitution and accumulation in the colonic lamina propria.
FACS-purified naïve $CD4^+CD25^-CD45RB^{Hi}$ T cells ($4x10^5$) isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ mice were transferred i.p. to *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease. Leukocytes were isolated from the colonic lamina propria, MLN and spleen and $CD4^+$ $TCR\beta^+$ cell frequencies analysed by flow cytometry.
A) Total numbers of WT or $Il18r1^{-/-}$ live $CD4^+$ $TCR\beta^+$ cells in the colonic lamina propria, MLN and spleen of *Rag1*$^{-/-}$ recipient mice.
B) Frequencies of WT or $Il18r1^{-/-}$ live $CD4^+$ $TCR\beta^+$ cells in the colonic lamina propria, MLN and spleen of *Rag1*$^{-/-}$ recipient mice.
Data shown represents 1 of 3 independent experiments with similar results, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

The T cell transfer model of colitis is characterised by accumulation of activated Th1 and Th17 cell populations within the colonic lamina propria (Hue et al. 2006, Ahern et al. 2010) and IL-18R1-signalling plays an auxiliary role in Th1 cell differentiation under inflammatory environments (Sims and Smith 2010). To assess the role of IL-18R1-signalling on $CD4^+$ T cell subset lineage specification *in vivo*, we isolated colonic lamina propria leukocytes from *Rag1*$^{-/-}$ recipient mice receiving WT or *Il18r1*$^{-/-}$ naïve $CD4^+$ T cells and identified Th1, Th17 and $Foxp3^+$ cell populations by intracellular staining and flow cytometry. We observed similar frequencies of IL-$17A^+$ Th17 and IL-$17A^+$ IFN-γ^+ double-producing $CD4^+$ T cells in the inflamed colons of *Rag1*$^{-/-}$ mice that received WT or *Il18r1*$^{-/-}$ naïve $CD4^+$ T cells (Figure 4.4A,B). By contrast, the frequencies of IFN-γ^+ Th1 cells were slightly decreased in the colons of *Rag1*$^{-/-}$ mice receiving *Il18r1*$^{-/-}$ naïve $CD4^+$ T cells when compared to those receiving WT naïve $CD4^+$ T cells, however, this difference did not reach statistical significance (Figure 4.4A,B). The emergence of $Foxp3^+$ iTreg cells was not dependent upon IL-18R1-signalling, as *Rag1*$^{-/-}$ recipient mice receiving WT or *Il18r1*$^{-/-}$ naïve $CD4^+$ T cells had equivalent frequencies of colonic $Foxp3^+$ Treg cells (Figure 4.4C,D).

Together, these data indicate that $CD4^+$ T cell intrinsic IL-18R1 signals are dispensable for induction of intestinal inflammation upon adoptive transfer of naïve $CD4^+$ T cells into a lymphopenic environment.

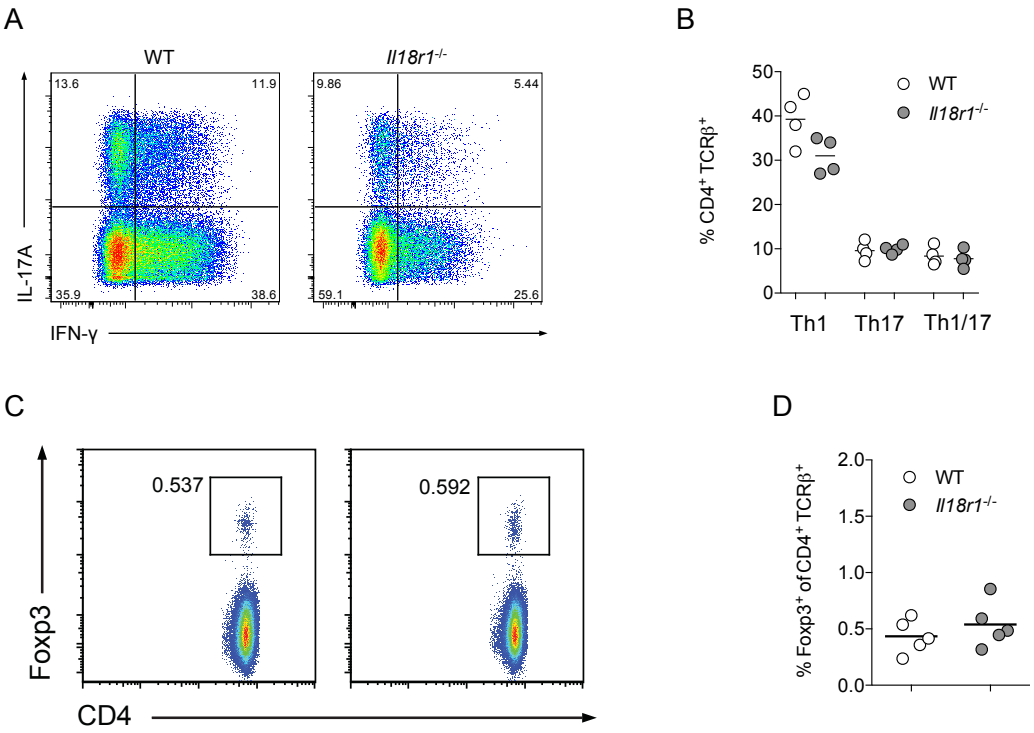


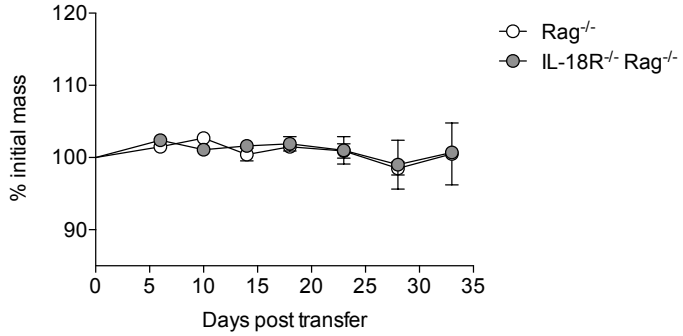
Figure 4.4 IL-18R1-signalling on CD4$^+$ T cells has little impact on colonic effector CD4$^+$ T cell differentiation during T cell transfer colitis.
FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) isolated from WT or *Il18r1*$^{-/-}$ mice were transferred i.p. to *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease. Colonic LPL were isolated and the frequencies of Th1, Th17 and Foxp3$^+$ Treg cell populations analysed by flow cytometry.
A) Representative FACS plots showing colonic Th1 and Th17 cell frequencies.
B) Frequencies of colonic Th1, Th17 and Th1/17 cell populations.
C) Representative FACS plots showing Foxp3$^+$ Treg cell frequencies.
D) Frequencies of colonic Foxp3$^+$ Treg cell populations.
All FACS-plots are gated on live CD4$^+$ TCRβ$^+$ cells. Data shown represents 1 of 3 independent experiments with similar results, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

4.2.2 IL-18R1 expression on host APC is not required for the induction of naïve CD4$^+$ T cell mediated colitis.

Whilst the role of IL-18R1-signalling in promoting inflammatory responses is widely associated with actions on T cells, a seminal study by *Gutcher et al*, demonstrated a requirement for IL-18R1-signalling on APC for induction of pathogenic Th17 cells within the central nervous system (CNS) (Gutcher et al. 2006). In this study, IL-18R1-signalling occurred independently of IL-18 and acted to promote IL-23 production from APC to drive tissue pathology in a model of EAE. We hypothesised that a similar pathway may be required for IL-23 production within the colonic lamina propria to promote induction of colitogenic Th17 cell responses. To formally examine the role of IL-18R1 signals on cells of the innate immune system, we again utilised the T cell transfer model of colitis. We generated recipient mice lacking IL-18R1 expression on host innate leukocytes by crossing *Rag1*$^{-/-}$ mice with *Il18r1*$^{-/-}$ mice to generate *Rag1*$^{-/-}$ *Il18r1*$^{-/-}$ mice. We found that adoptive transfer of WT naïve CD4$^+$ T cells elicited similar systemic inflammation in both *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ *Il18r1*$^{-/-}$ recipient mice, as evidenced by comparable wasting disease (Figure 4.5A) and splenomegaly (Figure 4.5B). Furthermore, adoptively transferred naïve CD4$^+$ T cells elicited severe colitis and moderate typhlitis in both *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ *Il18r1*$^{-/-}$ recipients, with no significant differences in disease severity or incidence (Figure 4.6A,B). Additionally, CD4$^+$ T cell reconstitution and accumulation within lymphoid tissues (spleen and MLN) and the colonic lamina propria occurred independently of host IL-18R1 signals (Figure 4.7A,B). Likewise, host derived IL-18R1-dependent signals were dispensable for differentiation of Th1, Th17 and Foxp3$^+$ CD4$^+$ T cell subsets within the inflamed colonic lamina propria (Figure 4.8A-D).

Together, these results demonstrate that IL-18R1 expression on innate immune and host tissue cells is not required for induction of intestinal inflammation upon adoptive transfer of naïve CD4$^+$ T cells to a lymphopenic environment.

A



B

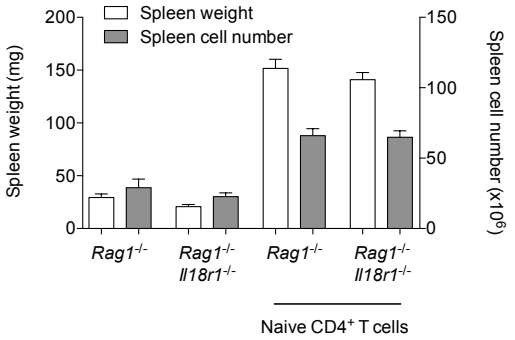
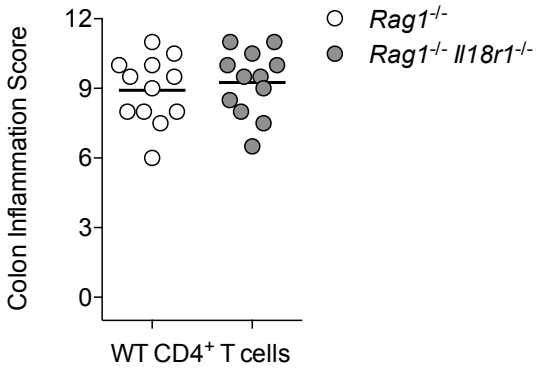


Figure 4.5 Innate IL-18R1-signalling is dispensable for systemic inflammation induced by naïve CD4$^+$ T cell transfer.
FACS-purified naïve CD4$^+$CD25$^-$CD45RB^{Hi} T cells (4x10^5) isolated from the spleens and peripheral lymph nodes of WT mice were transferred i.p. to *Rag1*$^{-/-}$ or *Il18r1*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease and assessed for systemic inflammation.
A) Representative weight loss curves, shown as percentage of initial mass.
B) Spleen weight (white bars) and spleen cell number (grey bars) at point of sacrifice. Data shown represents 1 of 2 independent experiments with similar results, each using n=5 mice/group. Data are represented as mean ± S.E.M. Statistical significance was determined using (A) 2-way ANOVA and (B) Mann-Whitney tests.

A



B

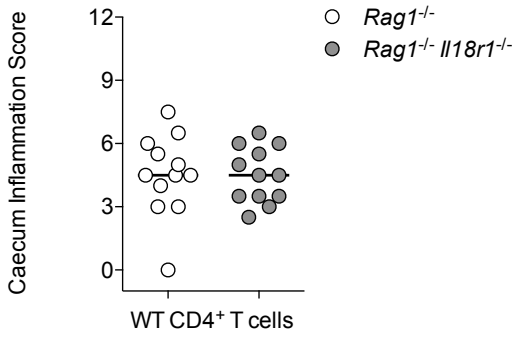


Figure 4.6 – Innate IL-18R1-signalling is dispensable for intestinal inflammation induced by naïve CD4$^+$ T cell transfer.
FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) isolated from the spleens and peripheral lymph nodes of WT mice were transferred i.p. to *Rag1*$^{-/-}$ or *Il18r1*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease and assessed for intestinal inflammation.
A) Mean inflammation scores for proximal, mid and distal colon tissue sections.
B) Inflammation scores for caecal tissue sections.
Data shown represents 2 pooled independent experiments with similar results, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

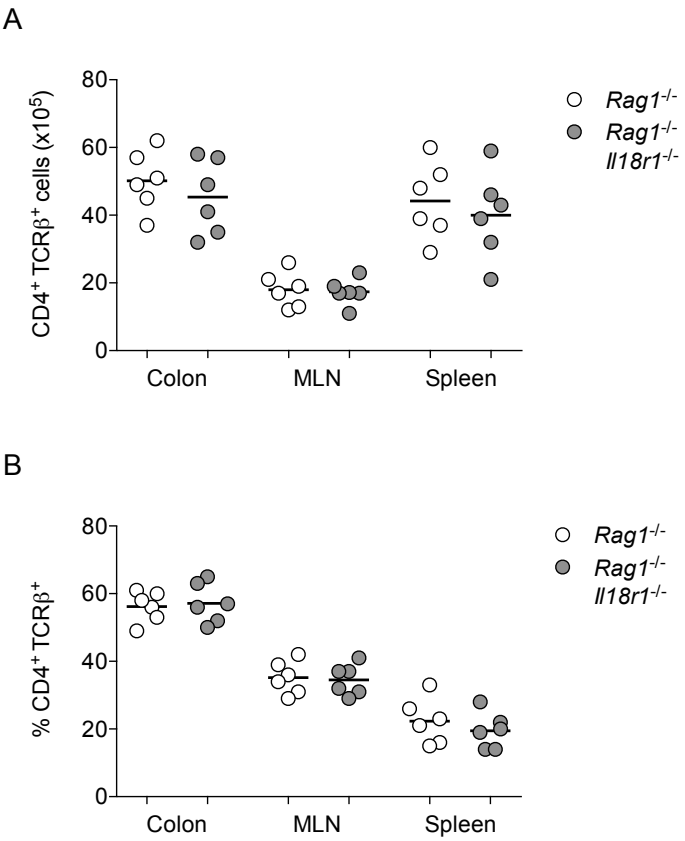


Figure 4.7 Innate IL-18R1-signalling is dispensable for lymphoid reconstitution and accumulation of CD4$^+$ T cells in the colonic lamina propria.
FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) isolated from the spleens and peripheral lymph nodes of WT mice were transferred i.p. to *Rag1*$^{-/-}$ or *Il18r1*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease. Leukocytes were isolated from the colonic lamina propria, MLN and spleen and frequencies of live CD4$^+$ TCRβ$^+$ cells analysed by flow cytometry.
A) Total numbers of WT live CD4$^+$ TCRβ$^+$ cells in the colonic lamina propria, MLN and spleen of *Rag1*$^{-/-}$ and *Il18r1*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice.
B) Frequencies of WT live CD4$^+$ TCRβ$^+$ cells in the colonic lamina propria, MLN and spleen of *Rag1*$^{-/-}$ and *Il18r1*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice.
Data shown represents 1 of 2 independent experiments with similar results, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

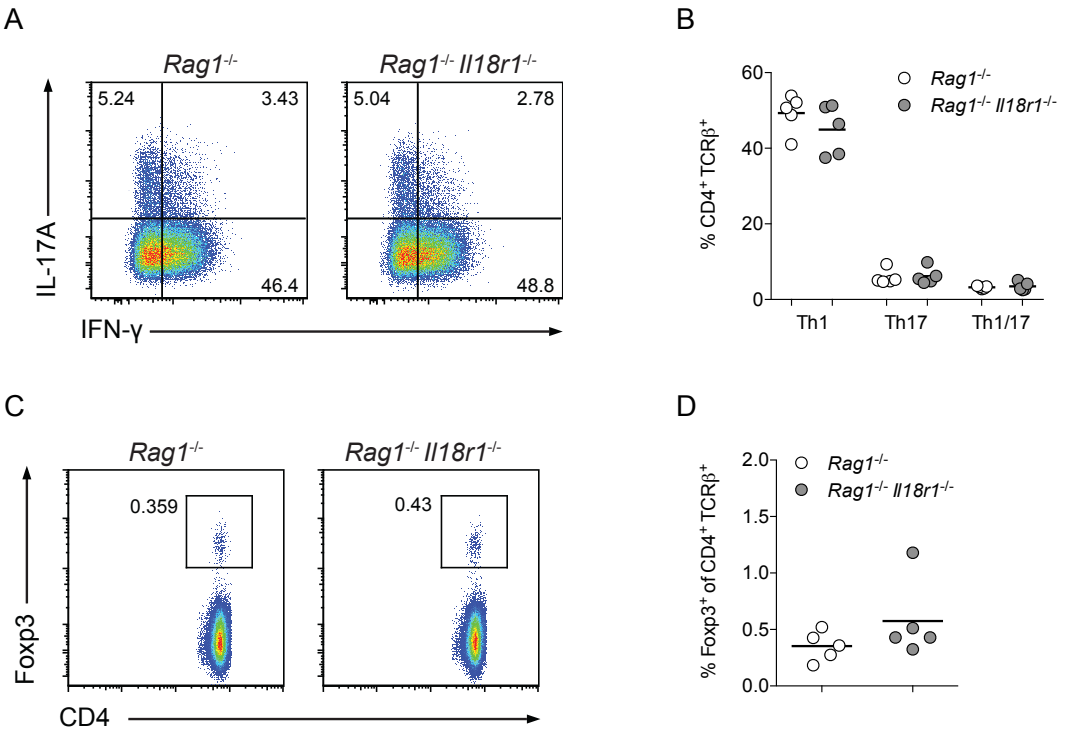


Figure 4.8 Innate IL-18R1-signalling is dispensable for colonic CD4$^+$ T cell lineage differentiation during T cell transfer driven colitis.
FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) isolated from WT mice were transferred i.p. to *Rag1*$^{-/-}$ and *Il18r1*$^{-/-}$ *Rag1*$^{-/-}$ recipient mice. Animals were sacrificed upon development of clinical signs of disease. Leukocytes were isolated from the colonic lamina propria and frequencies of Th1, Th17 and Foxp3$^+$ Treg cells analysed by flow cytometry.
A) Representative FACS plots of colonic Th1 and Th17 cell frequencies.
B) Frequencies of colonic Th1, Th17 and Th1/17 cell populations.
C) Representative FACS plots of colonic Foxp3$^+$ Treg cell frequencies.
D) Frequencies of colonic Foxp3$^+$ Treg cell populations.
All FACS-plots were gated on live CD4$^+$ TCRβ$^+$ cells.
Data shown represents 1 of 2 independent experiments with similar results, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

4.2.3 Induction of chronic intestinal inflammation in immune competent mice is IL-18R1-independent.

Reconstitution of lymphopenic hosts with naive $CD4^+$ T cells is associated with high levels of spontaneous proliferation and differentiation into effector T cells that may have masked any effects of IL-18R1 signalling on intestinal $CD4^+$ T cell differentiation (Kieper et al. 2005, Min et al. 2005, Surh and Sprent 2008). We therefore sought to investigate the requirement for IL-18R1-signalling during induction of chronic intestinal inflammation in the absence of lymphopenia. To address this, we utilised a recently developed IBD model, where intestinal pathology is induced in WT C57BL/6 mice by oral infection with *Helicobacter hepaticus* together with concomitant blockade of IL-10R-signalling by administration of blocking mAb (Kullberg et al. 2006). As expected, control cohorts of WT and *Il18r1*$^{-/-}$ mice that were either infected with *H. hepaticus* only, or treated with anti-IL-10R mAb alone, did not develop significant intestinal pathology (Figure 4.9A,B). By contrast, *H. hepaticus* infection in combination with anti-IL-10R mAb administration elicited severe typhlocolitis in both WT and *Il18r1*$^{-/-}$ mice (Figure 4.9A-C). However, no significant difference in disease incidence or severity was observed between WT and *Il18r1*$^{-/-}$ mice receiving both anti-IL-10R mAb and *H. hepaticus* infection (Figure 4.9A-C). Consistent with the comparable intestinal inflammation, systemic inflammation was not diminished by IL-18R1-deficiency in this model; *Il18r1*$^{-/-}$ and WT mice exhibited similar increases in splenic mass and cell number following *H. hepaticus* infection with anti-IL-10R mAb treatment (Figure 4.10A,B). In addition, accumulation of $CD4^+$ T cells within lymphoid tissues and the colonic lamina propria following *H. hepaticus* infection and anti-IL-10R mAb treatment was similar in *Il18r1*$^{-/-}$ and WT mice (Figure 4.11A,B). Finally, the frequencies of Th1, Th17 and $Foxp3^+$ Treg cells arising upon induction of chronic intestinal inflammation did not differ significantly between *Il18r1*$^{-/-}$ and WT mice (Figure 4.12A-D). Thus, IL-18R1-signalling is dispensable for induction of chronic intestinal inflammation in immune competent mice elicited by oral infection with *H. hepaticus* and treatment with anti-IL-10R mAb.

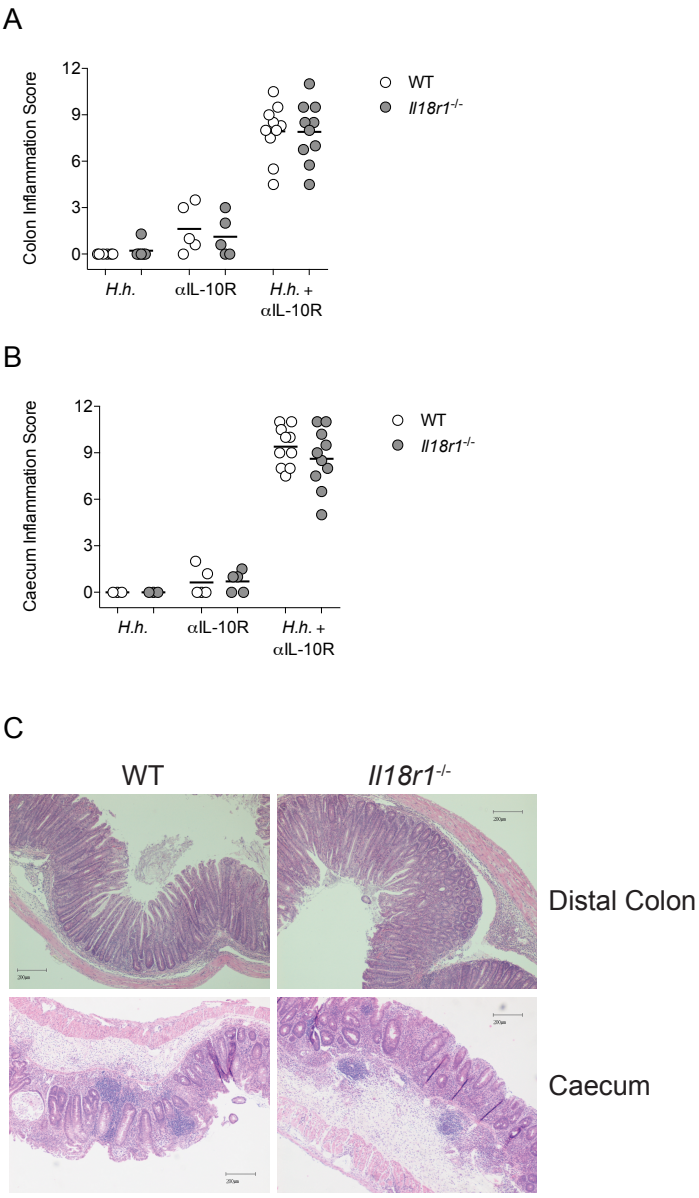


Figure 4.9 IL-18R1-signalling is dispensable for induction of chronic intestinal inflammation in lymphocyte replete mice

WT and *Il18r1*$^{-/-}$ mice were infected orally with *H. hepaticus* and treated with 1mg/week αIL-10R mAb i.p. (*H.h.* + αIL-10R) for 4 weeks. Control groups received only αIL-10R mAb treatment (αIL-10R) or *H. hepaticus* infection (*H.h.*). One week after the final mAb administration, mice were sacrificed and histologically assessed for intestinal inflammation.

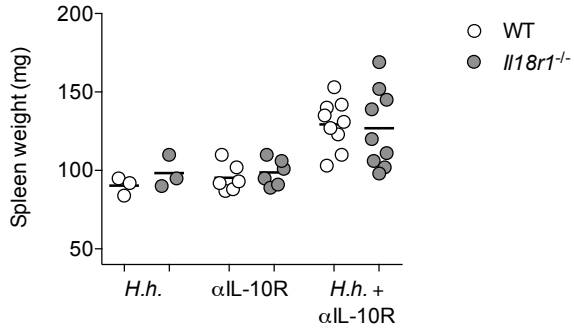
A) Mean inflammation scores from proximal, mid and distal colon sections.

B) Inflammation scores from caecal sections.

C) Representative photomicrographs of distal colon and caecal inflammation from WT and *Il18r1*$^{-/-}$ mice receiving *H.h.* + αIL-10R. Scale bar = 200μm.

Data shown represents 2-pooled independent experiments (n=4-5 mice/control group and n=10 mice/experimental group). Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

A



B

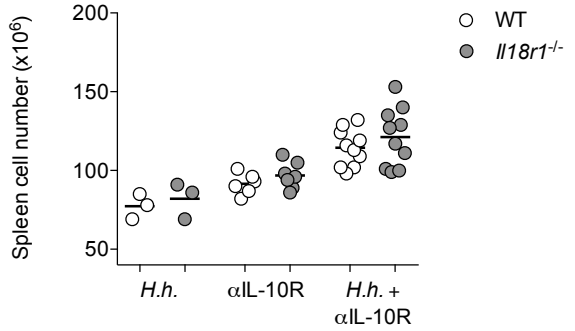
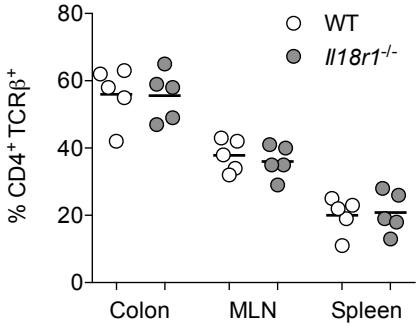


Figure 4.10 *H. hepaticus* infection with αIL-10R mAb treatment induces comparable systemic inflammation in both WT and *Il18r1*$^{-/-}$ mice.
WT and *Il18r1*$^{-/-}$ mice were infected orally with *H. hepaticus* and treated with 1mg/week αIL-10R mAb i.p. (*H.h.* + αIL-10R) for 4 weeks. Control groups received only αIL-10R mAb treatment (αIL-10R) or *H. hepaticus* infection (*H.h.*). One week after the final mAb administration, mice were sacrificed and assessed for systemic inflammation.
A) Spleen weights.
B) Spleen cell numbers.
Data shown represents 2-pooled independent experiments (n=4-5 mice/control group and n=10 mice/experimental group). Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

A



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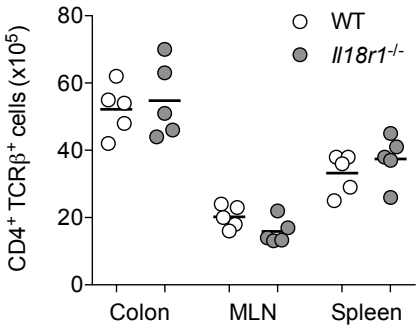


Figure 4.11 Comparable lymphoid and colonic CD4$^+$ T cell numbers in WT and *Il18r1*$^{-/-}$ mice after *H. hepaticus* infection with αIL-10R mAb treatment.
WT and *Il18r1*$^{-/-}$ mice were infected orally with *H. hepaticus* and treated with 1mg/week αIL-10R mAb i.p. (*H.h.* + αIL-10R) for 4 weeks. One week after the final mAb administration, mice were sacrificed and the frequencies of CD4$^+$ TCRβ$^+$ cells in different lymphoid tissues analysed by flow cytometry.
A) Frequencies of live CD4$^+$ TCRβ$^+$ cells in the colon, MLN and spleen.
B) Total numbers of CD4$^+$ TCRβ$^+$ cells in the colon, MLN and spleen.
Data shown represents 1 of 2 independent experiments with similar results, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

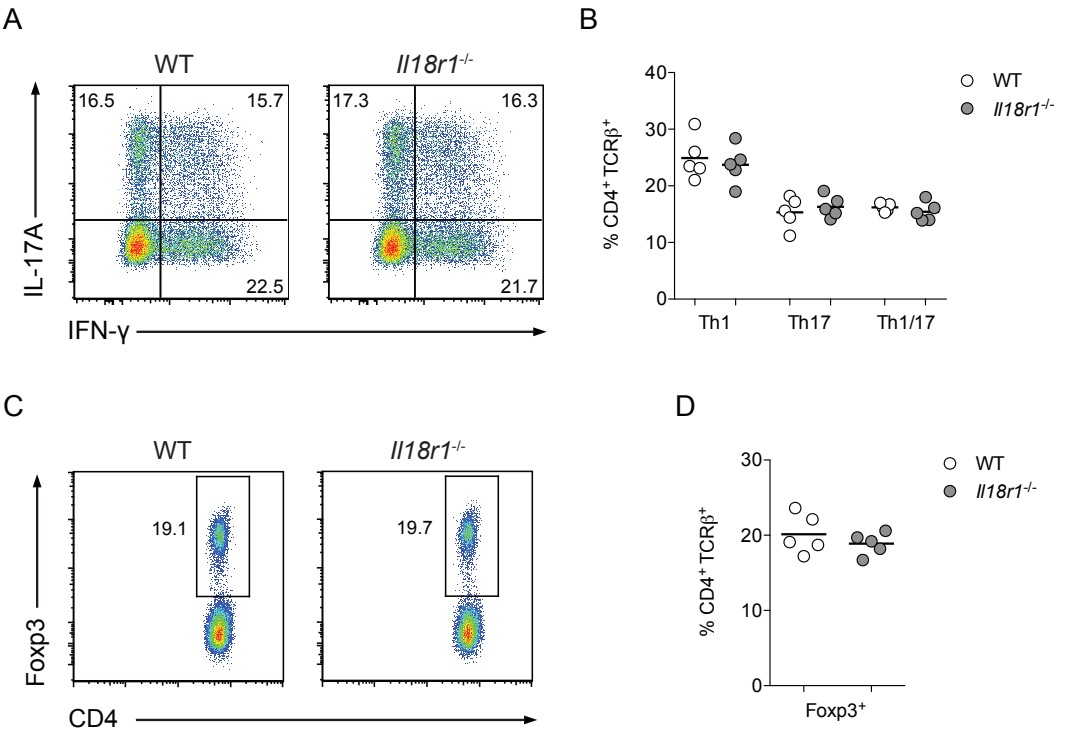


Figure 4.12 CD4$^+$ T cell differentiation during chronic intestinal inflammation is independent of IL-18R1-signalling.
WT and *Il18r1*$^{-/-}$ mice were infected orally with *H. hepaticus* and treated with 1mg/week αIL-10R mAb i.p. (*H.h.* + αIL-10R) for 4 weeks. One week after the final mAb administration, mice were sacrificed and the frequencies of colonic Th1, Th17 and Foxp3$^+$ Treg cells analysed by flow cytometry.
A) Representative FACS plots of colonic Th1 and Th17 cell frequencies.
B) Frequencies of colonic Th1, Th17 and Th1/17 cell populations.
C) Representative FACS plots of colonic Foxp3$^+$ Treg cell frequencies.
D) Frequencies of colonic Foxp3$^+$ Treg cell populations.
Data shown represents 1 of 2 independent experiments with similar results, each with n=5 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using the Mann-Whitney test.

4.2.4 Discussion

The role of IL-18 in promoting $CD4^+$ T cell responses in inflammatory environments is well established. However, whilst IL-18 production and IL-18R1-signalling have been implicated in both host protective and deleterious immune responses, the significance of these investigations to induction of intestinal inflammation remains poorly defined. The reported roles for IL-18R1-signalling in intestinal inflammation, derived primarily from studies using the DSS model of acute colitis, were often contradictory, and furthermore, the effect of IL-18R1-signalling on distinct cellular compartments and populations has not been investigated (Kanai et al. 2001, Siegmund et al. 2001, Sivakumar et al. 2002, Ishikura et al. 2003, Dupaul-Chicoine et al. 2010, Siegmund 2010, Zaki et al. 2010). Also worthy of note is that the DSS model of acute colitis is only partially T cell dependent (Axelsson et al. 1996), thus may not be the most suitable IBD model for investigating the requirement for IL-18R1-signalling on colitogenic $CD4^+$ T cell responses within the intestine.

In order to assess these requirements in induction of chronic intestinal inflammation, we exploited two distinct models of $CD4^+$ T cell mediated colitis, which allowed us to delineate the role of IL-18R1-signalling on innate and adaptive cell populations.

Initially, we used the T cell transfer model of $CD4^+$ T cell mediated colitis to investigate the requirement for $CD4^+$ T cell-intrinsic IL-18R1-signalling in promoting pathogenic effector responses with the intestine. We demonstrated that $CD4^+$ T cell-intrinsic IL-18R1-signalling was dispensable for induction of severe systemic or intestinal inflammation. The ability of transferred naïve $CD4^+$ T cells to accumulate within the lymphoid and non-lymphoid tissue of *Rag1*$^{-/-}$ recipient mice was not dependent upon IL-18R1-signalling. Further analyses revealed that whilst $CD4^+$ T cell-intrinsic IL-18R1-signalling was dispensable for accumulation of Th17 and Th1/17 populations within the colonic lamina propria, a minor component of the classical Th1 response (IFN-γ^+) was dependent upon functional responses to IL-18.

Furthermore, the induction of a small population of colonic Foxp3^{+} iTreg cells that occurs during naïve CD4^{+} T cell transfer colitis was independent of IL-18R1-signalling.

Recent studies have demonstrated a pathogenic role for IL-18R1-signalling in the experimental autoimmune encephalomyelitis (EAE) model of CNS autoimmunity (Gutcher et al. 2006). In addition, administration of IL-18BP was protective in experimentally induced and genetically driven models of CNS inflammation in both WT and *Il18*$^{-/-}$ mice (Millward et al. 2010). Gutcher *et al.* also demonstrated that IL-18R1-signalling could occur in an IL-18-independent manner and was required for differentiation of pathogenic Th17 cells within the brain and spinal cord. Furthermore, they determined that IL-18R1-signalling was required on cells of the innate immune system for production of IL-23 and the consequent differentiation and maintenance of pathogenic Th17 cells in the CNS (Gutcher et al. 2006). We therefore utilised the T cell transfer colitis model to address whether IL-18R1-signalling on innate immune cells was also required for generation of pathogenic Th17 cell populations within the colonic lamina propria tissue. However, despite the absence of IL-18R1-signalling on host innate immune cells in *Rag1*$^{-/-}$ *Il18r1*$^{-/-}$ mice, adoptive transfer of WT naïve CD4^{+} T cells elicited wasting disease, splenomegaly and severe intestinal inflammation to a similar extent as in *Rag1*$^{-/-}$ recipient control mice. In addition, differentiation of Th1, Th17 and Th1/17 effector cell populations within the colonic lamina propria was also independent of innate immune cell expression of IL-18R1.

Together, these results clearly show that IL-18R1-signalling on innate and adaptive immune cell populations is dispensable for induction of intestinal inflammation in the T cell transfer model of colitis. It would be reasonable to hypothesise that tissue restricted expression of alternative IL-18R1-ligands may reflect the requirement for this axis in driving pathogenic effector CD4^{+} T cells in the CNS. Alternatively, IL-18R1-signalling dependent pathways required for APC-production of IL-23 during

EAE may be functionally redundant in the intestinal environment, where polarisation of Th17 cells occurs readily during homeostasis and inflammation.

To explore the role of IL-18R1 signals in $CD4^+$ T cell mediated colitis in a non-lymphopenic environment, we exploited a model of chronic colitis triggered by *H. hepaticus* infection and IL-10 signalling blockade in WT mice. We found that upon infection with *H. hepaticus* and concomitant anti-IL-10R mAb administration, *Il18r1*$^{-/-}$ mice developed severe typhlocolitis comparable to WT control mice. This suggests that IL-18R1-signalling is dispensable for activation and effector potential of innate and adaptive immune cell populations within the colonic lamina propria. Previous findings from our lab have identified a key role for the common adaptor molecule MyD88 in induction of intestinal inflammation in the *H. hepaticus* plus anti-IL-10R model (Asquith et al. 2010). However, the intestinal pathology appears to be independent of signalling through individual TLR family members (TLR2, TLR4 and TLR5)[(Boulard et al. 2010)(Mark Asquith; D.Phil thesis)] or IL-1R1-signalling (Margherita Coccia; D.Phil thesis). We demonstrate that IL-18R1-signalling is not an essential part of the MyD88-dependent inflammatory response required for induction of chronic intestinal inflammation in this model and suggest that activation of alternative signalling pathways dependent upon MyD88, including other TLRs, singly or in combination, may be required for induction of colitis in this setting.

Of interest to us was the subtle difference in requirement for IL-18R1-signalling on Th1 cell differentiation in the different colitis models. Whilst IL-18R1-deficieny on donor naïve $CD4^+$ T cells slightly decreased the frequency of colonic IFN-γ^+ $CD4^+$ T cells isolated from *Rag1*$^{-/-}$ recipient mice, the absence of this axis did not affect $CD4^+$ T cell differentiation in the *H. hepaticus* plus anti-IL-10R model of chronic intestinal inflammation.

$CD4^+$ T cell proliferation occurs under homeostatic conditions, however $CD4^+$ T cells placed under lymphopenic conditions proliferate in distinct ways dependent upon the severity of lymphopenia (Surh and Sprent 2008). IL-7, IL-15 and self-ligands promote

slow “homeostatic” proliferation, maintenance of a naïve phenotype and IL-2 production, whilst “spontaneous” or chronic lymphopenia-induced proliferation (LIP) following adoptive transfer of naïve $CD4^+$ T cells to *Rag1*$^{-/-}$ recipient mice occurs independently of IL-7, leads to cellular activation and acquisition of a $CD44^+$ $CD62L^-$ effector phenotype, accompanied by production of IFN-γ (Min and Paul 2005, Min et al. 2005). Chronic LIP occurs in response to cognate antigens (Surh and Sprent 2008), including those derived from the commensal microbiota, as transfer of naïve $CD4^+$ T cells to germ-free *Rag1*$^{-/-}$ recipients does not elicit “spontaneous” chronic LIP (Kieper et al. 2005), or intestinal inflammation (Feng et al. 2010). The difference between lymphopenic and lymphocyte replete settings may underlie the differential requirements of IL-18R1-signalling in IFN-γ-production by $CD4^+$ T cells in our studies. Thus, the slight reduction in intestinal IFN-γ^+ $CD4^+$ T cells observed following transfer of naïve *Il18r1*$^{-/-}$ $CD4^+$ T cells suggests that IL-18R1-signalling may contribute to LIP-induced production of IFN-γ, rather than Th1 cell differentiation. Thus, assessment of future investigations into the role of IL-18R1-signalling in models of lymphopenia-induced proliferation and intestinal inflammation would benefit from analysis of transcription factor expression as well as cytokine production.

Alternatively, a severely lymphopenic environment favours the conversion of Th17 cells to those producing IFN-γ (Nurieva et al. 2009, Morrison et al. 2013). Thus, whether all $CD4^+$ IFN-γ^+ T cells emerging during T cell transfer colitis are of a *bona fide* Th1 cell phenotype is not clear. Indeed, during transfer of BDC2.5/NOD cells to NOD/*Scid* mice, myelin oligodendrocyte glycoprotein (MOG)-induced EAE and *H. hepaticus*-triggered intestinal inflammation, distinct developmental routes of IFN-γ producing $CD4^+$ T cells have been described (Bending et al. 2009, Hirota et al. 2011, Morrison et al. 2013). Elegant fate-mapping of the IL-17A locus using *Il17a*^{Cre} *Rosa26*^{eYFP} mice demonstrated that at the site of tissue pathology, either in the CNS or gut, a considerable proportion of IFN-γ producing $CD4^+$ T cells had previously expressed IL-17A, termed “ex-Th17” cells (Hirota et al. 2011, Morrison et al. 2013). As such, “ex-Th17” cells represent a distinct differentiation route to IFN-γ production

from that of presumably direct Th1 cell differentiation. How lymphopenia affects this progression from Th17 to ex-Th17/Th1 cell phenotypes within the colonic lamina propria and other tissues remains to be determined. With this in mind, whether the reduction in frequency of IFN-γ producing $CD4^+$ T cells upon transfer of IL-18R1-deficient naïve $CD4^+$ T cells to *Rag1*$^{-/-}$ recipients represents a decreased frequency of Th1 cells or "ex-Th17" cells would be interesting to determine, and in this instance we would be able to address this question through newly generated *Rag1*$^{-/-}$ *Il18*$^{-/--}$ recipient mice and transfer of naïve *Il17a*^{Cre} *Rosa26*^{eYFP} $CD4^+$ T cells.

Taken together, the data presented within this chapter demonstrate that induction of colitis in two complementary models of $CD4^+$ T cell mediated intestinal inflammation is independent of IL-18R1-signalling. Both *H. hepaticus* triggered and naïve $CD4^+$ T cell transfer colitis are reliant upon the absence or perturbation of regulatory mechanism, IL-10R-signalling and $Foxp3^+$ Treg cells, respectively. As such, in the absence of regulatory mechanisms, IL-18R1-signalling is dispensable for induction of colitogenic responses within the intestine.

Chapter 5 Critical requirement of IL-18R1 signalling in Foxp3$^+$ Treg cell function to prevent T cell driven colitis

5.1 Introduction

Through studies from our laboratory and the work of others, molecular mechanisms required for effective suppression of T cell transfer colitis have been evaluated, and been shown to require the ability of Treg cells to traffic to the GALT (Schneider et al. 2007, Yuan et al. 2007), interact with tissue resident APC (Annacker et al. 2005, Griseri et al. 2010) and produce anti-inflammatory cytokines, including IL-10, TGF-β_1 and IL-35 (Powrie et al. 1996, Asseman et al. 1999, Collison et al. 2007). Key to immune tolerance within the GI tract is an array of environmental factors, derived from a variety of sources including the diet (RA), microbiota (propionate), IEC (TSLP, TGF-β_1) and myeloid cells (IL-10). Cytokines are key regulators of Foxp3$^+$ Treg cell differentiation, survival and effector function, therefore our observations that colonic Foxp3$^+$ Treg cells express IL-18R1 under homeostatic conditions suggested that it might influence their activity within the gut. Furthermore, mutations in the *Il18r1-Il18rap* locus are associated with susceptibility to both adult and early-onset IBD (Barrett et al. 2008, Imielinski et al. 2009, Jostins et al. 2012). We have demonstrated that IL-18R1-signalling is dispensable for induction of chronic intestinal inflammation upon perturbation of regulatory pathways. We hypothesised that IL-18R1-signalling may promote regulatory responses within the intestinal microenvironment and aimed to determine the requirement for IL-18R1-signalling in Foxp3$^+$ Treg cell function.

5.2 Results

5.2.1 IL-18R1-signalling is dispensable for induction of Foxp3.

We observed equivalent percentages of Foxp3$^+$ Treg cells amongst CD4$^+$ T cells isolated from the peripheral lymphoid tissues and colonic lamina propria of WT, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice under homeostatic conditions (Chapter 3 Figure 3.4), demonstrating that IL-18 is not required for development or maintenance of peripheral Foxp3$^+$ Treg cells. In line with this observation, we found that *in vitro* differentiation of Foxp3$^+$ iTreg cells from naïve CD4$^+$ T cells, induced by activation in the presence of TGF-β_1 +/- IL-2 was unaffected by the addition of IL-18 (Figure 5.1A,B), despite high levels of *Il18r1* and *Il18rap* expression on the iTreg cells at these time points (Chapter 3, Figure 3.1).

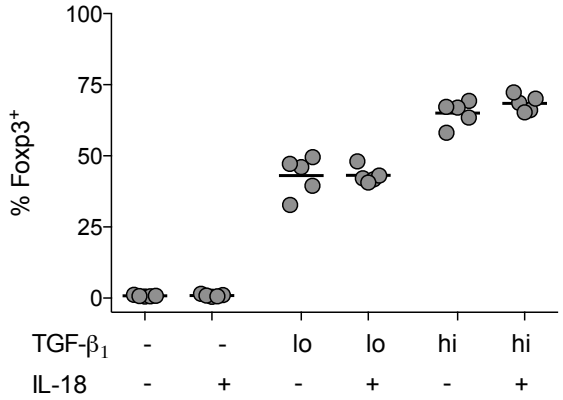
Previous reports from our laboratory have demonstrated that IL-23 inhibits induction of Foxp3$^+$ iTreg cells within the inflamed intestine through direct actions on CD4$^+$ T cells (Izcue et al. 2008, Ahern et al. 2010). Adoptive transfer of naïve CD4$^+$ T cells into IL-23-deficient *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipients resulted in elevated frequencies of Foxp3$^+$ Treg cells compared to transfer into IL-23-sufficient *Rag1*$^{-/-}$ recipients. In this setting, Foxp3$^+$ Treg cells restrained intestinal inflammation as transfer of Foxp3-deficient naïve CD4$^+$ T cells elicited severe intestinal inflammation in both IL-23-sufficient and -deficient environments (Izcue et al. 2008). Thus, in the absence of key regulatory responses naïve CD4$^+$ T cells are able to elicit IL-23-independent intestinal inflammation. We hypothesised that whilst dispensable for Foxp3$^+$ iTreg cell induction under homeostatic conditions, IL-18R1-signalling may be required for Foxp3$^+$ iTreg cell induction under an inflammatory/lymphopenic environment and/or for Foxp3$^+$ iTreg cell function. Thus, we transferred WT or *Il18r1*$^{-/-}$ naïve CD4$^+$ T cells into *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipient mice and compared induction of Foxp3 expression and the onset systemic and intestinal inflammation. As expected, adoptive transfer of WT or *Il18r1*$^{-/-}$ naïve CD4$^+$ T cells induced wasting disease and severe intestinal inflammation in *Rag1*$^{-/-}$ recipient mice (data not shown; Chapter 4, Figure 4.1, Figure 4.2). However, whilst adoptive transfer of WT or *Il18r1*$^{-/-}$ naïve CD4$^+$ T cells induced

wasting disease in *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipients, they did not elicit intestinal inflammation (Figure 5.2A,B).

As previously demonstrated (Hue et al. 2006, Ahern et al. 2010), accumulation of CD4^{+} T cells within the colonic lamina propria was dependent upon IL-23-production by *Rag1*$^{-/-}$ recipient mice (Figure 5.3A,B). Furthermore, no significant differences in colonic CD4^{+} T cell frequencies or numbers were observed between *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipients that received either WT or *Il18r1*$^{-/-}$ naïve CD4^{+} T cells (Figure 5.3A,B). As expected, the frequencies of Foxp3^{+} iTreg cells observed in the colonic lamina propria of *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipient mice were significantly elevated when compared to those found in *Rag1*$^{-/-}$ recipients, however, CD4^{+} T cell signalling via IL-18R1 was not required for induction of Foxp3 expression in this setting (Figure 5.4A,B).

Taken together, these results indicate that CD4^{+} T cell intrinsic IL-18R1-signalling does not promote expression of Foxp3 in homeostatic or inflammatory conditions and is not required for regulatory function of Foxp3^{+} iTreg cells in the absence of IL-23 signalling.

A



B

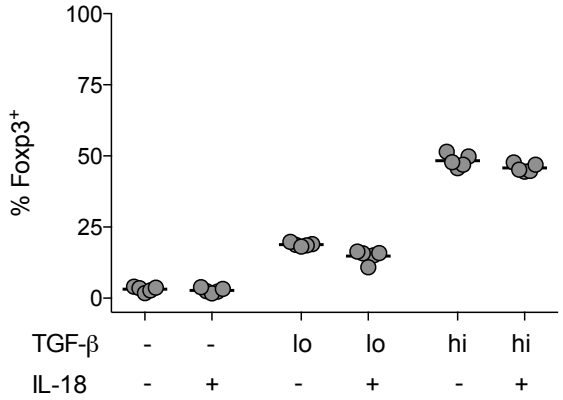


Figure 5.1 *In vitro* differentiation of Foxp3$^+$ CD4$^+$ T cells is not enhanced by IL-18

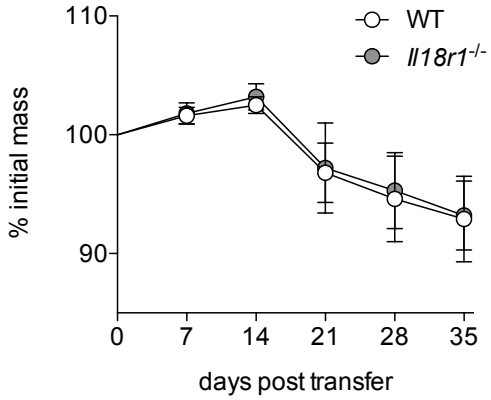
FACS-purified naïve CD4$^+$ CD25$^-$ CD44$^-$ CD62L$^+$ cells (2x10^5) isolated from the spleens and peripheral lymph nodes of WT mice were activated with plate-bound anti-CD3 (1µg/ml) and soluble anti-CD28 (1µg/ml) for 3 days under iTreg polarising conditions: with TGF-β_1 (250pg/ml (lo) or 2.5ng/ml (hi)); in the presence or absence of IL-18 (10ng/ml); and with (A) or without (B) IL-2 (100U/ml). Foxp3 expression was analysed by flow cytometry.

A) Frequencies of Foxp3$^+$ Treg cells observed under iTreg cell polarising conditions in the presence of IL-2.

B) Frequencies of Foxp3$^+$ Treg cells observed under iTreg cell polarising conditions in the absence of exogenous IL-2.

Data shown represent 2 pooled independent experiments each performed with 2-3 technical replicates. Horizontal bars represent the mean and each dot represents a technical replicate. Statistical significance was determined using Students t test.

A



B

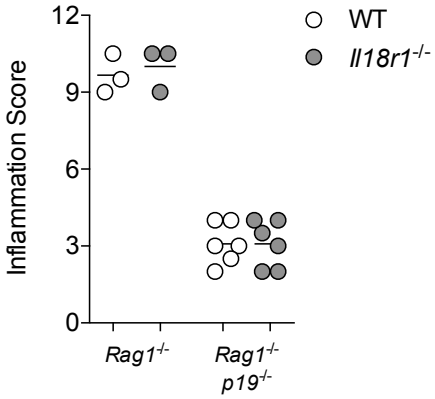
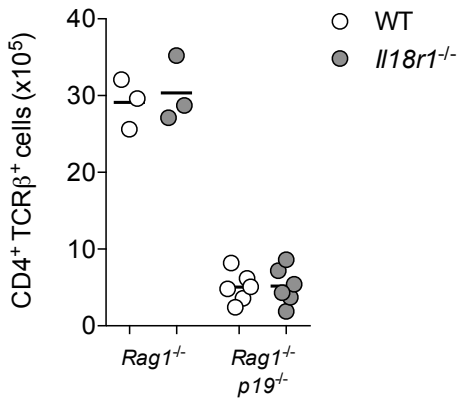


Figure 5.2 WT and *Il18r1*$^{-/-}$ CD4^{+} T cells require IL-23 to induce colitis
FACS-purified naïve CD4^{+} CD25^{-} CD45RB^{Hi} T cells isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ mice were transferred i.p. to *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipient mice. Mice were sacrificed upon development of clinical signs of disease in *Rag1*$^{-/-}$ recipient mice. Upon sacrifice mice were analysed for histological evidence of colitis.
A) Representative weight loss curves of *Rag1*$^{-/-}$ *p19*$^{-/-}$ receiving WT or *Il18r1*$^{-/-}$ naïve CD4^{+} T cells, shown as percentage of initial mass (mean ± S.E.M).
B) Mean inflammation scores from distal, mid and proximal colon sections. Each symbol represents an individual mouse and horizontal bars indicate the mean.
Data shown represents 1 of 2 independent experiments with similar results, each using n=3-6 mice/group. Statistical significance was determined using (A) 2-way ANOVA and (B) Mann-Whitney test.

A



B

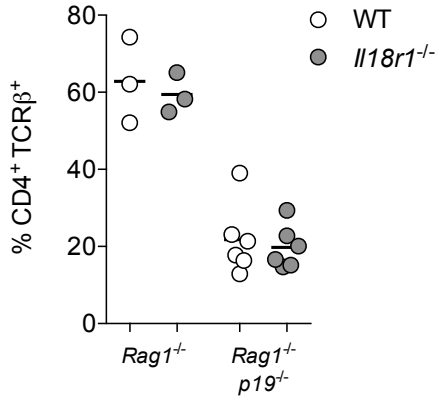
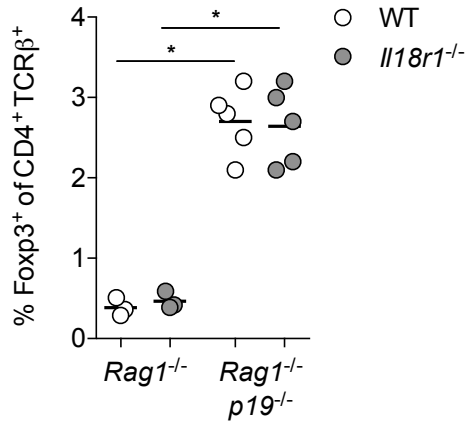


Figure 5.3 IL-18R1-signalling is dispensable for colonic accumulation of CD4$^+$ T cells.
FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ mice were transferred i.p. to *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipient mice. Mice were monitored throughout the experiment and sacrificed upon development of clinical signs of disease in *Rag1*$^{-/-}$ recipient mice. Colonic LPL were isolated and the frequencies of live CD4$^+$ TCRβ$^+$ cells analysed by flow cytometry.
A) Total numbers of CD4$^+$ T cells in the colonic lamina propria.
B) Frequencies of CD4$^+$ TCRβ$^+$ cells in the colonic lamina propria.
Data shown represents 1 of 2 independent experiments with similar results, each with n=3-6 mice/group. Horizontal bars represent the mean and each dot represents and individual mouse. Statistical significance was determined using the Mann Whitney test.

A



B

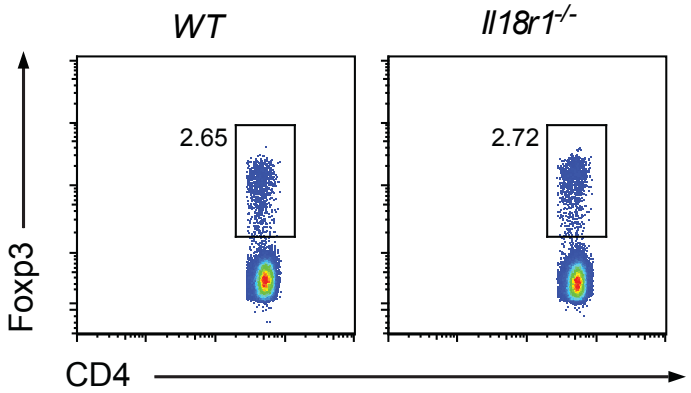


Figure 5.4 Colonic iTreg induction in the absence of IL-23 is independent of IL-18R1-signalling on CD4$^+$ T cells.
FACS-purified naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ mice were transferred i.p. to *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipient mice. Mice were monitored throughout the experiment and sacrificed upon development of clinical signs of disease in *Rag1*$^{-/-}$ recipient mice. Colonic LPL were isolated and the frequencies of Foxp3$^+$ Treg cells analysed by flow cytometry.
A) Frequencies of colonic Foxp3$^+$ Treg cell populations
B) Representative FACS plots of colonic Foxp3$^+$ Treg cell frequencies taken from *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipient mice receiving WT or *Il18r1*$^{-/-}$ naïve CD4$^+$ T cells.
FACS-plots were gated on live CD4$^+$ TCRβ$^+$ cells. Data shown represents 1 of 2 independent experiments with similar results, each with n=3-6 mice/group. Horizontal bars represent the mean and each dot represents and individual mouse. Statistical significance was determined using the Mann Whitney test.

5.2.2 Characterisation of colonic Foxp3$^+$ Treg cells.

Having determined that IL-18R1-signalling on Foxp3$^+$ Treg cells was dispensable for their induction under both homeostatic and inflammatory, we hypothesised that such signals might still contribute to their function within the colonic lamina propria. In order to facilitate the study of Foxp3$^+$ Treg cells and ensure high cellular purity when FACS-purifying these populations, we crossed *Il18r1*$^{-/-}$ mice with Foxp3^{hCD2} IL-10^{gfp} dual reporter mice (Kamanaka et al. 2006, Komatsu et al. 2009) to generate *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice, from which Foxp3$^+$ Treg cells can be isolated based on hCD2 expression.

We initially characterised intestinal Foxp3$^+$ Treg cell populations in WT Foxp3^{hCD2} IL-10^{gfp} mice. Analysing cell surface marker expression of colonic CD4$^+$ TCRβ$^+$ cell subsets allowed us to briefly interrogate the characteristics of Foxp3$^+$ IL-10$^-$ and Foxp3$^+$ IL-10$^+$ Treg cells. Whilst a few Foxp3$^-$ IL-10$^+$ Tr-1-like cells were detectable within the colonic lamina propria, their numbers were too few to allow reliable analysis. In WT Foxp3^{hCD2} IL-10^{gfp} mice, Foxp3$^+$ IL-10$^+$ cells appeared of similar phenotype to their Foxp3$^+$ IL-10$^-$ counterparts, as evidenced by equivalent expression of Foxp3$^+$ Treg cell associated cell surface markers GITR, ICOS, CD103, OX40 and PD-1 (Figure 5.5). Expression of CD62L, ICAM and CXCR3 were not distinguishably different between distinct Foxp3$^+$ IL-10$^+$ and Foxp3$^+$ IL-10$^-$ cell populations (Figure 5.5). Efforts to comprehensively analyse Foxp3$^+$ IL-10$^-$ and Foxp3$^+$ IL-10$^+$ cells are currently the focus of others within the Powrie laboratory.

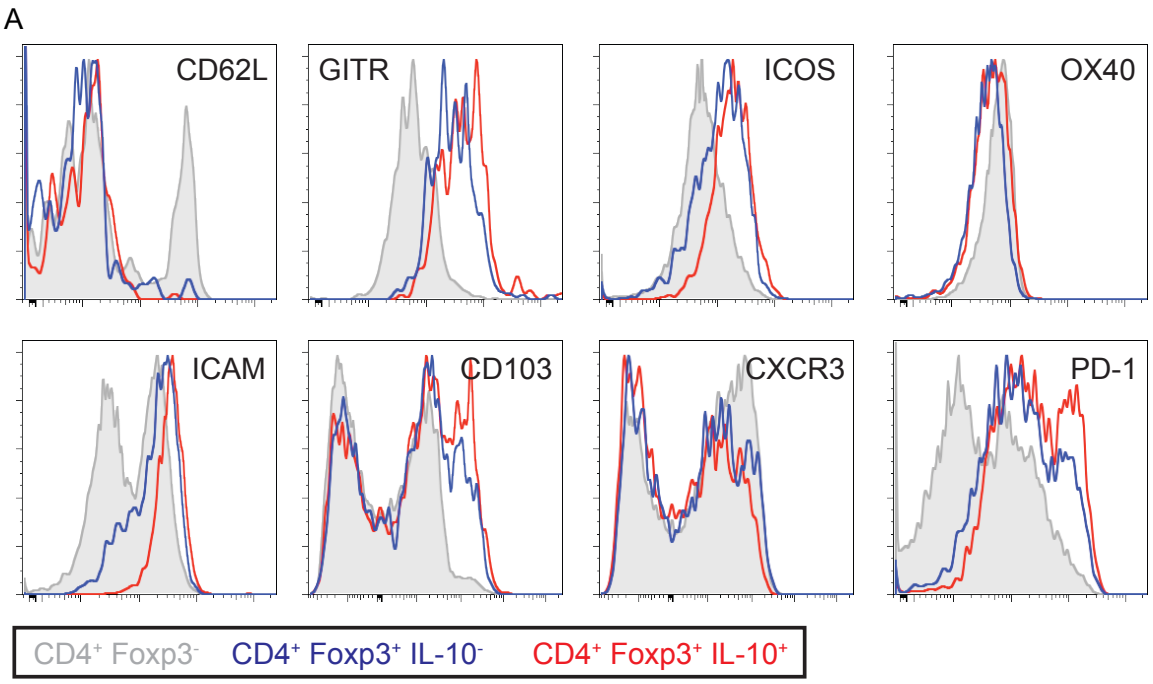


Figure 5.5 Characterisation of colonic Foxp3^{+} Treg cells
Colonic lamina propria leukocytes were isolated from steady state WT Foxp3^{hCD2} IL-10^{gfp} mice and the expression of various cellular surface antigens on Foxp3^{+} Treg cells analysed by flow cytometry.

Conventional Foxp3^{-} T (hCD2^{-}) cells (grey), Foxp3^{+} IL-10^{-} (hCD2^{+} gfp^{-}) (blue) and Foxp3^{+} IL-10^{+} (hCD2^{+} gfp^{+}) subsets were defined within the CD4^{+} TCRβ^{+} population. Tr-1-like cells (Foxp3^{-} IL-10^{+}; hCD2^{-} gfp^{+}) were too few in number to allow accurate analysis.

Representative concatenate histograms of cell surface marker staining (indicated) are shown, pooled from 4 WT Foxp3^{hCD2} IL-10^{gfp} mice, and data shown represent 1 of 2 independent experiments with similar results, each with 4 pooled mice.

Consistent with our earlier findings (Chapter 3, Figure 3.4), analyses of colonic Foxp3^{+} Treg cell populations from WT and *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice under homeostatic conditions demonstrated that IL-18R1-signalling was dispensable for peripheral Foxp3^{+} Treg cell induction or maintenance (Figure 5.6A,C). In addition, we found comparable frequencies of IL-10-producing Foxp3^{+} Treg cells within the intestines of WT and *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice, indicating that acquisition of IL-10 secretion was independent of IL-18R1-signalling (Figure 5.6B,C). Furthermore, WT and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells isolated from the colonic lamina propria exhibited near identical expression of Foxp3^{+} Treg cell associated cell surface markers (Figure 5.7).

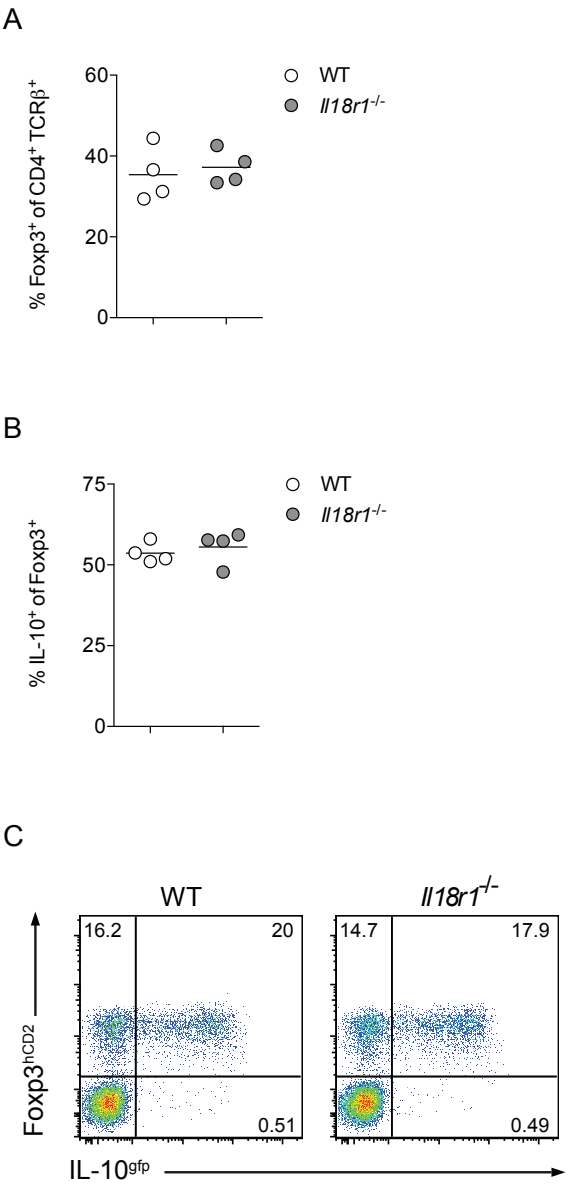


Figure 5.6 Colonic Foxp3$^+$ IL-10$^+$ Treg cell differentiation occurs independently of IL-18R1-signalling.

Colonic LPL were isolated from steady state WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice and frequencies of Foxp3$^+$ IL-10$^+$ Treg cells analysed by flow cytometry.

A) Frequencies of total colonic Foxp3$^+$ Treg cell populations.

B) Frequencies of colonic Foxp3$^+$ IL-10$^+$ Treg cell populations.

C) Representative FACS plots of Foxp3 and IL-10 expression by colonic lamina propria CD4$^+$ TCRβ$^+$ cells.

Data shown represents 1 of 2 independent experiments with similar results, each with n=4 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using Mann-Whitney test.

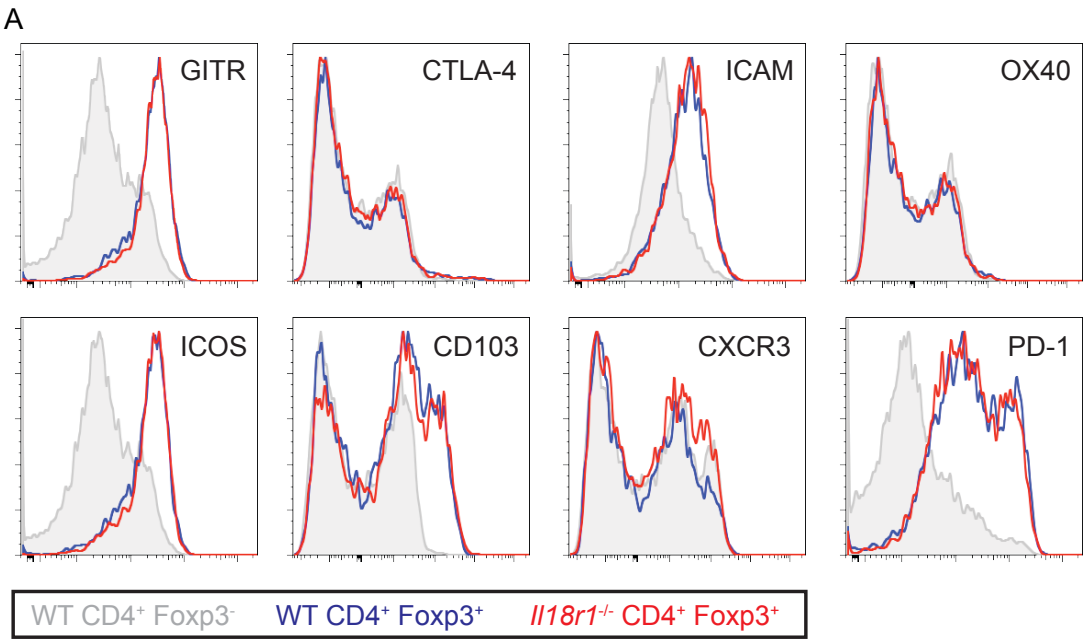


Figure 5.7 Expression of colonic Foxp3^{+} Treg cell surface markers is independent of IL-18R1-signalling.
Colonic LPL were isolated from steady state WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice and expression of distinct cell surface antigens by CD4^{+} TCRβ^{+} cells analysed by flow cytometry.

Conventional WT Foxp3^{-} T cells (hCD2^{-}; grey), WT Foxp3^{+} Treg cells (hCD2^{+}; blue) and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells (hCD2^{+}; red) were defined within the CD4^{+} TCRβ^{+} population. Tr-1-like cells (Foxp3^{-} IL-10^{+}; hCD2^{-} gfp^{+}) were too few in number to allow accurate analysis

Representative concatenate histograms of cell surface marker staining (indicated) are shown, pooled from 4 WT Foxp3^{hCD2} IL-10^{gfp} or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice, and data shown represent 1 of 2 independent experiments with similar results, each with 4 pooled mice.

We next assessed whether IL-18R1-signalling on Foxp3$^+$ Treg cells was required for their function in an inflammatory environment within the colonic lamina propria. To assess the regulation of IL-18R1-expression on Foxp3$^+$ Treg cells during active suppression of intestinal inflammation, we co-transferred FACS-purified WT CD45.1 naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells and WT CD45.2 CD4$^+$ Foxp3$^+$ Treg cells to *Rag1*$^{-/-}$ recipient mice. Four weeks after adoptive transfer, we isolated colonic lamina propria leukocytes and analysed IL-18R1 expression on CD45.2$^+$ Foxp3$^+$ Treg cells by flow cytometry. We found that the vast majority (>90%) of Foxp3$^+$ Treg cells during active regulation of intestinal inflammation expressed IL-18R1 (Figure 5.8), suggesting that IL-18R1-signalling may be playing a functional role in Foxp3$^+$ Treg cell mediated regulation of T cell transfer colitis.

A

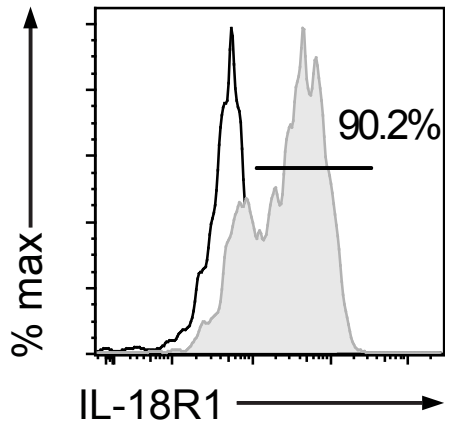


Figure 5.8 Colonic Foxp3^{+} Treg cells express IL-18R1 during active prevention of intestinal inflammation.
FACS-purified WT CD45.1 naïve CD4^{+} CD25^{-} CD45RB^{Hi} T cells (4x10^{5}) and CD45.2 CD4^{+} Foxp3^{+} Treg cells (2x10^{5}, isolated from Foxp3^{hCD2} IL-10^{gfp} mice) were co-transferred i.p. to *Rag1*$^{-/-}$ recipient mice. Four weeks post-transfer, colonic LPL were isolated and expression of IL-18R1 on CD45.2^{+} CD4^{+} Foxp3^{+} Treg cells was analysed by flow cytometry.
A) Representative FACS histogram of IL-18R1 expression (grey) on colonic Foxp3^{+} Treg cells (black = isotype). Gated on live CD4^{+} TCRβ^{+} Foxp3^{+} Treg cells.
Data shown represents a single experiment with n=5 mice.

5.2.3 IL-18R1-signalling is essential for Foxp3$^+$ Treg cell control of naive T cell transfer colitis

To formally assess the requirement for IL-18R1-signalling on Foxp3$^+$ Treg cells to control intestinal inflammation, cohorts of *Rag1*$^{-/-}$ recipient mice were adoptively transferred with congenically marked WT CD45.1 naïve CD4$^+$ T cells alone, or in combination with WT or *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells (both CD45.2). As expected, co-transferred WT Foxp3$^+$ Treg cells completely prevented the wasting disease and severe systemic and intestinal inflammation elicited by WT naïve CD4$^+$ T cells in *Rag1*$^{-/-}$ recipient mice (Figure 5.9, Figure 5.10A,B). By contrast, *Rag1*$^{-/-}$ recipient mice that received WT naïve CD4$^+$ T cells together with *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells developed systemic pathology and intestinal inflammation with a similar incidence and severity to that found in *Rag1*$^{-/-}$ recipient mice that received WT naïve CD4$^+$ T cells alone (Figure 5.9A, Figure 5.10A,B). In addition, *Rag1*$^{-/-}$ recipient mice receiving *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells alone developed neither systemic (Figure 5.9), nor intestinal inflammation (Figure 5.10A,B), demonstrating that *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells do not adopt a pathogenic effector phenotype upon transfer to lymphopenic *Rag1*$^{-/-}$ recipients. These results demonstrate that Foxp3$^+$ Treg cells require IL-18R1-signalling to prevent T cell transfer colitis.

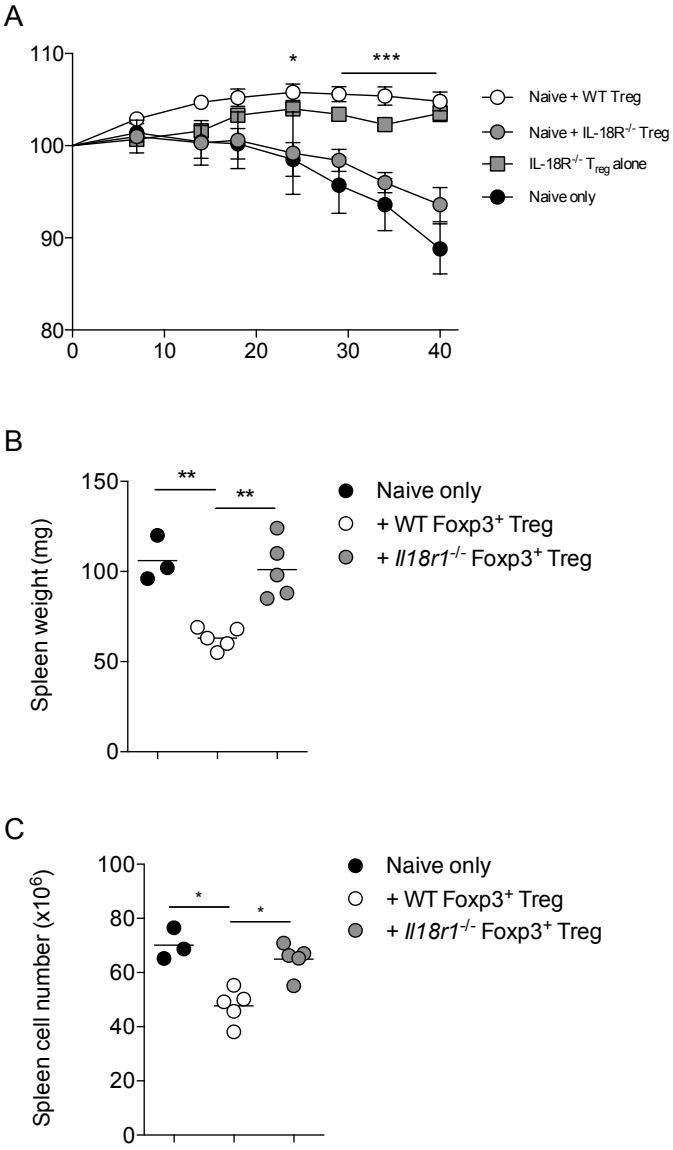
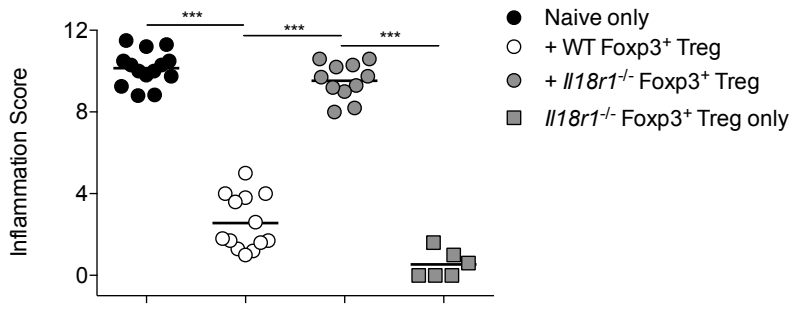


Figure 5.9 Cell-intrinsic IL-18R1-signalling is critical for Foxp3$^+$ Treg cell mediated control of systemic inflammation during T cell transfer colitis.
FACS-purified WT (CD45.1) naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) and CD4$^+$ Foxp3$^+$ Treg cells (2x10^5) isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice were co-transferred i.p. to *Rag1*$^{-/-}$ recipient mice. A control group of *Rag1*$^{-/-}$ recipient mice received *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells only. Upon development of clinical signs of disease in the group receiving naïve CD4$^+$ T cells, mice were sacrificed and analysed for systemic inflammation.
A) Representative weight loss curves, shown as percentage of initial mass (mean ± S.E.M).
B) Spleen weight (mg) and C) Spleen cell number. Horizontal bars represent the mean and each dot represents an individual mouse.
Data shown represents 1 of 3 independent experiments with similar results, each with n=3-6 mice/group. Statistical significance was determined using (A) 2-way ANOVA and (B,C) Mann-Whitney tests, (*=p<0.05, **=p<0.01, ***=p<0.001).

A



B

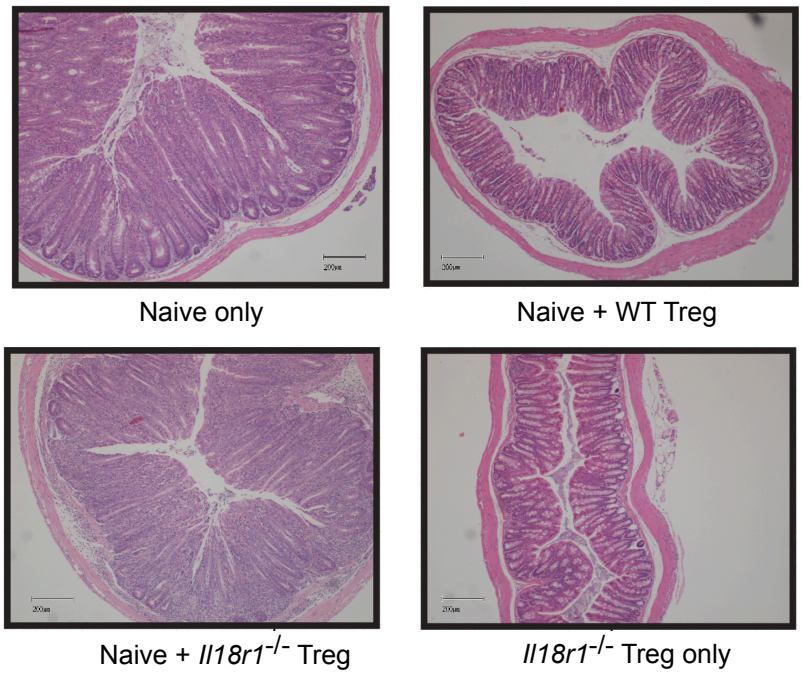


Figure 5.10 Cell-intrinsic IL-18R1-signalling is critical for Foxp3$^+$ Treg cell mediated control of intestinal inflammation.
FACS-purified WT (CD45.1) naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) and CD4$^+$ Foxp3$^+$ Treg cells (2x10^5) isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice were co-transferred i.p. to *Rag1*$^{-/-}$ recipient mice. A control group of *Rag1*$^{-/-}$ recipient mice received *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells only. Upon development of clinical signs of disease in the group receiving naïve CD4$^+$ T cells, mice were sacrificed and analysed for intestinal inflammation.
A) Mean inflammation scores from proximal, mid and distal colon sections. Data shown represents 3 pooled individual experiments, n=6-16 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using Mann-Whitney test. (*** = p<0.001).
B) Representative photomicrographs of distal colon histological sections.
Scale bar = 200µm.

Foxp3$^+$ Treg cell trafficking to distinct anatomical locations is critical for prevention of tissue pathology in multiple experimental settings, including infection, transplantation and models of autoimmunity (Lee et al. 2005, Suffia et al. 2005, Sather et al. 2007, Dudda et al. 2008, Koch et al. 2009). We therefore measured CD4$^+$ effector T cell and Foxp3$^+$ Treg cell accumulation within the colonic lamina propria of *Rag1*$^{-/-}$ recipients following co-transfer of WT CD45.1 naïve CD4$^+$ T cells with either WT or *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells. We observed that total numbers of isolated cLPL, as well as CD4$^+$ T cells of CD45.1 and CD45.2 progeny were elevated in the inflamed intestine of *Rag1*$^{-/-}$ recipient mice receiving WT naïve CD4$^+$ T cells and *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells (Figure 5.11A-C). However, the relative frequencies of Foxp3$^+$ Treg cells to naïve CD4$^+$ T cell progeny (CD45.2:CD45.1) was independent of the ability of Foxp3$^+$ Treg cells to respond to IL-18R1-signalling (Figure 5.11D). Thus, total numbers of *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells within the colonic lamina propria of *Rag1*$^{-/-}$ recipient mice were significantly higher than mice co-transferred with WT Foxp3$^+$ Treg cells (Figure 5.11C), reflecting the greatly increased total cell and CD4$^+$ T cell number within the inflamed intestine of these *Rag1*$^{-/-}$ recipient mice (Figure 5.11A,B). These results show that IL-18R1-signalling is not required for Foxp3$^+$ Treg cell accumulation within the colonic lamina propria.

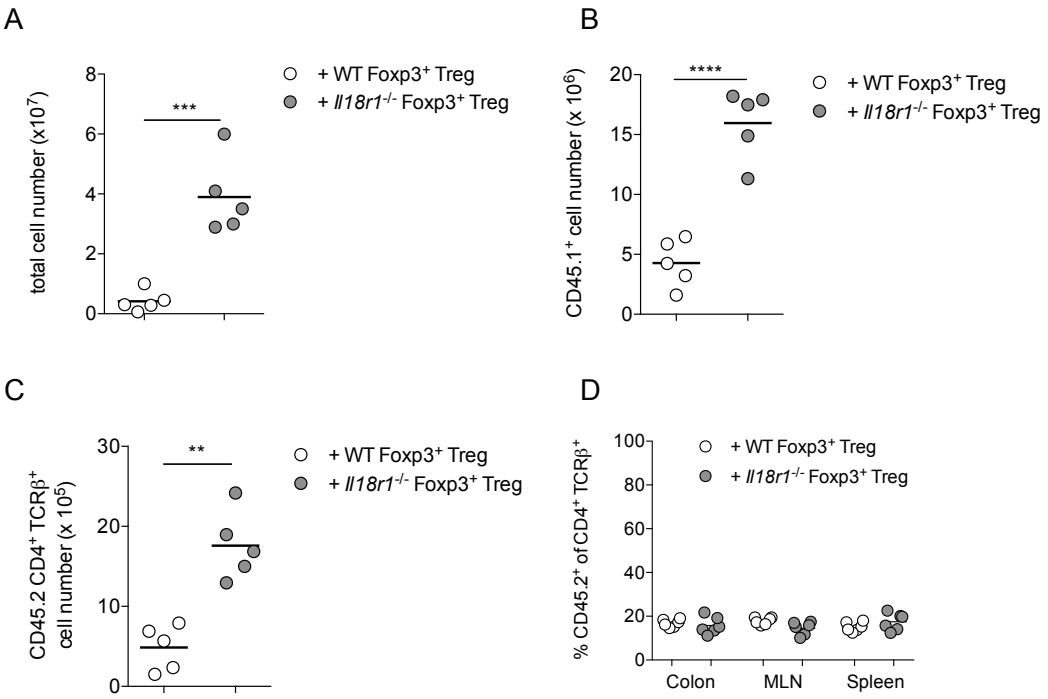


Figure 5.11 – *Il18r1*$^{-/-}$ Foxp3^{+} Treg efficiently accumulate in lymphoid and non-lymphoid organs during T cell mediated colitis.
FACS-purified WT (CD45.1) naïve CD4^{+} CD25^{-} CD45RB^{Hi} T cells (4x10^{5}) and CD4^{+} Foxp3^{+} Treg cells (2x10^{5}) isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice were co-transferred i.p. to *Rag1*$^{-/-}$ recipient mice. Upon development of clinical signs of disease in the group receiving naïve CD4^{+} T cells, colonic LPL were isolated and the frequencies of CD4^{+} TCRβ^{+} cells analysed by flow cytometry.
A) Total cell number of colonic lamina propria leukocytes.
B) Total cell number of colonic CD45.1^{+} CD4^{+} TCRβ^{+} cells.
C) Total cell number of colonic CD45.2^{+} CD4^{+} TCRβ^{+} cells.
D) Frequencies of CD45.2^{+} CD4^{+} TCRβ^{+} cells within lymphoid and non-lymphoid tissues.
Data shown represents 1 of 3 individual experiments with similar results, each with n=5-6 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using Mann-Whitney test. (**= p<0.01, ***= p<0.001, ****= p<0.0001).

We next tested whether the adoptively transferred Treg cells had retained expression of Foxp3. Crucially, both WT and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells that were co-transferred with naïve CD4^{+} T cells to *Rag1*$^{-/-}$ recipient mice maintained equivalently high frequencies (>95%) of Foxp3 expression (Figure 5.12B), indicating that IL-18R1-signalling is not required for maintenance of Foxp3 expression under lymphopenic/inflammatory conditions. Production of IL-10 by Foxp3^{+} Treg cells is critical for induction and maintenance of immune homeostasis within the colonic lamina propria (Rubtsov et al. 2008). However, we found that following co-transfer with naïve CD4^{+} T cells into *Rag1*$^{-/-}$ recipient mice both WT and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells were capable of IL-10 production, and the frequencies of Foxp3^{+} IL-10^{+} Treg cells were enriched within the GALT (Figure 5.12C). In fact, the frequencies of IL-10^{+} cells within the colonic lamina propria and MLN were significantly increased in *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells when compared to WT Foxp3^{+} Treg cells (Figure 5.12C), though this was most likely as a result of the on-going intestinal inflammation in this experimental group. Thus, whilst *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells are able to correctly traffic to the colonic lamina propria, maintain expression of Foxp3 and produce the regulatory cytokine IL-10, they are unable to prevent induction of intestinal inflammation following adoptive transfer of naïve CD4^{+} T cells to *Rag1*$^{-/-}$ recipients.

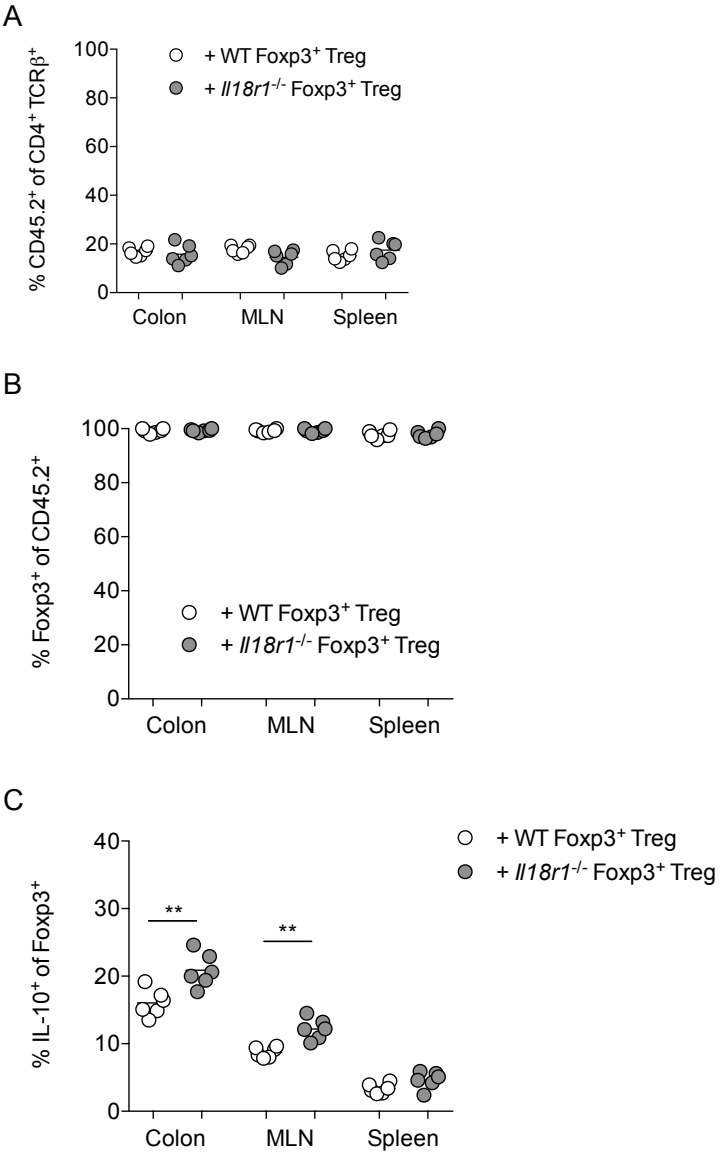


Figure 5.12 Foxp3 expression and IL-10 production are maintained by IL-18R1$^{-/-}$ Treg cells during intestinal inflammation.

FACS-purified WT (CD45.1) naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) and CD4$^+$ Foxp3$^+$ Treg cells (2x10^5) isolated from the spleens and peripheral lymph nodes of WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice were co-transferred i.p. into *Rag1*$^{-/-}$ recipient mice. Upon development of clinical signs of disease in the group receiving naïve CD4$^+$ T cells, leukocytes were isolated from different tissues and expression of Foxp3^{hCD2} and IL-10^{gfp} by CD4$^+$ TCRβ$^+$ cells was analysed by flow cytometry.

A) Frequencies of CD45.2$^+$ cells amongst all colonic CD4$^+$ TCRβ$^+$ cells.

B) Frequencies of Foxp3^{hCD2} expression by colonic CD45.2$^+$ CD4$^+$ TCRβ$^+$ cells

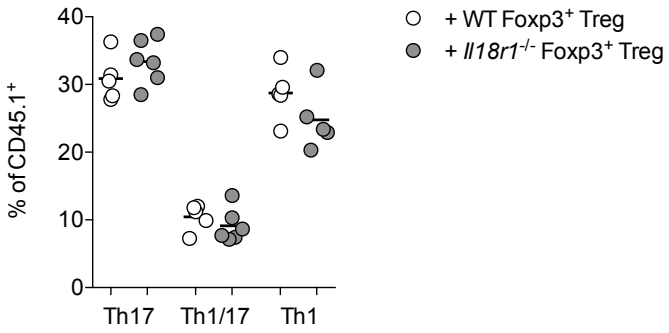
C) Frequencies of IL-10^{gfp} expression by CD45.2$^+$ CD4$^+$ TCRβ$^+$ Foxp3$^+$ Treg cells.

Data shown represents 1 of 2 independent experiments, with similar results, each with n=5-6 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using Mann-Whitney test. (** = p<0.01).

A recent concept in the biology of Foxp3$^+$ Treg cells is that tissue specific signals and inflammatory cytokines reflect contextual cues for induction of transcription factors classically associated with Th1, Th2 and Th17 cell differentiation, allowing the acquisition of specialised regulatory functions (Chaudhry et al. 2009, Koch et al. 2009, Zheng et al. 2009). We hypothesised that IL-18R1-signalling in Foxp3$^+$ Treg cells may be required for regulation of distinct effector CD4$^+$ T cells subsets within the colonic lamina propria. However, analysis of effector cytokine production by naïve CD4$^+$ T cell progeny for using intracellular staining revealed a similar distribution of Th1 and Th17 cell frequencies in *Rag1*$^{-/-}$ recipient mice receiving naïve CD4$^+$ T cells together with either WT or *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells (Figure 5.13A). Thus, it appears that IL-18R1-signalling in Foxp3$^+$ Treg cells is not required for the selective regulation of a particular pathogenic CD4$^+$ T cell effector subpopulation in the intestine.

Efficient prevention of T cell transfer colitis by Foxp3$^+$ Treg cells also requires deviation of a small proportion of the naïve CD4$^+$ T cell progeny to a Foxp3$^+$ iTreg cell phenotype (Haribhai et al. 2009). We therefore hypothesised that IL-18R1-signalling on Foxp3$^+$ Treg cells may be required *in trans* to generate Foxp3$^+$ iTreg cells from the naïve CD4$^+$ T cell pool. However, analysis of Foxp3 expression by naïve CD4$^+$ T cell progeny excluded this possibility as *Rag1*$^{-/-}$ recipient mice receiving naïve CD4$^+$ T cells and WT or *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells exhibited equivalent frequencies of Foxp3$^+$ iTreg cells within the colonic naïve CD4$^+$ T cell progeny (Figure 5.13B).

A



B

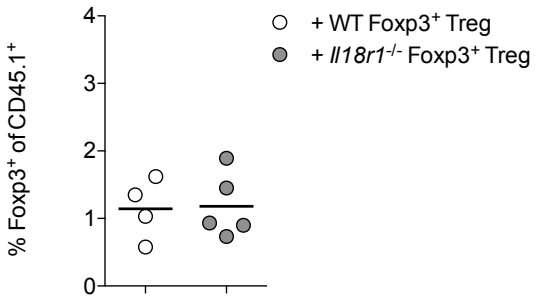
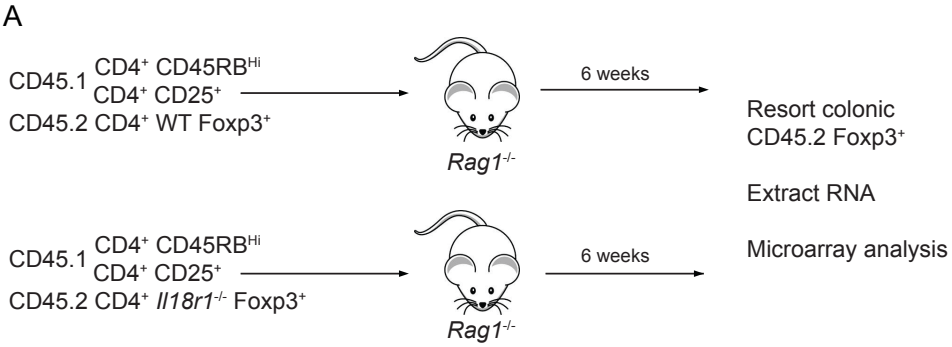
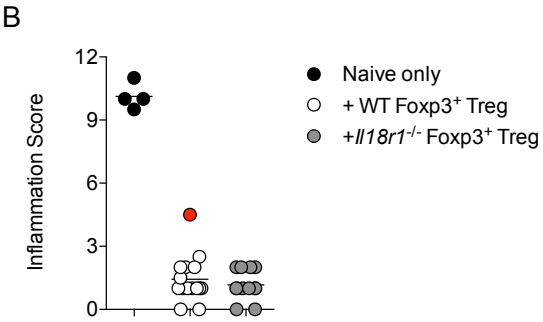
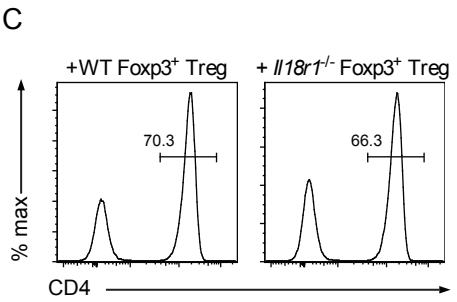


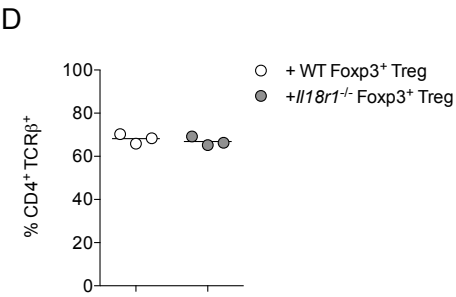
Figure 5.13 Analysis of naïve CD4$^+$ T cell progeny lineage specification
FACS-purified WT (CD45.1) naïve CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells (4x10^5) and CD4$^+$ Foxp3$^+$ Treg cells (2x10^5) isolated from WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice were co-transferred i.p. to *Rag1*$^{-/-}$ recipient mice. Upon development of clinical signs of disease in the group receiving naïve CD4$^+$ T cells, colonic LPL were isolated and frequencies of Th1, Th17 and Foxp3$^+$ iTreg cells amongst CD45.1$^+$ CD4$^+$ TCRβ$^+$ cells was analysed by flow cytometry.
A) Frequencies of colonic Th1, Th17 and Th1/17 cell populations.
B) Frequencies of colonic Foxp3$^+$ iTreg cell populations.
Data shown represents 1 of 2 independent experiments with similar results, each with n=4-6 mice/group. Horizontal bars represent the mean and each dot represents an individual mouse. Statistical significance was determined using Mann Whitney test.

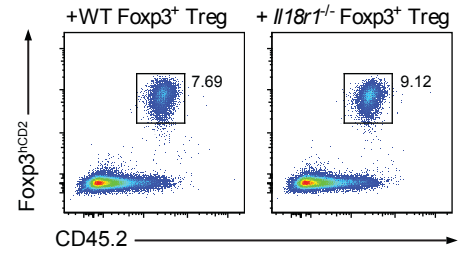
We chose to characterise IL-18R1-signalling regulated genes in colonic Foxp3^{+} Treg cells by comparing the gene expression profiles of WT and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells during Foxp3^{+} Treg cell mediated prevention of T cell transfer colitis. In order to ensure both WT and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells were derived from comparable inflammatory contexts, we constructed an experimental setup outlined in Figure 5.14A. Briefly, *Rag1*$^{-/-}$ recipient mice received congenically marked WT naïve CD4^{+} T cells (CD45.1), congenically marked WT regulatory T cells (CD25^{+} CD45RB^{lo}; CD45.1) and Foxp3^{+} Treg cells isolated from either WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice. As such, the dominant role of WT regulatory T cells prevented development of severe intestinal inflammation in *Rag1*$^{-/-}$ recipient mice, which was confirmed by histological analysis (Figure 5.14B). A single mouse that exhibited macroscopic intestinal pathology at the point of sacrifice was excluded from further analysis (red point, Figure 5.14A). Six weeks post adoptive transfer, colonic lamina propria leukocytes were isolated and FACS-purified to isolate either WT or *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells based on expression of TCRβ, CD4, CD45.2 and hCD2. Frequencies of effector and regulatory CD4^{+} T cell populations were comparable between experimental groups (Figure 5.14C-H) and sorted WT or *Il18r1*$^{-/-}$ Foxp3^{+} Treg were >98% pure, as assessed by post sort analysis (data not shown). Total RNA was extracted, amplified and subjected to whole transcriptome expression analysis on the Illumina Bead Array Platform.

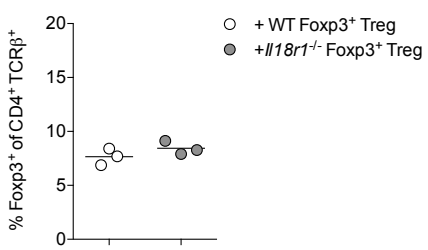

A
CD45.1 CD4+ CD45RBHi
CD4+ CD25+
CD45.2 CD4+ WT Foxp3+
Rag1-/-
6 weeks
Resort colonic
CD45.2 Foxp3+
Extract RNA
Microarray analysis
CD45.1 CD4+ CD45RBHi
CD4+ CD25+
CD45.2 CD4+ Il18r1-/- Foxp3+
Rag1-/-
6 weeks

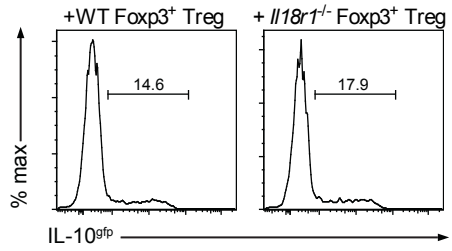

B
Inflammation Score
12
9
6
3
0
Naive only
+ WT Foxp3+ Treg
+Il18r1-/- Foxp3+ Treg


C
+WT Foxp3+ Treg
+ Il18r1-/- Foxp3+ Treg
70.3
66.3
% max
CD4


D
% CD4+ TCRβ+
100
80
60
40
20
0
+ WT Foxp3+ Treg
+Il18r1-/- Foxp3+ Treg


E
+WT Foxp3+ Treg
+ Il18r1-/- Foxp3+ Treg
7.69
9.12
Foxp3hCD2
CD45.2


F
% Foxp3+ of CD4+ TCRβ+
20
15
10
5
0
+ WT Foxp3+ Treg
+Il18r1-/- Foxp3+ Treg


G
+WT Foxp3+ Treg
+ Il18r1-/- Foxp3+ Treg
14.6
17.9
% max
IL-10gfp

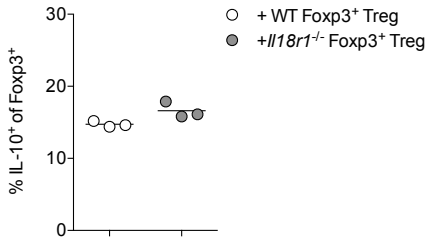

H
% IL-10+ of Foxp3+
30
20
10
0
+ WT Foxp3+ Treg
+Il18r1-/- Foxp3+ Treg

Figure 5.14 Transcriptional profiling of IL-18R1-dependent networks in Foxp3$^+$ Treg cells during prevention of intestinal inflammation.
Whole genome Illumina platform microarray analysis of gene expression of WT and *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells during prevention of colitis was undertaken.
Experimental groups utilised are outlined in (A). Briefly, *Rag1*$^{-/-}$ recipient mice were transferred with FACS-purified naïve CD45.1$^+$ CD4$^+$ T cells (4x10^5) and CD45.1$^+$ CD4$^+$ CD25$^+$ Treg cells (2x10^5) in addition to CD45.2$^+$ Foxp3$^+$ Treg cells obtained from either WT or *Il18r1*$^{-/-}$ Foxp3^{hCD2} IL-10^{gfp} mice (2x10^5). Six weeks after transfer, mice were sacrificed, macroscopically examined for intestinal inflammation, colonic lamina propria leukocytes isolated and viable CD4$^+$ TCRβ$^+$ CD45.2$^+$ CD45.1$^-$ hCD2$^+$ Treg cells FACS sorted and snap frozen.
A) Schematic of experimental approach to microarray.
B) Inflammation scores of distal colon sections. The red dot was excluded at the point of sacrifice following macroscopic analysis of intestinal inflammation.
C) Representative FACS plots of colonic CD4$^+$ TCRβ$^+$ cell frequencies, gated on live lymphocytes.
D) Frequencies of colonic CD4$^+$ TCRβ$^+$ cell populations.
E) Representative FACS plots of colonic CD45.2$^+$ Foxp3$^+$ Treg cell frequencies, gated on CD4$^+$ TCRβ$^+$ cells.
F) Frequencies of colonic CD45.2$^+$ Foxp3$^+$ Treg cell populations.
G) Representative FACS histograms of IL-10^{gfp} expression by colonic CD45.2$^+$ Foxp3$^+$ Treg cells.
H) Frequencies of colonic IL-10$^+$ Foxp3$^+$ cell populations.

Data shown represents 1 of 3 independent experiments. Horizontal bars represent the mean and each dot represents 4-5 pooled recipient mice. Statistical significance was determined using Mann-Whitney test.

Analysis of differences in transcriptome expression values between WT and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells isolated from similar inflammatory environments identified a total of 911 transcripts differentially expressed between the 2 groups. Of these, 910 were significantly down-regulated (≥2 fold), and 1 transcript was significantly up-regulated (≥2 fold) in *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells, indicating that IL-18R1-signalling into Foxp3^{+} Treg cells is almost uniquely positively effecting gene expression (Figure 5.15A). Amongst the transcripts differentially expressed between WT and *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells, using a gene-set enrichment analysis of transcripts previously associated with immune cell function, we identified a host of molecules previously implicated in Foxp3^{+} Treg cell function and/or IL-18R1-signalling, including *Tgfb1*, *Tnfrsf25* (*Dr3*), *Relb*, *Tnfrsf18* (*Gitr*), *Cd52*, *Foxp3*, *Icos*, *Tnfrsf4* (*OX40*) and *Gadd45b* (Figure 5.15B).

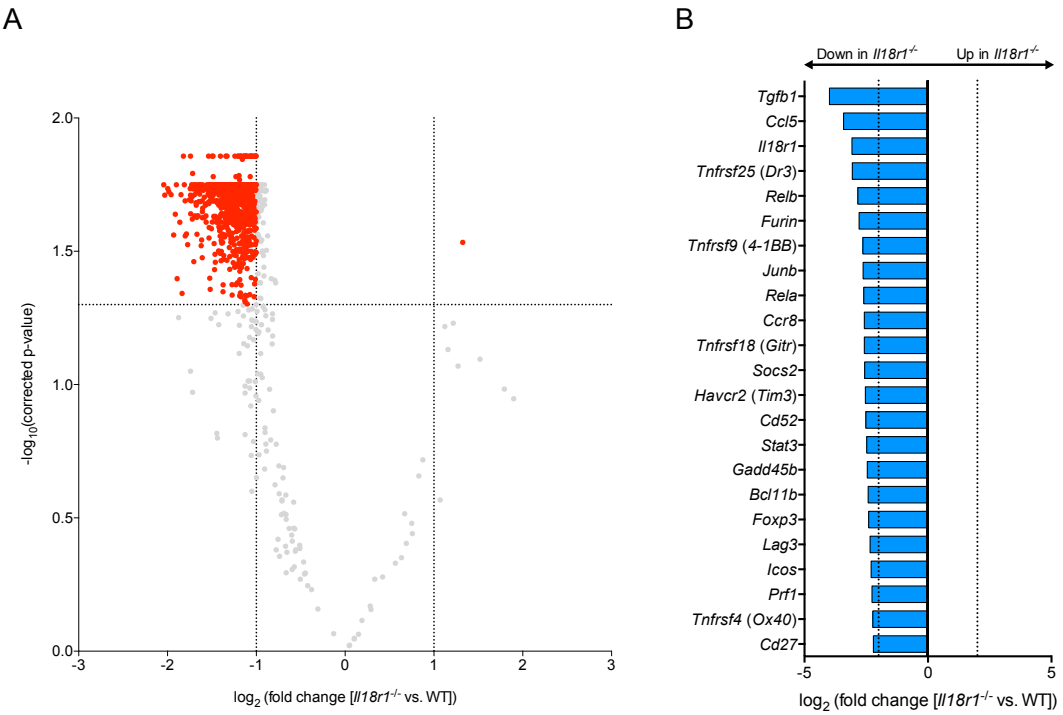


Figure 5.15 IL-18R1-signalling broadly promotes the Foxp3$^+$ Treg cell transcriptional programme.

Whole transcriptome analysis of IL-18R1-regulated genes in colonic Foxp3$^+$ Treg cells derived from the experimental strategy shown in Figure 5.14A.

A) Volcano plot analysis of gene expression (hierarchical, Pearson uncentered, average linked, unsupervised) of the 911 transcripts whose expression was significantly affected in the comparison of WT vs. *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells. Each dot corresponds to a single transcript, with red dots being ≥2 fold differentially expressed with a significance of $p<0.05$ by post-hoc testing for multiple comparisons. Genes significantly down-regulated in *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells appear in the top left quadrant.

B) Absolute fold change of genes identified through gene-set enrichment analysis previously associated with immune cell function which are ≥2 fold differentially regulated in colonic WT vs. *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells. Blue bars represent genes that are down regulated in *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells, if multiple transcripts were differentially regulated the mean expression change is represented.

Data represent results from a single experiment. Statistical significance was determined using an unpaired t-test followed by Benjamini Hochberg false discovery rate multiple testing correction. P value cut-off was set to 0.05.

Whilst the validation of gene expression changes and onwards analysis of their role in Foxp3^{+} Treg cell function are the subject of current and future investigations, together the data presented here demonstrate a critical requirement for Foxp3^{+} Treg cells to receive IL-18R1-dependent signals in order to prevent experimental intestinal inflammation.

5.3 Discussion

The results described within this chapter demonstrate a clear requirement for cell intrinsic IL-18R1-signalling in Foxp3$^+$ Treg cell mediated prevention of experimental intestinal inflammation. We were intrigued by the expression of IL-18R1 by Foxp3$^+$ Treg cells in steady state WT mice and took several approaches to assess the role of this axis in the regulation of CD4$^+$ T cell subset frequencies and function within the intestine. *In vitro* experiments demonstrated that IL-18 neither enhances nor inhibits induction of Foxp3 expression in activated CD4$^+$ T cells, despite their high *Il18r1* and *Il18rap* expression. A recent study reported that *Helicobacter pylori* (*H. pylori*) infection promoted gastric Foxp3$^+$ iTreg induction that was dependent upon DC-derived IL-18 (Oertli et al. 2012). Whilst these findings appear to contrast with our data regarding the requirements for IL-18 in Foxp3$^+$ iTreg cell induction, there are key differences in experimental setup, which may explain these disparities. In *Oertli et al*, neonatal mice (7 days) were utilised, whereas we used adult mice (6-10 weeks). Whilst we have not performed a kinetic analysis of Foxp3$^+$ iTreg cell induction in the colon, it remains possible that early in life, during initial colonisation by the resident intestinal microbiota, IL-18 may promote Foxp3$^+$ iTreg cell induction, but that IL-18R1 signals may not be required upon stabilisation of the microbiota/immune interactions. In addition, the microenvironment of the large and small intestines are uniquely amenable to Foxp3$^+$ iTreg induction (Bilate and Lafaille 2012), and as such, compensatory mechanisms (that are lacking in the in the stomach) may mask any requirement for IL-18 in the lower bowel.

However, a key difference between the experimental conditions is that we assayed the role of IL-18R1 signals during homeostasis, whereas *Oertli et al.* looked during *H. pylori* infection. We therefore hypothesised that IL-18R1-signalling may be required for Foxp3$^+$ iTreg cell differentiation under inflammatory conditions. To address this experimentally, we utilised the naive T cell transfer model of colitis. Previous studies in our laboratory had shown that, although intestinal pathology in this model was usually highly dependent on IL-23, when naive CD4$^+$ T cells that were unable to

differentiate into Foxp3 iTreg were used for adoptive transfer, they could mediate intestinal inflammation via an IL-23-independent mechanism (Izcue et al. 2008). However, whilst WT and *Il18r1*$^{-/-}$ naïve CD4^{+} T cells induced severe colitis in *Rag1*$^{-/-}$ recipient mice, *Rag1*$^{-/-}$ *p19*$^{-/-}$ recipients receiving either WT or Il18r1$^{-/-}$ naïve CD4^{+} T cells did not develop intestinal inflammation, demonstrating that IL-18R1-signalling is dispensable for Foxp3^{+} iTreg cell induction and function within the colonic lamina propria during inflammatory conditions. Furthermore, under homeostatic conditions, colonic Foxp3^{+} Treg cell production of IL-10 appeared independent of IL-18R1-signalling, in line with recent publications demonstrating that this pathway is independent of MyD88/Trif signalling (Atarashi et al. 2011, Geuking et al. 2011). In addition, expression of typical Treg cell surface markers appeared independent of IL-18R1-signalling, though in this setting any differences in Foxp3^{+} Treg cell phenotype may be difficult to observe, given that IL-18R1 is expressed by only a minority (40%) of colonic Foxp3^{+} Treg cells. Thus, we could find no requirement for IL-18R1 signalling in differentiation of Foxp3^{+} Treg cells.

This led us to speculate that IL-18R1 signalling may affect Foxp3^{+} Treg cell function rather than differentiation. Indeed, during *in vivo* suppression of intestinal inflammation the vast majority (>90%) of Foxp3^{+} Treg cells expressed IL-18R1. In this experimental setting, the requirements for IL-18R1-signalling for suppressive activity of Foxp3^{+} Treg cells were revealed. Co-transfer of WT Foxp3^{+} Treg cells prevented induction of systemic and intestinal inflammation elicited by naïve CD4^{+} T cells, whereas *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells did not. Whilst *Il18r1*$^{-/-}$ Foxp3^{+} Treg cells appeared to correctly traffic to peripheral and intestinal lymphoid tissues, maintained their frequencies relative to effector cells and prevented exacerbated differentiation of naïve CD4^{+} T cells to either Th17 or Th1 cell lineages, they were unable to prevent severe colitis in *Rag1*$^{-/-}$ recipient mice. Furthermore, IL-18R1-deficiency did not impair Foxp3^{+} Treg cell stability or their production of the anti-inflammatory cytokine IL-10.

To ascertain the effects of IL-18R1 signalling in intestinal Foxp3$^+$ Treg cells *in vivo*, we sorted WT and *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells from non-inflamed colons of *Rag1*$^{-/-}$ recipients that were co-transferred with naive and Treg cells, and performed comparative transcriptome analyses. Microarray-based analysis of gene expression in colonic Foxp3$^+$ Treg cells demonstrated that IL-18R1-signalling almost uniquely induces gene expression, with over 900 genes expressed at significantly higher levels in WT relative to *Il18r1*$^{-/-}$ Foxp3$^+$ Treg cells. At the time of writing this thesis, we are currently validating these data using qRT-PCR and Western blot analysis to better identify which candidate genes are direct and indirect targets of IL-18R1-signalling in Foxp3$^+$ Treg cells. A relatively large number of genes associated with Foxp3$^+$ Treg cell phenotype and function (Feuerer et al. 2010) were identified in our studies as being differentially regulated in the absence of IL-18R1-signalling. However, it is unlikely that IL-18R1-signalling directly promotes expression of all these genes, more likely induction of a key transcriptional regulator further promotes expression of a transcriptional module of Foxp3$^+$ Treg cell associated genes (Bettini et al. 2012, Darce et al. 2012, Fu et al. 2012). We will focus firstly on validating differentially expressed genes implicated in immune function that were identified through gene set enrichment analyses of the transcriptional data. These include the gene encoding zinc-finger protein B-cell leukaemia/lymphoma 11b (Bcl11b) and Growth arrest and DNA-damage-inducible β (Gadd45β), both of which are essential in CD4$^+$ T cells for maintenance of self-tolerance (Liu et al. 2005, Vanvalkenburgh et al. 2011). Furthermore, previous studies demonstrate Gadd45β to be directly induced in CD4$^+$ T cells by IL-18 (Yang et al. 2001).

The identification of several TGF-β_1 pathway components is also of great interest in our hunt for the mechanistic explanation of the requirement for IL-18R1-signalling in Foxp3$^+$ Treg cells. Expression of both TGF-β_1 and its converting enzyme, furin, appear dependent upon IL-18R1-signalling. Both Foxp3$^+$ Treg cell production of TGF-β_1 and naïve CD4$^+$ T cell expression of TGF-βR is required for prevention intestinal inflammation during T cell transfer colitis (Fahlen et al. 2005, Li et al. 2006,

Li et al. 2007). However, *Il18r1*$^{-/-}$ mice do not develop spontaneous lymphoproliferative or T cell mediated fatal autoimmune syndromes observed in mice deficient for TGF-β_1/TGF-βR (Shull et al. 1992, Diebold et al. 1995, Letterio et al. 1996, Kobayashi et al. 1999), as such the validity of these gene expression changes remains to be confirmed.

As described previously and addressed directly in Chapter 3 with respect to the regulation of colonic Th17 cell frequencies by IL-18, non-canonical IL-18R1-signalling has also been described (Gutcher et al. 2006). Foxp3$^+$ Treg cell expression of IL-18R1 is critical for prevention of intestinal tissue pathology in the T cell transfer colitis model. However, whether signalling via IL-18R1 in this instance represents canonical signalling dependent upon IL-18, or upon non-canonical ligands, remains to be confirmed. Therefore, we have recently crossed *Rag1*$^{-/-}$ mice with *Il18*$^{-/-}$ mice to generate *Rag1*$^{-/-}$ *Il18*$^{-/-}$ recipient mice in order to test the requirement for IL-18 during Foxp3$^+$ Treg cell-mediated prevention of intestinal inflammation. Furthermore, whilst production of IL-18 within in the homeostatic intestine is derived from intestinal epithelial cells, phenotypic shifts in the cellular sources of IL-18 during chronic intestinal inflammation have been described, with lamina propria mononuclear cells suggested as a key source during Crohn's disease (Pizarro et al. 1999). As such, in combination with IHC analysis of inflamed tissue, we will functionally determine the cellular source of IL-18 during intestinal inflammation through the generation of bone marrow chimeric recipient mice, enabling the selective ablation of IL-18 production from haematopoietic or non-haematopoietic compartments in *Rag1*$^{-/-}$ recipient mice. In addition, the mechanism by which pro-IL-18 is converted to its mature bioactive form under inflammatory conditions remains to be determined. Parallel studies in our lab have found that CD25$^+$ Treg cells are fully able to prevent intestinal inflammation during adoptive transfer of WT naïve CD4$^+$ T cells to *Rag1*$^{-/-}$ *Asc*$^{-/-}$ recipient mice (Naren Srinivasan, D.Phil thesis). The requirement for IL-18 in this scenario though is unclear, as the absence of other inflammasome-processed factors and cytokines

may qualitatively alter the naïve $CD4^+$ T cell response in this setting, and reduce the required regulatory capacity of transferred $CD25^+$ Treg cells. Indeed, we have previously demonstrated that IL-1R1-signalling drives colitis in this model through direct actions on $CD4^+$ T cells (Coccia et al. 2012). However, the metalloproteinases, meprin A and meprin B, which are encoded within IBD-susceptibility loci, are highly expressed within the gastrointestinal tract and are also able to process pro-IL-18 to its active form (Banerjee and Bond 2008, Banerjee et al. 2009).

As alluded to previously, whilst the epigenetic and transcriptional modifications required for Th1 cell expression of IL-18R1 are well characterised, the requirements for expression of IL-18R1 by $Foxp3^+$ Treg cells are not understood. Stimulation of naïve $CD4^+$ T cells with TGF-β_1 leads to induction of both Foxp3 and IL-18R1, though the relative contribution of direct TGF-βR signalling and adoption of a Foxp3-dependent transcriptional network to *Il18r1* expression are difficult to interpret without the use of $Foxp3^{-/Y}$ cells. However, recent proteomic analysis of $Foxp3^+$ Treg cells indicates that Foxp3 mediates its effects at a molecular level through large multi-protein complexes, where it directly regulates expression of many of its interacting partners, including GATA3 (Rudra et al. 2012). Chromatin-immunoprecipitation in combination with high-throughput DNA sequencing (ChIP-Seq) data from $Foxp3^+$ Treg cells indicates Foxp3 and GATA3 to have both independent and shared binding sites throughout the genome, of which *Il18r1* represents a co-bound site (Samstein et al. 2012). Functional data demonstrate that GATA3 is critical for $Foxp3^+$ Treg cell function at environmental interfaces, as genetic deletion of GATA3 from $Foxp3^+$ Treg leads to development of dermatitis and intestinal inflammation (Rudra et al. 2012). The direct regulation of IL-18R1-expression by Foxp3 and/or GATA3 in these settings would be of great interest. We attempted pilot ChIP experiments to assess direct interactions between Foxp3 and coding and conserved non-coding sequences of the *Il18r1-Il18rap* locus at higher resolution than ChIP-Seq, however, these were not technically successful and are being optimised.

Whilst we have conclusively demonstrated a requirement for IL-18R1-signalling in Foxp3^{+} Treg cells to prevent T cell transfer in a lymphopenic environment, whether Foxp3^{+} Treg cells require IL-18R1-signalling at extra-intestinal sites and under lymphocyte replete settings will be of interest for future studies. In a similar vein to generating better tools to understand the role of IL-18R1-signalling specifically in Th17 cells through the use of IL-17A^{Cre} x IL-18R1$^{fl/fl}$ mice, the use of Foxp3^{iCre} x IL-18R1$^{fl/fl}$ mice would be an ideal tool to further the studies of IL-18R1-signalling in Foxp3^{+} Treg cell function. However, in the absence of these tools it would be interesting to generate mixed bone marrow chimeric mice by transferring a 50:50 mixture of T cell depleted *Il18r1*$^{-/-}$ and *scurfy*/*Foxp3*$^{-/Y}$ bone marrow to irradiated *Rag1*$^{-/-}$ recipients, as such, the only cellular population unable to express IL-18R1 would be Foxp3^{+} Treg cells. The anatomical location of autoimmune lesions in these mice would be highly interesting, and presumably related to tissues with high expression of IL-18, including the gastrointestinal tract, skin and lungs.

Chapter 6 General discussion

6.1 Summary

Data in this thesis demonstrate the role of a single cytokine receptor, encoded by an IBD susceptibility gene, in intestinal immune regulation. In order to experimentally investigate the contribution of IL-18R1 to intestinal homeostasis, we focussed our efforts on determining its expression on, and modulation of, intestinal $CD4^+$ T cell subsets. Strikingly, the absence of canonical IL-18R1-signalling resulted in elevated frequencies of intestinal Th1 and Th17 cell populations within the colonic lamina propria. Moreover, intestinal epithelial cells represented the major source of IL-18 under homeostatic conditions, whereupon IL-18 acted directly on $CD4^+$ T cells to limit Th17 cell differentiation within the colonic lamina propria. As such, IL-18 may represent a key regulatory cytokine within the homeostatic intestinal microenvironment to limit $CD4^+$ effector T cell differentiation. Furthermore, using two complementary models of chronic intestinal inflammation, we demonstrated that IL-18R1-signalling on innate and adaptive immune cells was dispensable for induction of colitis, with $CD4^+$ T cell accumulation, differentiation and function ultimately occurring independently of IL-18R1-signalling. Finally, we were able to demonstrate the perhaps surprising role for IL-18R1-signalling in promoting $Foxp3^+$ Treg cell function in intestinal immune homeostasis under inflammatory conditions, a novel description for this cytokine in $Foxp3^+$ Treg cell biology. The aim of this chapter is to place these findings in the wider context of emerging themes within the field of mucosal immunology, and where possible, postulate on where we intend to extend these studies.

6.2 Inflammasome activation in the intestine

A vital aspect of the mammalian immune system in maintenance of host survival is that of self/non-self discrimination. Within this remit, distinguishing harmful non-self (pathogenic microbes), harmless self (auto-antigens), harmless non-self (food

antigens, commensal microbes, transplants) and harmful self (cancerous cells) is key to host defence, all whilst minimising immune-mediated tissue pathology. This is especially true within the intestine, where aberrant responses to commensal microbes cause significant inflammation, tissue destruction and morbidity (Xavier and Podolsky 2007, Maloy and Powrie 2011). With this in mind, it is perhaps worth highlighting the distinctions between “autoimmunity”, clonal responses against self-antigens mediated by T and B cells and “autoinflammation”, aberrant innate immune activation not requiring adaptive cell contribution (gout, cryopyrin-associated periodic syndrome) (Chervonsky 2010). Additionally, “diseases of the activated immune system” can be added to these disorders, where aberrant adaptive cell responses to non-self antigens result in chronic inflammation, as in IBD (Chervonsky 2010, Chervonsky 2013). Autoimmunity is triggered upon loss of immunological tolerance to self-antigens. By contrast, whilst the immune system is not strictly speaking “tolerant” of the commensal microbiota, a combination of regulatory pathways, compartmentalisation and hypo-responsiveness to commensal bacteria and their products results in an active state of mutualism between flora and host. Experimental dissection of how the mucosal immune system distinguishes commensal and pathogenic species of bacteria is key to better understanding how this functional tolerance is maintained within the healthy gastrointestinal tract. Recent research into the molecular composition and activation of distinct inflammasomes has identified the role of these PRR assemblies in host defence from pathogens and noxious stimuli, at both intestinal and extra-intestinal sites (Schroder and Tschopp 2010). Current dogma dictates that inflammasome activation occurs under situations of cellular stress, damage or danger, with production of IL-1β and IL-18 routinely used as a surrogate for inflammasome activation. Recent studies have reported that infection with an enteric pathogen, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) triggers maturation of pro-IL-1β downstream of the NLRC4 inflammasome within resident intestinal macrophage populations; and this was postulated to be a key distinguishing factor between commensal and pathogenic

bacterial populations within the gastrointestinal tract (Franchi et al. 2012). Indeed, IL-1β production promotes defence against pathogenic bacteria, as IL-1R1-deficient mice are often hyper-susceptible to challenge with mucosal pathogens (Raupach et al. 2006, Lebeis et al. 2009). However, several caveats give us reason to query findings in this study, not least some contradictory findings obtained in parallel studies by the same group (Shaw et al. 2012). In Franchi *et al*., infection of mice or isolated intestinal macrophages with S. Typhimurium triggered release of mature IL-1β, and this was dependent upon the NLRC4-containing inflammasome. However, Shaw *et al*. demonstrated that colonisation of GF mice with commensal bacteria was sufficient to trigger IL-1β production from intestinal macrophages and drive intestinal Th17 cell differentiation, though the requirement for inflammasome activation in this setting was not demonstrated. Whilst the introduction of commensal microbiota to GF mice does not entirely recapitulate natural microbial colonisation occurring following birth, these two studies appear to claim different microbial requirements for maturation of IL-1β within the intestine. Genetic differences between BALB/c and C57BL/6 mouse strains used in these studies may account for some of the observed differences, which in itself puts the generalisation of such findings into question, but more likely a contributing factor is the choice of experimental enteric pathogen. S. Typhimurium exhibits a tropism for macrophages, whereas commensal bacteria often lack the required virulence factors to productively infect this cellular population. The use of extracellular pathogens, including *C. albicans* and *C. rodentium* will be key in determining whether NLRC4 inflammasome activation in intestinal macrophages is a general mechanism of distinguishing pathogenic and commensal microbes, or is specific to the experimental setting of intracellular pathogens such as S. Typhimurium. Recent studies in our laboratory have found that infection with *C. rodentium* does not result in elevated tissue levels of IL-1β (Naren Srinivasan, D.Phil thesis), suggesting distinct mechanisms may be required for discriminating between commensals and pathogens within the gastrointestinal tract. The simplest explanation would be related to tissue localisation of bacteria within the intestine,

where bacterial translocation beyond the IEC layer would pose a danger to host health, triggering inflammasome activation in myeloid cells. This could occur during pathogenic infections, or following translocation of commensal species following perturbation of the IEC barrier.

A second major issue with the current understanding of inflammasome activation is that the vast majority of studies in this area have been performed *in vitro* using LPS-primed bone marrow-derived macrophages. Thus, the mechanisms and consequences of inflammasome activation within other cell types, especially non-haematopoietic cells such as intestinal epithelial cells, have not been well characterised. It is imperative that such studies be extended to IEC, given their frequent role as a portal for pathogen invasion. It is possible that inflammasome activation within distinct cellular populations within the intestinal microenvironment results in different immunological responses, as has been described for TLR signalling (Asquith and Powrie 2010, Pott and Hornef 2012). For example, tonic TLR/MyD88-signalling within IEC drives cell-intrinsic proliferation, survival, AMP production and fortification of intercellular tight junctions, limiting bacterial translocation to the lamina propria (Hill and Artis 2010). By contrast, chronic TLR/MyD88-signalling in haematopoietic cells drives intestinal inflammation in a variety of innate and adaptive immune mediated models of IBD (Rakoff-Nahoum et al. 2004, Nenci et al. 2007, Asquith et al. 2010). It would seem reasonable to hypothesise that tonic inflammasome activation within IEC maintains intestinal homeostasis in a manner akin to that of TLR-signalling. Our data demonstrate that intestinal epithelial cells constitute a physiological source of mature IL-18 under homeostatic conditions, and unpublished data from our laboratory demonstrate this to be dependent upon the central inflammasome adaptor protein, Asc. (N. Srinivasan, D.Phil thesis). Thus, inflammasome activation in IEC appears to be constitutive in the presence of a commensal microbiota and in the absence of infection. As such, it is interesting to hypothesise that this axis plays a part in the establishment and maintenance of mutualism between host and commensal

microbiota. Indeed, in the absence of the inflammasome/IL-18 axis, elevated representation by bacterial phyla *Prevotella* and TM7 predispose mice to exacerbated experimental colitis (Elinav et al. 2011). Whilst we have focussed on the modulation of $CD4^+$ T cell populations by this innate immune axis, the role of IL-18 in promoting IEC fortification against commensals has also been postulated (Siegmund 2010). Whilst no direct evidence exists for expression of IL-18R components by IEC, the regulation of IEC homeostasis is highly sensitive to IFNγR signalling, which aids in fortification of tight-junctions (Nava et al. 2010). Intraepithelial lymphocytes (IEL), which include γδ T cell populations, also aid barrier function within the intestine (Ismail et al. 2011). Furthermore, in response to combinations of IL-23 and IL-18, or IL-12 and IL-18, γδ T cells produce IL-17A and IFN-γ in the absence of TCR-stimulation (Lalor et al. 2011). Therefore, production of IL-18 from the intestinal epithelium may act to promote IFN-γ production from γδ T cells contained within the IEL, which in turn feeds back to promote fortification of the intestinal epithelial barrier (Figure 6.1).

Our observation of tonic inflammasome activation in IEC in the absence of infection may require revision of the current dogma of inflammasome activation within the intestine, that is; that pathogenic bacteria, but not commensal species, are able to trigger this cellular sensor.

6.3 $Foxp3^+$ Treg and IL-18R1 as an example of plasticity?

Foxp3 mediates its effects at a molecular level through formation of multimeric protein complexes that directly regulate both the epigenetic landscape and gene transcription program of Treg cells (Samstein et al. 2012). Additionally, Foxp3 directly regulates the expression of many of its interacting partners, including GATA3 (Rudra et al. 2012). Co-operation between Foxp3 and GATA3 promotes the Treg transcriptional program, cell function and subset stability (Wang et al. 2011, Wohlfert et al. 2011, Rudra et al. 2012). A direct binding consensus sequence is yet to be

identified for Foxp3; however, interestingly, ChIP-Seq experiments have demonstrated the *Il18r1* locus to be occupied by both Foxp3 and GATA3 in Foxp3^{+} Treg cells (Rudra et al. 2012).

Whilst we have not examined the role for GATA3 in regulating expression of IL-18R1 within Foxp3^{+} Treg cells, it is possible that GATA3 could be acting in concert with Foxp3 to promote *Il18r1* gene expression, which, as we have demonstrated, is key to their function within the intestine. As such, GATA3-mediated control of IL-18R1 expression may represent an interesting example of the ability of distinct transcription factors to promote distinct subsets of gene expression under different cellular contexts, i.e. plasticity of CD4^{+} T cells; whereby GATA3 limits IL-18R1 expression in Th2 cells, yet promotes expression in Foxp3^{+} Treg cells. Experiments utilising Foxp3^{Cre} GATA3$^{fl/fl}$ mice would be key to determining the requirement for GATA3 in regulation of IL-18R1 expression by Foxp3^{+} Treg cells *in vivo*.

Continuing the idea of Foxp3^{+} Treg cell specialization, it is important to note that expression levels of effector T cell transcription factors by Foxp3^{+} Treg cells are low relative to their Foxp3^{-} counterparts, suggesting that Foxp3^{+} Treg cells may utilise this strategy to adopt some, but not all, aspects of distinct effector programs. For example, low level expression of T-bet by Foxp3^{+} Treg cells promotes acquisition of the CXCR3 homing receptor, without production of the pro-inflammatory cytokine IFN-γ, which allows the Foxp3^{+} Treg cells to migrate to and suppress Th1-driven pathologies (Koch et al. 2009, Hall et al. 2012). This is interesting in the context of IL-18R1-signalling, which in differentiating Th1 cells promotes IL-12Rβ2 expression, and likewise, IL-12 stimulation promotes IL-18R1 expression, together bolstering Th1 cell differentiation (Xu et al. 1998). However, whilst IL-18 is able to induce the expression of IL-12Rβ2 on conventional Th1 cells, this appears not to be the case in Foxp3^{+} Treg cells where the *Il12rb2* locus is epigenetically repressed, preventing adoption of an effector Th1 phenotype by Foxp3^{+} Treg cells in inflammatory environments. The mechanism by which *Il12rb2* is epigenetically repressed in Foxp3^{+}

Treg is not known, neither is the reliance of the mechanism on Foxp3 itself, though it has been suggested that the role of Foxp3 in Treg cells may be to allow partial plasticity whilst limiting full effector cell commitment and production of effector cytokines, with the latter scenario observed only under conditions of lethal infection or severe experimental autoimmunity (Oldenhove et al. 2009, Zheng et al. 2010, Ouyang et al. 2012).

Whilst around 40% of colonic Foxp3^{+} Treg cells express IL-18R1 under homeostatic conditions, during active regulation of proliferating and differentiating naïve CD4^{+} T cells in the colon, the vast majority of Foxp3^{+} Treg cells express IL-18R1. This observation is in itself interesting with respect to studies of Foxp3^{+} Treg cell biology, where activation status of this homogeneous population is often disregarded. The understanding of the role of Foxp3 within Foxp3^{+} Treg cells to date has employed the use of transcriptional and epigenetic analyses from experiments with "resting" Foxp3^{+} Treg cells. However, it would be interesting to compare these data with those derived from experiments carried out on physiologically activated Foxp3^{+} Treg cells. It is unlikely that any particular *in vitro* stimulation will sufficiently mimic the complex array of signals received by a specific cell population during inflammation *in vivo.* As such, perhaps the most physiological manner to isolate activated Foxp3^{+} Treg cells would be through the use of graded depletion of these cells *in vivo*, followed by isolation of remaining Foxp3^{+} Treg cells actively suppressing autoimmunity. Currently available tools would allow this comparison, including the use of female Foxp3$^{DTR/hCD2}$ mice (Foxp3^{DTR} x Foxp3^{hCD2}), where random X-inactivation would render 50% of Foxp3^{+} Treg cells susceptible to reproducible diphtheria toxin induced depletion, whilst allowing the isolation and functional characterisation of the remaining physiologically activated Foxp3^{hCD2+} cells. It would be interesting to examine whether the constituent members of the Foxp3-containing multimeric transcription factor assemblies required for histone modification and transcriptional regulation are altered during Treg cell

activation, as well as the targets of distinct transcription factors associated with Foxp3^{+} Treg cell function, including GATA3, IRF4, Eos and T-bet.

6.4 Fishing off the edge of the map - Non-canonical IL-18R1 ligands

Throughout these studies we have attempted to account for the possibility of non-canonical ligands for IL-18R1, however, we would ideally seek to identify these factors. Human IL-1F7 has been identified as a ligand of hIL-18R1 (Kumar et al. 2002), and also interacts with hIL-18BP (Bufler et al. 2002), however, the murine orthologue of IL1F7 appears to have been lost following genetic diversion between human and mouse (Sims and Smith 2010). As such, *in silico* efforts to discover the gene encoding murine IL-1F7 have so far been unsuccessful. However, a key piece of evidence suggesting that non-canonical IL-18R1 ligands may have biological functions in mice, is the observation that antibody-mediated blockade of IL-18R1 is sufficient to prevent disease induction in the EAE model, in both WT and *Il18*$^{-/-}$ mice (Gutcher et al. 2006). Further to this, in separate studies, administration of IL-18BP was also sufficient to limit immunopathology in the EAE model, again, in both WT and *Il18*$^{-/-}$ mice (Millward et al. 2010). As such, IL-18BP may prove a useful tool to identify alternative IL-18R1-ligands. One approach would be to use tagged recombinant IL-18BP to precipitate potential ligands from serum isolated from susceptible *Il18*$^{-/-}$ mice undergoing induction of EAE. Analysis of interacting partners could then be assessed through mass-spectrometry and proteomic approaches. Potential issues with this approach include the ability to "map back" identified peptides to the annotated murine proteome, given the current absence of an identified murine orthologue of *Il1f7*. However, peptides can also be mapped back to other species still carrying orthologues of *Il1f7*, including humans, rhesus monkey, cow and dog (http://www.ncbi.gov). Ideally any candidates would be confirmed to interact with murine IL-18R1 and/or IL-18RaP both biochemically and via surface plasmon resonance (Biacore) before generation of genetically deficient mouse strains and blocking monoclonal antibodies for future experimentation.

6.5 IL-18R1-signalling, a conserved innate axis?

We have thus far focussed our attention on the regulation of $CD4^+$ T cell populations by IL-18/IL-18R1. However, as alluded to earlier, a recently identified cellular population of innate lymphoid cells (ILC) that are now termed ILC3 (Spits et al. 2013), bears a more than passing resemblance to Th17 cells, and appears to have similar requirements for their differentiation and function. Similar to Th17 cells, these innate Rorγt$^+$ ILC3 have been implicated in maintenance of intestinal immunity during both homeostatic (Sonnenberg et al. 2012, Hepworth et al. 2013) and infection conditions (Sonnenberg et al. 2011), but can also contribute to tissue pathology in models of IBD and colitis-associated cancer (CAC) (Buonocore et al. 2010, Kirchberger et al. 2013). Interestingly, in preliminary experiments we have identified expression of IL-18R1 by the majority of colonic ILC3 (>75%) under homeostatic conditions, and have found that *Rag1*$^{-/-}$ *Il18r1*$^{-/-}$ mice exhibit elevated susceptibility to agonistic anti-CD40 mAb mediated colitis, which is mediated by ILC3 (data not shown)(Buonocore et al. 2010). As such, it will be of great interest for future experiments to determine whether absence of IL-18R1-signalling results in elevated frequencies of intestinal Rorγt$^+$ ILC3, representing a conserved pathway with Th17 cells, which then results in exacerbated innate immunity mediated intestinal inflammation.

6.6 Conclusions

The complexity of cytokine networks within the intestinal environment is testament to the delicate balance required to ensure immune homeostasis whilst maintaining appropriate defence from invading pathogens. Although the role of IL-18 in IBD pathogenesis has long been implicated as promoting inflammation and deleterious responses within the tissue, this knowledge has been limited by superficial investigation of cellular populations involved, and the use of acute chemically driven or infectious models of colitis. Through the functional assessment of IL-18 and IL-

18R1 in intestinal immune regulation, we contribute to the mounting evidence that tissue-specific factors are key to maintaining tolerance to the resident intestinal microbiota.

Finally, it is worth considering our findings in the context of potential therapies for use in the clinical setting of IBD. Our data suggest that administration of IL-18 may promote regulatory pathways within the intestine, though it would be worth stratifying the target patient population to exclude those bearing mutations within the IL18R1-IL18RAP locus, where the response would likely be lost. Furthermore, whilst exogenous IL-18 may act to limit the frequency of intestinal Th17 cells, it is worth noting that recent clinical trials using an anti-IL-17A mAb in IBD were ineffective and associated with elevated incidence of adverse events (Hueber et al. 2012). Further work is required to fully appreciate the appropriateness of modulating the IL-18 axis in treatment of IBD.

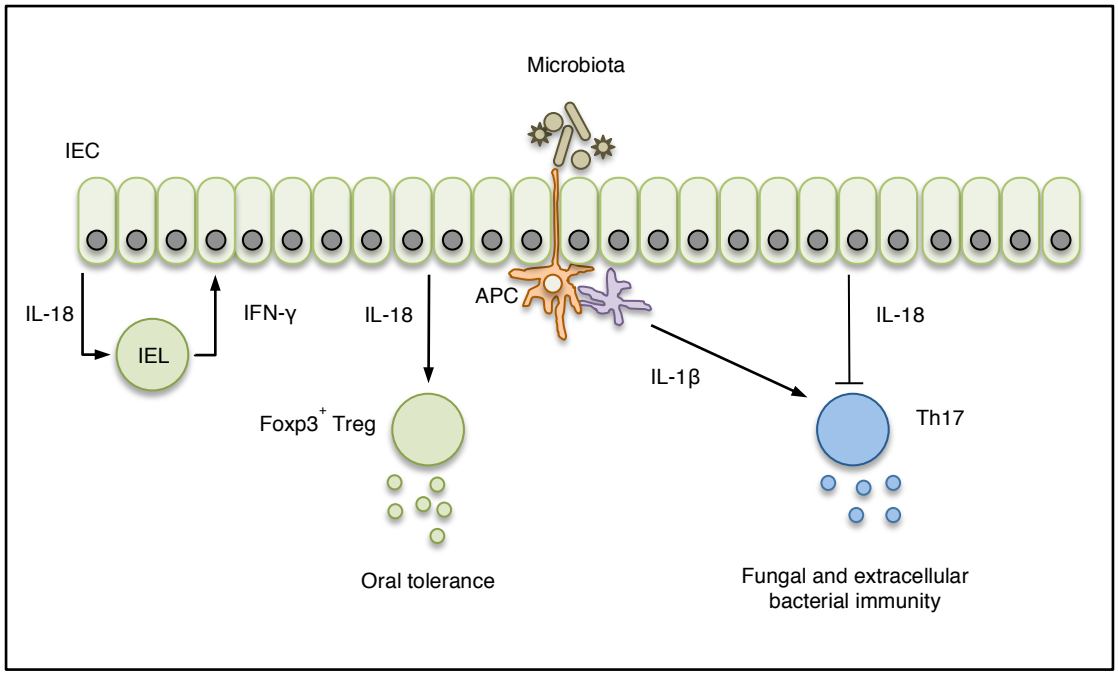


Figure 6.1 The role of IL-18 in intestinal immune regulation
Intestinal epithelial cell derived IL-18 acts directly on $CD4^+$ T cells within the colonic lamina propria to limit Th17 cell differentiation under steady state conditions, whilst promoting $Foxp3^+$ Treg cell function to prevent T cell mediated intestinal inflammation. Functions of IL-18 independent of $CD4^+$ T cells may promote IEC barrier integrity through innate lymphoid cells.

Chapter 7 References

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