Identification of the cellular and molecular mechanisms of IL-23 driven intestinal inflammation

Chris Schiering

Lincoln College

University of Oxford

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Abstract

IL-23 is an essential mediator of chronic intestinal inflammation in experimental models of colitis. Polymorphisms in the *IL23R* locus are associated with IBD susceptibility in humans. The biological activity of IL-23 has been linked to Th17 cells but little is known about the cellular and molecular mechanism by which IL-23 drives intestinal inflammation. The work presented herein has identified that direct IL-23 signalling into $CD4^+$ T cells was not only required for the accumulation of Th17 cells in the intestine but also modulated their phenotype. Through direct cell intrinsic effects on T cells, IL-23 drove the emergence of an IL-$17A^+$IFN-γ^+ population of T cells that co-expressed RORγ and T-bet. Interestingly, we found that expression of RORγ but not T-bet by T cells was required for the development of intestinal inflammation. Furthermore, colitis induced by T-bet deficient T cells was dependent on IL-17A, and showed a unique inflammatory phenotype, thus demonstrating that pathogenic intestinal Th17 responses can develop independently of T-bet. In addition, using transcriptional profiling we identified a core set of genes that is regulated by direct cell-intrinsic IL-23 signals into intestinal $CD4^+$ T cells. This revealed a previously unrecognised role for IL-23 in suppressing Th2 associated genes, such as GATA3 and IL-33R. Functional experiments demonstrated that expression of GATA3 in $CD4^+$ T cells limited their colitogenic potential, suggesting that IL-23-mediated inhibition of GATA3 might contribute to the development of intestinal inflammation. Finally, we described a novel function for IL-33 as a factor that promotes $Foxp3^+$ iTreg differentiation *in vitro* and *in vivo* through direct effects on T cells. This activity of IL-33 was inhibited in the presence of IL-23, providing a mechanistic link for the known role of IL-23 in restraining iTreg generation. Collectively, these data suggest that IL-23 promotes acquisition of a pathogenic effector T cell phenotype through multiple mechanisms. This indicates that therapeutic blockade of IL-23 is likely to reduce pro-inflammatory mediators while also facilitating the expansion of regulatory pathways that might help to re-establish intestinal homeostasis.

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Abbreviations

APC	Antigen presenting cells
BM	Bone marrow
BSA	Bovine serum albumin
CD	Crohn's disease
CFU	Colony forming units
CIA	Collagen induced arthritis
CTLA4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cells
DSS	Dextran sulphate sodium
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
Foxp3	forkhead box P3
GALT	Gut associated lymphoid tissue
GATA3	GATA-binding protein 3
GM-CSF	Granulocyt-macrophage colony stimulating factor
GWAS	Genome-wide association studies
H&E	Haematoxylin and eosin
HPRT	Hypoxanthine phosphoribosyltransferase
i.p.	Intraperitoneal
i.v.	Intravenous
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immunedysregulation polyendocrinopathy enteropathy X-linked
IRF	Interferon regulatory protein
iTreg	Induced regulatory T cells
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAMP	microbe associated molecular pattern
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MyD88	Myeloid differentiation response gene 88
NLR	NOD-like receptor
nTreg	Natural regulatory T cell
PP	Peyer's Patch
PRR	pathogen recognition receptor
qPCR	quantitative polymerase chain reaction

RA	Retinoic acid
RAG	Recombinase activating gene
RORγ	RAR-related orphan receptor gamma
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
SNPs	Single nucleotide polymorphisms
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
T-bet	T-cell specific T-box transcription factor
TCR	T-cell receptor
TGF	Transforming growth factor
TGF	Transforming growth factor
TLR	Toll like receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
WT	Wild type

Chapter 1: General Introduction

1.1 The intestinal immune system

The intestine of healthy individuals harbours an incredibly diverse commensal flora composed of up to 10^{14} organisms [3] separated from the host by a single layer of epithelial cells. This vast collection of microbes, referred to as the microbiota, provides numerous benefits to their hosts. The intestinal microbiota directs the maturation of the immune system and contributes to nutrient metabolism, and also protects against invasion or outgrowth of pathogenic organisms through competition for space and nutrient sources [4-9]. This beneficial co-existence has shaped the evolution of the intestinal immune compartment, which normally does not mount pro-inflammatory responses against the microbiota, despite their expression of a wide array of microbe associated molecular patterns (MAMPs). The intestine represents a specialised anatomical niche that favours the induction of tolerance, or maintenance of ignorance to commensals in order to maintain homeostasis [10]. Elaborate networks of functionally specialised hematopoietic and non-hematopoietic cells have evolved to maintain this host-microbe mutualism. In individuals suffering from inflammatory bowel diseases (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), this balanced immunity is perturbed, and inappropriate immune reactivity toward components of the intestinal flora results in chronic intestinal inflammation [11, 12].

Intestinal epithelial cells (IECs) comprise a number of distinct cell types with specialised functions [13]. Goblet cells produce mucus components that form a thick layer covering the mucosal surface of the gastrointestinal tract. This protective mucus layer does not only act as a physical barrier but also contains high concentrations of antimicrobial peptides and secretory IgA, thereby forming a matrix that helps prevent penetration of the epithelial barrier by intestinal microbes [14-16]. IECs also express pattern recognition receptors (PRRs) that allow them to directly sense microbes [13]. PRRs are germline encoded receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) that recognise MAMPs as well as endogenous danger signals released in response to tissue injury or stress [17, 18]. Although IECs are known to exclude TLRs from their luminal surface to avoid overt immune activation [19], tonic PRR signalling is important for the maintenance of intestinal barrier integrity. Defects in PRRs or PRR signalling components within IECs have been shown to lead to bacterial translocation and altered microbiota composition, demonstrating the essential function of IECs in the maintenance of intestinal homeostasis [20-23]. Finally a specialised type of IEC called M cells reside in the follicular associated epithelium (FAE) overlying Peyer's Patches (PP) of the small intestine. M cells are an important cell type in the uptake of antigens and microbes in the intestine, which they can then pass to other cells such as dendritic cells (DCs) [24, 25]. The lamina propria is the main effector site of the intestinal immune system, while organised structures such as the mesenteric lymph nodes (MLN), Peyer's Patches, isolated lymphoid follicles and cryptopatches act as inductive sites for the initiation of adaptive immune responses [26-28]. Collectively these structures are referred to as the gut associated lymphoid tissue (GALT).

Myeloid antigen presenting cells (APCs) of the intestine are a heterogeneous population consisting of dendritic cells (DCs) and macrophages and can be broadly distinguished by their expression of CD103 and CX3CR1 [29, 30]. Intestinal macrophages are highly phagocytic cells with potent bacteriocidal activity. However, engulfment of microbes does not induce pro-inflammatory cytokine release by these cells, a feature which is crucial for the maintenance of homeostasis [29]. DCs can act as a bridge with the adaptive immune system. Rather than directly killing microbes as macrophages do, DCs can acquire antigen in the intestine and migrate to the MLN where they prime the activation of naive $CD4^+$ T cells [31-34]. T cell activation in the MLN leads to the upregulation of the gut homing receptors CCR9 and $\alpha4\beta7$ allowing T cells to migrate specifically to the intestine [35-37]. A unique subset of intestinal DCs express CD103 and appears especially endowed with the ability to promote tolerance in the intestine. Indeed, $CD103^+$ DCs in the intestine are potent inducers of $Foxp3^+$ regulatory T cells (Tregs) through TGF-β and retinoic acid (RA) dependent mechanisms [38, 39]. Furthermore, resident $CX3CR1^+$ APCs are important for promoting local proliferation of Tregs in the lamina propria [40] and might also contribute to the maintenance of Foxp3 expression by Treg cells [41], demonstrating cooperativity between distinct intestinal myeloid cell subsets. DCs are not the only regulators of intestinal homeostasis. B cells producing IgA also represent an important component which helps to prevent unwarranted access of intestinal microbes, and serves to limit local T cell proliferation [42]. Both Tregs [42] and microbe-bearing intestinal DCs [43] can lead to priming of IgA responses in the GALT, highlighting the various layers maintaining our peaceful co-existence with our intestinal microbiota.

Conditioning factors produced locally in the intestine such as thymic stromal lymphopoietin (TSLP) and prostaglandin E2 (PGE2), which can be produced by IECs, help to maintain the tolerogenic state of intestinal DC in the face of a vast microbiota [44-46]. However not all APCs in the intestine are tolerogenic, and some can also prime effector $CD4^+$ T cell responses [5, 47, 48]. APCs express a variety of PRRs which allow them to respond to microbial stimulation, and integrate this information to instruct the immune decision-making process [34]. Indeed, $CX3CR1^+CD70^{high}$ APCs in the lamina propria express high levels of pro-inflammatory cytokines IL-23 and IL-6, which are important for the generation of T helper (Th) 17 cells [5]. Th17 cells are abundant in the intestinal lamina propria [49] and are essential for host protective immunity against extracellular bacteria and fungi. However, Th17 cells are also implicated as major drives of chronic intestinal inflammation [50] and APC-derived IL-23 and IL-6 may promote this further. Furthermore, intestinal $CD103^+$ DCs have been shown to produce IL-23 in response to bacterial flagellin challenge [51], and can favour the induction of effector T cells rather than Tregs when isolated from an inflamed environment, highlighting their capacity to respond to cues in the intestine and prime the appropriate immune response [52].

Breakdown of the mechanisms that serve to reinforce intestinal homeostasis leads to the accumulation of pathogenic effector T cells and a concomitant reduction in Tregs in the intestine, which drives chronic inflammatory responses [12]. The colitogenic potential of intestinal effector T cells is revealed in experimental systems when regulatory pathways such as IL-10 [53, 54] and TGF-β [55, 56] are blocked. Thus, there is a delicate balance of pro and anti-inflammatory responses in the intestine, and

although breakdown of this balance is remarkably infrequent, perturbation results in chronic intestinal inflammation and immune system-mediated damage to the host [11].

The intestine harbours a variety of additional cells types such as intraepithelial $CD8^+$ T cells and γδ T cells, distinct populations of innate lymphoid cells and a dense network of stromal cells. All of these populations have important functions in the maintenance of homeostasis and also contribute to host defence and chronic intestinal inflammation. However these cells have not been the focus of this thesis and will not be discussed in detail.

1.2 $CD4^+$ T cell activation and differentiation

$CD4^+$ T cell activation requires the interaction between the T cell receptor (TCR) and cognate antigen in the context of MHC class II. TCR and CD4 ligation by peptide MHC class II complexes is sufficient to trigger early signalling events that result in the recruitment and activation of Lck and Zap70 [57]. These protein kinases mediate the tyrosine phosphorylation of TCR associated signalling proteins TCRζ, LAT and PLC-γ. This leads to a cascade of events culminating in high intracellular levels of the second messenger $Ca2^+$, and activation of Ras-MAPK and PKCθ signalling pathways. In addition, ligation of the T cell costimulatory molecule CD28 by CD80 and CD86 expressed on APCs strongly reinforces TCR signalling events through activation of the PI3K signalling pathway [58-60]. Together these events lead to cell cycle progression and provision of survival signals. Additional molecules with costimulatory capacity include CD28 family members ICOS and TNF receptor family members CD40L, CD27, OX40, 4-1BB and RANKL/TRANCE [61].

Activation of $CD4^+$ T cells in the presence of polarising cytokines determines their differentiation into T helper cell (Th) subsets, which are characterised by the production of a distinct set of effector cytokines, and mediate protective as well as pathogenic immune responses. Since the initial identification of Th1 and Th2 cells by Mossman and Coffman [62] several other "lineages" of $CD4^+$ T cells, such as Treg cells [63] and Th17 cells [50], have been described. Complex networks of cytokines and transcription factors contribute to T helper cell differentiation and will be described in detail in this section.

1.2.1 Th1 cells

Th1 cells are characterised by the expression of IFN-γ, IL-2, lymphotoxin, TNF-α and GM-CSF [64, 65]. Th1 responses are important for protection against intracellular parasites, bacteria and viruses. IFN-γ induces the secretion of chemokines and cytokines required for the recruitment and activation of myeloid cells, stimulates IgG2a production by B cells and enhances recruitment of Th1 cells to the site of inflammation.

TCR stimulation of naïve $CD4^+$ T cells in the presence of IFN-γ promotes STAT1 dependent induction of T-bet [66, 67], which is encoded for by *Tbx21*. T-bet drives the upregulation of IL-12Rβ2 [67, 68], which forms a functional receptor for IL-12 by pairing with the IL-12Rβ1. The IL-12Rβ1 is expressed at low levels on naïve $CD4^+$ T cells and its expression is further enhanced during Th1 differentiation by IFN-γ-inducible transcription factor IRF1 [69]. IL-12 signalling through STAT4 further enhances T-bet expression and IFN-γ production, and results in full commitment to the Th1 programme [70-72]. IL-2 also plays a functional role in Th1 differentiation by augmenting expression of IL-12Rβ2 and T-bet in a STAT5 dependent manner [73]. IL-12-STAT4 signalling also leads to the induction of the IL-18R, and IL-12 and IL-18 can synergise to promote TCR-independent IFN-γ production by Th1 cells [74-76]. T-bet deficient T cells express higher levels of the Th2 transcription factor GATA3 [77, 78], demonstrating that repression of the Th2 programme is an essential factor in Th1 commitment. Indeed T-bet has been shown to suppress GATA3 expression [77] and directly interacts with GATA3 to inhibit its ability to bind to target genes [79, 80]. T-bet deficient mice develop spontaneous Th2 mediated asthma like disease [81] [82], indicating that T-bet is essential in limiting

Th2 type responses *in vivo*. T-bet and/or STAT4 also induce expression of additional transcription factors Runx3 [78], Etv5 [83] and Hlx1 [84]. Runx3 is a transcriptional repressor that binds directly to the IL-4 locus and inhibits IL-4 expression. Similar to Etv5 and Hlx1, Runx3 also cooperates with T-bet to drive IFN-γ production. T-bet deficient T cells are not completely impaired in their ability to produce IFN-γ, and the related T-box transcription factor eomesodermin has been shown to compensate for IFN-γ production in the absence of T-bet [85, 86]. However, aberrant Th1 responses have also been associated with autoimmune and chronic inflammatory diseases such as IBD [87, 88].

1.2.2 Th2 cells

Th2 cells produce a variety of effector cytokines such as IL-4, IL-5, IL-13, IL-9 and IL-25, and are essential for host defence against extracellular parasites [89]. Th2 derived cytokines regulate B cell antibody class switching to IgE, promote recruitment of eosinophils and mast cells to mucosal surfaces, induce mucus production by epithelial cells, and promote a distinct activation state in macrophages. Dysregulated Th2 responses result in allergic inflammatory diseases such as asthma [90].

The factors required for Th2 differentiation have been characterised extensively *in vitro*. TCR stimulation of naïve $CD4^+$ T cells in the presence of IL-4 promotes phosphorylation of STAT6, which induces expression of the transcription factor GATA3 [91, 92]. Enforced expression of GATA3 is sufficient to drive IL-4 production in committed Th1 cells [93] and GATA3 deficient T cells fail to produce Th2 associated cytokines [94, 95], demonstrating the central role for GATA3 in Th2

specification. The IL-2-STAT5 pathway is also essential for Th2 commitment through induction and maintenance of IL-4Rα expression, and modulation of chromatin accessibility at the Th2 locus [96]. Additional transcription factors involved in Th2 cell development are induced upon TCR stimulation and include NFAT family members [97], IRF4 [98], c-MAF [99, 100], Gfi-1 [101] and TCF-1 [102]. Recently STAT3, activated through IL-6 or IL-21, was shown to cooperate with STAT6 in the induction of Th2-associated cytokines and transcription factors [103].

As with Th1 cells, Th2 transcription factors and cytokines also promote Th2 development through inhibition of alternative T cell fates. For example, GATA3 inhibits Th1 development through suppression of STAT4 [70] and IL-12Rβ2 [93], and prevents IFN-γ production through direct physical interaction with Runx3 [104]. IL-4 has been shown to inhibit Th1, Th17 and regulatory T cell (Treg) differentiation, probably in part through activation of the transcriptional repressor Gfi-1 [105, 106]. Despite the essential role of the IL-4-STAT6 pathway in Th2 differentiation *in vitro*, the development of Th2 responses *in vivo* can occur independently of IL-4 [107, 108]. Additional cytokines such as TSLP, IL-25 and IL-33, which are induced by parasites or allergens, contribute to the induction and amplification of Th2-associated cytokines *in vivo* [89]. Moreover, the host genetic background has an important role in determining the balance between Th2 and Th1 cells, and is a critical determinant of susceptibility or resistance to infectious agents [109, 110]. For example, in response to TCR stimulation, $CD4^+$ T cells from BALB/c mice produce much greater amounts of IL-4 compared to $CD4^+$ T cells from C57BL/6 mice [111-114]. This genetic bias is due in part to the differential expression of the transcriptional repressor MINA,

which has been shown to bind to the *Il4* promoter and repress IL-4 production [114]. Therefore, the Th2 bias of certain strains must be kept in mind when interpreting experimental results obtained on different genetic backgrounds.

1.2.3 Th17 cells

Th17 cells are characterised by the expression of signature cytokines such as IL-17A, IL-17F, IL-22 [115-118] and the cell surface receptors IL-23R and CCR6 [119, 120]. Th17 cells and their associated cytokines are critical for host defence against a variety of pathogens including extracellular and intracellular bacteria and fungi [172]. However, Th17 cells are also thought to be essential players in several chronic inflammatory and autoimmune diseases [50].

The differentiation of Th17 cells is driven by TGF-β1 in combination with pro-inflammatory cytokines IL-6, IL-21 and/or IL-1β [121-128]. The Th17 cell transcriptional programme is coordinated by RORγt, and mice deficient in this transcription factor are almost completely devoid of Th17 cells [49]. Forced expression of RORγt in naive $CD4^+$ T cells is sufficient to drive IL-17A production in the absence of exogenous TGF-β1 and IL-6 [49]. In addition neither Th1 nor Th2 cells express detectable levels of RORγt, demonstrating the specific requirement for RORγt in the development of Th17 cells. IL-6 induces the expression of IL-2 family cytokine IL-21 in developing Th17 cells, and both IL-6 and IL-21 activate STAT3, which promotes the induction of RORγt [125-127, 129]. Furthermore IL-6 and IL-21 alone, or in combination with low concentrations of TGF-β1, induce the expression of the receptor for IL-23 [130]. Although IL-23 is dispensable for the differentiation of

Th17 cells, it is thought to be important for Th17 cell accumulation and acquisition of effector functions [1, 116, 117, 122, 124, 131]. Indeed, IL-23 has been shown to promote expression of pro-inflammatory cytokines such as IFN-γ [132] and GM-CSF [133, 134] by Th17 cells. In addition to RORγt and STAT3, several other transcription factors have been shown to play a role in Th17 development. These include Runx1 [135], BATF [136], IRF4 [137] and Aiolos [138], and nuclear receptors RORα [139], RORγ [140] and AHR [141, 142]. They also include the NF-κB factors Rel [140], IKKα [143] and IκBζ [144] and metabolic sensors such as HIF1-α [145, 146]. These transcription factors can act upstream of RORγt and induce its expression and/or promote IL-17A transcription. Whether these transcription factors merely act to reinforce the RORγt driven transcriptional programme or modulate specific gene signatures in Th17 cells has not yet been established.

The *in vivo* source of TGF-β1, and the mechanism by which it controls Th17 differentiation, have received much attention. TGF-β1 is a potent activator of the SMAD transcription factors SMAD2, SMAD3 and SMAD4 [148]; however, induction of *Rorc*, the gene that encodes RORγt, appears to be independent of this signalling pathway [149, 150]. It has been demonstrated that TGF-β1 promotes Th17 differentiation through repression of the Th1 associated transcription factor eomesodermin in a SMAD independent manner [151]. In this study, TGF-β1 was found to inhibit eomesodermin-mediated suppression of *Rorc* and *Il17a* transcription, suggesting an indirect role for TGF-β1 in Th17 differentiation. In addition, it has been shown that $CD4^+$ T cells deficient in STAT6 and T-bet do not require TGF-β1

to differentiate into Th17 cells, further suggesting that TGF-β1 promotes Th17 development indirectly through inhibition of Th1 and Th2 differentiation [152]. In keeping with this finding, TGF-β1-mediated SMAD-dependent signalling has been shown to suppress IFN-γ, IL-2 and IL-4, all of which are potent inhibitors of Th17 differentiation [149]. By contrast, TGF-β1 was shown to activate the serine-threonine kinase ROCK2, which promoted phosphorylation of IRF4, an essential factor for the induction of RORγt [153], suggesting a direct role of TGF-β1 in promoting Th17 differentiation. Thus it appears that TGF-β1 promotes Th17 differentiation through both direct and indirect mechanisms. Recently, the absolute requirement for TGF-β1 in Th17 differentiation has been challenged by the finding that Th17 cells could be differentiated in the presence of IL-6, IL-1β and IL-23 in the absence of TGF-β1 [154]. These Th17 cells differed markedly from Th17 cells cultured in the presence of IL-6 and TGF-β1 in that they expressed high levels of Th1 associated factors such as T-bet, CXCR3, Hlx1 and IL-18R. This finding is in stark contrast to early studies that described an essential role for T cell derived TGF-β1 [155], and $CD4^+$ T cell responsiveness to TGF-β1 [156], in the generation of Th17 cells *in vivo*. In addition a recent study showed that TGF-β1 deletion from activated $CD4^+$ T cells and Treg cells, but not Treg cells alone, abrogated the generation of Th17 cells, suggesting that Th17 cells themselves are potent producers of TGF-β1 [157]. These conflicting findings are at first sight difficult to reconcile but the activation of TGF-β1 is tightly controlled *in vivo* [158] and variations in the local concentration of TGF-β1 could have differential effects on Th17 differentiation

Several cytokines and transcription factors associated with Th1 and Th2 type responses negatively regulate Th17 differentiation. These include IFN-γ, IL-2, IL-4, IL-13 [159, 160] [161, 162] [163], T-bet [164], Gfi-1 [105] and Ets-1 [165]. In addition, Foxp3, which is essential for the function of Treg cells, also negatively regulates Th17 differentiation. In the early phase of T cell differentiation, TGF-β1 induces the expression of both RORγt and Foxp3 in TCR activated $CD4^+$ T cells [49, 125, 130, 166]. In the absence of pro-inflammatory cytokines, Foxp3 inhibits Th17 differentiation through direct physical interactions with RORγt, RORα and Runx1 [130, 135, 150]. This inhibitory effect of Foxp3 is overcome in the presence of STAT3-activating cytokines IL-6 and IL-21, which shift the balance towards Th17 differentiation [121, 125-127]. Despite this antagonism of Foxp3 and RORγt at the molecular level, Treg cells and Th17 cells have also been shown to cooperate *in vivo*. A recent study showed that Treg cells promote Th17 differentiation early in the immune response through consumption of IL-2, which might be important for the ability of Treg cells to expand and limit Th17 responses at a later stage [167].

Expression of Th17 signature cytokines is not restricted to $CD4^+$ T cells. Other populations of lymphocytes such as $CD8^+$ T cells [168, 169], γδ T cells [170] and innate lymphoid cells [171] have also been shown to express RORγt and produce IL-17A and other Th17 associated cytokines.

1.2.4 Treg cells

Early studies identified a population of $CD4^+$ T cells characterised by high expression of the IL-2Rα chain (CD25) that could suppress chronic inflammation induced by neonatal thymectomy [173], or transfer of naïve $CD4^+CD45RB^{hi}$ cells into

lymphopenic hosts [174]. Although $CD4^+CD25^+$ regulatory T cells (Treg) do not produce IL-2 themselves, their survival and expansion was found to be critically dependent on IL-2 produced by effector T cells [175-180], suggesting a negative feedback loop by which Treg cells expand alongside effector T cells during the course of an immune response. The identification of Foxp3 as an essential transcription factor for Treg specification and maintenance has led to the acceptance of Treg cells as a specialised subset of $CD4^+$ T cells with immunosuppressive properties [181-183]. The dominant role of Treg cells in the maintenance of homeostasis is demonstrated by the identification of a loss-of-function mutation in the human *FOXP3* gene, which leads to immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX). This results in widespread multiorgan autoimmune manifestations, including intestinal inflammation [184]. In addition, genetic deletion of Foxp3 specifically in $CD4^+$ T cells was shown to lead to autoimmune disease indistinguishable from that observed in mice with a germ-line deletion of Foxp3 [179]. Together, these findings highlight the non-redundant role of Foxp3 expression by Treg cells in reinforcing peripheral tolerance and homeostasis.

Two distinct origins of Foxp3 expressing Treg cells have been described. Treg cells can either arise in the thymus (nTreg) [185] or can be induced in the periphery from $Foxp3^-$ naïve $CD4^+$ T cells (iTreg) [186, 187]. Thymic development of Treg cells is dependent on TCR:MHCII interactions [185, 188-190] in the context of CD28 costimulatory signals [191, 192]. This leads to activation of factors such as PKCθ, BLC10 [193, 194], CARMA1 [195-197], TAK1 [198, 199] and IKK [200], that link TCR activation to the NF-κB pathway. The NF-κB family member c-Rel seems to be particularly important in Foxp3 induction through binding to the conserved non-

coding sequence CNS3 in the Foxp3 locus [201] [193, 202-204]. Additional transcription factors such as NFAT [205], CREB-ATF-1 [206], and Foxo1 and Foxo3 [207] are also thought to contribute to Foxp3 expression. On the contrary, activation of the PI3K/Akt pathway inhibits Foxp3 expression [208-211]. In addition to TCR signals, exposure to common γ-chain cytokines IL-2, and to a lesser extent IL-7 and IL-15, mediate activation of STAT5 and further facilitate induction of Foxp3 [212-214]. The maintenance of Foxp3 expression in progeny of mature Treg cells is also regulated by Foxp3 itself through Cbf-β-Runx1 dependent binding to the regulatory element CNS2 within the Foxp3 locus [204].

$Foxp3^+$ Treg cells are also generated extra-thymically from naïve T cell precursors. Differentiation of naïve $CD4^+$ T cells into $Foxp3^+$ Treg cells *in vitro* requires TGF-β1 and IL-2, and is enhanced in the presence of retinoic acid [38, 39]. The specialised microenvironment of the GALT appears to favour iTreg induction, and this is thought to contribute to the maintenance of intestinal homeostasis [34, 215]. In support of this, colonic $Foxp3^+$ Treg cells appear to have a distinct TCR repertoire as compared to $Foxp3^+$ Treg cells in systemic tissues, suggesting an important role for the microbiota in shaping the peripheral Treg population [216]. Generation of transgenic mice expressing bacteria-reactive TCRs from colonic $Foxp3^+$ Treg cells showed that these TCRs could not facilitate thymic Treg development, suggesting that colonic Treg cells can be induced in an antigen-specific manner in the periphery. Interestingly, approximately 50% of colonic Treg cells' TCRs were reactive to colonic contents, indicating that iTreg cells feature prominently in the colonic $Foxp3^+$ Treg pool. Although nTreg and iTreg cells have been shown to have different transcriptional signatures [217, 218], they are hard to distinguish once generated.

However, recent studies have given some insight into the relative contribution of nTreg and iTreg to homeostasis. Specific deletion of the conserved non-coding sequence CNS1 in the Foxp3 locus demonstrated that this element was specifically required for iTreg induction, whereas its deletion did not affect nTreg development [204]. Surprisingly, loss of Foxp3 CNS1 led to exacerbated Th2 type immunopathology in the lung and intestine, demonstrating that iTreg cells are particularly important for restraining Th2 type responses at mucosal sites [219]. In addition, it has been reported that cooperation between iTreg and nTreg cells is required for protection from the autoimmune disease that develops in Foxp3 deficient mice [220], and protection from colitis induced by transfer of naïve $CD4^+$ T cells [218]. However, the molecular basis of this cooperation is currently unknown.

Treg cells exert their suppressive effects through multiple mechanisms. Treg mediated suppression of effector T cell responses can affect T cell priming and differentiation in the lymph node [221], migration of effector T cells to their target tissue [222], and effector T cell function at the site of inflammation [223, 224]. In order to control the diverse array of effector T cell responses, Treg cells have been shown to co-opt transcription factors whose expression was previously thought to be restricted to effector T cells. For example, T-bet expression by $Foxp3^+$ Treg cells is required for upregulation of CXCR3 on Treg cells and their ability to home to sites of Th1-mediated inflammation [225]. In addition, targeted deletion of IRF4 [226] or STAT3 [227] in $Foxp3^+$ Treg cells leads to spontaneous development of Th2 or Th17 driven mucosal immunopathology respectively, demonstrating an essential role for the functional specialisation of Treg cells in the control of specific types of immune responses. In addition, Treg cells also express cell surface receptors such as CTLA4

[174, 191, 228], GITR [229, 230] and OX40 [231], which are important for their suppressive function and ability to compete for survival signals *in vivo*. Additional mechanisms of suppression include direct killing of targets cells [232], growth factor deprivation [233] and suppression of DC maturation [234]. Much attention has focused on the immunosuppressive cytokines IL-10, TGF-β1 and IL-35 released by Treg cells. Deletion of IL-10 specifically in Tregs leads to spontaneous colitis in *Helicobacter* infected mice [235], indicating an essential role for this cytokine in the maintenance of intestinal homeostasis. IL-10 has also been implicated in the maintenance of Foxp3 expression [41], and IL-10 induced STAT3 activation in Treg cells was shown to further enhance IL-10 production by Tregs. These data demonstrate that in addition to dampening immune responses directly, IL-10 is also an essential amplifier of immunoregulatory circuits [236]. Recently, it was suggested that IL-10 predominantly suppresses Th17 cells through direct effects on T cells, while regulation of other effector T cell responses may occur indirectly through effects on APCs [237]. TGF-β1 is an anti-proliferative cytokine [238], and effector T cell responsiveness to TGF-β1 is essential for Treg mediated control of intestinal inflammation [239]. IL-35 suppresses effector T cell proliferation but can also convert naïve $CD4^+$ T cells into suppressive Treg cells that do not require Foxp3 for their function [240, 241]. Indeed $Foxp3^-$ IL-10 producing regulatory T cells, termed Tr1 cells, are abundant in the lamina propria of the small intestine and have been shown to have potent immunosuppressive capacity *in vivo* [237, 242].

1.2.5 Follicular helper T cells

T_{FH} cells are characterised by the expression of cell surface markers CXCR5, PD-1, ICOS and CD40L, and produce high levels of IL-21 [243-247]. Their primary

function is to promote germinal centre (GC) formation and provide help for GC B cell class switching and survival [247]. The STAT3 activating cytokines IL-6 and IL-21 are thought to promote T_{FH} differentiation [248], however recent reports have suggested the existence of STAT3 independent pathways for the generation of T_{FH} *in vivo* [249] [250]. The transcriptional repressor BCL6 has been shown to be essential for the differentiation of T_{FH} by antagonising Th1, Th2 and Th17 effector T cell differentiation [251]. Recent findings, however, suggest that T_{FH} cells are capable of expressing low levels of IFN-γ, IL-4 and IL-17A within germinal centres [247]. The precise lineage relationship between T_{FH} and other effector $CD4^+$ T cell subsets has not been well defined. A recent report showed that Th1 cells go through a transitional Th1/T_{FH} phase early during differentiation and that IL-12-STAT4 signals could promote expression of BCL6 and IL-21 in these cells [252]. Although T-bet repressed expression of *Bcl6* and *Il21* genes at later stages of differentiation, the *Bcl6* locus remained accessible in differentiated Th1 cells. Thus it remains unclear whether T_{FH} represent a distinct T helper cell subset or whether transitional T_{FH} type cells can arise under various T helper cell polarizing conditions.

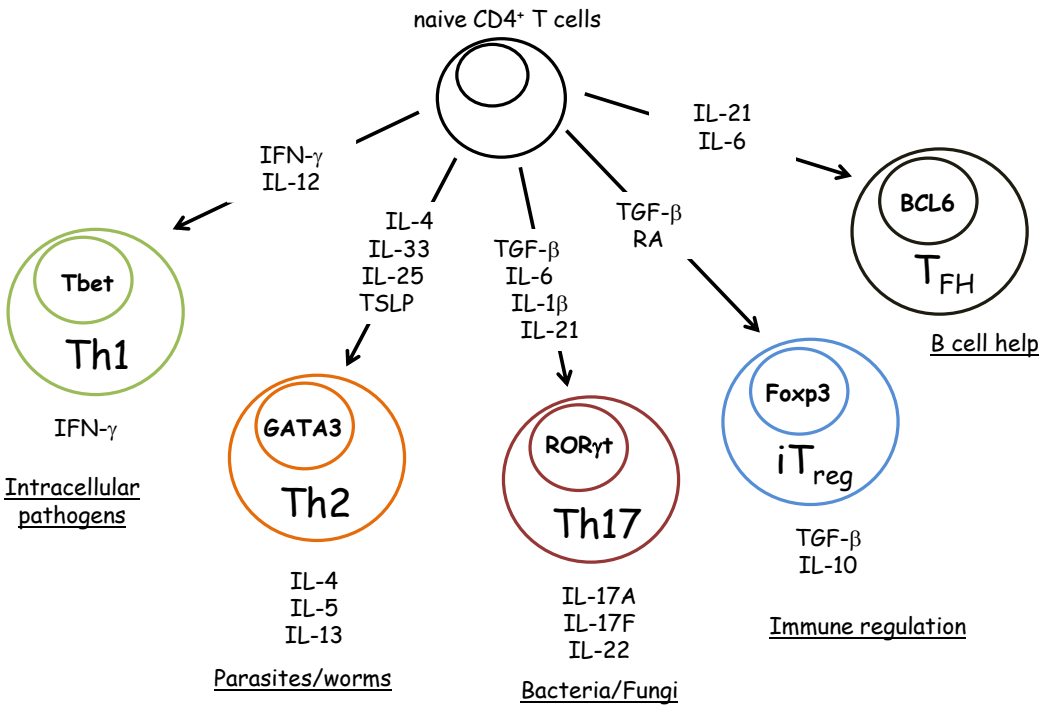

Figure 1.1 T helper cell differentiation and function
In the presence of appropriate polarising cytokines naive $CD4^+$ T cells express specific transcription factors, which promote their differentiation into distinct subsets of T helper cells. Each subset is characterised by the secretion of signature cytokines that mediate unique effector functions.

1.2.6 $CD4^+$ T cell plasticity

In recent years it has become evident that T helper cells are not terminally differentiated effector cells and can exhibit functional plasticity [253]. In addition, the concept of distinct T helper cells lineages has been challenged by the finding that many transcription factors classically associated with particular T cell subsets can be coexpressed and do not always antagonise each other, but can also cooperate in shaping T helper cell effector function. Recent work has demonstrated cooperativity between Foxp3 and what have classically been defined as lineage specific transcription factors, such as T-bet, GATA3, IRF-4, BCL6 and STAT3 [225-227, 254, 255]. Expression of these transcription factors by $Foxp3^+$ Treg cells is essential for their ability to effectively control the immune response. In addition, $ROR\gamma t^+Foxp3^+$ T cells are highly enriched within the intestine, and it has been shown that this population remains stable during acute inflammatory responses and may represent a regulatory counterpart of Th17 cells [130, 256]. IL-17A-producing Treg cells have also been described [150, 257, 258], although the functional role of these cells has not yet been defined. Moreover it has been shown that $Foxp3^+$ Treg cells can lose Foxp3 expression, acquire the ability to produce pro-inflammatory cytokines, and in some cases mediated pathogenic immune responses [258-261]. However, loss of Foxp3 expression is not always associated with pathology, as $Foxp3^+$ Treg cells have also been shown to convert into T_{FH} cells in the Peyer's patches of the small intestine, and promote GC formation and IgA production in the gut [262]. Epigenetic analyses have revealed that Th17 cells are inherently unstable, and that the loci for *Tbx21* and *Ifng* are associated with permissive histone modifications in Th17 cells, suggesting that Th17 cells are "poised" to express T-bet and IFN-γ [260, 263]. Indeed the cytokines IL-12 and IL-23 have been shown to promote conversion of established

Th17 cells into Th1-like cells *in vitro* [132], and populations of IL-17A$^+$IFN-γ$^+$ T cells arise at sites of inflammation in both mice and humans [264-267]. In addition, T-bet expression in Th17 cells and their ability to acquire IFN-γ production has been linked to Th17 cell pathogenicity *in vivo* [132, 154, 268, 269]. Similarly, infection with the lymphocytic choriomeningitis virus (LCMV) was shown to reprogram committed GATA-3$^+$ Th2 cells into GATA3$^+$T-bet$^+$ T cells in an IL-12 and interferon dependent manner [270]. However, in contrast to the pathogenic role for T-bet expression in Th17 cells described above, GATA3$^+$T-bet$^+$ T cells induced less immunopathology, suggesting that T-bet limits the pathogenic potential of Th2 cells in that setting. Thus, considerable heterogeneity exists among T helper subsets, and a better understanding of the physiological relevance of T helper cell plasticity is required to accurately assess the role of functional adaptation in health and disease.

1.3 The microbiota shapes the intestinal T cell compartment

The intestinal microbiota has profound effects on CD4 T cell responses (Figure 1.2). Colonisation of germ free (GF) mice with conventional specific pathogen free (SPF) microbiota leads to the accumulation of CD4$^+$ T cells in the intestinal lamina propria (LP), and induction of both pro-inflammatory and regulatory gene signatures [6, 7], demonstrating that microbial signals directly affect the composition and function of the intestinal T cell compartment. In addition, the appearance of Th17 and Treg cells in the intestinal LP during ontogeny correlates with the onset of bacterial colonisation shortly after birth [8, 271]. Th17 cells are generally more frequent in the lamina propria of the small intestine (SI LP) [49, 130, 271], while Tregs are more abundant in the lamina propria of the large intestine (LI LP) [8, 272], suggesting that the signals required for the generation of these subsets might be linked to differences in

the composition of the local microbiota. Comparison of mice kept under SPF versus GF conditions has revealed that the latter almost completely lack Th17 cells in the intestinal LP [271]. Mono-colonisation experiments showed that a commensal, the *Clostridium*-related segmented filamentous bacteria (SFB), specifically drives the emergence of IL-17 and IL-22 producing $CD4^+$ T cells in the lamina propria [7]. However, a related study found that SFB had a broader effect and enhanced accumulation of Th1 and Treg cells as well as Th17 cells [6]. This discrepancy might reflect differences in the SFB strains used. Analysis of transcriptional programs induced by SFB colonisation revealed a marked induction of serum amyloid A (SAA), and SAA could induce expression of IL-6 and IL-23 by lamina propria DCs *in vitro*[7]. However, the source of SAA and the cellular populations it acts on *in vivo* to create a Th17 permissive environment have not yet been defined. SFB colonises the terminal ileum of the SI where it tightly adheres to epithelial cells [6, 7, 273, 274]. This intimate contact with the epithelium is rather unconventional for a commensal. Given that SFB is also a strong inducer of the intestinal IgA response [275], which is required to limit its outgrowth, SFB might be a particularly potent inducer of intestinal effector responses by virtue of its close association with its host. Although the intestine is thought to be a site that favours iTreg generation [34], several studies have found unchanged or even raised frequencies of Treg cells in the SI LP, MLN and peripheral LNs of GF mice [8, 271, 276]. In contrast, Treg cell accumulation in the LI LP is severely impaired in the absence of a commensal microbiota [8], suggesting that the LI LP represents a specialised microenvironment for iTreg cell generation. The emergence of colonic Treg cells upon colonisation was linked to certain indigenous Clostridial strains (*Clostridium* cluster IV and XIVa), which form constituents of the commensal flora of the caecum and proximal colon [8].

Colonisation of GF mice with a cocktail of *Clostridium* species mimicked the response to colonisation with SPF flora in that it induced selective Treg accumulation in the colon. *Clostridium* species might favour Treg emergence in the colon by enhancing TGF-β1 production by epithelial cells. The majority of Treg cells that appeared upon colonisation were negative for Helios, a transcription factor associated with nTreg cells, suggesting that these cells represent extra-thymically-derived Tregs. In addition to the local effect on Treg accumulation, *Clostridium* species increased Treg cell IL-10 production in the colon, as well as at extra-intestinal sites. Interestingly, dysbiosis of the microbiota in IBD patients is associated with reduced Clostridial groups IV and XIVa, suggesting that bacterial species may also be involved in maintenance of intestinal homeostasis in humans [277, 278]. Although colonisation of GF mice with a single bacterial species, or group of related species, provides valuable insights into host-microbe dialogue, this reductionist approach might result in atypical colonisation patterns and immune responses due to the absence of interspecies competition for intestinal niches. To address this issue, Geuking et al examined intestinal Treg responses upon colonisation of GF mice with the altered Schaedler flora (ASF), a defined commensal microbiota consisting of eight distinct bacterial species [279]. Colonisation of GF mice with ASF induced Helios$^-$ Treg accumulation specifically in the colon and promoted IL-10 expression in colonic CD4$^+$ T cells. This induction of IL-10 had a functional role in establishing a dominant suppressive tissue environment, because blockade of IL-10 signalling at the time of colonisation led to the emergence of IL-17 and IFN-γ producing T cells in the LI LP. Importantly, ASF does not contain SFB, demonstrating that Th17 responses can be generated in the absence of SFB when regulatory pathways are defective. Thus, it appears that the ability to induce Treg or Th17 responses is not restricted to single

bacterial species. Indeed, Atarashi et al have shown that the reduced frequency of intestinal Th17 cells in GF mice could be restored by rectal administration of adenosine 5'-triphosphate (ATP) [5]. The authors argued that luminal ATP is mostly derived from commensal microbiota and acts on a specific subset of lamina propria DCs that exhibits a non-migratory phenotype ($CX3CR1^{+}CD70^{high}$), and expresses high levels of the receptors for ATP. As compared to other subsets of DCs, $CX3CR1^{+}CD70^{high}$ DCs express high levels of IL-23, IL-6 and TGF-β-activating integrins, suggesting that this subset of DCs is particularly suited to promote Th17 responses. Bacterial products have also been shown to act directly on T cells. Colonisation of GF mice with the human commensal *Bacteriodes fragilis* leads to the induction of IL-10 producing Treg cells, and this is dependent on the *B. fragilis* derived polysaccharide A (PSA) [280, 281] . PSA acts directly through TLR2 on $CD4^{+}$ T cells and colonisation with PSA deficient *B. fragilis* results in the generation of Th17 responses instead [282]. Interestingly, this PSA induced suppression of Th17 differentiation seems to be important for the ability of *B. fragilis* to occupy a particular intestinal niche.

The ability of the microbiota to alter the balance of intestinal T cell responses can influence the ability of the mucosal immune system to respond to microbial challenge or injury. For example, colonisation with the human commensal *Bacteroides fragillis* protects from experimental colitis induced by *Helicobacter hepaticus* [280]. This protection is mediated by *B. fragillis* derived polysaccharide A (PSA), which suppresses IL-17 production by T cells, and induces IL-10 producing T cells. In addition, induction of iTregs by *Clostridium* species belonging to cluster IV and XIVa was shown to protect mice from epithelial damage induced by administration of

the chemical agent DSS [8]. Furthermore, the heightened Th17 response in SFB colonised mice has been shown to confer protection against infection with the murine pathogen *Citrobacter rodentium* [7]. Thus dynamic host-microbe interactions favour induction of adaptive regulatory responses, but also serve to maintain basal effector T cell responses. However, constituents of the microbiota have also been shown to exacerbate intestinal as well as systemic inflammatory responses. Colonisation with SFB can promote chronic inflammatory responses that are driven by Th17 cells in models of arthritis [283], experimental autoimmune encephalomyelitis (EAE) [284] and intestinal inflammation [285]. By contrast, host-microbiota interactions have been shown to have a protective role in murine models of type 1 diabetes [286]. Thus the microbiota has a profound impact on shaping the intestinal T cell compartment, and a better understanding of how the microbiota influences the pathogenesis of IBD will be of utmost importance for unravelling the complex aetiology of this disease.

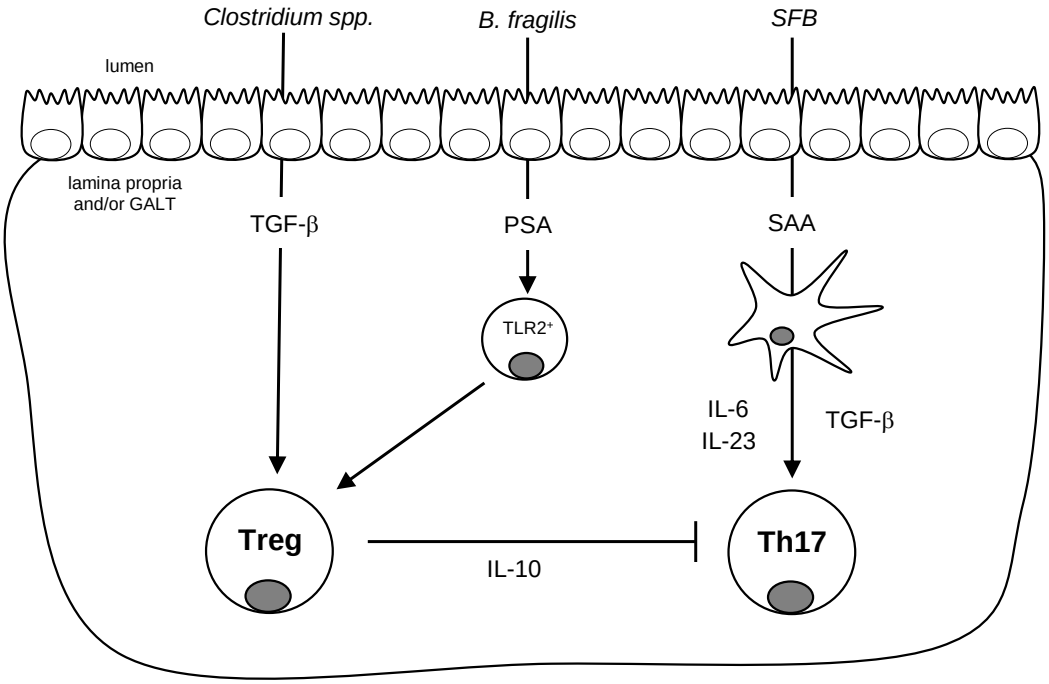

Figure 1.2. The microbiota shapes the intestinal T cell compartment

The microbiota directs the accumulation of both Treg cells and Th17 cells in the intestinal lamina propria. *Clostridium* groups IV and XIVa (*Clostridium* spp.) induce iTregs possibly through increasing TGF-β production by epithelial cells, and also induce IL-10 expression by iTregs. Colonisation with the human commensal *Bacteroides fragillis* leads to induction of iTregs and IL-10 production through PSA-TLR2 interactions. Segmented filamentous bacteria (SFB) promote the induction of Th17 responses, possibly by promoting expression of Th17 inducing cytokines from APCs through serum amyloid A (SAA). The relative balance of Treg and Th17 cells can alter the outcome of local as well as systemic immune responses.

1.4 IL-23 and intestinal inflammation

1.4.1 IL-12 and IL-23: cytokine and receptor expression

IL-23 is a member of the IL-12 family of cytokines. IL-12 is a heterodimeric cytokine composed of IL-12p35 and IL-12p40 [287], while IL-23 is comprised of IL-12p40 and the IL-23p19 subunit [288]. Macrophages and dendritic cells have been shown to express p19, p35 and p40 in response to several PRR agonists, as well as CD40-CD40L interactions. Thus environmental sensors, as well as T cells, can augment the production of IL-12 and IL-23 [289-291]. Despite their structural relationship, IL-12 and IL-23 have profoundly different roles in regulating inflammatory responses. Therefore, it is not surprising that certain stimuli induce differential expression of these cytokines. For example, stimulation of DCs with the TLR2 and NOD agonist peptidoglycan was shown to preferentially enhance IL-23 induction [292, 293], whereas TLR4 agonists such as LPS are more potent inducers of IL-12 rather than IL-23 [294]. In addition, endogenous danger signals such as PGE_2 [295] and ATP [296] have also been shown to preferentially induce IL-23 expression. Interestingly, ATP derived from the commensal microbiota also induces IL-23 expression in a specific subset of lamina propria DCs [5]. Recently, activation of ER stress in combination with TLR agonists was shown to enhance expression and secretion of IL-23, but not IL-12, by DCs. In that study the IL-23p19 gene was found to be a direct target of the ER stress-induced transcription factor C/EBP homologous protein (CHOP) [297]. Furthermore, signalling through the C-type lectin receptor Dectin-1 induces secretion of large amounts IL-23 but not IL-12 [298]. Together these data suggest that various microbial stimuli, as well as endogenous danger signals, cooperate to promote preferential induction of IL-23 over IL-12. There is also emerging evidence of a positive feedback loop whereby Th17 derived cytokines such as GM-CSF [133] and

IL-17A [299] enhance expression of IL-23 by myeloid cells. In addition, IL-23 is constitutively expressed by intestinal DCs [300], and is highly induced at this site during intestinal inflammation [291], suggesting a tissue specific role for this cytokine.

The receptor for IL-12 is comprised of the IL-12Rβ1 and IL-12Rβ2 subunits [301]. It exerts its effects through STAT4 [287, 302], and is expressed by various cell types such as NK cells, DCs and αβ T cells [301, 303]. The IL-23R consists of IL-12Rβ1 and the IL-23R subunit, and signals predominantly through STAT3 but can also weakly activate STAT1, STAT4 and STAT5 [304]. The functional receptor for IL-23 is expressed on a variety of immune cells such as myeloid cells, innate lymphoid cells, NKT cells, γδ T cells and αβ T cells [1, 304]. Expression of the IL-12Rβ1 subunit is regulated by IRF-1 [69], and IL-12Rβ2 expression is promoted by T-bet [67]. The expression of IL-23R is regulated by STAT3 activating cytokines IL-6 and IL-21, and has been shown to require RORγt [125]. The expression of the IL-23R is further regulated in a dose-dependent manner by TGF-β1. At low concentrations TGF-β1 promotes IL-21 and IL-6 driven IL-23R induction, but high concentrations of TGF-β1 repress IL-23R [130]. Recent studies on human T cells have demonstrated an additional layer of complexity in the regulation of IL-23 responsiveness. The microRNA Let-7f inhibits IL-23R expression in human memory Th17 cells [305], and a soluble truncated splice variant of human IL-23R has been described that inhibits IL-23 induced STAT3 phosphorylation [306, 307].

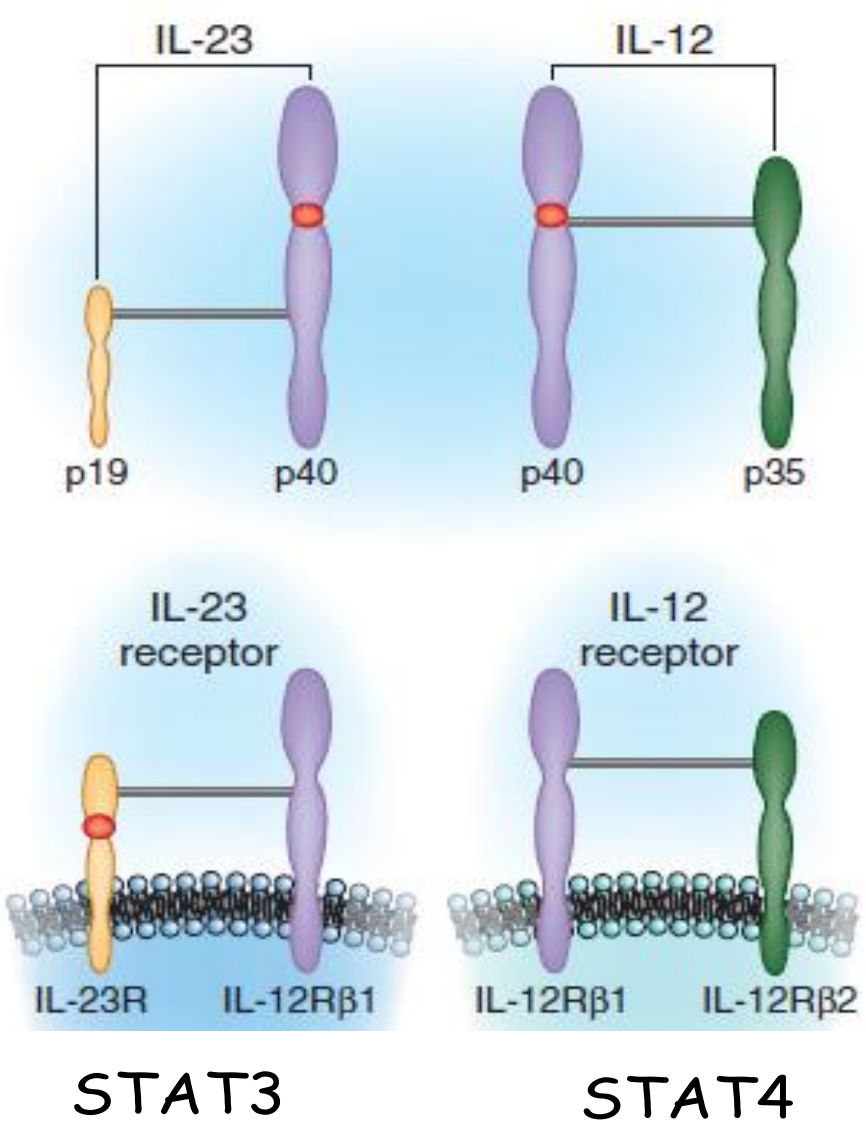

Figure 1.3 IL-12 and IL-23 and their receptors

IL-12 is comprised of IL-12p40 and IL-12p35 and binds to the IL-12R which is composed of IL-12Rβ1 and IL-12Rβ2. IL-23 consists of the IL-12p40 and IL-23p19 subunits and binds to the IL-23R comprised of IL-23R and IL-12Rβ1. IL-12 signals through STAT4 while IL-23 signals predominantly through STAT3. Figure modified from Blauvelt *et al.* [2]

1.4.2 IL-23 is an essential driver of chronic inflammatory responses

Prior to the discovery of IL-23, several autoimmune and chronic inflammatory disease such as experimental autoimmune encephalomyelitis (EAE) [308], collagen induced arthritis (CIA) [309] and colitis [310] were thought to be driven by IL-12. However these findings were based on the use of neutralizing antibodies to IL-12p40, or IL-12p40 deficient mice, and several conflicting observations called the role of IL-12 into question. Mice deficient in critical components of the Th1 pathway such as IL-12Rβ2 [311], IFN-γ [312, 313] IFN-γR [314], STAT1 [315] or IL-12p35 [316, 317] were susceptible to EAE, and in fact developed exacerbated pathology in some cases. Indeed, administration of exogenous IL-12 could ameliorate EAE through induction of IFN-γ [318]. The first demonstration that IL-23, rather than IL-12, is a key driver of chronic inflammation came from a study by Cua *et al*, which demonstrated that IL-23p19 deficient mice were resistant to the development of EAE [319]. This finding was later extended to several other chronic inflammatory diseases such as CIA [116], psoriasis [320, 321], experimental autoimmune myocarditis [322] and intestinal inflammation [264, 265, 291, 323], highlighting the importance of IL-23 as mediator of pathogenic immune responses. Because Th17 cells have been implicated in development of disease in these models it was originally thought that IL-23 is necessary for the generation of Th17 cells [117, 161, 163]. However, it is now clear that IL-23 is dispensable for Th17 differentiation [121, 122, 124] and emerging data suggest that rather than driving Th17 development IL-23 modulates Th17 effector function and pathogenicity [131, 154].

1.4.3 IL-23 drives T cell dependent intestinal inflammation

As mentioned above, it has now been well established that IL-23 rather than IL-12 mediates chronic intestinal inflammation. Development of T cell dependent colitis in IL-10 deficient hosts, and in mice treated with *Helicobacter hepaticus* in the absence of IL-10 signalling, has been shown to be dependent on IL-23 [264, 323]. Furthermore, adoptive transfer of $CD4^+$ T cells into IL-23 deficient *Rag1*$^{-/-}$ recipients does not lead to the development of colitis, whereas IL-12 deficient *Rag1*$^{-/-}$ hosts develop intestinal inflammation comparable to that observed upon $CD4^+$ T cell transfer into *Rag1*$^{-/-}$ mice [265]. In the absence of IL-23, several pro-inflammatory cytokines associated with Th1 and Th17 type responses such as IL-17A, IFN-γ, IL-1β and TNF-α are decreased at the tissue level. Similarly, administration of exogenous IL-23 leads to enhanced expression of IL-17A, IFN-γ, IL-1β and TNF-α in the colon of *Rag1*$^{-/-}$ hosts transferred with $CD4^+$ T cells from IL-10 deficient mice [323]. In addition to its role in the induction of colitis, IL-23 has also been shown to be required for the maintenance of intestinal inflammation, as neutralization of IL-23 reversed established colitis [324].

Together, these results demonstrate that IL-23 promotes both Th1 and Th17 type responses. Accordingly, accumulation of both Th1 and Th17 cells in the inflamed colon is a common feature of T cell dependent models of colitis [264, 265], and there is abundant evidence that transcription factors and cytokines associated with the differentiation and function of these cells contribute to the induction of disease. For example, blockade of IFN-γ prevents *Helicobacter hepaticus* driven disease in IL-10 deficient mice [54], and also blocks colitis induced by T cell transfer [87]. In addition, T-bet [88] and IRF-1 [69] are required for development of T cell transfer

colitis and STAT4 deficient T cells induce a late onset disease [325]. Genetic ablation of factors involved in Th17 differentiation such as RORγ [326] or IRF4 [327], or antibody mediated blockade of IL-6 [328, 329], inhibit T cell transfer colitis. Moreover, transfer of *in vitro* differentiated Th17 cells into *Rag1*$^{-/-}$ hosts was shown to lead to the development of IL-23 dependent colitis [132, 324]. Although Th1 and Th17 cells are capable of inducing colitis, it has not been established which T cell derived effector cytokines mediate the inflammatory response. Several groups have shown that T cell deficiency in IFN-γ [325], IL-17A [326, 329, 330], IL-17F [326], IL-22 [326, 331] or GM-CSF [332] did not prevent the development of intestinal inflammation. This suggests that a combination of these cytokines, or as yet unidentified factors, may contribute to the pathogenicity of the IL-23 driven CD4^{+} T cell responses in this model.

Recently it was shown that IL-23 can promote intestinal inflammation indirectly through inhibition of Foxp3^{+} Treg differentiation [330]. As described previously, transfer of CD4^{+} T cells into IL-23p19 deficient *Rag1*$^{-/-}$ hosts does not lead to colitis. However, concomitant neutralisation of TGF-β or IL-10 resulted in the development of colitis, suggesting that IL-23 independent intestinal inflammation can develop when immunoregulatory pathways are blocked. In addition, the lack of intestinal inflammation in IL-23p19 deficient *Rag1*$^{-/-}$ hosts was associated with an increased frequency of Foxp3^{+} Treg cells, which was also observed in IL-12p40 but not IL-12p35 deficient mice [333]. This demonstrates that IL-23, but not IL-12, restrains the differentiation of Foxp3^{+} Treg cells. Moreover, transfer of Foxp3 deficient CD4^{+} T cells into IL-23p19 deficient *Rag1*$^{-/-}$ hosts results in intestinal inflammation similar to that observed in IL-23 sufficient *Rag1*$^{-/-}$ mice. Interestingly, Foxp3 deficient CD4^{+} T

cells also induce colitis in IL-12p35 deficient mice that lack IL-12 alone, but not IL-12p40 deficient mice, which lack both IL-12 and IL-23. This demonstrates that IL-12 is only sufficient for the development of colitis in the absence of IL-23-mediated inhibition of $Foxp3^+$ Treg cell differentiation. Collectively, these findings show that IL-23 promotes intestinal inflammation through induction of pro-inflammatory cytokines and inhibition of regulatory T cell responses, which results in the accumulation of Th1 and Th17 cells in the intestine.

1.4.4 IL-23 drives innate intestinal inflammation

In addition to its role in T cell dependent models of colitis, IL-23 has been shown to drive innate intestinal inflammation in *Rag1*$^{-/-}$ mice, which lack an adaptive immune system. In the anti-CD40 model of colitis, administration of an agonistic anti-CD40 monoclonal antibody (mAb) leads to the development of intestinal as well as systemic inflammation [291]. Comparison of the inflammatory response in IL-12 and IL-23 deficient *Rag1*$^{-/-}$ mice revealed a differential role for IL-12 and IL-23 in this model. Systemic disease was driven by IL-12, but not IL-23, while intestinal inflammation was dependent on IL-23 and did not require IL-12. This tissue specific role of IL-23 correlated with markedly greater induction of IL-23p19, as compared to IL-12p35, in colonic DCs, and higher levels of expression of IL-23 in the colon compared to the spleen. This compartmentalization of IL-23 expression during acute intestinal inflammation is in keeping with the finding that IL-23 is constitutively expressed by DCs in the terminal ileum of steady state mice [300]. Together these studies suggest a tissue specific function for IL-23 in the intestine. Another model of innate colitis that is bacterially driven has also been shown to be dependent on IL-23. Infection of *Rag1*$^{-/-}$ mice with *Helicobacter hepaticus* leads to the development of intestinal

inflammation that can be prevented by administration of IL-23 blocking antibody [265]. In both these models of innate colitis, depletion of IL-23 results in decreased levels of pro-inflammatory cytokines such as IFN-γ, IL-17A, TNF-α, IL-6 and IL-1β. Recently it was shown that IL-23 promotes *Helicobacter hepaticus* driven innate colitis through induction of IL-17A and IFN-γ production by a novel population of IL-7Rα^{+} Thy1^{hi} SCA-1^{+}CD4^{-} NKp46^{-} innate lymphoid cells (ILCs) [171]. Depletion of Thy1^{+} cells prevented the development of colitis in mice infected with *Helicobacter hepaticus* or treated with agonistic anti-CD40 antibody, suggesting that the IL-23 responsive ILCs contribute to the inflammatory response in both models. This population of ILCs also expressed high levels of the transcription factor RORγ□, which had a functional role in disease as *Rag1*$^{-/-}$*Rorc*$^{-/-}$ failed to develop the innate colitis induced by anti-CD40 injection. Importantly, a similar population of IL-23 responsive innate lymphoid cells was found to be increased in the inflamed intestine of patients with IBD [334]. Together these findings suggest that IL-23 drives intestinal inflammation through conserved pathways in both innate and T cell dependent models of colitis.

1.4.5 The IL-23/Th17 axis in host defence

Despite the association of the IL-23-Th17 axis with autoimmune and chronic inflammatory responses, Th17 associated cytokines have been shown to be essential for mucosal host defence against pathogenic bacteria and fungi. IL-23 is required for protection from oral infection with *Citrobacter rodentium* [122]. and this host protective function of IL-23 appears to be mediated by IL-22 [335] through induction of antimicrobial peptides RegIIIβ and RegIIIγ. In addition, IL-17A and IL-17F induce the production of anti-bacterial β-defensins in colonic epithelial cells, and both cytokines are required for protective immunity to *C. rodentium* and *Staphylococcus*

aureus [336]. In addition, host protective immunity to the lung pathogen *Klebsiella pneumonia* has been shown to require IL-23 driven IL-22 expression [337]. Th17 cells are also associated with protective immune responses to the fungal pathogen *Candida albicans* through recruitment of neutrophils and induction of antimicrobial factors [338]. A tissue protective role for IL-23 has also been described in an acute model of colitis induced by treatment of *Rag1*$^{-/-}$ with the chemical agent DSS [339]. The authors linked this to IL-23 mediated induction of IL-22, which is known to stimulate intestinal epithelial cell proliferation [340] and mucus production [341]. A similar protective role for IL-17A and IL-22 has also been described in immunocompetent mice treated with DSS [342, 343]. In addition, T cell deficiency in IL-22 [331] or IL-17A [344] has been shown to exacerbate some aspects of T cell transfer colitis, suggesting a protective role for these cytokines in chronic inflammation. Thus, IL-23 and its downstream mediators have an important host protective function through their effects on neutrophil recruitment, antimicrobial peptide production and maintenance of epithelial integrity.

These data highlight the complexity of IL-23 driven immune responses and demonstrate that IL-23 can mediate host protective as well as pathogenic immunity. So far the study of IL-23 driven immune responses has focused on a discrete set of inflammatory mediators downstream of IL-23, however, little is known about the signature of IL-23 driven immune responses beyond these known factors. Elucidating the transcriptional targets and cellular programs controlled by IL-23 in innate and adaptive cells is likely to provide insight into the mechanisms by which IL-23 modulates immune cell function in pathogenic and host protective immunity.

1.5 IL-23 and inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises Crohn's disease (CD) and ulcerative colitis (UC), which are chronic relapsing inflammatory diseases of the intestine. IBD affects 1 in 1000 people in Europe [345] with a peak incidence in young adults between 15- 30 years of age [346]. CD can affect any region of the intestine but commonly affects the ileum and colon. Inflammation is discontinuous and transmural, and is associated with complications such as granulomas, fistulas and strictures. UC is restricted to the rectum and colon, and involves continuous inflammation that is typically confined to the mucosa and submucosa. The aetiology of IBD is unknown but is thought to be a result of inappropriate immune reactivity toward components of the intestinal flora in genetically susceptible hosts [347]. This is supported by the findings of genome-wide association studies (GWAS) that have identified several genetic risk loci, many of which are associated with epithelial barrier function, bacterial handling and regulation of innate and adaptive immunity [347-350]. These include polymorphisms in *IL23R as well as NOD2* and *ATG16L*, which are involved in bacterial sensing and autophagy respectively. Much effort has focused on how mutations in these genes contribute to the pathogenesis of IBD. Furthermore, IBD is associated with marked changes in the composition of the microbiota characterised by a reduction in *Firmicutes* and *Bacteriodetes*, and increased numbers of *Enterobacteriaceae* [351, 352]. However, whether this dysbiosis contributes the development of disease, or is the result of heightened immune reactivity towards the commensal flora, is currently unresolved. Studies in experimental models of IBD have shown that innate immune defects can lead to the development of colitis and outgrowth of particular bacterial species [23, 353]. Transfer of this imbalanced mircobiota to wild type animals could transmit colitis, demonstrating that alterations

in the microbiota caused by host genetic defects have the potential to contribute to the development of disease.

With regard to the adaptive immune system, CD was classically thought of as a Th1 driven disease as IL-12 [354, 355], IFN-γ [356] and T-bet [88] are increased in inflammatory lesions of CD patients. By contrast, UC is typically associated with increased levels of IL-5 and IL-13 and is thought to be a Th2 mediated disease [356, 357]. However, GWAS studies have linked several single nucleotide polymorphisms in *IL23R* to increased susceptibility to both UC and CD, suggesting that these diseases also share common features [348]. Several signalling components downstream of the IL-23R pathway such as *TYK2*, *JAK2* and *STAT3*, *IL17A* and *IL17F* have also been identified as IBD susceptibility genes [349, 350, 358, 359]. In addition, $CD14^+$ intestinal macrophages, which produce high levels of IL-23, have been identified in CD patients [360], and levels of the Th17 associated cytokines IL-17A [361-363], IL-21 [364] and IL-22 [365] are also increased in patients with IBD. Together, these findings strongly suggest that the IL-23/Th17 axis is an important determinant in the pathogenesis of IBD. However, little is known about how polymorphisms in *IL23R* affect responsiveness to IL-23. Schmechel *et al.* linked CD-associated variants of *IL23R* to increased serum levels of IL-22, suggesting that these mutations represent a gain of function, and lead to increased responsiveness to IL-23 [366]. In support of this, a recent study found that an IBD associated variant that affects the 3'-untranslated region of the *IL23R* gene could not be regulated by microRNAs. This resulted in increased levels of IL-23R mRNA expression and protein production [367]. Interestingly, GWAS studies also identified the *IL23R* variant R381Q as associated with protection from CD [348]. This mutation results in

an arginine to glutamine substitution at position 381 in the cytoplasmic domain of IL-23R. Two independent reports have shown that IL-23 stimulation led to reduced STAT3 phosphorylation and IL-17A secretion in Th17 cells from carriers of the protective allele, suggesting that the R381Q variant represents a loss of function mutation. [368, 369].Thus differential responsiveness to IL-23 might modulate disease outcome through effects on Th17 type responses.

Current treatment regimens for IBD involve blockade of TNF-α, but not all patients respond to this treatment and there are associated side-effects, including heightened susceptibility to secondary infection [370]. Thus, more targeted therapies are required for the treatment of IBD. Clinical trials with anti-IL-12p40 monoclonal antibody, which blocks both IL-12 and IL-23, have shown promising results [371]. Given that IL-23 expression is prominent in the intestine, and appears to have a tissue specific role in driving intestinal inflammation [291], blockade of IL-23 rather than IL-12 might be a more specific therapeutic strategy for the treatment of IBD in some patients.

1.6 Animal models of IBD

A variety of animal models of intestinal inflammation exist that recapitulate certain aspects of IBD. These can be broadly classified as acute and chronic inflammatory models, which are driven by the innate or adaptive immune system. Although none of these models accurately reflect the complexity of human IBD, they provide useful tools for the dissection of the cellular and molecular mechanisms that drive intestinal inflammation. Throughout this thesis two distinct models of T cell dependent colitis have been used and these are described in detail here.

1.6.1 *Helicobacter hepaticus*/anti-IL-10R model of colitis

Helicobacter hepaticus is a gram negative, microaerophilic bacterium that colonizes the crypts of the caecum and colon of mice [372]. It was first isolated from the liver of A/JCr mice [373] and has since been shown to promote intestinal inflammation in a variety of immune deficient mice, including mice deficient in IL-10 [264, 374-376]. Although *Helicobacter hepaticus* can establish persistent infection in WT mice, it does not cause disease. There is evidence that *Helicobacter hepaticus* infection drives the emergence of IL-10 producing $CD4^+$ T cells with regulatory activity, suggesting that host deficiency in IL-10 removes a crucial regulatory component that would normally prevent intestinal inflammation upon infection with *Helicobacter hepaticus* [377]. Similar to IL-10 deficient mice, infection of WT mice with *Helicobacter hepaticus* and concomitant administration of IL-10R blocking antibody leads to the development of severe intestinal inflammation that is restricted to the colon and caecum [264]. Administration of IL-10R blocking antibody alone does not lead to the development of colitis, demonstrating that the endogenous microbiota is not sufficient to drive intestinal inflammation, and that infection with *Helicobacter hepaticus* is

required to trigger disease. The intestinal inflammation that develops in this model is characterized by high levels of IL-17A, IL-17F and IFN-γ with IFN-γ playing a functional role in colonic but not caecal inflammation. In addition, restimulation of single cell suspension from the MLN of inflamed mice with soluble *Helicobacter hepaticus* antigen leads to bacterial specific induction of IL-17A and IFN-γ. IL-23 is essential for driving inflammation in this model as IL-12p40 deficient mice, but not IL-12p35 deficient mice, were protected from development of colonic and caecal inflammation. This model of colitis is useful as it allows the study of effector pathways in mice that are fully competent in both innate and adaptive immune cells. However, careful dissection of the contribution of individual cellular populations or components to disease development can only be achieved by using antibody mediated or conditional genetic ablation of specific cell types or molecules.

1.6.2 T cell transfer model of colitis

Early studies revealed the presence of two distinct populations of $CD4^+$ T cells capable of promoting or preventing the development of colitis when transferred into immune deficient recipients [378-381]. Adoptive transfer of $CD4^+CD45RB^{hi}$ into *Rag1*$^{-/-}$ or SCID recipients leads to the induction of colitis, splenomegaly and systemic wasting disease [379-381]. Histologically, the intestinal inflammation is characterised by dense cellular infiltrate in the lamina propria, epithelial cell hyperplasia and depletion of goblet cells. These pathological changes occur along the length of the colon, and intestinal inflammation is associated with high levels of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-17A. Both IFN-γ and TNF-α play a functional role, as antibody mediated neutralisation of these cytokines prevented induction of colitis [265, 330]. In addition, transfer of T-bet deficient $CD4^+$

T cells did not lead to colitis [88], which led to the notion that Th1 cells are the major population driving disease in this model. However, concomitant inactivation of IL-17A and IL-17F, or transfer of RORγt deficient $CD4^+$ T cells into *Rag1*$^{-/-}$ recipients, also prevented the onset of disease, demonstrating a role for Th17 cells in the pathogenesis of T cell transfer colitis [326]. The relative contribution of Th1 and Th17 cells to the development of colitis has been addressed in detail in this thesis. Importantly, intestinal inflammation, but not systemic wasting disease, is dependent on IL-23 in this model [265]. In addition, colitis in this model is bacterially driven as immune deficient mice maintained under germ-free conditions do not develop disease upon naive $CD4^+$ T cell transfer [285].

Early studies found that colitis induced by $CD4^+CD45RB^{hi}$ T cells can be prevented by co-transfer of $CD4^+CD45RB^{low}$ cells [379, 380]. The suppressive activity among this pool of antigen experienced cells was later found to be enriched among the $CD4^+CD25^+$ fraction [174]. $CD4^+CD25^+$ Treg cells prevent systemic wasting disease as well as the intestinal inflammation induced by transfer of $CD4^+CD45RB^{hi}$ T cells. The control of colitis by $CD4^+CD25^+$ Treg cells requires responsiveness of $CD4^+CD45RB^{hi}$ T cells to TGF-β [239], and expression of particular cell surface receptors such as CTLA4 [174, 382], OX40 [231] or CCR6 [383] on $CD4^+CD25^+$ Treg cells. In addition, transfer of $CD4^+CD25^+$ Treg cells can also cure established disease, demonstrating the potent immunoregulatory role of these cells [384, 385].

The T cell transfer model is a useful tool for dissecting the contribution of innate and adaptive immune cells to the development of colitis, as genes can be selectively ablated in the *Rag1*$^{-/-}$ recipient or the T cell donor. However, transfer of naïve $CD4^+$ T

cells into *Rag1*$^{-/-}$ recipients results in rapid lymphopenia induced proliferation and acquisition of effector function [386]. In addition, many cell types such as B cells, CD8^{+} T cells and Foxp3^{+} Treg cells are absent in *Rag1*$^{-/-}$ hosts, and this is likely to alter the requirements for T cell transfer colitis.

1.7 Aims

Evidence from experimental models and genetic studies in humans support an essential role for the IL-23-Th17 axis in the development of chronic intestinal inflammation. However, little is known about the cellular and molecular mechanisms by which IL-23 promotes T cell driven intestinal inflammation. Therefore the aims of this thesis were:

(1) To determine the functional consequences of IL-23 signalling into T cells in the context of intestinal inflammation

(2) To identify the transcription factor modules required for IL-23 driven T cell dependent inflammation

(3) To characterise the transcriptional targets of IL-23 signalling into T cells

Chapter 2: Materials and Methods

2.1 Mice

Wild-type (WT) C57BL/6 (B6), B6.*Rag1*$^{-/-}$, B6.*Il23r*$^{-/-}$, B6.*Il23a*$^{-/-}$*Rag1*$^{-/-}$, B6.SJL-*Cd45.1*, B6.IL-23R$^{gfp/gfp}$, B6.IL-23R$^{gfp/+}$, B6.*Rag1*$^{-/-}$IL-23R$^{gfp/gfp}$, B6.*Tbx21*$^{-/-}$ and B6.*Rorc*$^{-/-}$ mice were maintained in individually ventilated cages in a specific pathogen-free (SPF) animal facility at the Biomedical Services Facility, John Radcliffe Hospital, University of Oxford. B6.*Rorc*$^{-/-}$ mice were originally obtained from Dan Littman (NYU, USA). B6.*Il23r*$^{-/-}$ and B6.*Il23a*$^{-/-}$*Rag1*$^{-/-}$ mice were originally obtained from Dan Cua (Schering Plough, USA). B6.IL-23R$^{gfp/gfp}$ and B6.*Rag1*$^{-/-}$IL-23R$^{gfp/gfp}$ were obtained from Vijay Kuchroo and Mohamed Oukka (Harvard Medical School, USA). Cells from B.6.*Il33r*$^{-/-}$ (B.6T1/ST2$^{-/-}$) mice were a kind gift from Padraic Fallon (Trinity College Dublin, Ireland). GATA3$^{fl/fl}$ OX40Cre mice were kept at National Institute of Health (NIH) and experiments were performed at the NIH.

2.2 Isolation of cells

2.2.1 Mesenteric lymph node and spleen

Single cell suspensions were prepared in in PBS/0.1% BSA by passing individual MLN or spleens through 70µm cell strainers (BD Biosciences). Following centrifugation at 1500rpm for 5 minutes ACK lysis buffer (150 mM NH_4CL, 10 mM, $KHCO_3$, 0.1 mM NA_2.EDTA, pH 7.2-7.4) was added to spleen cell preparations for 3 minutes at room temperature to lyse red blood cells. Thereafter 10 volumes of PBS/0.1% BSA was added to cells and cells were filtered through 70µm cell strainer and washed once to remove residual ACK lysis buffer. Finally, cells were resuspended in PBS/0.1% BSA and put on ice.

2.2.2 Colonic lamina propria leukocyte (LPL) isolation

Luminal contents were removed and colons first cut longitudinally with scissors and then cut into approx. 0.5 cm pieces with scalpel and placed in a 50 ml tube (BD Biosciences) containing 10ml PBS/0.1% BSA and put on ice immediately. Tissue was allowed to settle and PBS/0.1% BSA was aspirated followed by addition of 10 ml pre-warmed (37^{o}C) complete RPMI 1640 containing 5mM EDTA (VWR International UK) to each sample. Samples were incubated for 40 minutes at 37^{o}C in a shaking incubator at 200rpm. Supernatant was discarded and tissue was incubated for a further 40 minutes at 37^{o}C in a shaking incubator at 200 rpm. Thereafter, Samples were incubated in 20ml pre-warmed (37^{o}C) compete RPMI 1640 containing 15mM HEPES (PAA) for 10 minutes at room temperature to neutralize the activity of EDTA, which would otherwise inhibit collagenase activity in the next step. Supernatant was aspirated and 10ml of RPMI 1640 containing 15mM HEPES (PAA) and 0.4-0.6 mg/ml type VIII collagenase (Sigma Aldrich) was added to each sample. Samples were incubated for 1 hour at 37^{o}C while shaking at 200 rpm. Supernatants were filtered through a 70µm cell strainer and washed in cold PBS/0.1% BSA. Percoll (GE) gradients consisting of a bottom layer of 3 ml 75% Percoll solution in PBS (P75) and a middle layer of 4 ml 40% Percoll solution in RPMI 1640 (P40) were prepared in a 50ml tube. Cells released from the colon tissue by digestion were resuspended in 3 ml of 30% Percoll solution in PBS (P30) and gently placed on top of the 40% Percoll layer. Gradients were then centrifuged at 1800 rpm without brake. The P30/P40 layer was aspirated and the colonic lamina propria leukocytes isolated from the P40/P75 interface. Cells were washed in PBS/0.1% BSA to remove excess Percoll and cell suspensions were resuspended in cold PBS/0.1% BSA and kept on ice.

2.3 Flow cytometry and antibodies

2.3.1 Restimulation for intracellular cytokine staining

Cell suspensions ($1x10^6$ cells/well) were restimulated in a 96 well round bottom plates (Corning) for 3.5-4 hours at 37^oC in 200µl complete IMDM (Gibco) containing 10% FCS (Gibco), 0.05mM 2-mercaptoethanol (VWR International), 100 Units penicillin/streptomycin (PAA) with 0.1µg/ml PMA and 1µg/ml ionomycin (both Sigma Aldrich) and Brefeldin A (eBioscience) or Monensin (BD Biosciences). Monensin was used for intracellular staining panels involving IL-13 and IL-4. Thereafter cells were washed at least twice in 200µl PBS and centrifuged at 1500rpm for 5 minutes.

2.3.2 Surface staining

Following restimulation, and prior to surface staining, cell suspensions ($1x10^6$ cells/well) were stained with Fixable Viability Dye e780 (eBioscience) in PBS (Gibco) for 10 minutes on ice in a V bottom 96 well plate (Corning) followed by two wash steps in PBS at 1500rpm for 5 minutes. Thereafter all incubations were performed in 50µl PBS supplemented with 0.1% BSA (Sigma Aldrich). Cell suspensions were first incubated with anti-CD16/32 (eBioscience) to block Fc receptors and prevent non-specific binding of subsequent antibodies. Cells were washed at least twice by addition of 200µl PBS/0.1% BSA and centrifugation at 1500rpm for 5 minutes. Cells were then incubated with relevant panel of antibodies for 15 minutes on ice in the dark. Biotinylated primary antibodies were detected by secondary staining using fluorescently labelled streptavidin conjugates. Samples were washed at least twice in 200µl PBS/0.1% BSA and centrifuged at 1500rpm for 5 minutes. Stained samples were resuspended in 200µl PBS/0.1% BSA for analysis or fixed in 100µl PBS/2% PFA for 10 minutes on ice, washed twice and resuspended in

200µl PBS/0.1% BSA. Fixed samples were analysed no later than 2 days following staining.

2.3.3 Intracellular staining

Following cell surface staining, cell suspensions were resuspended in 100µl permeabilisation buffer (BD Biosciences or eBioscience) and incubated for 30 minutes in the dark at 4^{o}C. Cells were centrifuged at 1750rpm for 3 minutes resuspended in 50µl of relevant panels of antibodies prepared in permeabilisation buffer and incubated at 4^{o}C for 30-45 minutes in the dark. Cells were washed four times by addition of 100-200µl of permeabilisation buffer and centrifuged at 1750 rpm for 3 minutes. After the final wash, cells were resuspended in 200µl PBS/0.1% BSA for analysis.

2.3.4 CD4^{+} T cell enrichment and FACS sorting

Single cell suspensions were prepared from spleens and red blood cells lysed as described above. Cells were resuspended in CD4 enrichment mix containing antibodies to CD8, MHCII, CD11b and B220 for 20 minutes in PBS/10% FCS on ice. Thereafter cells were washed once in PBS/0.1% BSA and resuspended at a concentration of 1x108 cells/ml and mixed with anti-rat IgG coated beads (Dynal, Invitrogen) at a concentration of 1x108 beads/ml and incubated at 4^{o}C on a rotator for 20 minutes. Suspensions were placed on a magnet for approx. 30-60 seconds to allow separation of beads and cells and CD4 enriched cells, depleted of beads, were poured into a 50ml tube trough a cell strainer. Cell suspensions were then washed in ice cold PBS/0.1% BSA and resuspended at a concentration of 1x108 cells/ml. Next cells were incubated with antibodies directed against CD4, CD25 and CD45RB for 20 minutes at 4^{o}C followed by two wash steps in PBS/0.1% BSA. Stained cells were resuspended at a concentration of 2.5-5x10^{7}cells/ml. Naive CD4^{+}CD25^{-}CD45RB^{hi} T

cells and $CD4^+CD25^+CD45RB^{lo}$ regulatory T cells were sorted using a FACS Aria (BD Biosciences).

2.3.5 Antibodies

Antibodies used for FACS staining, *in vitro* stimulation and *in vivo* blockade are depicted in the table below.

Specificity	**Clone**	**Source**	**Fluorochrome**	**Concentration**
CD4 enrichment mix				
CD4	YTA 3.1	In-house	N/A	~ 10 µg/ml
CD4	GK1.5	In-house	N/A	~ 10 µg/ml
CD8	YTS169	In-house	N/A	~ 10 µg/ml
MHCII	TIB120	In-house	N/A	~ 10 µg/ml
Mac-1 (CD11b)	M1/70	In-house	N/A	~ 10 µg/ml
B220	RA36B2	In-house	N/A	~ 10 µg/ml
***In vitro* stimulation**				
CD3 (agonistic)	1-45-2C11	eBioscience	N/A	5 µg/ml
CD28 (agonistic)	37.51	eBioscience	N/A	1-5µg/ml
anti-IFN-γ (blocking)	XMG1.2	BioXCell	N/A	10µg/ml
anti-IL-4 (blocking)	11B11	BioXCell	N/A	10µg/ml
***In vivo* depletion/neutralisation**				
IL-10R (blocking)	1B1.2	In-house	N/A	1 mg/week
IL-17A (blocking)	17F3	BioXCell	N/A	2mg/week
MOPC-21 (isotype control)	MOPC-21	BioXCell	N/A	2mg/week
Anti Thy1.1	19E12	In-house	N/A	1mg/week
Surface Staining				
Fc Block (CD16/32)	93	eBioscience	N/A	5 µg/ml
CD4	GK1.5	eBioscience	FITC/PE	1 µg/ml
CD4	RM4-5	eBioscience	PercpCy5.5	1 µg/ml
CD45.2	104	eBioscience	PercpCy5.5/PE	1 µg/ml
CD45.1	A20	eBioscience	PercpCy5.5/PE	1 µg/ml
TCR-β	H57-597	eBioscience	PE/APC	1 µg/ml
CD11b	M1/70	eBioscience	PercpCy5.5/APC	1 µg/ml
GR1	RB6-8C5	eBioscience	PE	0.5 µg/ml
Thy1.2	30-H12	Biolegend	APC/ PercpCy5.5	1 µg/ml
CD25	7D4	BD Bioscience	FITC	1 µg/ml
CD45RB	C363-16A	Biolegend	APC	1 µg/ml
IL-33R	DJ8	mdbioproducts	biotinylated	10 µg/ml
Streptavidin	N/A	BD Bioscience	PerCp/PE/APC	0.5 µg/ml

Intracellular Staining				
IL-17A	ebio17B7	eBioscience	PE/FITC/APC	2 µg/ml
IFN-γ	XMG1.2	eBioscience	FITC/APC/e450	2 µg/ml
IL-17F	9D3.1C8	Biolegend	PE	2µg/ml
IL-22	1H8PWSR	eBioscience	PE	2µg/ml
GM-CSF	MP-122E9	BD Bioscience	PE	2µg/ml
TNF-α	MP6-XT22	eBioscience	e450	2µg/ml
IL-13	Ebio13A	eBioscience	Alexa 647	2µg/ml
IL-4	11B11	eBioscience	PE	2µg/ml
Foxp3	FJK-16S	eBioscience	FITC/APC/e450	2 µg/ml
T-bet	O4-46	BD Bioscience	Alexa 647	N/A
RORg(t)	AFKJS-9	eBioscience	PE	4 µg/ml
Eomes	21Mags8	eBioscience	PE	2 µg/ml
Ki67	B56	BD Bioscience	FITC	N/A
Fixable Viability Dye	N/A	eBioscience	e780	N/A
Isotype Controls				
IgG1	R3-34	BD Bioscience or eBioscience	FITC/PE/APC	2-5 µg/ml
IgG1	A110-1	BD Bioscience or eBioscience	APC, Alexa 647	5 µg/ml
IgG1	B56	BD Bioscience or eBioscience	FITC/PercpCy5.5	N/A
IgG2a	R35-95	BD Bioscience or eBioscience	FITC/PE/APC	2 µg/ml
IgG2b	A95-1	BD Bioscience or eBioscience	PE, e450	5 µg/ml

Stained cells were acquired on a FACS Calibur or FACS LSR2 flow cytometer and analysed using FlowJo software (TreeStar Inc.).

2.4 T cell differentiation assays

For *in vitro* cultures $CD4^{+}CD44^{lo}CD62L^{hi}$ T cells were sorted from the spleen and peripheral lymph nodes as described above. Cells were plated in complete IMDM (10% FCS) at a concentration of $2x10^5$ cells/well. Th17 differentiation experiments were performed in flat bottomed 96 well plates coated with agonistic antibodies to CD3 and CD28 (both eBioscience) at a concentration of 5μg/ml. Cytokines were added at the following concentrations, TGF-β1 (R&D, 200pg/ml), IL-23 (R&D; 20ng/ml), IL-6 (Peprotech; 20 ng/ml), IL-1β (R&D, 20ng/ml), anti-IL-4 (BioXCell, 10μg/ml) and anti-IFN-γ (BioXCell, 10μg/ml). After 3 days cultured cells were restimulated with PMA and ionomycin and intracellular cytokine staining was performed as described above. For Foxp3 induction assays $CD4^{+}CD44^{lo}CD62L^{hi}$ $Foxp3^{-}$ naive T cells were FACS sorted from $Foxp3^{gfp}$ mice. Cells were plated at $2x10^5$ cells/well in flat bottomed 96 well plates coated with anti-CD3 (5μg/ml) in complete IMDM (10% FCS). Antibodies and cytokines were added at the following concentrations, anti-CD28 (2μg/ml), TGF-β1 (R&D, 500pg/ml), IL-2 (Peprotech, 100U/ml), IL-23 (R&D, 20ng/ml), IL-33 (Biolegend, 20ng/ml). After 3 days of culture GFP expression was analysed by flow cytometry.

2.5 Induction of intestinal inflammation

2.5.1 T cell transfer colitis and Treg mediated protection from colitis

Unless indicated otherwise $4x10^5$ $CD4^{+}CD25^{-}CD45RB^{hi}$ T cells were injected via the intra-peritoneal (i.p.) route into *Rag1*$^{-/-}$ recipients [379]. In co-transfer experiments $2x10^5$ $CD4^{+}CD25^{-}CD45RB^{hi}$ T cells from each source were mixed and injected i.p. into *Rag1*$^{-/-}$ hosts. For Treg mediated protection from colitis $4x10^5$ $CD4^{+}CD25^{-}$ $CD45RB^{hi}$ T cells and $2x10^5$ $CD4^{+}CD25^{+}CD45RB^{lo}$ were mixed and injected i.p. into

Rag1$^{-/-}$ hosts [174]. Weight was recorded once weekly throughout the course of the experiment and mice were sacrificed when weight loss approached 20% of the original body weight at the start of the experiment. Following sacrifice the whole colon was removed and pieces of colon approximately 1 cm in length were taken from the proximal, mid and distal portions. Samples were transferred immediately into 3.6% (w/v) formaldehyde solution.

2.5.2 *Helicobacter hepaticus* culture

Helicobacter hepaticus NCI-Frederick isolate 1A was grown at 37^{o}C on blood agar plates made with laked horse blood containing trimethoprim (5μg/ml), vancomycin (10 μg/ml) and polymyxin B (25 IU/ml) (TVP, Oxoid) under microaerophilic conditions (10% CO_2, 10% H_2, balance $N_{2)}$ and maintained in vented CampyPak jars (Oxoid). Following 2-3 days of culture on blood agar plates bacteria were transferred to liquid cultures of TSB supplemented with TVP and 10% FCS (Gibco) in vented Erlenmeyer flasks (Corning) and were grown under microaerophilic conditions at 37^{o}C on a shaking incubator set to 175rpm for a maximum of 24 hours. Cultures were inoculated at OD_{600} 0.05. Bacteria were pelleted using an ultracentrifuge at 7500rpm for 20 minutes. Viability of bacteria was assessed by a fluorescent Live/Dead assay (Molecular Probes) through visualisation on a fluorescent microscope.

2.5.3 Induction of *Helicobacter hepaticus*/anti-IL-10R colitis

Mice were fed 1x10^{8} cfu *Helicobacter hepaticus* by oral gavage with a 22G curved needle on day 0, day 1 and day 2 of the experiment. In addition mice received 1mg of an anti-IL-10R blocking antibody by i.p. injection once weekly starting at the day of *Helicobacter hepaticus* infection [264]. Mice were sacrificed at indicated time points and colon and caecum washed in PBS/0.1% BSA and fixed in a 3.6% (w/v) formaldehyde solution.

2.5.4 Generation of mixed bone marrow chimaeras

Rag1$^{-/-}$ mice were irradiated twice with 550 RAD from a ^{60}Co source with an interval of 3-4 hours between each course of irradiation. Single cell suspensions of bone marrow were prepared by flushing the femurs and tibias of mice with PBS/0.1% BSA. Bone marrow cells were passed through a 100µm cell strainer and centrifuged at 1500rpm for 5 minutes. Then ACK lysis buffer (150 mM NH_4CL, 10 mM, $KHCO_3$, 0.1 mM NA_2.EDTA, pH 7.2-7.4) was added for 3 minutes at room temperature to lyse red blood cells and thereafter cells were washed twice in PBS/0.1% BSA. Single cell suspension were mixed at a 1:1 ratio and 5-10x10^6 cells (2.5-5x10^6 of each population) were injected intravenously (i.v.) into irradiated *Rag1*$^{-/-}$ recipients. Mice were allowed to rest for at least 8 weeks to allow for full reconstitution of the hematopoietic system. Colitis was induced by infection with *Helicobacter hepaticus* as described above.

2.5.5 Histological assessment of colitis

Sections (4.0 μm) were prepared from fixed tissue samples using a microtome and stained with haematoxylin and eosin. Scoring was performed by two researchers in a blinded fashion according to the criteria described below. Proximal, mid and distal colon sections were scored individually and the average score was plotted as colitis score. Caecum scores were calculated separately. Images of sections were taken on a CoolScope microscope (Nikon).

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%)

INFLAMMATION IN LAMINA PROPRIA

0	None - few leucocytes
1	Mild - some increase in leucos at tips of crypts OR many lymphoid follicles
2	Moderate - marked infiltrate (notable broadening of crypt)
3	Severe - dense infiltrate throughout

AREA AFFECTED (% of section)

0	None
1	up to 25%
2	25-50%
3	>50%

MARKERS OF SEVERE INFLAMMATION

0	None
1	Submucosal inflammation OR Few crypt abscesses (<5)
2	Submucosal inflammation & Few Crypt Absecesses (<5) Many crypt abscesses (<5) OR Extensive submucosal inflamm OR Crypt branching (*Hh)*
3	Many crypt abscesses (<5) & Extensive submucosal inflammation / Crypt branching (*Hh)* Ulceration OR Extensive fibrosis

2.6 RNA extraction, RT-qPCR and microarray preparation

2.6.1 RNA extraction

RNA from single cell suspensions was extracted using RNeasy Mini kit (Qiagen) according to the manufacturers' protocol. A DNAase step was always included to minimize genomic DNA contamination. For RNA extraction from whole tissue, 0.25 cm pieces of proximal, mid and distal colon were snap frozen in liquid nitrogen and stored at -80^{o}C. Tissue was homogenised in TRI Reagent (Ambion) in Lysing Matrix D Tubes (MP Biomedicals) at 6.5 m/s for 15s in a FastPrep^{TM} 24 homogeniser (MP Biomedicals). Total RNA was then extracted using RiboPure™ Kit (Ambion) according to the manufacturers' protocol. RNA was quantified using a NanoDrop (Thermo Scientific) and in some cases RNA quality was assessed using the Agilent 2100 Bioanalyzer. RNA was snap frozen on dry ice and stored at -80^{o}C immediately.

2.6.2 Real-time quantitative PCR analysis

cDNA was synthesised using 1-5µg of total RNA with Superscript III reverse transcriptase and oligodT primers (Both from Invitrogen), according to the manufacturer's instructions. RT-qPCR reactions were performed using TaqMan Gene Expression Assays purchased from Applied Biosystems. All probes were labelled with FAM/TAMRA. RNA that was not reverse transcribed was used as a negative control to assess DNA contamination. Samples were assayed in triplicate in a final reaction volume of 20µl using the Bio-Rad CFX96 RT-qPCR machine. Gene expression levels for each individual sample were normalized to HPRT. Mean relative gene expression was determined and expressed as $2^{-\Delta C(t)}$ ($\Delta C_{(t)}=C_{(t)\text{gene of interest}}-C_{(t)Hprt}$) x 10000.

2.6.3 Microarray preparation and analysis

RNA was prepared as described above, RNA quality was assessed using the Agilent 2100 Bioanalyzer and only samples with RNA integrity number (RIN) values above 7 were used for further processing. Biotinylated cRNA was prepared from total RNA according to the manufacturers protocol (Illumina) and hybridised to MouseWG-6 v2.0 Expression BeadChip (Illumina). Preparation of biotinylated cRNA, hybridisation to BeadChips and sample acquisition were performed by the microarray core facility at the Wellcome Trust Center for Human Genetics, Oxford. Raw data was exported from Bead Studio and all further analysis was performed by the author of this thesis. 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.

2.7 Statistics

Non-parametric tests were used for all statistical analyses except analysis of weight loss and time course data for which a repeated measure ANOVA followed by Bonferroni post-hoc test was applied. The Mann Whitney U test or Wilcoxon matched-pairs signed rank test was used for comparison of two unpaired or paired groups respectively. For comparison of more than two groups a Kruskal Wallis one-way analysis of variance was performed followed by a Dunn's post-hoc test. All statistical analysis was performed in Prism (Graphpad). Data is depicted as the mean ± standard error of the mean (SEM). Differences were considered to be statistically significant when $p \leq 0.05$. Lacking data points are due to technical errors and do not represent intended data exclusion.

Chapter 3: IL-23 drives intestinal inflammation through direct effects on T cells

3.1 Introduction

Inflammatory bowel diseases (IBD), comprising Crohn's disease and ulcerative colitis, are chronic inflammatory diseases of the intestine that are thought to be a result of genetic defects in the host immune system that lead to inappropriate immune reactivity toward components of the intestinal flora [11]. Current treatment regimens for IBD are associated with considerable side-effects and more specific therapeutic targets are required for the successful treatment of IBD [370]. Genome-wide association studies in patients have linked polymorphisms in the *IL23R* gene locus to susceptibility or resistance to IBD [348, 387], suggesting that responsiveness to IL-23 is an important factor in determining disease outcome.

IL-23, which is composed of IL-23p19 and IL-12p40 [288], has been shown to be a key driver of pathology in various murine models of autoimmune and chronic inflammatory disease such as experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA) and intestinal inflammation [116, 264, 265, 291, 319, 323]. The functional receptor for IL-23 is composed of IL-23R and IL-12Rβ1 [304], signals predominantly through STAT3, and is expressed on a variety of immune cells such as myeloid cells, innate lymphoid cells, NKT cells, γδ T cells and αβ T cells [1, 304].

The functional activity of IL-23 has been studied extensively in the context of Th17 cell responses. Differentiation of naive $CD4^+$ T cells in the presence of TGF-β1 and pro-inflammatory cytokines IL-6, IL-21 or IL-1β promotes the expression of RORγt,

which drives expression of Th17 signature cytokines IL-17A and IL-17F and also promotes expression of IL-23R [121-128]. Although IL-23 is not necessary for the differentiation of Th17 cells *in vitro* [116, 117, 122, 124], protective [122] as well as pathogenic immune responses [116, 264, 265, 291, 319, 323] that have been linked to the activity of Th17 type cytokines are known to be dependent on IL-23. Indeed, direct signalling of IL-23 into $CD4^+$ T cells was recently shown to be necessary for the maintenance of IL-17A production by Th17 cells *in vivo* [131]. In the context of intestinal inflammation, genetic ablation or antibody mediated neutralisation of IL-23p19 have demonstrated an essential role for IL-23 in the development of T cell dependent colitis such as that induced by naïve T cell transfer [265, 324]. In this model RORγt was shown to be necessary for the development of colitis and this was linked to its role in promoting the induction of IL-17A and IL-17F [326]. By contrast, early studies demonstrated that the Th1 transcription factor T-bet [88] and the cytokine IFN-γ [87] contribute to pathology suggesting a link between IL-23 and IFN-γ production, although how IL-23 promotes Th1 responses in this setting is currently unknown. Recent studies have demonstrated that the Th17 phenotype is intrinsically unstable and acquisition of IFN-γ production by Th17 cells has been shown to contribute to their pathogenicity [132, 268]. In particular, IL-23 has been shown to drive IFN-γ-expression by Th17 cells in a T-bet dependent manner *in vitro* [132]. However, whether IL-23 signals directly into T cells to modulate the Th17 cell phenotype during intestinal inflammation is currently unclear because IL-23 has also been shown to induce the secretion of cytokines by innate immune cells that can modulate T cell responses, such as IL-1β, TNF-α, IL-6 and IFN-γ.

Here, we have utilised IL-23R reporter mice to track and characterise IL-23-responsive cells during the course of intestinal inflammation, and used selective ablation of IL-23R in the innate and T cell compartments to identify the cell types and mechanisms through which IL-23 drives intestinal inflammation.

3.2 Results

3.2.1 Validation of IL-23R reporter mice

The importance of IL-23 as a potent driver of intestinal inflammation is well documented [264, 265, 291, 323]. However, little is known about the cellular populations that are responsive to IL-23. This is partly due to the lack of monoclonal antibodies that reliably detect IL-23R expression. Recently, Awasthi et al generated an IL-23R GFP reporter strain to track IL-23 responsive cells *in vivo* [1]. In these reporter mice an IRES-GFP cassette has been inserted between exon 8 and 10 of the endogenous *Il23r* locus (Figure 3.1A). This knock-in results in a functional knock-out when mice are bred as homozygotes (IL-23R$^{gfp/gfp}$) but allows tracking of IL-23 responsive cells in heterozygote mice (IL-23R$^{gfp/+}$). To verify the validity of this reporter strain, IL-23R^{+} and IL-23R^{-} cells were FACS sorted from the mesenteric lymph nodes of naive IL-23R$^{gfp/+}$ mice (Figure 3.1B). As expected, *Il23r* mRNA expression was confined to the IL-23R^{+} fraction (Figure 3.1C) and stimulation with IL-23 induced expression of *Il22* mRNA in IL-23R^{+} but not IL-23R^{-} cells (Figure 3.1D). Thus, IL-23R$^{gfp/+}$ mice reliably report IL-23R expression as assessed in this assay while retaining the ability to respond to IL-23.

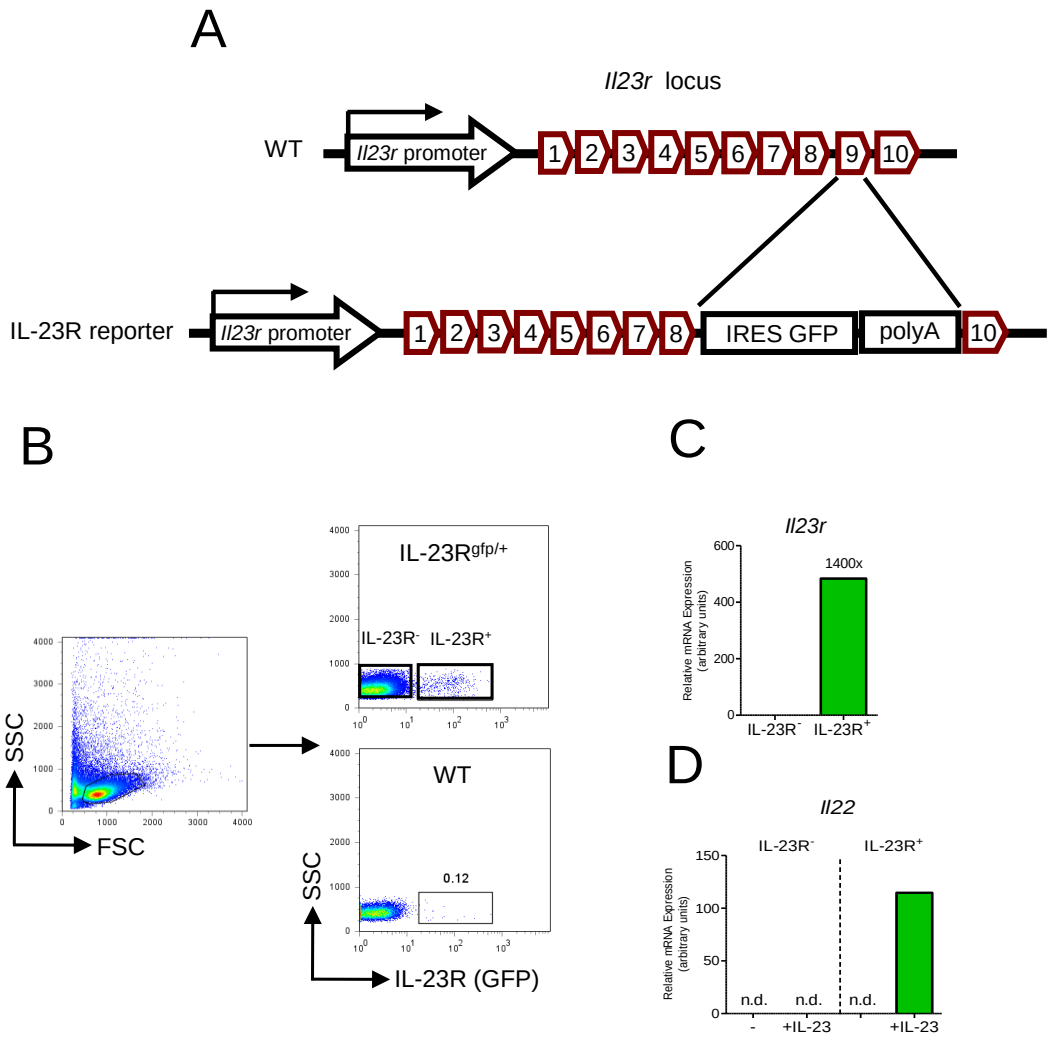

Figure 3.1. Validation of IL-23R reporter mice

(A) IL-23R reporter mice have an IRES-EGFP cassette introduced between exon 8 and 10 of the endogenous IL-23R gene [1].

(B) Representative FACS plots showing the gating strategy for sorting of GFP^+ cells from the MLN of heterozygous IL-23R reporter mice ($IL\text{-}23R^{gfp/+}$).

(C) *Il23r* mRNA expression in $IL\text{-}23R^-$ and $IL\text{-}23R^+$ cells sorted as described in B. Transcript levels were normalised to *Hprt*. n=1.

(D) $5x10^4$ $IL\text{-}23R^-$ and $IL\text{-}23R^+$ cells were incubated overnight with or without IL-23 (20ng/ml) and *Il22* mRNA expression was determined. Transcript levels were normalised to *Hprt* and n=1 mouse Transcripts below the limit of detection are labelled n.d. (not detectable).

3.2.2 IL-23R$^+$CD4$^+$ T cells accumulate during intestinal inflammation

Next we set out to identify IL-23R expressing cells in the steady state and during chronic intestinal inflammation. To this end we induced colitis in IL-23R$^{gfp/+}$ mice by oral infection with *Helicobacter hepaticus (Hh)* and concomitant administration of IL-10R blocking antibody (Figure 3.2A) [264]. Importantly, there was no difference between the colitis scores in IL-23R$^{gfp/+}$ mice and published colitis scores in WT IL-23R$^{+/+}$ mice [264], suggesting that one functional IL-23R allele is sufficient for the development of intestinal inflammation in this model. We focused our analysis of IL-23R$^+$ cells on lymphocytes as we could not reliably detect IL-23R expression among non-lymphocyte populations (data not shown). Interestingly, the frequency of IL-23R$^+$ lymphocytes in the colon did not increase significantly during inflammation as compared to steady state control mice (Figure 3.2B). However, we observed marked differences in the cellular composition of colonic IL-23R$^+$ lymphocytes. In steady state control mice, CD4$^+$ T cells constituted one third of IL-23R$^+$ lymphocytes while approximately 90% of IL-23R$^+$ lymphocytes in the inflamed colon were CD4$^+$ T cells (Figure3.2C). In addition, we observed a marked increase in the total number of IL-23R expressing CD4$^+$ T cells during the course of colitis and this accumulation correlated with the kinetics of pathological change in the colon as indicated by colitis scores (Figure 3.2D-E). In summary, IL-23R expressing CD4$^+$ T cells accumulate during the development of colitis and represent the dominant population of IL-23R$^+$ lymphocytes in the inflamed colon.

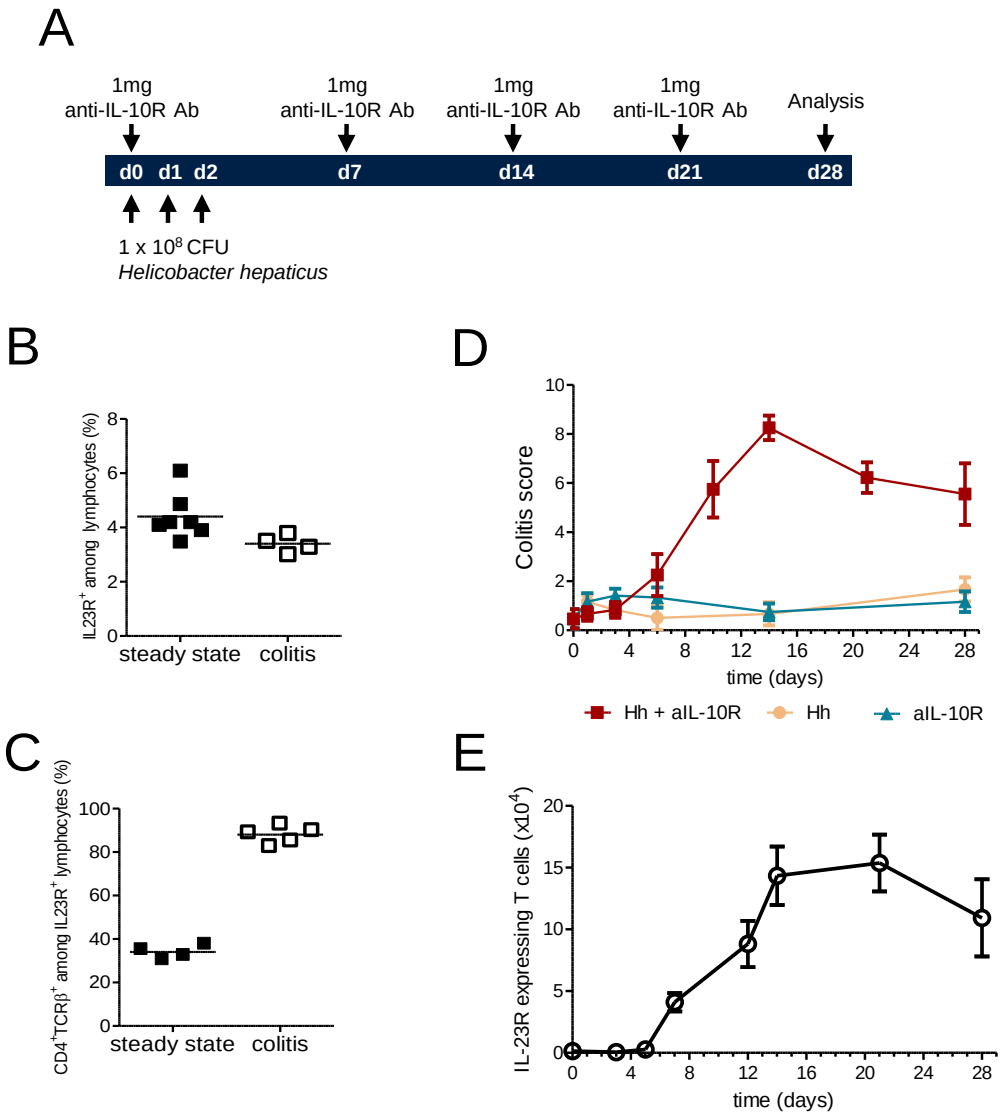

Figure 3.2. IL-23R expressing CD4$^+$ T cells accumulate during chronic intestinal inflammation

C57BL/6 IL-23R$^{gfp/+}$ mice were sacrificed without treatment (steady state) or infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* for three consecutive days with concomitant *i.p.* injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at indicated time points and intestinal inflammation was assessed by histological analysis.

(A) Schematic showing the experimental protocol for the induction of *Helicobacter hepaticus*/anti-IL- 10R colitis.

(B) Frequency of colonic CD45$^+$IL-23R$^+$ cells in the steady state or 4 weeks post induction of colitis. Cells were gated on lymphocytes (defined by size and granularity). Bars represent the mean ±SEM. (n=5 mice).

(C) Frequency of CD4$^+$ T cells among CD45$^+$IL-23R$^+$ lymphocytes in the colon lamina propria. Bars represent the mean ±SEM. (n=5 mice).

(D) Histological analysis of colitis at the indicated time points. Mice were either infected with *Helicobacter hepaticus* (Hh), treated with anti-IL-10R (aIL-10R) or received both *Helicobacter hepaticus* and anti-IL-10R (Hh + aIL-10R). Symbols represent the mean ±SEM (n=4 mice at each time point).

(E) Total number of IL-23R$^+$ CD4$^+$ T cells in the colon at indicated time points post induction of colitis. Bars represent the mean ±SEM (n=3-11 mice).

3.2.3 IL-23R is preferentially expressed by colonic $CD4^+$ T cells in steady state and inflamed mice

Previous work from our group [291] and others [300] showed that IL-23 is preferentially expressed in the colon rather than secondary lymphoid organs and this expression pattern correlates with its ability to promote intestinal but not systemic inflammation [291]. We hypothesized that the same concept could apply to the regulation of IL-23R on $CD4^+$ T cells. Therefore we analyzed the frequency of IL-23R expressing $CD4^+$ T cells in the spleen, MLN and colon of inflamed and steady state control mice. In the steady state, the highest proportion of IL-23R expressing $CD4^+$ T cells was found in the colon (~15%) whereas less than 1% of $CD4^+$ T cells in spleen and MLN expressed IL-23R (Figure 3.3A). Upon induction of intestinal inflammation with *Helicobacter hepaticus* and IL-10R blockade the frequency of IL-23R expressing $CD4^+$ T cells was markedly increased across all analyzed organs (Figure 3.3B). However, the proportion of IL-23R expressing $CD4^+$ T cells remained the highest in the colon with approximately 25% of cells expressing the IL-23R as compared to 3% - 5% in the spleen and MLN, respectively. Thus, IL-23R is preferentially expressed on colonic $CD4^+$ T cells and its expression is further induced in the context of inflammation.

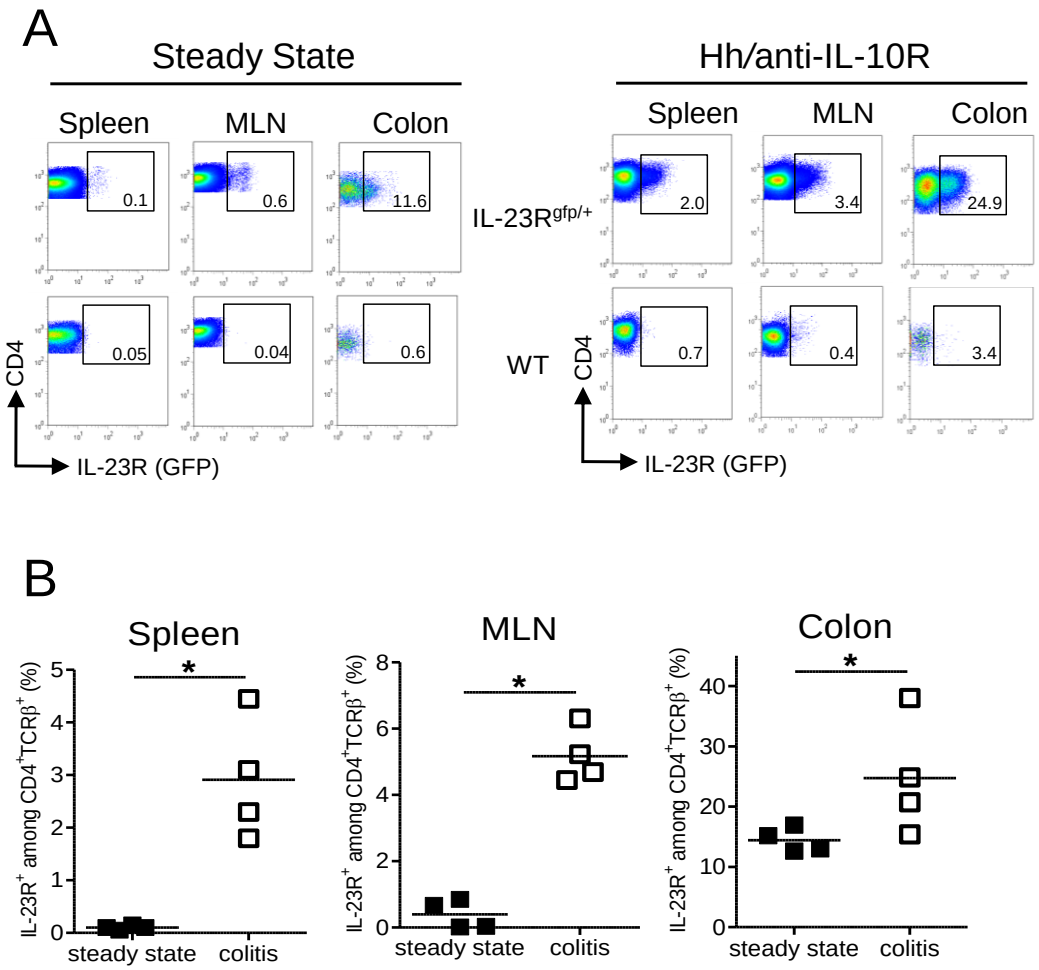

Figure 3.3. IL-23R$^+$CD4$^+$ T cells are enriched in the colon of naïve and inflamed mice

C57BL/6 WT or IL-23R$^{gfp/+}$ mice were sacrificed without treatment (steady state) or following infection by oral gavage with ~10^8 cfu *Helicobacter hepaticus* (Hh) for three consecutive days with concomitant *i.p.* injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at 4 weeks post infection and cells were isolated from indicated organs and analysed by FACS.

(A) Representative FACS plots of CD4$^+$ T cells from the spleen, MLN and colon from steady state and inflamed WT or IL-23R$^{gfp/+}$ mice. Numbers in plots represent frequencies of IL-23R$^+$ cells.

(B) Frequency of IL-23R$^+$ cells among CD4$^+$ T cells from the spleen, MLN and colon from steady state and inflamed IL-23R$^{gfp/+}$ mice. Bars represent the mean ±SEM.

Data represent results from a single experiment (n=4 mice per group). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$.

3.2.4 IL-23R expressing CD4$^+$ T cells produce IL-17A and IFN-γ

IL-23R expression is highly enriched on Th17 cells [117] and IL-23 signalling has been shown to be required for sustained expression of IL-17A *in vivo* [131]. Although the role of IL-17A in *Helicobacter hepaticus*/anti-IL-10R induced colitis has not been investigated, expression of this pro-inflammatory cytokine is increased in the inflamed colon of *Hh*$^+$ mice [264]. In addition, IFN-γ deficiency ameliorates colitis in anti–IL-10R–treated *Hh*$^+$ mice and IL-23 has been linked to induction of IFN-γ production by CD4$^+$ T cells in this model [264]. To assess whether IL-23R expression is associated with IL-17A and/or IFN-γ producing CD4$^+$ T cells we assessed the expression of these cytokines in IL-23R$^+$ CD4$^+$ T cells of inflamed mice. Due to the quenching of the GFP signal by the fixation and permeabilisation procedure required for assessment of intracellular cytokine levels we FACS sorted IL-23R$^+$CD4$^+$ T cells from the spleen, MLN and colon prior to analysis. We found three distinct populations of CD4$^+$ effector T cells based on the expression of IFN-γ and IL-17A (Figure 3.4A). The IL-23R$^+$ fraction of CD4$^+$ T cells was highly enriched in IL-17A$^+$IFN-γ$^-$ cells in all analyzed organs (Figure 3.4B). Interestingly, colonic CD4$^+$ T cells contained a higher proportion of IL-17A$^+$IFN-γ$^+$ and IL-17A$^-$IFN-γ$^+$ cells as compared to IL-23R$^+$ CD4$^+$ T cells in the spleen and MLN, suggesting that colonic IL-23R$^+$ T cells are phenotypically distinct from IL-23R$^+$ cells in secondary lymphoid organs.

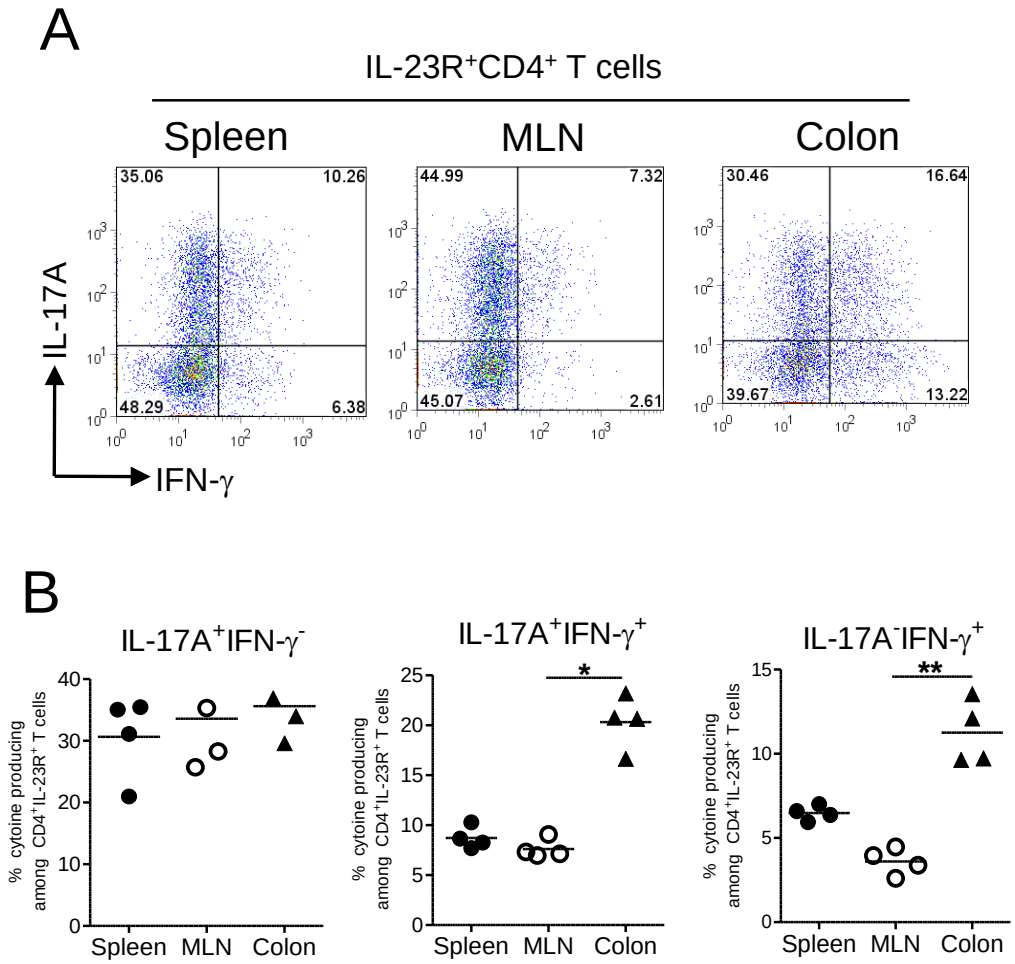

Figure 3.4. IL-17A and IFN-γ expression by IL-23R$^+$CD4$^+$ T cells

C57BL/6 IL-23R$^{gfp/+}$ mice were infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* for three consecutive days with concomitant *i.p*. injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at 4 weeks post infection and viable IL-23R$^+$TCRβ$^+$CD4$^+$ T cells were FACS sorted on the basis of GFP expression from the indicated organs. Cells were re-stimulated with PMA and Ionomycin in the presence of Brefeldin A for 4 hours.

(A) Representative FACS plots showing cytokine production by IL-23R$^+$ CD4$^+$ T cells from the indicated organs.

(B) Frequency of IL-17A$^+$ and/or IFN-γ$^+$ T cells among IL-23R$^+$CD4$^+$ T cells in various organs. Bars represent the mean and each point represents an individual mouse.

Data represent results from a single experiment (n= 4 mice). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test.* $p \leq 0.05$.

3.2.5 IL-23R is required for the development of *Helicobacter hepaticus*/anti-IL-10R colitis

Given the accumulation of IL-23R-expressing $CD4^+$ T cells capable of producing pro-inflammatory cytokines in the inflamed colon we decided to probe the requirement for IL-23R signalling in the development of intestinal inflammation using *Il23r*$^{-/-}$ mice. WT mice infected with *Helicobacter hepaticus* and treated with anti-IL-10R antibody developed severe intestinal inflammation in the colon as well as the caecum (Figure 3.5A-B). By contrast, *Il23r*$^{-/-}$ mice were protected from the development of colitis and only showed mild signs of inflammation in the caecum. This reduced intestinal pathology correlated with significantly reduced numbers of $CD4^+$ T cells in the colon while no difference in total number of $CD4^+$ T cells was observed in the spleen (Figure 3.5C). Next we assessed the impact of IL-23R deficiency on cytokine expression by intestinal $CD4^+$ T cells. Despite our previous observation that IL-17A expressing cells are highly enriched among IL-$23R^+CD4^+$ T cells we found similar frequencies of IL-$17A^+$IFN-γ^- cells among WT and *Il23r*$^{-/-}$ $CD4^+$ T cells in the colon (Figure 3.6A-B). By contrast, the frequencies of IL-$17A^+$IFN-γ^+ and IL-$17A^-$IFN-γ^+ cells were significantly reduced among *Il23r*$^{-/-}$ $CD4^+$ T cells in the intestine suggesting a role for IL-23 in the differentiation of these particular cells. In addition, the total number of all three cytokine producing $CD4^+$ T cell populations was significantly reduced in the absence of IL-23R signalling (Figure 3.6C) demonstrating that IL-23 is required for the accumulation of effector $CD4^+$ T cells in the colon regardless of their cytokine profile.

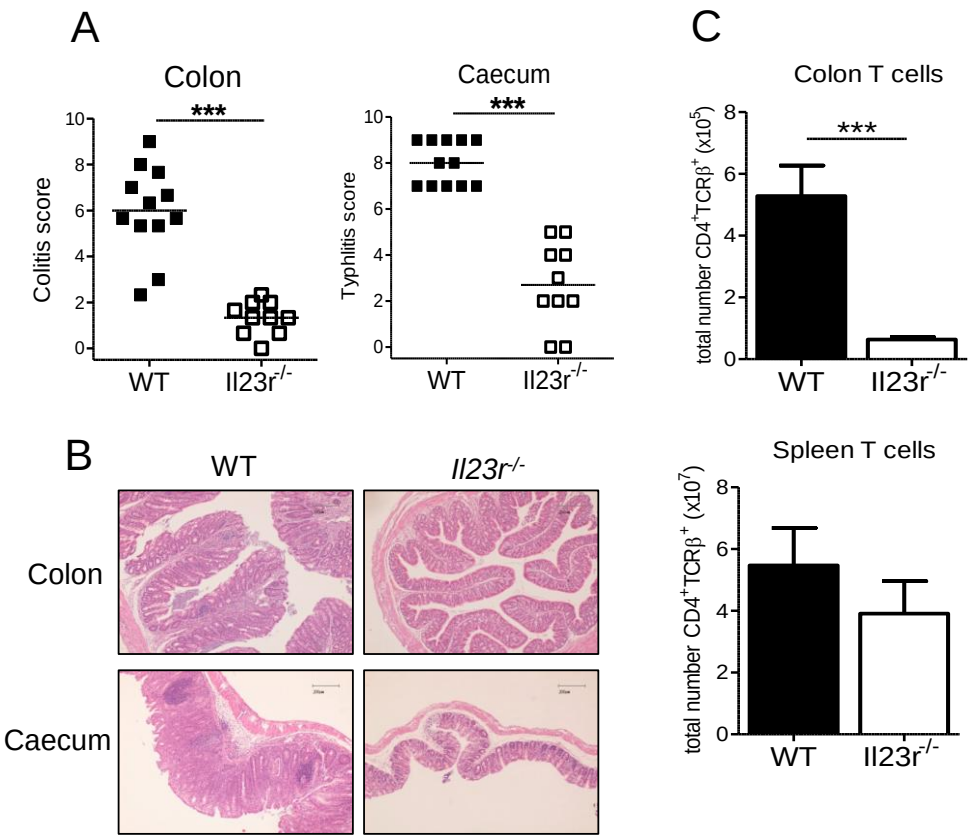

Figure 3.5. IL-23R is required intestinal inflammation in the *Helicobacter hepaticus* /anti-IL-10R model of colitis

C57BL/6 WT or *Il23r*$^{-/-}$ mice were infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* for three consecutive days with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at 4 weeks post infection and intestinal inflammation assessed.

(A) Histological analysis of colonic and caecal inflammation. Bars represent the mean and each point represents an individual mouse.

(B) Representative photomicrographs of proximal colon and caecum sections (50X magnification).

(C) Total number of $CD4^+$ T cells in the colon or spleen. Bars represent the mean ±SEM.

Data represents pooled results from two independent experiments (n=12 mice for WT, n=10 mice for *Il23r*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. *** $p \leq 0.001$.

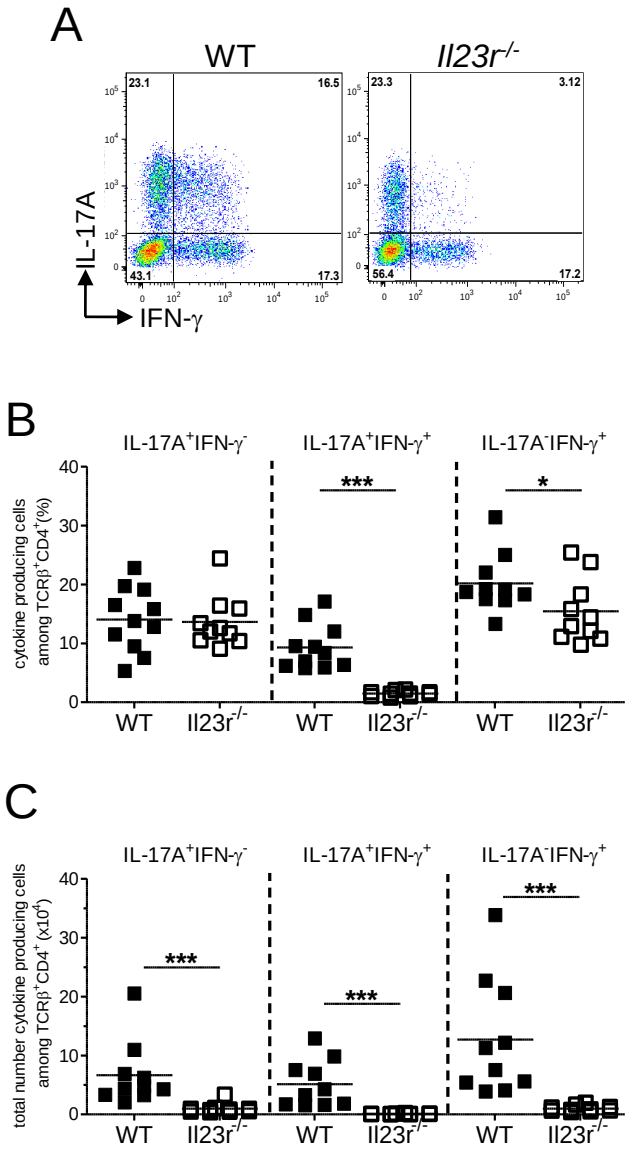

Figure 3.6. IL-23 drives the emergence of IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells

C57BL/6 WT or *Il23r*$^{-/-}$ mice were infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* for three consecutive days with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at 4 weeks post infection and cells from the colonic lamina propria re-stimulated with PMA and Ionomycin in the presence of Brefeldin A for 4 hours.

(A) Representative FACS plots of colonic lamina propria cells, gated on viable CD4$^+$ T cells.

(B) Frequency of IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells present in the colon. Bars represent the mean and each point represents an individual mouse.

(C) Total number of IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells present in the colon. Bars represent the mean and each point represents an individual mouse.

Data represents pooled results from two independent experiments (n=11 mice for WT, n=10 mice for *Il23r*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$, *** $p \leq 0.001$.

3.2.6 IL-23 promotes the emergence of IL-17A$^+$IFN-γ$^+$ T cells in a cell intrinsic manner

A major caveat of comparing CD4$^+$ T cell differentiation in WT and *Il23r*$^{-/-}$ mice is the lack of intestinal inflammation in the absence of IL-23R. Consequently, we could not exclude that the difference in the frequency of cytokine producing CD4$^+$ T cells between WT and *Il23r*$^{-/-}$ mice is secondary to the lack of inflammation. To circumvent this problem we generated mixed bone marrow chimaeras by reconstituting irradiated *Rag1*$^{-/-}$ mice with WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) bone marrow cells at 1:1 ratio (Figure 3.7A). Following 8 weeks of rest, to allow for bone marrow reconstitution of the hematopoietic system, we induced intestinal inflammation through infection with *Helicobacter hepaticus* and administration of IL-10R blocking antibody. Importantly, intestinal pathology in mixed bone marrow chimaeras was indistinguishable from that in WT mice (Figure 3.7B & Figure 3.5A) and thus allowed us to investigate WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the same inflammatory environment. We found similar frequencies and total numbers of WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the colon indicating that *Il23r*$^{-/-}$ CD4$^+$ T cells can accumulate normally in the presence of WT bone marrow cells (Figure 3.7C). By contrast, the reduced frequency of IL-17A$^+$IFN-γ$^+$ cells among *Il23r*$^{-/-}$ CD4$^+$ T cells was still evident in this inflammatory context (Figure 3.7D). However, we no longer observed a difference in IL-17A$^-$IFN-γ$^+$ cells between WT and *Il23r*$^{-/-}$ CD4$^+$ T cells. This data strongly suggest that IL-23 controls the differentiation of IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells through cell intrinsic effects.

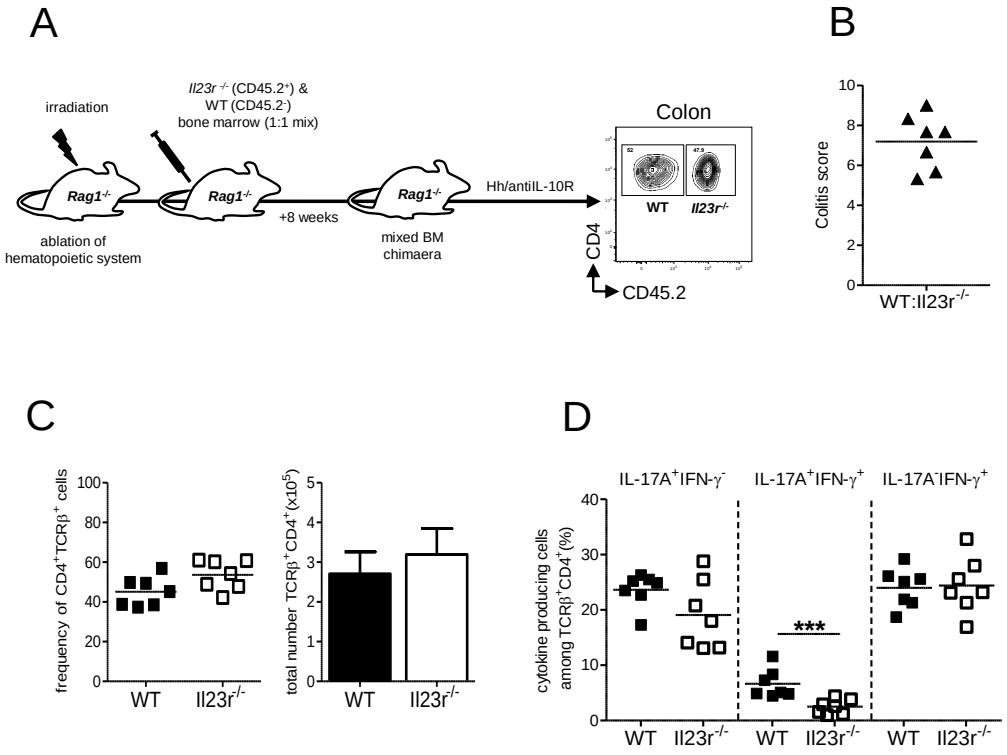

Figure 3.7. IL-23 drives the emergence of IL-17A$^+$IFN-γ^+ CD4$^+$ T cells in a direct cell intrinsic manner

Mixed bone marrow chimaeras were generated by lethal irradiation of C57BL/6 *Rag1*$^{-/-}$ mice followed by i.v. injection of 2.5x10^6 WT (CD45.2$^-$) and 2.5x10^6 *Il23r*$^{-/-}$ (CD45.2$^+$) bone marrow cells. Mice were allowed to rest for 8 weeks to allow re-constitution of the hematopoietic system. Re-constituted mice were infected orally with ~10^8 cfu *Helicobacter hepaticus* and received weekly i.p. injections of IL-10R blocking antibody. Mice were sacrificed at 2 weeks post infection and cells were isolated from the colonic lamina propria. Single cell suspensions were re-stimulated with PMA and Ionomycin in the presence of Monensin for 4 hours. Expression of cytokines was determined by FACS.

(A) Schematic explaining the generation of mixed bone marrow chimaeras, FACS plot is representative.

(B) Histological analysis of colitis. Bars represent the mean and each point represents an individual mouse.

(C) Frequency and total number of WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the colon of mixed bone marrow chimaeras. Bars represent the mean ±SEM.

(D) Frequency of IL-17A$^+$ and/or IFN-γ^+ CD4$^+$ T cells present in the colon. Bars represent the mean and each point represents an individual mouse.

Data represents results from a single experiment (n=7 mice). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. *** $p \leq 0.001$.

3.2.7 IL-23R signalling into CD4$^+$ T cells but not innate cells is required for the development of T cell transfer colitis

Although the *Helicobacter hepaticus*/anti-IL-10R model of colitis is an important tool for the analysis of intestinal inflammation in lymphocyte replete mice, it does not provide the means to restrict IL-23 responsiveness to particular cell types. Therefore, we utilised the T cell transfer model of colitis [379] to determine whether IL-23 exerts its effects on CD4$^+$ T cell accumulation and differentiation by direct signalling into CD4$^+$ T cells or indirectly through actions on innate immune cells. In this model, naive CD4$^+$CD25$^-$CD45RB^{hi} T cells from WT donors are adoptively transferred into *Rag1*$^{-/-}$ mice, which lack any endogenous T or B cells. This results in a rapid proliferative response of the transferred CD4$^+$ T cells accompanied by the acquisition of effector function and subsequent development of severe intestinal inflammation [174, 379]. Because T cell donors and recipient mice are different entities we could restrict IL-23 responsiveness to the innate compartment by adoptive transfer of *Il23r*$^{-/-}$ CD4$^+$ T cells into *Rag1*$^{-/-}$ hosts; or to the T cell compartment by adoptive transfer of WT CD4$^+$ T cells into *Il23r*$^{-/-}$*Rag1*$^{-/-}$ hosts. We found that transfer of WT CD4$^+$ T cells into either *Rag1*$^{-/-}$ or *Il23r*$^{-/-}$*Rag1*$^{-/-}$ recipients resulted in comparable intestinal pathology and CD4$^+$ T cell accumulation in the colon (Figure 3.8A-B). There was a trend towards reduced colitis in *Il23r*$^{-/-}$*Rag1*$^{-/-}$ hosts transferred with WT CD4$^+$ T cells but this was not statistically significant. By contrast, transfer of naive CD4$^+$ T cells from *Il23r*$^{-/-}$ donors into *Rag1*$^{-/-}$ hosts only led to mild intestinal inflammation which correlated with reduced numbers of CD4$^+$ T cells in the colon. In addition, deficiency of IL-23R in T cells or innate cells did not affect CD4$^+$ T cell accumulation in the spleen (Figure 3.8C). Similar to our findings in the *Helicobacter hepaticus*/anti-IL-10R model the frequency of IL-17A$^+$IFN-γ^+ T cells, but not IL-17A or IFN-γ

producing cells, was significantly reduced in *Il23r*$^{-/-}$ CD4^{+} T cells transferred into *Rag1*$^{-/-}$ recipients (Figure 3.8D). By contrast, WT CD4^{+} T cells transferred into either *Rag1*$^{-/-}$ or *Il23r*$^{-/-}$*Rag1*$^{-/-}$ mice showed no defect in the differentiation of IL-17A and/or IFN-γ producing CD4^{+} T cells. Thus, IL-23 signalling into CD4^{+} T cells, but not innate cells, is required for the development of T cell transfer colitis and the emergence of IL-17A^{+}IFN-γ^{+} T cells.

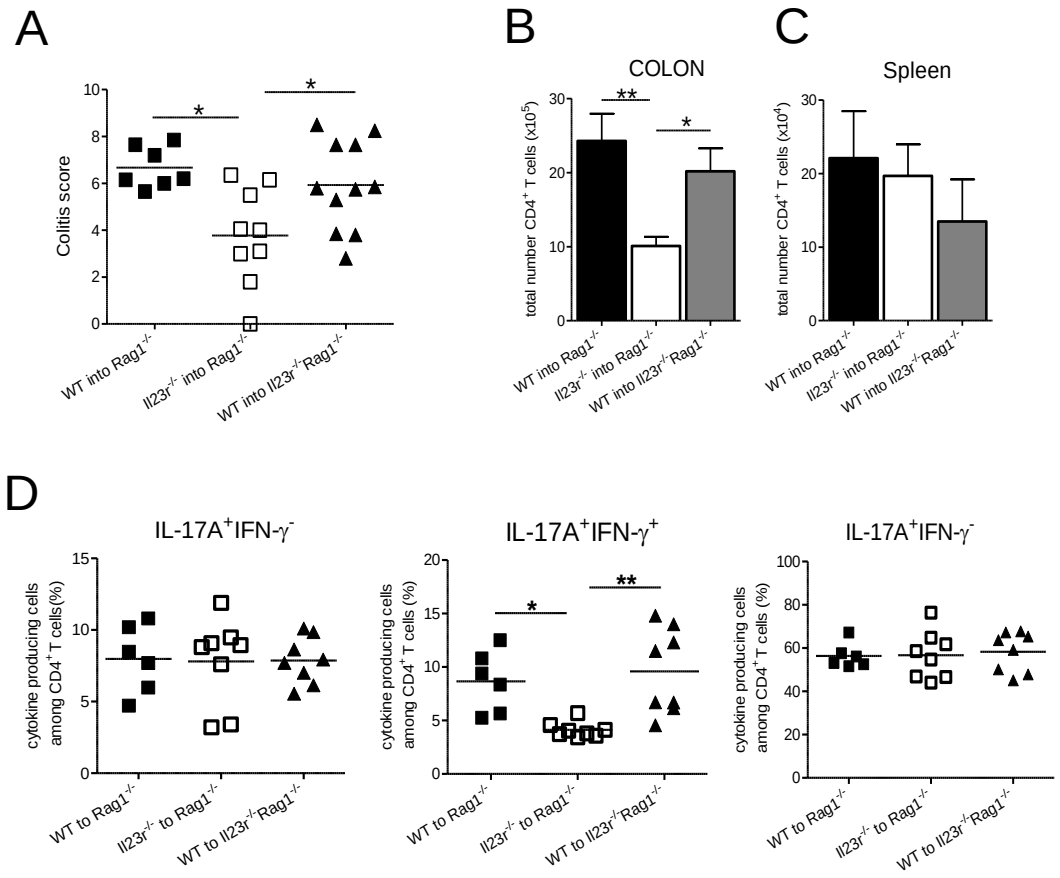

Figure 3.8. IL-23R signalling into CD4$^+$ T cells but not innate cells is required for the development of T cell transfer colitis

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT or *Il23r*$^{-/-}$ donors. In addition, C57BL/6 *Il23r*$^{-/-}$*Rag1*$^{-/-}$ mice were injected with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from C57BL/6 WT donors. Mice were sacrificed when recipients of WT T cells developed clinical signs of disease (6-8 weeks) and assessed for intestinal inflammation. Single cell suspensions from the colonic lamina propria were re-stimulated with PMA and Ionomycin in the presence of Brefeldin A for 4 hours and expression of cytokines determined by FACS.

(A) Histological analysis of colitis. Bars represent the mean and each point represents an individual mouse.

(B) Total number of TCRβ$^+$ cell in the colon. Bars represent the mean ±SEM.

(C) Frequency of IL-17A$^+$ and/or IFN-γ$^+$ T cells in the colonic lamina propria. Bars represent the mean and each point represents an individual mouse.

Data represents results from a single experiment (n=6-7 mice for WT to *Rag1*$^{-/-}$, n=8-9 mice for *Il23r*$^{-/-}$ to *Rag1*$^{-/-}$, n=8-10 mice for WT to *Il23r*$^{-/-}$ *Rag1*$^{-/-}$). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test (B). * $p \leq 0.05$, ** $p \leq 0.01$.

3.2.8 IL-23 promotes the differentiation of IL-17A$^+$IFN-γ$^+$ T cells through direct cell intrinsic effects on CD4$^+$ T cells.

Our experiments in *Hh*$^+$ anti-IL-10R-treated lymphoreplete mice strongly suggest that IL-23 controls the differentiation of IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells through cell intrinsic effects. To assess whether IL-23 drives the emergence of this population through direct cell intrinsic effects on CD4$^+$ T cells we utilised an experimental approach that allowed us to transfer WT and *Il23r*$^{-/-}$ CD4$^+$ T cells into the same *Rag1*$^{-/-}$ recipient. To achieve this, we transferred an equal number of WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) naive CD4$^+$ T cells into *Rag1*$^{-/-}$ recipients (Figure 3.9A). As a control we also transferred WT (CD45.2$^-$) and WT (CD45.2$^+$) naive CD4$^+$ T cells at a 1:1 ratio into a separate cohort of *Rag1*$^{-/-}$ hosts. Recipients of WT:WT or WT:*Il23r*$^{-/-}$ mixtures developed comparable intestinal inflammation and similar proportions of WT and *Il23r*$^{-/-}$ CD4$^+$ T cells were present in the inflamed intestines (Figure 3.9B-C), suggesting that the presence of IL-23R$^+$ WT CD4$^+$ T cells is sufficient to promote accumulation of *Il23r*$^{-/-}$ CD4$^+$ T cells in the colon. We observed equivalent differentiation of IL-17A$^+$IFN-γ$^-$ and IL-17A$^-$IFN-γ$^+$ cells among WT and *Il23r*$^{-/-}$ CD4$^+$ T cells (Figure 3.9D). By contrast, the frequency of IL-17A$^+$IFN-γ$^+$ T cells was significantly reduced among the *Il23r*$^{-/-}$ CD4$^+$ T cells. Thus, direct cell intrinsic IL-23 signals into CD4$^+$ T cells are required for the emergence of IL-17A$^+$IFN-γ$^+$ T cells as the impaired differentiation of this population in *Il23r*$^{-/-}$ CD4$^+$ T cells cannot be rescued by the presence of IL-23R responsive CD4$^+$ T cells within the same recipient.

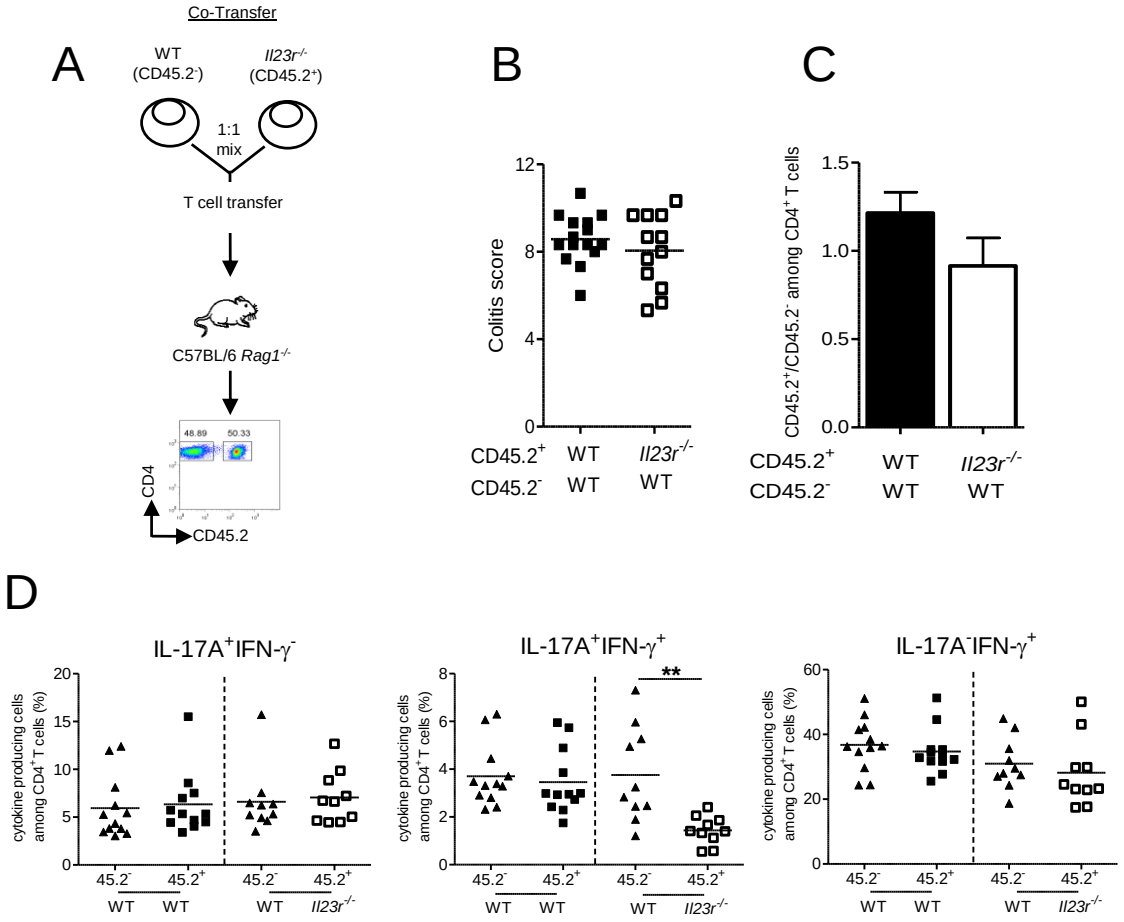

Figure 3.9. IL-23 drives the emergence of IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells through direct cell intrinsic effects

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 1:1 mixtures of WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) or WT (CD45.2$^-$) and WT (CD45.2$^+$) CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed upon development of clinical signs of inflammation (~8 weeks). Extend of colonic pathology was assessed and single cell suspensions from the colonic lamina propria were re-stimulated with PMA and Ionomycin in the presence of Brefeldin A for 4 hours.

(A) Schematic explaining the experimental strategy. FACS plot is representative.

(B) Histological analysis of colitis. Bars represent the mean and each point represents an individual mouse.

(C) Ratio of CD45.2$^+$ over CD45.2$^-$ CD4$^+$ T cells from the colon. Bars represent the mean ±SEM.

(D) Frequency of IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells in the colonic lamina propria gated on either WT or *Il23r*$^{-/-}$ CD4$^+$ T cells as indicated. Bars represent the mean and each point represents an individual mouse.

Data represent pooled results from two independent experiments (n=12 mice (WT:WT), n=10 mice (WT:*Il23r*$^{-/-}$). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. ** $p \leq 0.01$.

3.2.9 IL-23R signalling into CD4$^+$ T cells is not required for T cell homing to the intestine

Next, we hypothesised that IL-23 may mediate its effect on CD4$^+$ T cell accumulation through effects on T cell homing to the intestine. To assess this we transferred *Rag1*$^{-/-}$ mice with WT or *Il23r*$^{-/-}$ CD4$^+$ T cells and sacrificed recipients 12 days after transfer, prior to the onset of inflammation. We found equal numbers of CD4$^+$ T cells in the spleen and colon suggesting that *Il23r*$^{-/-}$ T cells are not deficient in their ability to home to the intestine at this early time-point (Figure 3.10A-B). In addition, colonic CD4$^+$ T cells isolated from *Rag1*$^{-/-}$ recipients of WT or *Il23r*$^{-/-}$ T cells did not differ in their mRNA expression of pro-inflammatory chemokine receptors *Ccr6, Cxcr3, Ccr2* and *Ccr5* (Figure 3.10C). Although IL-23 did not control chemokine receptor expression it could affect recruitment of T cells to the intestine through the modulation of chemokine expression by CD4$^+$ T cells. However, we did not observe impaired expression of pro-inflammatory chemokines *Ccl20, Cxcl10, Ccl3* and *Ccl5* in *Il23r*$^{-/-}$ CD4$^+$ T cells at the mRNA level (Figure 3.10C). Together, these results suggest that *Il23r*$^{-/-}$ CD4$^+$ T cells are not defective in their ability to home to the intestine.

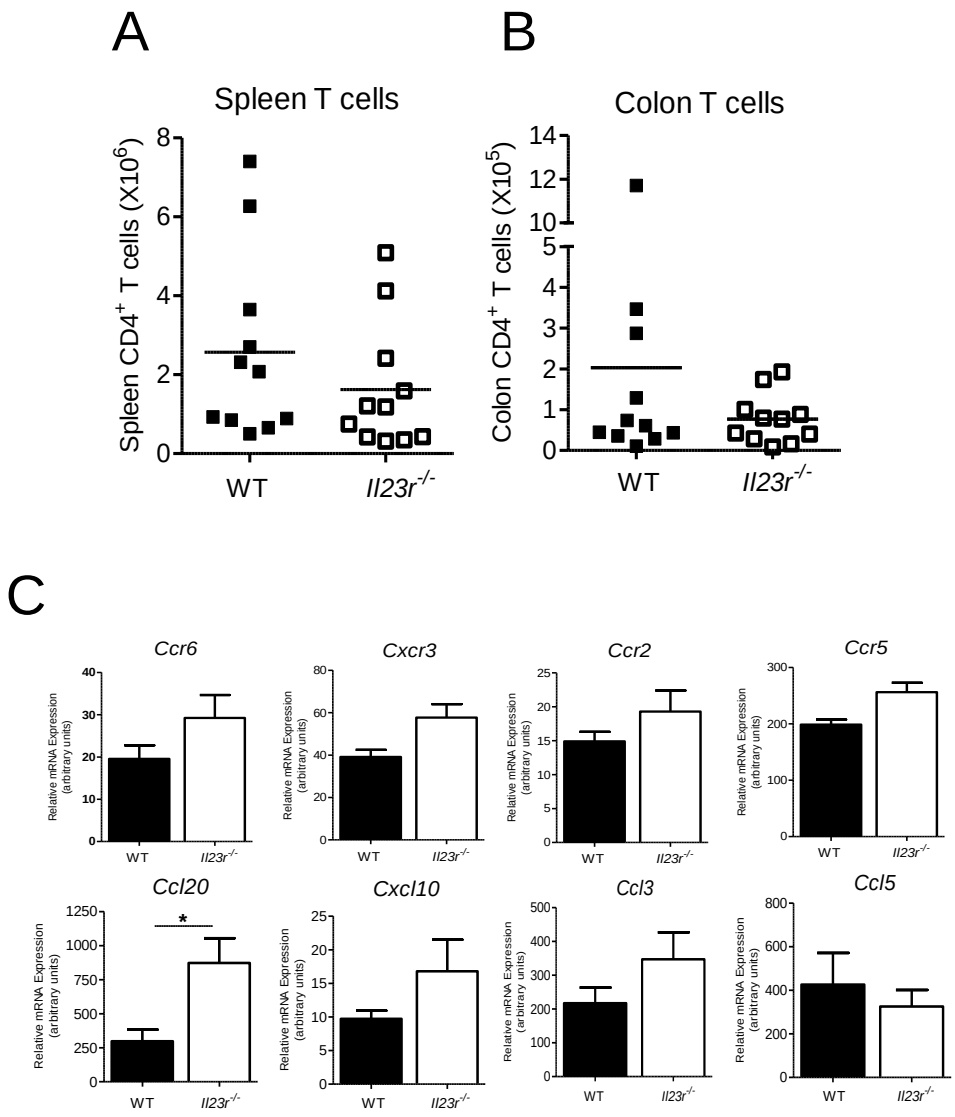

Figure 3.10. IL-23R is not required for CD4$^+$ T cell homing to the intestine

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^6 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT or *Il23r*$^{-/-}$ donors and sacrificed 12 days after transfer. In addition, a second cohort of C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT or *Il23r*$^{-/-}$ donors and sacrificed when recipients of WT T cells developed clinical signs of disease (6-8 weeks).

(A) Total number of CD4$^+$ T cells in the colonic lamina propria at day 12 post transfer. Bars represent the mean and each point represents an individual mouse.

(B) Total number of CD4$^+$ T cells in the spleen at day 12 post transfer. Bars represent the mean and each point represents an individual mouse.

(C) RT-qPCR analysis of FACS sorted WT or *Il23r*$^{-/-}$ colonic CD4$^+$ T cells 8 weeks post transfer. Transcript levels were normalised to *Hprt*. Bars represent the mean ±SEM.

Data represents results from a single experiment. (n=5-11 mice for WT, n=5-11 mice for *Il23r*$^{-/-}$) Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$.

3.2.10 IL-23 promotes intestinal CD4$^+$ T cell proliferation through cell extrinsic effects

To test whether IL-23 promotes T cell accumulation through effects on cellular proliferation we compared the frequency of Ki-67$^+$ CD4$^+$ T cells in *Rag1*$^{-/-}$ mice transferred with WT or *Il23r*$^{-/-}$ CD4$^+$ T cells. We found a trend towards reduced frequencies and significantly decreased numbers of Ki-67$^+$ cells among IL-17A$^+$IFN-γ^-, IL-17A$^-$IFN-γ^+ and IL-17A$^+$IFN-γ^+ in *Il23r*$^{-/-}$ compared to WT CD4$^+$ T cell (Figure 3.11A-C). Thus, it appears that IL-23R is required for T cell accumulation at late but not early time-points after T cell transfer into *Rag1*$^{-/-}$ hosts. This differential requirement for IL-23 at different time points was not due to differences in IL-23R expression as colonic CD4$^+$ T cells from WT donors expressed similar levels of *Il23r* at both early and late time points after T cell transfer into *Rag1*$^{-/-}$ recipients (Figure 3.12). To assess whether cell intrinsic IL-23R signalling is required for the accumulation of T cells in the intestine we transferred an equal number of WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$ T cells into *Rag1*$^{-/-}$ recipients. In this setting, the frequency of proliferating Ki-67$^+$ cells among all three colonic effector CD4$^+$ T cell populations was indistinguishable between recipients of *Il23r*$^{-/-}$ and WT T cells (Figure 3.13). These data suggest that IL-23 signalling into WT T cells rescues the proliferation defect observed in *Il23r*$^{-/-}$ T cells. We hypothesised that the presence of WT T cells could modulate the expression of anti-inflammatory factors by *Il23r*$^{-/-}$ CD4$^+$ T cells. To test this, we transferred *Rag1*$^{-/-}$ mice with WT or *Il23r*$^{-/-}$ T cells (single transfer) or a 1:1 mixture of WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) T cells (co-transfer) and measured the levels of the anti-inflammatory cytokine IL-10 (Figure 3.14). Interestingly, colonic *Il23r*$^{-/-}$ CD4$^+$ T cells from single transfer experiments expressed significantly greater amounts of *Il10* mRNA and secreted higher amounts

of IL-10 protein than WT T cells. By contrast, *Il23r*$^{-/-}$ T cells isolated from the colon of mice co-transferred with WT T cells did not exhibit enhanced IL-10 expression and secretion. In summary, *Il23r*$^{-/-}$ CD4^{+} T cells exhibit reduced proliferative capacity that can be rescued by extrinsic signals conveyed by IL-23 responsive WT T cells.

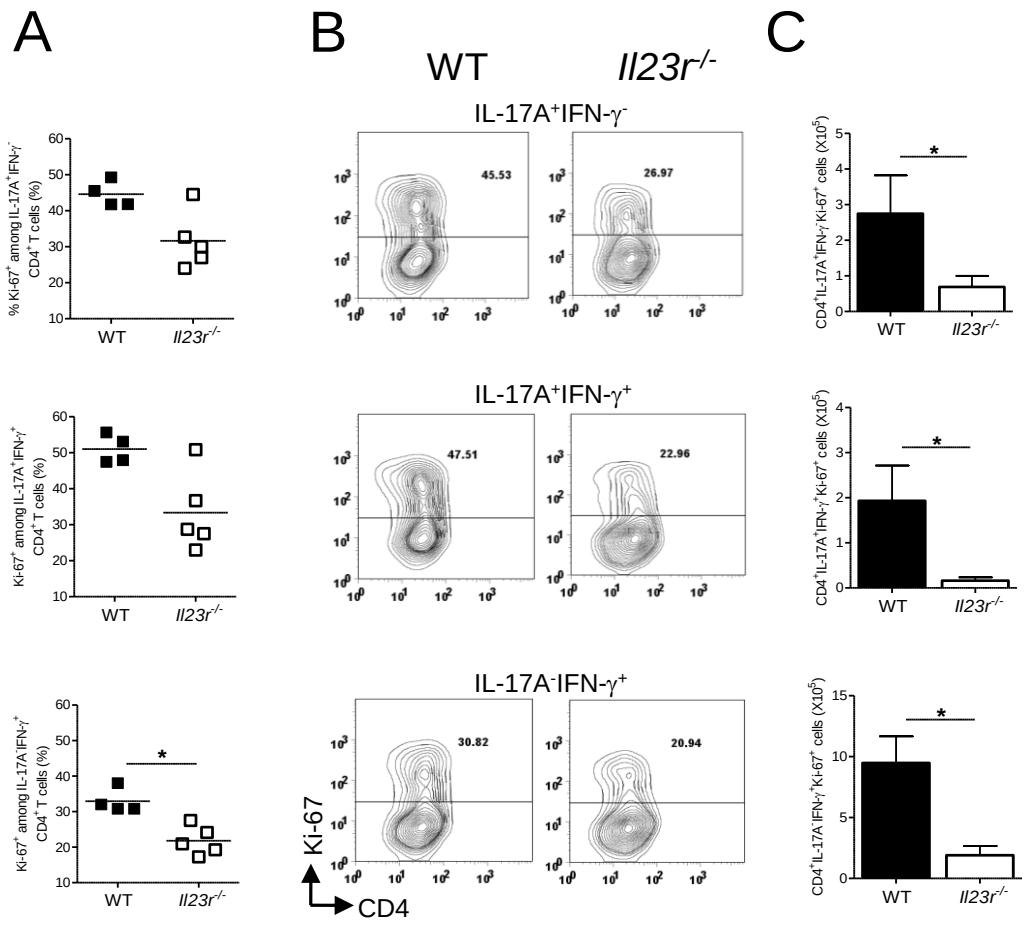

Figure 3.11. IL-23 promotes intestinal CD4$^+$ T cell proliferation

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT or *Il23r*$^{-/-}$ donors. Mice were sacrificed when recipients of WT T cells developed clinical signs of disease (6-8 weeks). Single cell suspensions from the colonic lamina propria were re-stimulated with PMA and Ionomycin in the presence of Brefeldin A for 4 hours and expression of intracellular markers determined by FACS.

(A) Frequency of Ki-67$^+$ cells among IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells in the colonic lamina propria. Bars represent the mean and each point represents an individual mouse.

(B) Representative FACS plots of colonic lamina propria cells, gated on CD4$^+$ T cells.

(C) Total number of Ki-67$^+$ cells among IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells in the colonic lamina propria. Bars represent the mean ±SEM.

Data represents results from a single experiment (n=4 mice for WT, n=5 mice for *Il23r*$^{-/-}$) Statistical significance was determined using a Mann Whitney U Test. * p≤0.05.

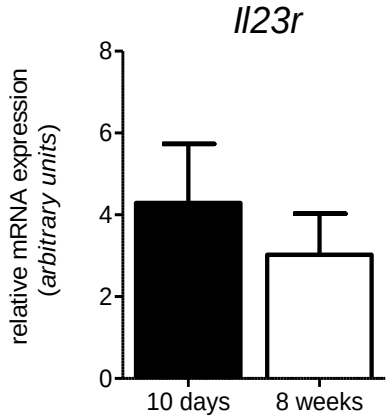

Figure 3.12. CD4$^+$ T cells express similar levels of *Il23r* early and late after T cells transfer

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^6 CD4$^+$CD25$^-$CD45RB^{hi} WT T cells and sacrificed 12 days after transfer. In addition, a second cohort of C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} WT T cells and sacrificed at 8 weeks post tranfser. WT colonic CD4$^+$ T cells were FACS sorted at day 12 or 8 weeks post transfer and *Il23r* mRNA expression assayed by RT-qPCR. Transcript levels were normalised to *Hprt*. Bars represent the mean ±SEM.

Data represents results from a single experiment (n=5 mice for 12 days, n=7 mice for 8 weeks) Statistical significance was determined using a Mann Whitney U Test. No significant difference was observed.

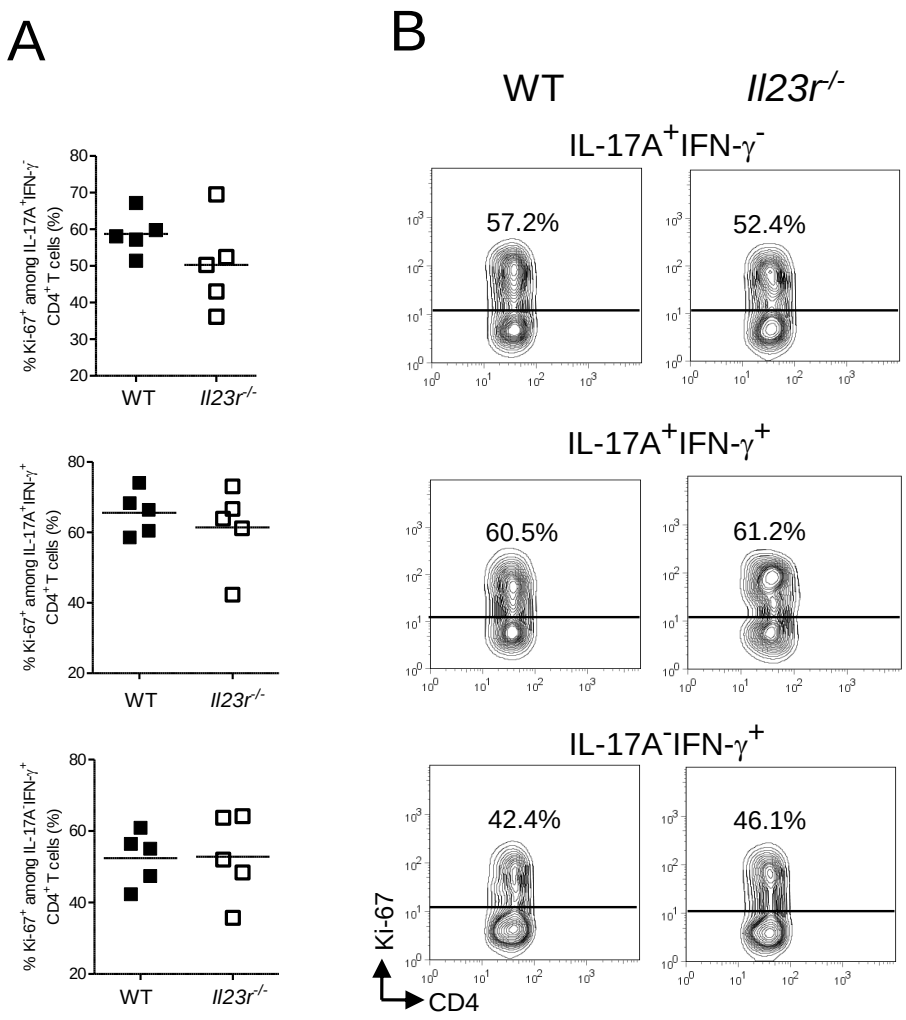

Figure 3.13. The proliferative defect of IL-23R deficient CD4$^+$ T cells is rescued by the presence of WT CD4$^+$ T cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with a 1:1 mixture of WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed upon development of clinical signs of inflammation (~8 weeks). Single cell suspensions were re-stimulated with PMA and Ionomycin in the presence of Brefeldin A for 4 hours and expression of intracellular markers determined by FACS.

(A) Frequency of Ki-67$^+$ cells among IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells in the colonic lamina propria. Bars represent the mean and each point represents an individual mouse.

(B) Representative FACS plots of colonic lamina propria cells, gated on CD4$^+$ T cells.

Data represents results from a single experiment (n=5 mice). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. No statistically significant difference was found.

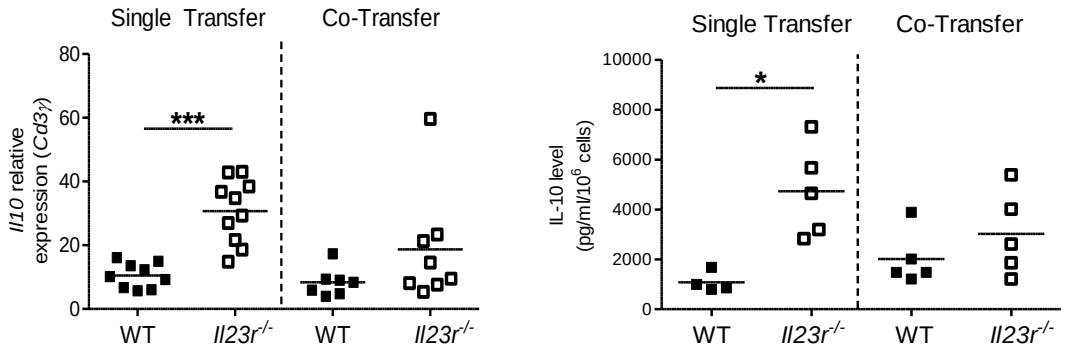

Figure 3.14. IL-23 controls IL-10 expression by intestinal CD4$^+$ T cells through a cell extrinsic mechanism

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 WT or *Il23r*$^{-/-}$ CD4$^+$CD25$^-$CD45RB^{hi} T cells (single transfer) or transferred with a 1:1 mixture of WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$CD25$^-$ CD45RB^{hi} T cells (co-transfer). Mice were sacrificed upon development of clinical signs of inflammation (6-8 weeks). CD4$^+$ T cells from the colonic lamina propria were FACS sorted and re-stimulated with PMA and Ionomycin. *Il10* mRNA levels and IL-10 protein levels in supernatants were assayed. Bars represent the mean and each point represents an individual mouse.

Data represents pooled results from two independent experiments (n=7-10 mice for *Il10* mRNA) or from a single experiment (n=4-5 mice for IL-10 protein). Statistical significance was determined using a Mann Whitney U Test (singel transfer) or a Wilcoxon matched-pairs signed rank test (co-transfer). * $p \leq 0.05$, *** $p \leq 0.001$.

3.2.11 IL-23 is not required for the maintenance of T cell transfer colitis

So far our data demonstrate that IL-23 signals into T cells are required for the induction of chronic intestinal inflammation. However, some of the defects we observed in *Il23r*$^{-/-}$ T cells, such as impaired accumulation, were overcome in the presence of WT T cells. Therefore an important question is whether *Il23r*$^{-/-}$ T cells that arise in the presence of WT T cells acquire pathogenic potential that does not require sustained IL-23R signalling. To address this we transferred Thy1.2^{+} *Rag1*$^{-/-}$ mice with an equal number of WT (Thy1.1^{+}) and *Il23r*$^{-/-}$ (Thy1.2^{+}) CD4^{+} T cells and selectively depleted WT T cells through injection of anti-Thy1.1 mAb starting at 4 weeks post T cell transfer (Figure 3.15A). As a control we also transferred WT (Thy1.1^{+}) and WT (Thy1.2^{+}) CD4^{+} T cells at a 1:1 ratio into a separate cohort of *Rag1*$^{-/-}$ hosts. Prior to the start of depletion we confirmed that both cohorts of mice had developed a similar degree of intestinal pathology (Figure 3.15B). Notably, Thy1.1^{+} WT CD4^{+} T cells were more effective at reconstitution of the *Rag1*$^{-/-}$ host regardless of the genotype of the co-transferred Thy1.2^{+} CD4^{+} T cells (Figure 3.15C-D). Although a previous study confirmed that a single dose of anti-Thy1.1 mAb efficiently depleted Thy1.1^{+} cells within 7 days [388] we continued injection of mAb once a week to ensure complete depletion of Thy1.1^{+} T cells throughout the course of the experiment. Four weeks after the start of anti-Thy1.1 injection, Thy1.1^{+} WT CD4^{+} T cells were almost below the limit of detection and Thy1.2^{+} WT or *Il23r*$^{-/-}$ CD4^{+} T cells constituted the majority of cells in the colon (Figure 3.15F-G). Interestingly, WT and *Il23r*$^{-/-}$ CD4^{+} T cells were able to sustain inflammation and promoted the development of comparable intestinal pathology in *Rag1*$^{-/-}$ recipients (Figure 3.15E). This data suggest that IL-23R signalling into T cells is not required for the maintenance of intestinal inflammation in this model.

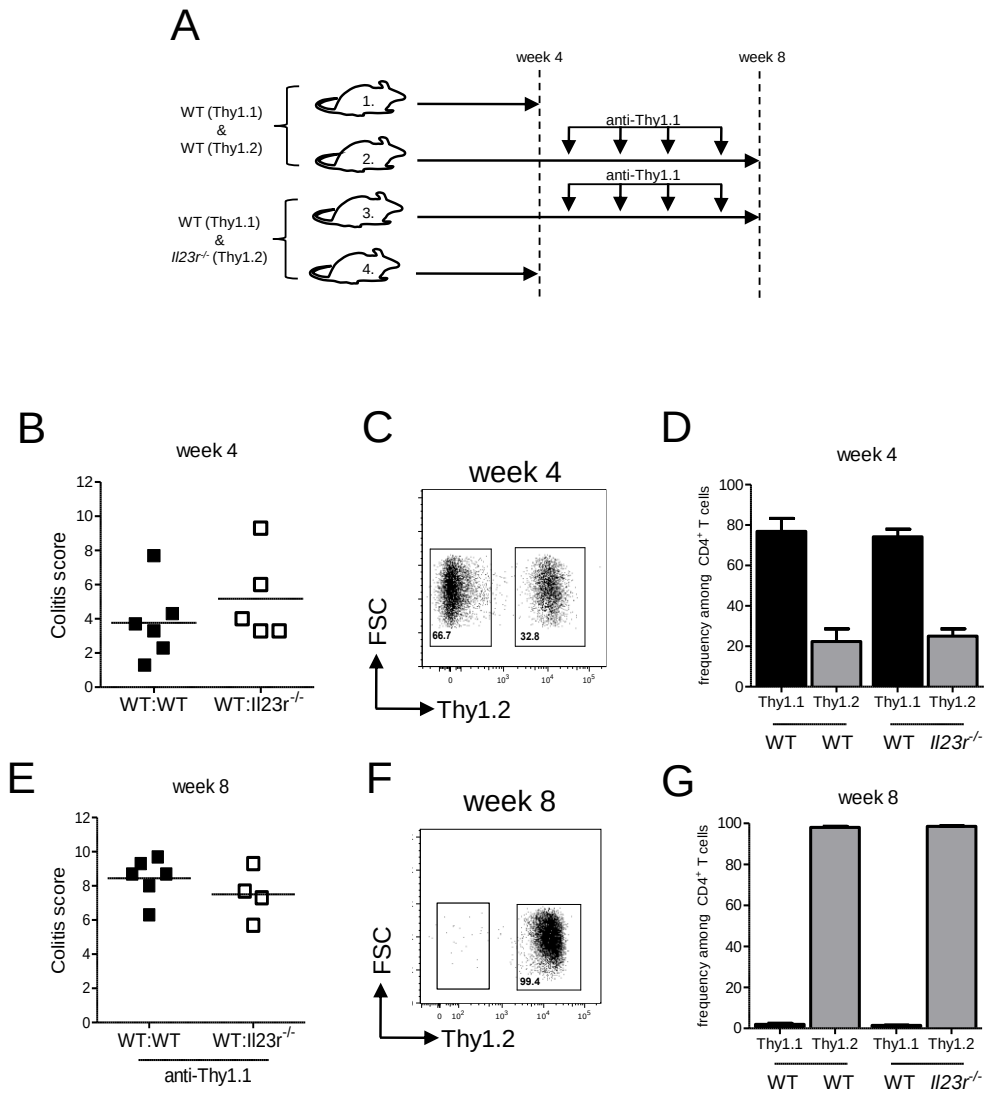

Figure 3.15. IL-23R signalling into CD4$^+$ T cells is not required for the maintenance of T cell transfer colitis

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 1:1 mixtures of WT (Thy1.1) and *Il23r*$^{-/-}$ (Thy1.2) or WT (Thy1.1) and WT (Thy1.2) CD4$^+$CD25$^-$CD45RB^{hi} T cells. At week 4 post transfer, one cohort of mice was sacrificed and intestinal inflammation was assessed. A second cohort of mice received Thy1.1 depleting mAb (1mg/ml/week) or isotype control mAb starting at 4 weeks post transfer and mice were sacrificed upon development of clinical signs of inflammation (~8 weeks).

(A) Schematic explaining the experimental strategy.

(B & E) Histological analysis of colitis at week 4 or week 8 post transfer. Bars represent the mean and each point represents an individual mouse.

(C & F) Representative FACS plots of CD4$^+$ T cells in the colonic lamina propria at week 4 or week 8 post transfer. Plots are gated on viable CD4$^+$ T cells.

(D & G) Frequency of CD4$^+$ T cells in the colonic lamina propria at week 4 or week 8 post transfer. Bars represent the mean ±SEM.

Data represents results from a single experiment (n=6 mice for WT:WT and n=4-5 mice for WT:*Il23r*$^{-/-}$ at each time point).

3.3 Discussion

IL-23 has been shown to drive chronic inflammatory responses in innate and T cell dependent models of colitis [264, 265, 291, 323]. However, which cell types IL-23 acts on to promote inflammatory responses is not well defined. Here we used a T cell dependent model of colitis to track IL-23R expressing lymphocytes during the development of chronic intestinal inflammation. We found that IL-23R$^+$CD4$^+$ T cells accumulated over the course of the inflammatory response and constituted the majority of IL-23 responsive lymphocytes at the peak of intestinal pathology. By contrast, CD4$^+$ T cells represented only a minor fraction of the IL-23R$^+$ population in the steady state suggesting that the expansion of IL-23R$^+$CD4$^+$ T cells during the course of inflammation occurred at the expense of other IL-23 responsive lymphocyte. Further analysis showed that γδ T cells are the major population of IL-23R$^+$ lymphocytes in the steady state colon (Matthew Shale, DPhil thesis, unpublished). The role of lamina propria γδ T cells in intestinal homeostasis and inflammation has not been elucidated in detail. However, the switch from γδ T cell to CD4$^+$ T cell dominance within the IL-23R$^+$ lymphocyte compartment in response to inflammation could suggest a role for γδ T cells in intestinal homeostasis. However, we did not observe spontaneous development of colitis in *Il23r*$^{-/-}$ mice under specific pathogen free (SPF) conditions (data not shown) excluding a role for IL-23 signalling into γδ T cells, or other IL-23R$^+$ lymphocytes, in the maintenance of normal tissue homeostasis. On the other hand, it is feasible that IL-23 exerts its pro-inflammatory effects on both γδ T cells and CD4$^+$ T cells at different phases of the immune response. In support of this, γδ T cells were shown to be the first cells to respond to IL-23 during autoimmune inflammation of the CNS and this early response was essential for the propagation of the antigen specific effector T cell response [389].

Thus, although $CD4^+$ T cells represent the dominant population of IL-$23R^+$ lymphocytes in the inflamed colon they are unlikely to be the only target of the pro-inflammatory activities of IL-23.

Our tracking experiments with the IL-$23R^{gfp/+}$ reporter strain also revealed that IL-$23R^+CD4^+$ T cells are highly enriched in the colon of naïve mice. In inflamed mice, IL-23R expression was further enhanced among $CD4^+$ T cells in the colon and at distal sites such as the MLN and spleen. This may be due to an increased availability of circulating IL-6 and IL-21 during inflammation which are potent inducers of IL-23R expression [125, 129]. However, IL-23R expression remained enriched among intestinal $CD4^+$ T cells even in an inflammatory context suggesting that a tissue specific signal in the colon further enhances IL-23R expression. Interestingly, the abundance of IL-$23R^+$ cells in the intestine correlates with the increased availability of IL-23 in the steady state intestine [300] and during chronic inflammation of the colon [291]. Indeed, IL-23 has been shown to enhance its own receptor expression *in vitro* [130] suggesting that IL-23 itself may be the tissue specific factor responsible for the selective expression of IL-23R in the colon.

The importance of IL-23 in driving intestinal inflammation is well documented and our finding that genetic ablation of IL-23R prevented development of disease in *Hh*+ anti-IL-10R-treated mice further supports a pathogenic role for IL-23 in colitis. This finding is also in line with our previous report that *Il12p40*$^{-/-}$ but not *Il12p35*$^{-/-}$ mice are resistant to inflammation in this model [264]. We further showed that IL-23 acts directly on T cells as transfer of naïve $CD4^+$ T cells from *Il23r*$^{-/-}$ mice into *Rag1*$^{-/-}$ hosts only led to mild intestinal pathology. IL-23 is thought to signal predominantly

through STAT3 [304] and a recent study described an essential role for T cell intrinsic STAT3 in the development of intestinal and systemic disease in T cell transfer colitis [390]. By contrast, we found that IL-23 drives T cell accumulation in the intestine but not the spleen suggesting that IL-23 independent STAT3 signals are sufficient for systemic T cell accumulation. This differential requirement for IL-23 could be a result of low levels of IL-23 in the serum and/or the small frequency of IL-23R^{+}CD4^{+} T cells found in the spleen. In addition, IL-6 and IL-21 also signal through STAT3 and have been shown to be required for T cell transfer colitis [329] (Matthew Shale, DPhil thesis, unpublished). Thus it is likely that a combination of STAT3 activating cytokines is required for the development of intestinal pathology and blockade of any individual one will result in impaired STAT3-dependent inflammatory gene expression and reduced pathology. This is perhaps not surprising as STAT3 activating cytokines are known to cross-regulate each other. For instance, IL-6 and IL-21 are potent inducers of IL-23R expression [125, 129] and IL-23 drives IL-6 secretion by intestinal T cells [323]. Importantly, blockade of IL-6 prevents the development of wasting disease in T cell transfer colitis [329] while IL-23 deficiency has no impact on systemic inflammation in this model [391]. This suggests that IL-23 may represent a more attractive therapeutic target among STAT3 activating cytokines due to its tissue-specific effects on inflammation.

In our T cell transfer experiments we noted that *Rag1*$^{-/-}$ recipients of *Il23r*$^{-/-}$ T cells developed only mild intestinal inflammation. However, the pathology induced by *Il23r*$^{-/-}$ CD4^{+} T cells was more pronounced than that previously observed in *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ hosts transferred with WT CD4^{+} T cells [265] suggesting that IL-23 signals into innate cells contribute to intestinal inflammation in this model. Therefore, we

were surprised to find that IL-23R deficiency in the innate compartment did not have a more significant impact on the severity of T cell transfer colitis. However, we did observe a trend towards lower intestinal inflammation in *Il23r*$^{-/-}$*Rag1*$^{-/-}$ recipients of WT CD4^{+} T cells as compared to *Rag1*$^{-/-}$ mice transferred with WT CD4^{+} T cells. Although we failed to establish a significant contribution of IL-23 responsive innate cells to T cell transfer colitis several innate models of colitis are known to be dependent on IL-23 [265, 291]. Thus, IL-23 can promote pathogenic immune responses through its action on innate immune cells but this may not be easily revealed in the presence of the overwhelming T cell response that develops in immunodeficient mice transferred with large numbers of T cells. Alternatively, it is possible that IL-23 signals into CD4^{+} T cells and innate cells have opposing consequences on the development of intestinal inflammation. In support of this a recent study in the DSS model of colitis found that the pathogenic potential of IL-23 was only revealed in immune competent hosts while IL-23 had a tissue protective role when colitis was induced in immunodeficient *Rag1*$^{-/-}$ mice [339]. The authors linked this to the ability of IL-23 to induce IL-22 production from ILCs which was required for the recovery from epithelial damage. Although these results were obtained in a model of acute inflammation induced by epithelial injury rather than a chronic inflammatory setting, they highlight the complexity of the IL-23 pathway. Interestingly, another report also described a protective role for innate-derived IL-22 in T cell transfer colitis as *Il22*$^{-/-}$*Rag1*$^{-/-}$ recipients developed more severe pathology upon CD4^{+} T cell transfer than IL-22 sufficient *Rag1*$^{-/-}$ hosts [331]. Thus, the inflammation observed in *Rag1*$^{-/-}$ recipients of *Il23r*$^{-/-}$ CD4^{+} T cells may not necessarily be mediated through IL-23 dependent effects on innate cells. However, our results do not support a protective role for IL-23 signals into innate cells as *Il23r*$^{-/-}$

Rag1$^{-/-}$ mice did not develop worse colitis than *Rag1*$^{-/-}$ mice transferred with WT CD4^{+} T cells.

One of the most pronounced effects of IL-23 was its tissue-specific role in T cell accumulation. While *Il23r*$^{-/-}$ CD4^{+} T cells were able to accumulate normally in the spleen they failed to accumulate in the intestine either under lymphopenic conditions or in lymphoreplete hosts. *Il23r*$^{-/-}$ CD4^{+} T cells were not impaired in their ability to home to the intestine but failed to proliferate within the colonic lamina propria. This effect of IL-23 on T cell accumulation required direct signalling into T cells and demonstrates that IL-23 provides an important proliferative signal for CD4^{+} T cells in the intestine. Interestingly, the proliferative defect and impaired accumulation of *Il23r*$^{-/-}$ CD4^{+} T cells could be overcome by the presence of WT CD4^{+} T cells within the same host. Thus, *Il23r*$^{-/-}$ CD4^{+} T cells do not have a cell intrinsic competitive disadvantage over WT CD4^{+} T cells and their accumulation defect can be rescued through paracrine signals conveyed by IL-23 responsive WT CD4^{+} T cells. However, the requirement for IL-23 signals in T cell accumulation appears to be context dependent as another study found that the defective accumulation of *Il23r*$^{-/-}$ CD4^{+} T cells in the CNS could not be rescued by the presence of WT T cells [131]. Furthermore, the reduced accumulation of *Il23r*$^{-/-}$ CD4^{+} T cells was associated with increased expression of IL-10. This result is consistent with our previous finding that IL-10 mediated immune suppression plays a functional role in inhibiting the development of colitis in *Il23a*$^{-/-}$*Rag1*$^{-/-}$ mice transferred with WT T cells [330]. Importantly, increased IL-10 expression by *Il23r*$^{-/-}$ CD4^{+} T cells was abrogated in the presence of WT CD4^{+} T cells. We did not elucidate the mechanism by which WT CD4^{+} T cells mediate this effect but it is likely that IL-23 induces factors in WT

$CD4^+$ T cells that can limit IL-10 expression by $CD4^+$ T cells in a paracrine manner. These factors could be soluble mediators that either suppress IL-10 production or alter the local microenvironment to restrain differentiation of IL-10 producing T cells. Furthermore, the cellular source of IL-10 among *Il23r*$^{-/-}$ T cells remains to be identified. Interestingly, we previously showed that IL-23 restrains the differentiation of $Foxp3^+$ Tregs [330] and these cells have been shown to be an important source of IL-10, especially in the intestine [235]. In addition, all known effector T cell subset can produce IL-10 [392]. Regardless of the source of IL-10, IL-23 may drive intestinal T cell accumulation, at least in part, through limiting the production of T cell-derived IL-10. Recently, it was suggested that IL-10 predominantly suppresses Th17 cells through direct effects on T cells while regulation of Th1 cells may occur indirectly through effects on antigen presenting cells [237]. As we observed reduced accumulation of effector T cells regardless of IL-17A or IFN-γ expression, it is plausible that IL-10 acts on multiple cellular populations to limit T cell accumulation in the absence of IL-23 signals into T cells.

In addition to effects on accumulation, we found that IL-23R deficiency had profound effects on intestinal T cell differentiation. We showed in two distinct models of colitis that direct cell intrinsic signalling into $CD4^+$ T cells is required for the differentiation of IL-$17A^+$IFN-γ^+ but not IL-$17A^+$IFN-γ^- or IL-$17A^-$IFN-γ^+ cells in the intestine. This specific role for IL-23 in promoting the emergence of IL-$17A^+$IFN-γ^+ T cells was somewhat surprising because $CD4^+$IL-$23R^+$ T cells in the inflamed colon contained a mixture of IL-17A and IFN-γ producing cells. Interestingly, we found that IL-$23R^+$ $CD4^+$ T cells in the MLN and spleen contained mostly IL-$17A^+$IFN-γ^- cells whereas colonic IL-$23R^+$ $CD4^+$ T cells contained a higher proportion of IL-$17A^+$IFN-γ^+ and

IL-17A$^-$IFN-γ^+ cells. This suggests that local exposure to IL-23 in the intestine could drive the acquisition of IFN-γ production by Th17 cells. Although we could not formally prove that IL-17A$^+$IFN-γ^+ arise from Th17 cells, recent evidence from fate mapping experiments of IL-17A producing T cells during chronic inflammation of the CNS support this conclusion [393]. In that study it was shown that the majority of IL-17A$^+$IFN-γ^+ as well as IL-17A$^-$IFN-γ^+ T cells that arise during EAE were derived from CD4$^+$ T cells that had once expressed IL-17A, and importantly this conversion was dependent on IL-23. However, we did not observe a difference in the frequency of IL-17A$^-$IFN-γ^+ CD4$^+$ T cells in the colon suggesting that these cells can arise independently of IL-23 signals into T cells during intestinal inflammation. In summary, the emergence of IL-17A$^+$IFN-γ^+ T cells correlates with inflammation and their dependency on IL-23 suggests a potential pathogenic role for this population in the development of colitis. In support of this IL-17A$^+$IFN-γ^+ CD4$^+$ T cells have also been isolated from intestinal lesions of Crohn's disease patients [266, 361]. However, a recent study found that IL-17A$^+$IFN-γ^+ CD4$^+$ T cells were also highly enriched in the non-inflamed intestines of mice protected from T cell transfer colitis by the co-transfer of CD25$^+$ Treg cells [394] demonstrating that an increased frequency of IL-17A$^+$IFN-γ^+ CD4$^+$ T cells does not always correlate with inflammation. In addition, it remains to be determined whether IL-17A and IFN-γ expression contribute to the potential pathogenic role of IL-17A$^+$IFN-γ^+ CD4$^+$ T cells or whether these cytokines serve as markers of a polyfunctional population of CD4$^+$ T cells capable of producing a variety of pro-inflammatory factors. Interestingly, several groups have shown that T cell deficiency in IFN-γ [325], IL-17A [326, 329, 330], IL-17F [326], IL-22 [326, 331] or GM-CSF [332] did not prevent the development of intestinal inflammation

suggesting that a combination of these cytokines, or as yet unidentified factors, may contribute to the pathogenicity of $CD4^+$ T cells in this model.

Despite the crucial role of IL-23 in the induction of intestinal inflammation we found that sustained IL-23 signalling into T cells was not required for the maintenance of established inflammatory responses in the colon. This observation is in contrast to published results showing that established colitis can be reversed by administration of an IL-23 neutralizing antibody [324]. A key difference between this study and ours is the source of $CD4^+$ T cells. Instead of naïve $CD4^+$ T cells the authors used a bacterial antigen-specific T cell line. In addition, rather than neutralizing IL-23 we only "blocked" IL-23 signalling into T cells in mice with established colitis. In support of our findings, a recent report demonstrated that both $ROR\gamma^-$ and $ROR\gamma^+$ effector $CD4^+$ T cells isolated from the colon induced a similar degree of colitis when transferred into *Rag1*$^{-/-}$ hosts [394]. Thus, IL-23 signals into T cells are necessary for the induction of colitis but IL-23 signals into innate cells, or IL-23 independent signals, may be sufficient for the maintenance of established colitis. Although these results are rather preliminary, they suggest that blockade of IL-23 in IBD patients with active disease might not be the ideal therapeutic strategy. Instead, blockade of IL-23 in IBD patients that are in remission might prove more successful at preventing relapse of disease. However, there are currently no animal models of IBD which address its remitting and relapsing nature and therefore the contribution of IL-23 to relapse of the disease is not known.

In conclusion, we showed that $CD4^+$ T cells capable of producing IL-17A and IFN-γ represent the dominant population of IL-23 responsive lymphocytes in the inflamed colon. Selective ablation of IL-23R in the innate or T cell compartment demonstrated that IL-23 signalling directly into $CD4^+$ T cells was required for their accumulation in the intestine and contributed to the emergence of IL-$17A^+$IFN-γ^+ T cell at this site. However, IL-23 signals into $CD4^+$ T cells were dispensable for the maintenance of established T cell dependent colitis, which could have potential implications for the treatment of IBD.

Chapter 4: RORγ but not T-bet expression by T cells is required for the development of T cell transfer colitis

4.1 Introduction

Th17 cells are enriched at mucosal sites [395], produce high levels of IL-17A, IL-17F and IL-22 [117, 118] and have an essential role in mediating host protective immunity against a variety of extracellular pathogens [396-399]. However, Th17 cells have also been implicated in a variety of autoimmune and chronic inflammatory conditions, including intestinal inflammation [116, 264, 265, 291, 319, 323]. The Th17 cell programme is driven by RORγt, which is encoded for by *Rorc*, and several other transcription factors that act either upstream of RORγt or cooperate with RORγt in the induction of Th17 signature genes [49, 400]. Additionally, RORγt is also required for the induction and maintenance of the receptor for IL-23 in differentiating Th17 cells [125, 401]. Until recently it was thought that the combination of TGF-β1 with STAT3-activating cytokines IL-6 or IL-21 is a minimal requirement for the differentiation of naïve T cells into Th17 cells [400]. However, IL-23 in concert with IL-6 and IL-1β was also shown to promote TGF-β1-independent Th17 differentiation [154]. In addition, IL-23 signalling into T cells is necessary for the terminal differentiation and acquisition of effector function by Th17 cells *in vivo* [131].

Currently, it is not well understood what controls the acquisition of pathogenic functions by Th17 cells. Recent work in regulatory T cells (Treg) has demonstrated cooperativity between FoxP3 and what have classically been defined as lineage specific transcription factors for Th2 and Th17 cells, i.e. GATA3, IRF-4 and STAT3 respectively [226, 227, 254]. This highlights the multifunctional role played by these

transcription factors in specification of $CD4^+$ helper T cell effector function. There is growing evidence that T-bet might play a similar functional role in Th17 cells. First, experimental autoimmune encephalomyelitis (EAE), which is thought to be a Th17 driven pathology, requires both RORγt and T-bet [49, 315]. Second, several studies have demonstrated that IL-23 is required to unleash pathogenic Th17 cell activity in EAE [117, 131, 402] and IL-23 stimulation of Th17 cells was shown to lead to an increase in T-bet expression [154]. In addition, a more recent study using a T cell transfer model of EAE demonstrated that T-bet expression in Th17 cells was required for their pathogenicity [269]. Finally, exposure of *in vitro* differentiated Th17 cells to IL-23 has been shown to promote the acquisition of IFN-γ production in a T-bet dependent manner [132] and this ability of Th17 cells to convert into IFN-γ producing cells has been linked to their pathogenicity [132, 268]. A similar picture is emerging in the intestine, where IL-23 drives T cell dependent intestinal pathology [265, 391] which has been shown to depend on T-bet and RORγ [88, 326]. This is supported by epigenetic analyses, which have revealed that the loci for *Tbx21* (coding for T-bet) and *Ifng* are associated with permissive histone modifications in Th17 cells, suggesting that Th17 cells are "poised" to express T-bet which could then drive IFN-γ production [260, 263] . Consistent with these findings we previously demonstrated that IL-23 signals into T cells drive the emergence of IL-$17A^+$IFN-γ^+ T cells in the intestine [391]. Moreover, the emergence of a population of T cells capable of producing both IL-17A and IFN-γ has also been described in intestinal biopsies of IBD patients [266, 361]. Collectively these studies suggest a model whereby RORγ drives differentiation of Th17 cells in response to various signals *in vivo* and subsequently IL-23 drives T-bet expression to generate IL-$17A^+$IFN-γ^+ T cells that contribute to IL-23 driven intestinal inflammation.

However this hypothesis has not been formally tested and it remains poorly defined in the intestine whether the requirement for RORγ and T-bet reflect a requirement for both Th17 cells and Th1 cells respectively or whether Th17 cells require T-bet to exert their pathogenic effector function. In this study, we used the T cell transfer model of colitis to delineate the contribution of RORγ and T-bet to the differentiation and pathogenicity of $CD4^+$ T cells.

4.2 Results

4.2.1 IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells express RORγ and T-bet

CD4$^+$ T cells capable of producing both IL-17A and IFN-γ are a shared feature of T cell driven models of colitis [264, 265, 330]. Based on the co-expression of IL-17A and IFN-γ we hypothesized that these CD4$^+$ T cells also express the transcription factors RORγ and T-bet, which are known to drive IL-17A [49] and IFN-γ [79]expression, respectively. To test this we induced colitis in C57BL/6 WT mice by oral infection with *Helicobacter hepaticus* and concomitant administration of IL-10R blocking antibody and assessed the expression of RORγ and T-bet among IL-17A and/or IFN-γ producing colonic CD4$^+$ T cells by intracellular FACS staining (Figure 4.1A). As expected IL-17A$^+$IFN-γ$^-$ T cells expressed RORγ and IL-17A$^-$IFN-γ$^+$ T cells expressed T-bet whereas co-expression of both RORγ and T-bet was limited to IL-17A$^+$IFN-γ$^+$ T cells (Figure 4.1B-C). Interestingly, IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells expressed similar levels of RORγ on a per cell basis as CD4$^+$ T cells that produced only IL-17A (Figure 4.1B). In addition, we observed high levels of T-bet expression in IL-17A$^+$IFN-γ$^+$ and IL-17A$^-$IFN-γ$^+$ T cells. Although there was a tendency for lower T-bet expression among IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells this difference was not statistically significant (Figure 4.1C). Thus, IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells that arise in the inflamed colon of lymphocyte replete mice co-express RORγ and T-bet.

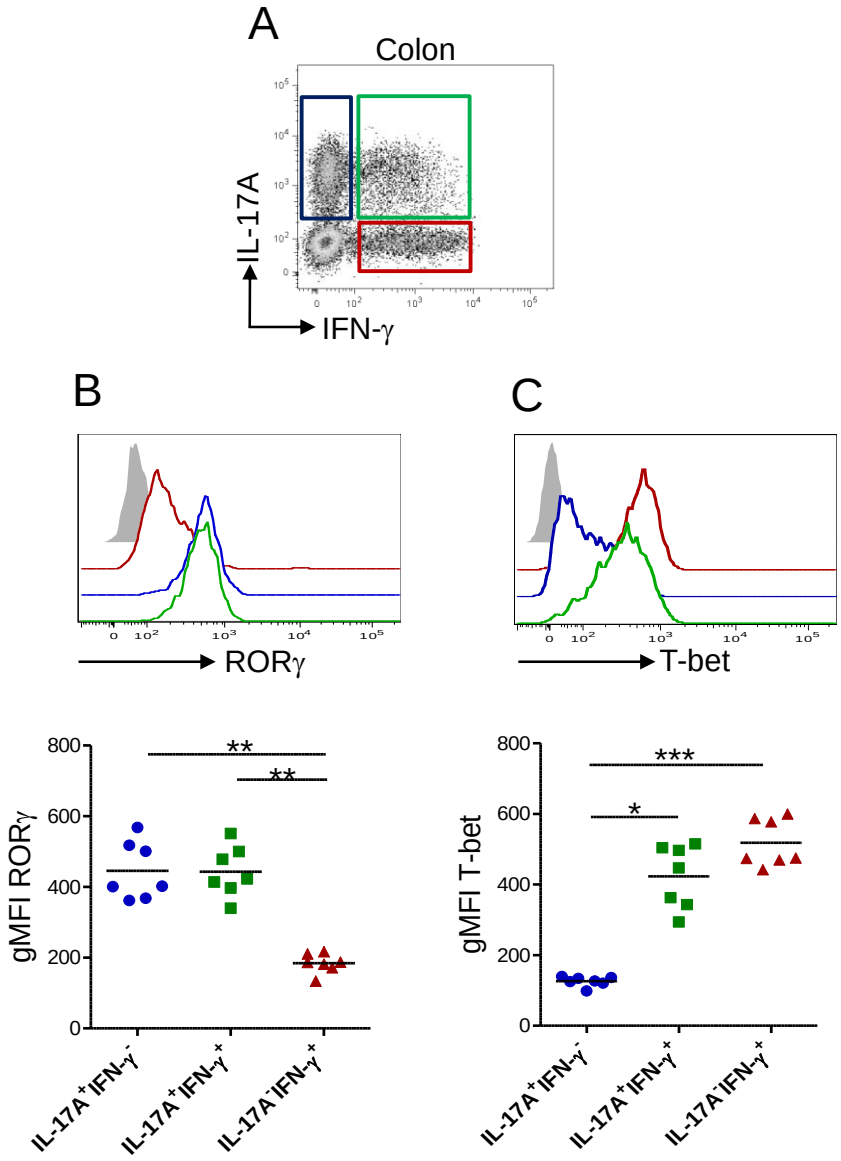

Figure 4.1. IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells express RORγ and T-bet

C57BL/6 mice were infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* on three consecutive days with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed 2 weeks post infection and cells were isolated from the colonic lamina propria. Single cell suspensions were re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of the indicated cytokines and transcriptions factors was determined by FACS.

(A) Representative FACS plot from the colon gated on viable CD4$^+$ T cells showing IL-17A$^+$IFN-γ$^-$ (blue), IL-17A$^+$IFN-γ$^+$ (green) and IL-17A$^-$IFN-γ$^+$ T cells (red).

(B) Representative histogram of RORγ expression in IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells (top) and summary of RORγ protein expression levels across all mice as measured by geometric MFI (bottom). Bars represent the mean and each point represents an individual mouse.

(C) Representative histogram of T-bet expression in IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells (top) and summary of T-bet protein expression levels as measured by geometric MFI (bottom). Bars represent the mean and each point represents an individual mouse.

Data represent results from a single experiment (n=7 mice/group). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

4.2.2 IL-23 does not regulate RORγ or T-bet expression in CD4$^+$ T cells

We showed previously that IL-23 promotes the emergence of IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells through direct cell intrinsic activity on CD4$^+$ T cells [391], however the mechanism by which IL-23 achieves this is not known. Evidence obtained from *in vitro* studies suggests that IL-23 drives the acquisition of IFN-γ production by Th17 cells in a T-bet dependent manner [132]. Furthermore, IL-23 has been shown to enhance RORγ expression in naïve CD4$^+$ T cells transduced with IL-23R expressing retrovirus [125]. Thus, IL-23 may drive the differentiation of IL-17A$^+$IFN-γ$^+$ T cells by virtue of its ability to induce and/or maintain expression of RORγ and T-bet in these cells. To test this we generated mixed bone marrow chimaeras by reconstituting irradiated *Rag1*$^{-/-}$ mice with WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) bone marrow cells at 1:1 ratio (Figure 4.2A). After 8 weeks, to allow for bone marrow reconstitution of the hematopoietic system, we induced intestinal inflammation through infection with *Helicobacter hepaticus* and administration of an IL-10R blocking antibody. Importantly, we found similar numbers of WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the inflamed colon (Figure 4.2B) and mice developed severe intestinal pathology (Figure 4.2C) which allowed us to compare WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the same inflammatory environment. However, no differences were detected in RORγ or T-bet expression among IL-17A and/or IFN-γ producing WT and *Il23r*$^{-/-}$ CD4$^+$ T cells (Figure 4.2C-D) suggesting that cell intrinsic IL-23 signals into T cells are not required to induce and/or maintain the expression of these transcription factors in intestinal CD4$^+$ T cells.

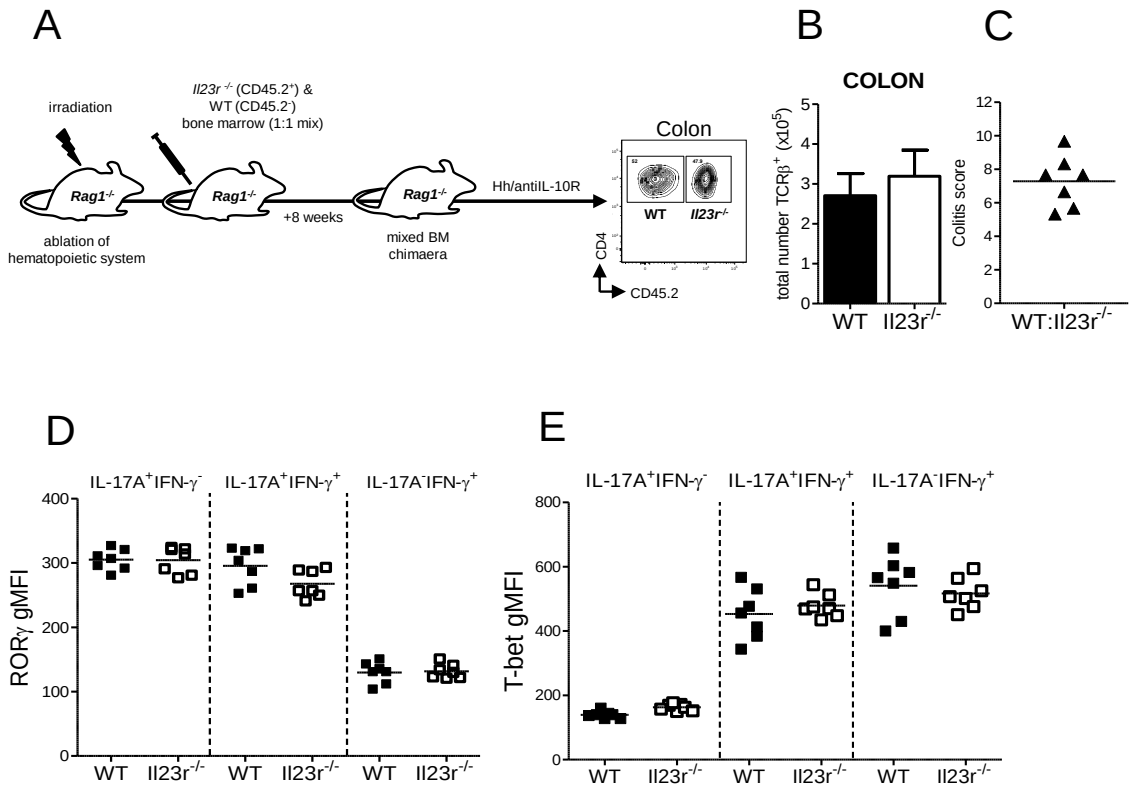

Figure 4.2. RORγ and T-bet expression are not controlled by direct IL-23 signalling into CD4$^+$ T cells

Mixed bone marrow chimaeras were generated by lethal irradiation of C57BL/6 *Rag1*$^{-/-}$ mice followed by i.v. injection of 2.5x10^6 WT (CD45.2$^-$) and 2.5x10^6 *Il23r*$^{-/-}$ (CD45.2$^+$) bone marrow cells. Mice were allowed to rest for 8 weeks to allow full re-constitution of the hematopoietic system. Re-constituted mice were infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* on three consecutive days with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at 2 weeks post infection and cells were isolated from the colonic lamina propria. Single cell suspensions were re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of cytokines and transcriptions factors was determined by intracellular FACS.

(A) Schematic outlining the generation of mixed bone marrow chimaeras with a representative FACS plot of colonic CD4$^+$ T cells showing the distribution of WT and *Il23r*$^{-/-}$ cells.

(B) Total number of WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the colon of mixed bone marrow chimaeras. Bars represent the mean, error bars represent SEM.

(C) Histological analysis of colitis. Bars represent the mean and each point represents an individual mouse.

(D) Levels of RORγ protein expression as measured by geometric MFI in IL-17A$^+$ and/or IFN-γ$^+$ WT and *Il23r*$^{-/-}$ colonic CD4$^+$ T cells. Bars represent the mean and each point represents an individual mouse.

(E) Levels of T-bet protein expression as measured by geometric MFI in IL-17A$^+$ and/or IFN-γ$^+$ WT and *Il23r*$^{-/-}$ colonic CD4$^+$ T cells. Bars represent the mean and each point represents an individual mouse.

Data represent results from a single experiment (n=7 mice/group). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. None of the comparisons yielded statistical significance.

4.2.3 RORγ but not T-bet expression by T cells is required for the development of T cell transfer colitis

The emergence of IL-17A^{+}IFN-γ^{+} T cell correlates with inflammation and their IL-23 dependency suggests that this population contributes to T cell driven colitis. Furthermore, two independent studies showed that CD4^{+} T cells deficient in T-bet or RORγ fail to induce colitis in the T cell transfer model [88, 326]. These findings suggested two distinct possibilities. First, both T-bet^{+} Th1 cells and RORγ^{+} Th17 cells are required for intestinal inflammation and thus a loss of either prevents onset of disease. Second, CD4^{+} T cells require the activity of both T-bet and RORγ within individual cells for pathogenic effector function, as appears evident in EAE [269]. Before addressing the second possibility we set out to confirm the earlier studies. We therefore transferred *Rag1*$^{-/-}$ mice with naive CD4^{+} T cells from C57BL/6 WT, *Tbx21*$^{-/-}$ or *Rorc*$^{-/-}$ donors and assessed the development of wasting disease and colitis. In keeping with previous reports *Rorc*$^{-/-}$ CD4^{+} T cells did not induce significant signs of weight loss (Figure 4.3A) and developed only mild colitis upon transfer into *Rag1*$^{-/-}$ recipients (Figure 4.3B-C). However, *Rag1*$^{-/-}$ recipients of *Rorc*$^{-/-}$ T cells were not completely devoid of inflammation suggesting a role for RORγ^{+} innate cells, or RORγ independent pathways within CD4^{+} T cells, in the development of colitis in this model. Strikingly and to our surprise, transfer of *Tbx21*$^{-/-}$ CD4^{+} T cells led to the development of a similar degree of intestinal inflammation as that observed in *Rag1*$^{-/-}$ recipients of WT T cells (Figure 4.3B-C). In addition, *Tbx21*$^{-/-}$ T cells induced severe early onset wasting disease which was not observed in *Rag1*$^{-/-}$ recipients of WT T cells (Figure 4.3A).

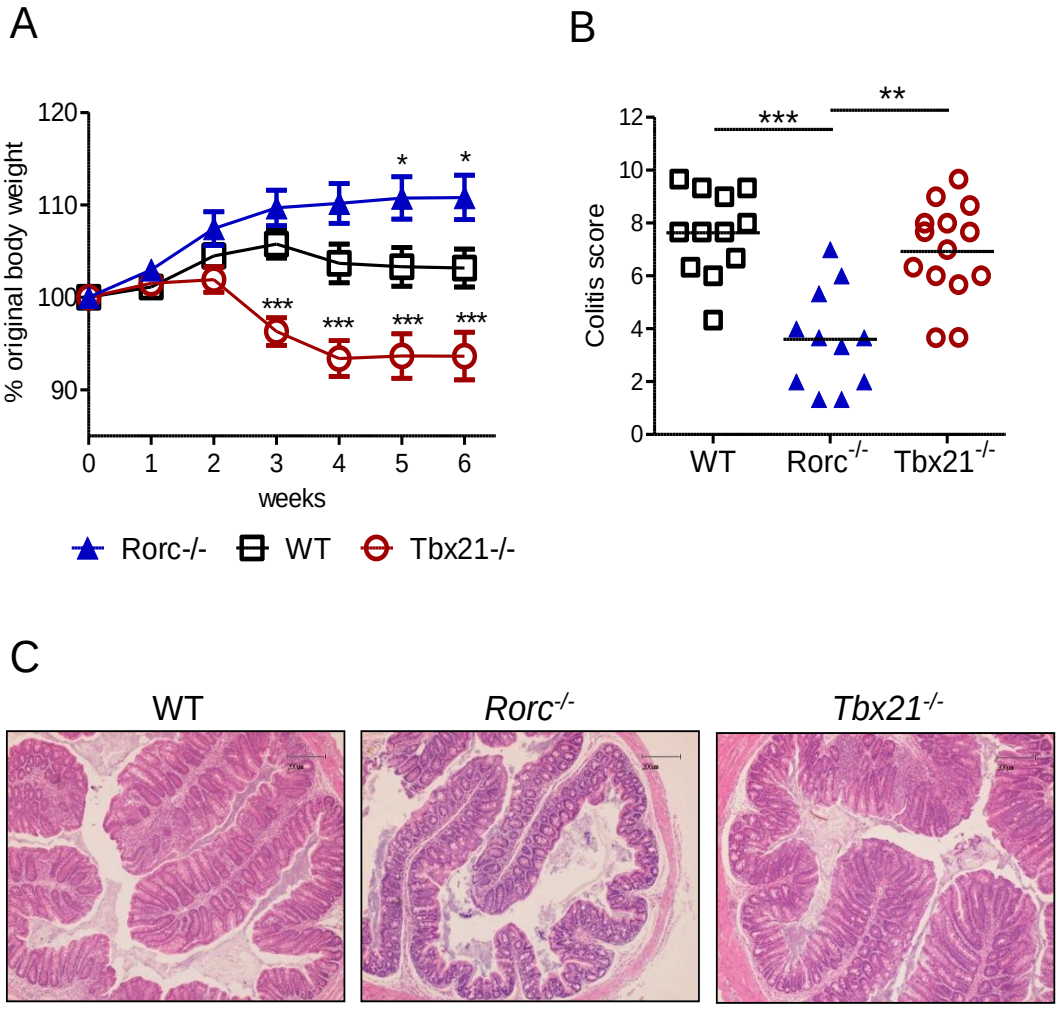

Figure 4.3. RORγ but not T-bet expression by CD4$^+$ T cells is required for the induction of T cell transfer colitis

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT, *Rorc*$^{-/-}$ or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (4-6 weeks) and assessed for intestinal inflammation.

(A) Weight loss during the course of the experiment shown as percentage of original weight at day 0. Points represent the mean ±SEM.

(B) Histological analysis of colonic inflammation. Bars represent the mean and each point represents an individual mouse.

(C) Representative photomicrographs of proximal colon sections (50X magnification).

Data represent pooled results from two independent experiments (n=17 mice for WT, n=14 mice for *Tbx21*$^{-/-}$, n=12 mice for *Rorc*$^{-/-}$). Statistical significance was determined using a repeated measure ANOVA followed by Bonferroni post-hoc test (A) and a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test (B). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Thus, while we confirmed that RORγ expression by T cells is required for the development of colitis, we found, contrary to published results, that T cell transfer colitis is independent of T-bet expression by $CD4^+$ T cells, which is in stark contrast to the situation observed in EAE.

4.2.4 T-bet expression by T cells contributes to IFN-γ expression by Th17 cells but is not necessary for the emergence of IL-17A$^+$IFN-γ$^+$ cells

The ability of Th17 cells to acquire IFN-γ expression has been linked to their pathogenic potential *in vivo* [268] and T-bet is thought to be important for driving this process [132]. Therefore, we assessed the effect of T-bet or RORγ deficiency on intestinal $CD4^+$ T cell differentiation. As expected, the differentiation and accumulation of IL-17A$^+$IFN-γ$^-$ T cells was impaired in *Rorc*$^{-/-}$ $CD4^+$ T cells whereas the frequency and total number of IL-17A$^-$IFN-γ$^+$ was significantly reduced in T-bet deficient T cells in the colon, MLN and spleen (Figure 4.4A-C & Figure 4.5). This result confirms the important role for RORγ [49] and T-bet [79] in the differentiation of Th17 and Th1 cells respectively. However, it should be noted that IL-17A was detectable in *Rorc*$^{-/-}$ $CD4^+$ T cells and *Tbx21*$^{-/-}$ $CD4^+$ T cells gave rise to IFN-γ producing cells, demonstrating that Th1 and Th17 cells can arise in the absence of these transcription factors. These findings are in keeping with published reports demonstrating a role for additional transcription factors such as RORα [139] and Eomes [85, 86] in Th17 and Th1 cell development respectively. In addition to its role in Th1 differentiation, T-bet has also been shown to negatively regulate the Th17 program [161, 164, 322, 403, 404]. In support of this notion, we found that colonic *Tbx21*$^{-/-}$ T cells gave rise to significantly increased frequencies and total numbers of

IL-17A^{+}IFN-γ^{-} cells as compared to WT or *Rorc*$^{-/-}$ T cells (Figure 4.4A-C). In addition, as compared to WT CD4^{+} T cells, the frequency of IL-17A^{+}IFN-γ^{+} cells was decreased in *Rorc*$^{-/-}$ T cells but enhanced among *Tbx21*$^{-/-}$ T cells. However, the proportion of Th17 cells expressing IFN-γ was reduced in *Tbx21-/-* CD4^{+} T cells suggesting that the transition from IL-17A^{+}IFN-γ^{-} to IL-17A^{+}IFN-γ^{+} involves T-bet (Figure 4.6B). These data suggest that RORγ is required for the differentiation of IL-17A^{+}IFN-γ^{-} and IL-17A^{+}IFN-γ^{+} T cells and although T-bet contributes to IFN-γ production by Th17 cells it is not necessary for the differentiation of IL-17A^{+}IFN-γ^{+} T cells.

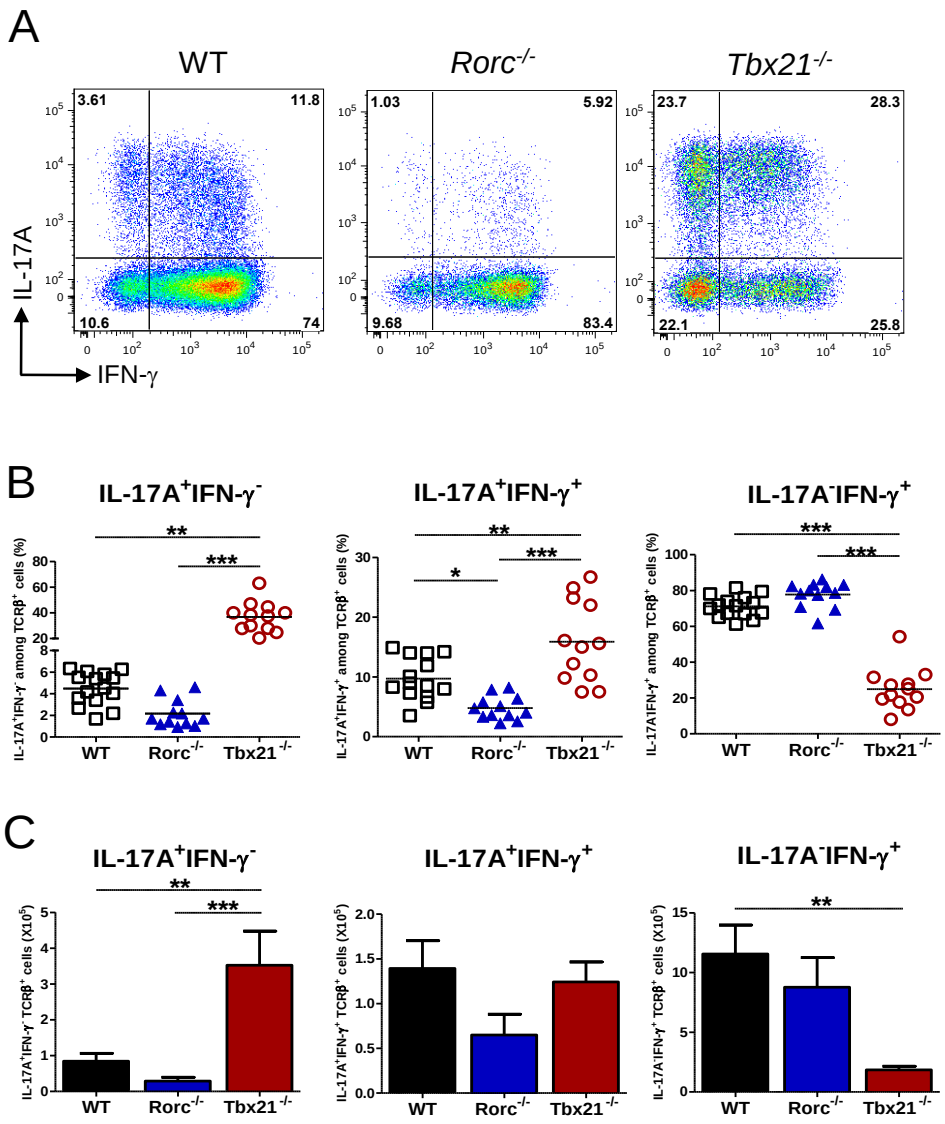

Figure 4.4. Effects of RORγ and T-bet deficiency on the differentiation of IL-17A$^+$ and/or IFN-γ$^+$ colonic CD4$^+$ T cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT, *Rorc*$^{-/-}$ or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (4-6 weeks). Single cell suspensions from the colonic lamina propria were re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of cytokines was determined by intracellular FACS.

(A) Representative FACS plots of colonic lamina propria cells, gated on viable CD4$^+$ T cells.

(B) Frequency of IL-17A$^+$ and/or IFN-γ$^+$ cells among CD4$^+$ T cells present in the colon. Bars represent the mean and each point represents an individual mouse.

(C) Total number of IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells in the colon.

Data represent pooled results from two independent experiments Bars represent the mean, error bars represent SEM (n=15 mice for WT, n=12 mice for Tbx21$^{-/-}$, n=12 mice for Rorc$^{-/-}$). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * p≤0.05, ** p ≤ 0.01, *** p ≤ 0.001

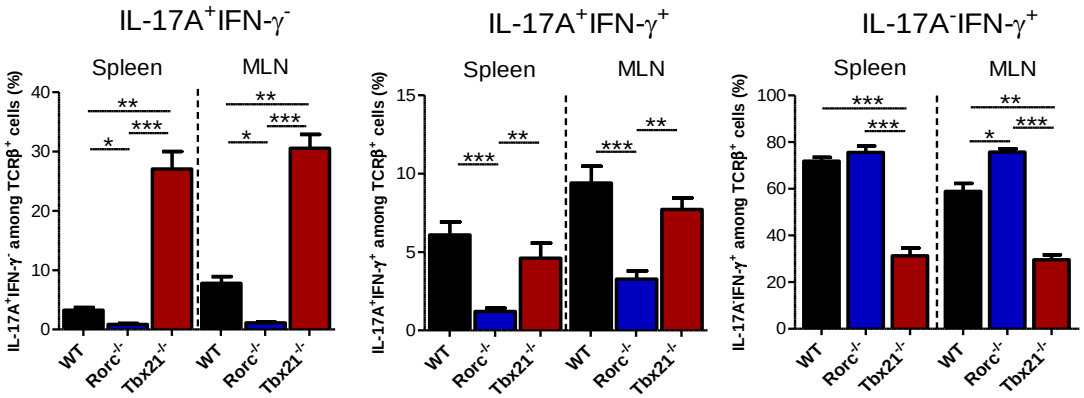

Figure 4.5. Effects of RORγ and T-bet deficiency on the differentiation of IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells in spleen and MLN.

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT, *Rorc*$^{-/-}$ or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ *T* cells developed clinical signs of disease (4-6 weeks). Single cell suspensions were prepared from indicated organs and re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of cytokines was determined by intracellular FACS.

Frequency of IL-17A$^+$ and/or IFN-γ$^+$ cells among CD4$^+$ T cells in the indicated organs. Bars represent the mean, error bars represent SEM.

Data represents pooled results from two independent experiments (n=15 mice for WT, n=12 mice for *Tbx21*$^{-/-}$, n=12 mice for *Rorc*$^{-/-}$). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

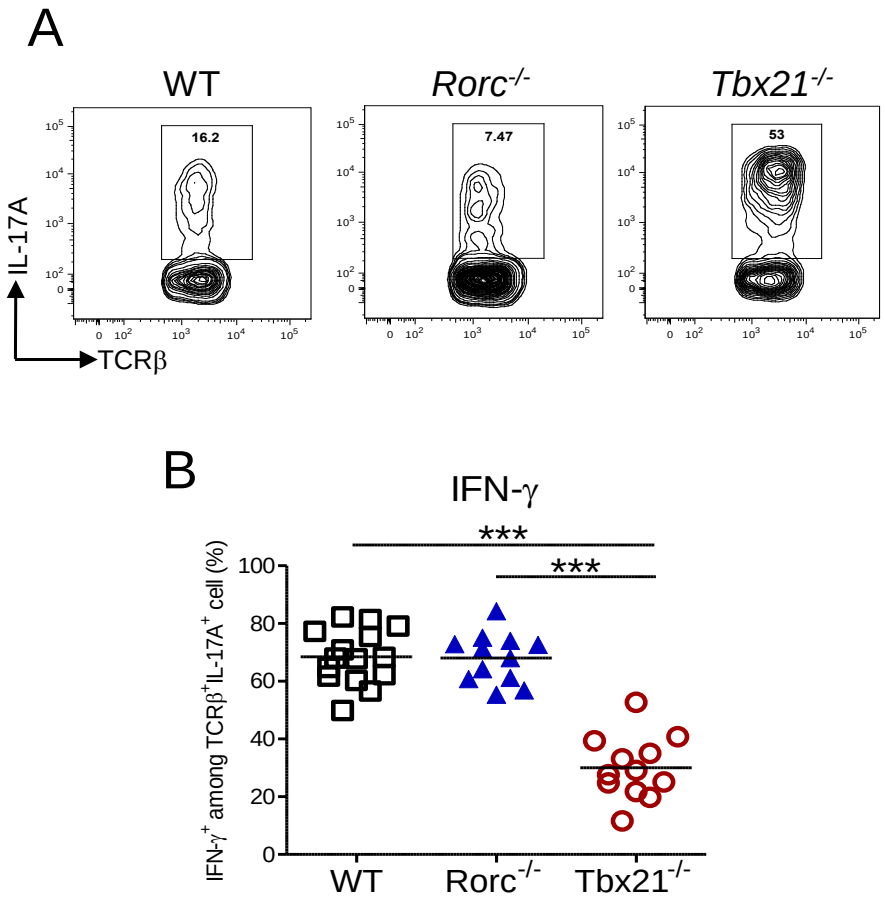

Figure 4.6. T-bet controls IFN-γ expression by intestinal Th17 cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT, *Rorc*$^{-/-}$ or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (4-6 weeks). Single cell suspensions from the colonic lamina propria were re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of cytokines was determined by intracellular FACS.

(A) Representative FACS plots of colonic lamina propria cells showing IL-17A expression, gated on viable CD4$^+$ T cells.

(B) Frequency of IFN-γ$^+$ cells among Th17 cells in the colonic lamina propria. Bars represent the mean and each point represents an individual mouse.

Data represents pooled results from two independent experiments (n=15 mice for WT, n=12 mice for *Tbx21*$^{-/-}$, n=12 mice for *Rorc*$^{-/-}$). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. *** $p \leq 0.001$.

4.2.5 Eomes is highly expressed among T-bet deficient CD4$^+$IFN-γ$^+$ T cells

Although relative to WT controls the frequency of IFN-γ producing cells was significantly decreased among *Tbx21*$^{-/-}$ CD4$^+$ T cells, approximately 40% of *Tbx21*$^{-/-}$ CD4$^+$ T cells expressed IFN-γ, suggesting the existence of a T-bet independent pathway for the generation of CD4$^+$ T cells that resemble Th1 cells. Eomes, a paralogue of T-bet, has been shown to regulate IFN-γ expression in both CD8$^+$ T cells [405] and CD4$^+$ T cells [85, 86]. Thus we hypothesized that IFN-γ-producing Tbx21$^{-/-}$ CD4$^+$ T cells would express high levels of Eomes. To test this we transferred *Rag1*$^{-/-}$ mice with WT or *Tbx21*$^{-/-}$ CD4$^+$ T cells and upon development of intestinal inflammation we isolated cells from the colonic lamina propria and analyzed Eomes expression in CD4$^+$ T cells by intracellular FACS staining. We found that approximately 20% of IL-17A$^-$IFN-γ$^+$ WT CD4$^+$ T cells expressed Eomes in the inflamed colon and Eomes expression was restricted to this population as IL-17A$^+$IFN-γ$^-$ and IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells did not express this transcription factor (Figure 4.7A-B). Interestingly, the frequency of Eomes$^+$ cells was increased among *Tbx21*$^{-/-}$ CD4$^+$ T cells in all three cytokine producing populations. However, less than 5% of IL-17A$^+$IFN-γ$^-$ and IL-17A$^+$IFN-γ$^+$ *Tbx21*$^{-/-}$ T cells expressed Eomes compared to approximately 75% of IL-17A$^-$IFN-γ$^+$ T cells (Figure4.7A-B). Thus, Eomes is preferentially expressed by Th1 cells in the inflamed colon and the majority of IFN-γ-producing *Tbx21*$^{-/-}$ T cells express Eomes, suggesting that this transcription factor may drive IFN-γ expression in the absence of T-bet.

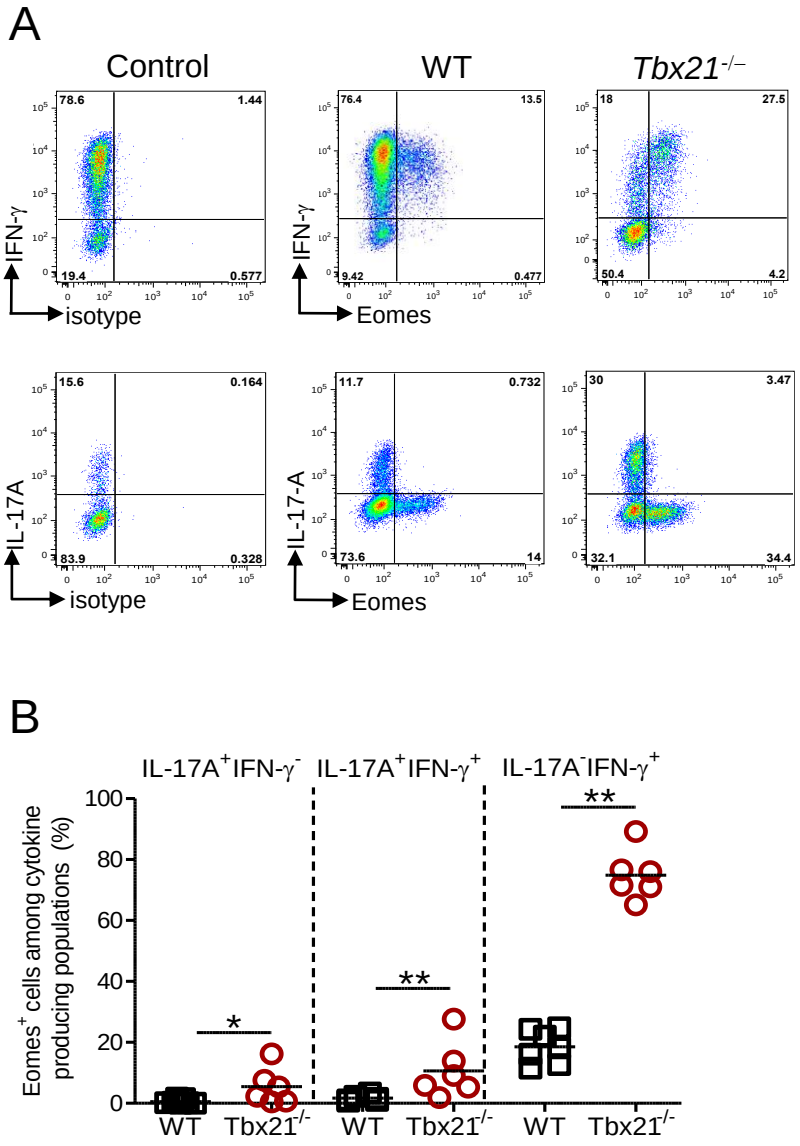

Figure 4.7. EOMES is highly expressed among T-bet deficient CD4$^+$IFN-γ$^+$ T cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from C57BL/6 WT or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (3-6 weeks). Single cell suspensions from the colonic lamina propria were re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of transcription factors and cytokines was determined by intracellular FACS.

(A) Representative FACS plots of the colon gated on viable CD4$^+$ T cells.

(B) Frequency of EOMES$^+$ cells among IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells isolated from the colon. Bars represent the mean and each point represents an individual mouse.

Data represents results from a single experiments (n=7 mice for WT, n=6 mice for *Tbx21*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$, ** $p \leq 0.01$.

4.2.6 Control of Th2 and Treg differentiation by RORγ and T-bet does not explain the differing colitogenic potential of *Rorc*$^{-/-}$ and *Tbx21*$^{-/-}$ CD4^{+} T cells

It has been shown that Foxp3^{+} Tregs and Th2 associated cytokines inhibit the development of T cell driven intestinal inflammation [160, 330, 406]. Therefore, we hypothesized that differential effects of T-bet or RORγ deficiency on Th2 or Treg differentiation might contribute to the reduced pathogenic potential of *Rorc*$^{-/-}$ versus *Tbx21*$^{-/-}$ T cells. To address this, we analyzed expression of IL-4, IL-13 and Foxp3 in the colon of *Rag1*$^{-/-}$ hosts transferred with WT, *Rorc*$^{-/-}$ or *Tbx21*$^{-/-}$ CD4^{+} T cells. Compared to WT CD4^{+} T cells the frequency of IL-4 and IL-13 expressing cells was increased among *Tbx21*$^{-/-}$ and *Rorc*$^{-/-}$ CD4^{+} T cells suggesting that RORγ and T-bet are negative regulators of Th2 responses in the colon (Figure 4.8A-B). In addition we observed a minor, but significant, increase in the frequency of Foxp3^{+} Treg cells among *Tbx21*$^{-/-}$ T cells compared to WT T cells (Figure 4.8C). However, there was no significant difference in the frequency of Foxp3^{+} Treg cells between *Tbx21*$^{-/-}$ and *Rorc*$^{-/-}$ T cells. Thus, dysregulated Th2 or Treg differentiation is unlikely to account for the difference in colitogenic potential of T-bet and RORγ deficient T cells.

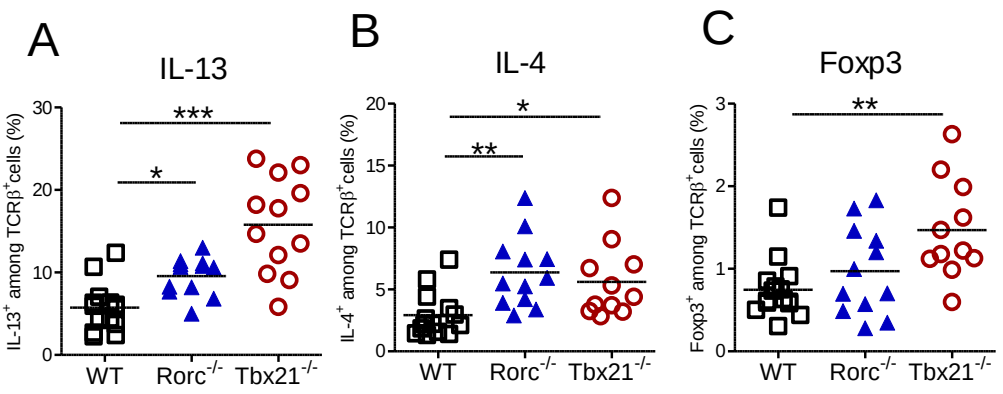

Figure 4.8. Effects of T-bet or RORγ deficiency on Th2 and Treg differentiation

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT, *Rorc*$^{-/-}$ or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (4-6 weeks). Single cell suspensions were prepared from the colonic lamina propria and re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of the indicated molecules was determined by intracellular FACS.

Frequency of IL-13 producing cells (A), IL-4 producing cells (B) and Foxp3$^+$ cells (C) among CD4$^+$ T cells in the colon. Bars represent the mean and each point represents an individual mouse.

Data represents pooled results from two independent experiments (n=15 mice for WT, n=12 mice for *Tbx21*$^{-/-}$, n=12 mice for *Rorc*$^{-/-}$). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

4.2.7 T-bet deficient CD4$^+$ T cells induce an inflammatory response distinct to that induced by transfer of WT T cells.

Th17 cells are characterized by the production IL-17A but also produce other cytokines such as IL-17F, IL-22, TNFα and GM-CSF [117, 118, 133, 134]. As we previously observed a striking increase in the frequency of IL-17A$^+$IFN-γ$^-$ cells in *Tbx21*$^{-/-}$ T cells we set out to determine the effect of T-bet deficiency on other Th17 associated cytokines. Therefore, we transferred WT or *Tbx21*$^{-/-}$ CD4$^+$ T cells into *Rag1*$^{-/-}$ recipients and measured the expression of IL-17A, IL-17F, GM-CSF and TNFα by CD4$^+$ T cells in the colon and spleen. We found significantly increased frequencies of IL-17A, IL-17F and IL-22 producing cells among *Tbx21*$^{-/-}$ T cells as compared to WT T cells, in both colon and spleen (Figure 4.9A-B & Figure 4.10). By contrast the frequencies of GM-CSF$^+$ and TNFα$^+$ CD4$^+$ T cells were reduced among *Tbx21*$^{-/-}$ T cells in the colon. Moreover, the frequencies of IL-22 and IL-17F-expressing cells among IL-17A$^+$CD4$^+$ T cells were increased, whereas frequencies of GM-CSF and TNFα–expressing cells were decreased, suggesting that T-bet deficiency has opposing consequences on production of cytokines by Th17 cells (Figure 4.9C). Next, we compared the expression of genes that are downstream of IL-17A, IL-17F and IL-22 signalling [118, 172, 335, 337] in colon homogenates of *Rag1*$^{-/-}$ recipients of WT or *Tbx21*$^{-/-}$ T cells. Transcript levels of *Csf3*, *Cxcl1*, *Cxcl2*, *Reg3g*, *S100a8* and *S100a9* were significantly increased in the colon of *Rag1*$^{-/-}$ recipients of *Tbx21*$^{-/-}$ T cells (Figure 4.11A) and consequently we also found increased numbers of neutrophils in the colonic lamina propria of these mice (Figure 4.11B). In summary, T cell transfer colitis induced by *Tbx21*$^{-/-}$ T cells is characterized by high levels of IL-17A, IL-17F and IL-22 expression, which results in the development of intestinal inflammation distinct from that induced by transfer of WT T cells.

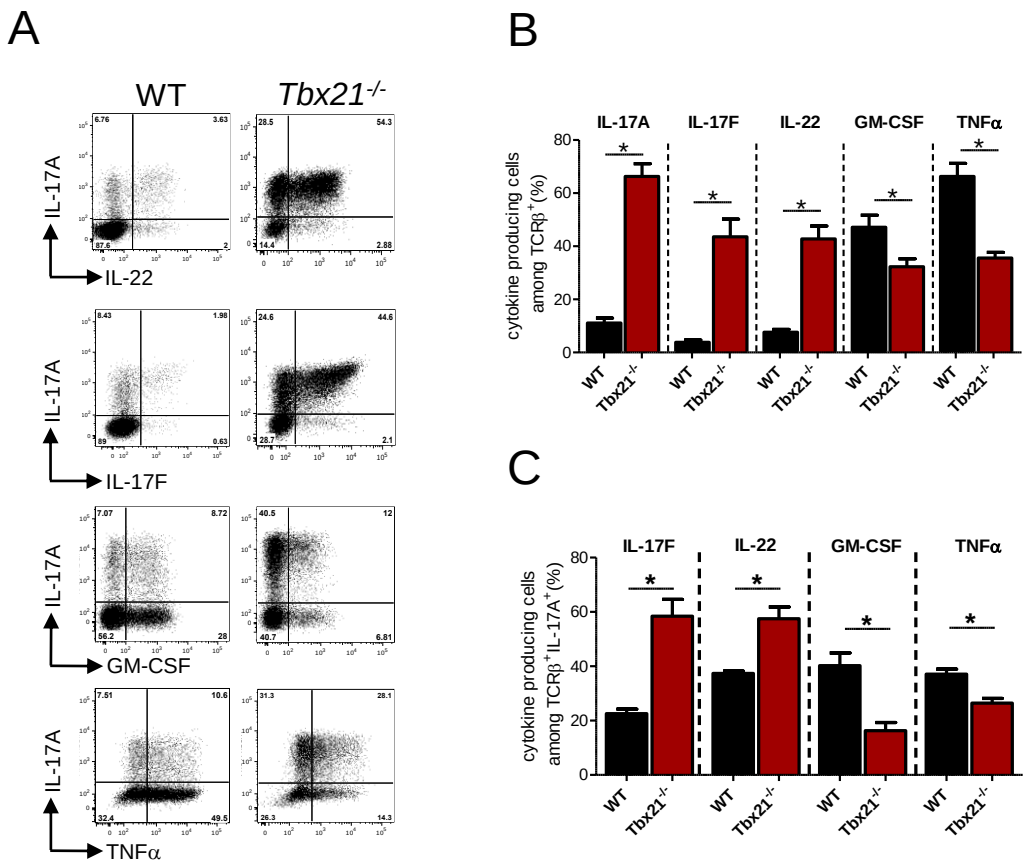

Figure 4.9. Effects of T-bet deficiency on the cytokine profile of intestinal CD4$^+$ T cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 WT or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (4-6 weeks). Single cell suspensions from the colonic lamina propria were re-stimulated with PMA and ionomycin in the presence of Monensin for 4 hours. Expression of cytokines was determined by intracellular FACS.

(A) Representative FACS plots from the colonic lamina propria, gated on viable CD4$^+$ T cells.

(B) Frequency of IL-17A$^+$, IL-17F$^+$, IL-22$^+$, GM-CSF$^+$ or TNF-α$^+$ cells among CD4$^+$ T cells in the colon. Bars represent the mean ±SEM.

(C) Frequency of IL-17F$^+$, IL-22$^+$, GM-CSF$^+$ or TNF-α$^+$ cells among IL-17A$^+$ CD4$^+$ T cells in the colon. Bars represent the mean, error bars represent SEM.

Data represents results from a single experiment (n=4 mice for WT, n=5 mice for *Tbx21*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. * p≤0.05.

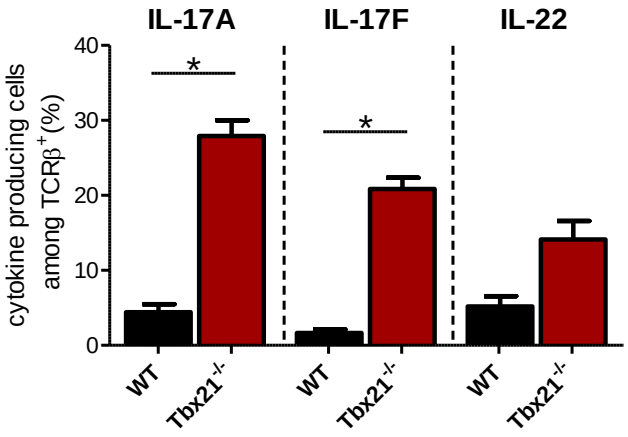

Figure 4.10. T-bet deficient $CD4^+$ T cells mount exacerbated systemic Th17 responses

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with $4x10^5$ $CD4^+CD25^-CD45RB^{hi}$ T cells from C57BL/6 WT or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (4-6 weeks). Single cell suspensions were prepared from the spleen and re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of cytokines was determined by intracellular FACS.

Frequency of IL-$17A^+$, IL-$17F^+$ or IL-22^+ among $CD4^+$ T cells in the spleen. Bars represent the mean and error bars represent SEM.

Data represents results from a single experiments (n=4 mice for WT, n=5 mice for *Tbx21*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$.

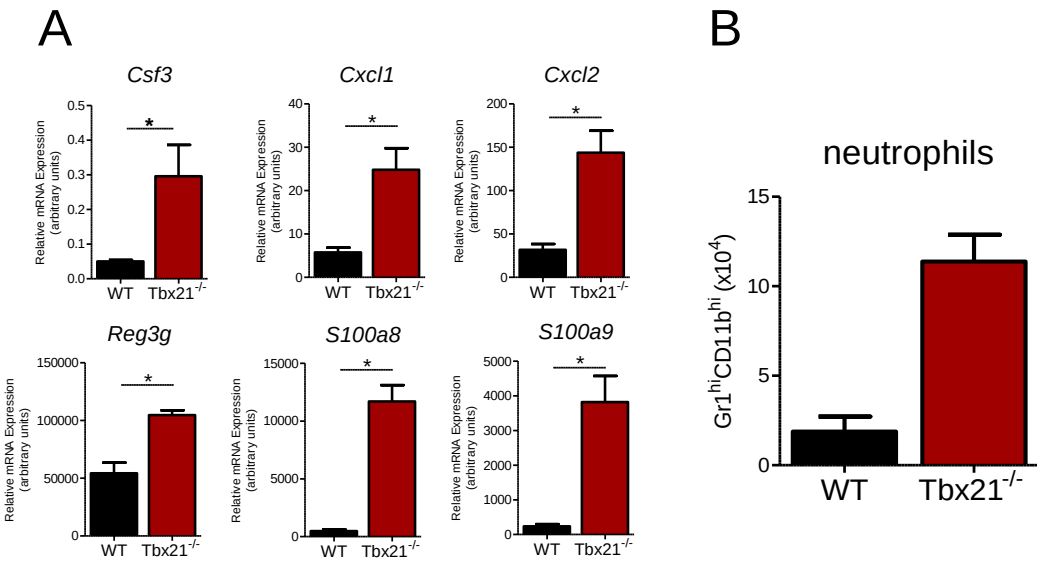

Figure 4.11. Increased expression of Th17 target genes and neutrophil accumulation in the colon of recipients of T-bet deficient CD4$^+$ T cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated C57BL/6 WT or *Tbx21*$^{-/-}$ donors. Mice were sacrificed when recipients of *Tbx21*$^{-/-}$ T cells developed clinical signs of disease (3-6 weeks).

(A) cDNA was generated from RNA extracted from colon homogenates and gene expression levels assayed by qRT-PCR. Transcript levels were normalised to *Hprt*. Bars represent the mean, error bars represent SEM.

(B) Total number of Gr1^{hi}CD11b^{hi} cells (neutrophils) in the colonic lamina propria. Bars represent the mean, error bars represent SEM.

Data represents results from a single experiments (n=4 mice for WT, n=5 mice for *Tbx21*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$.

4.2.8 Blockade of IL-17A attenuates wasting disease and prevents colitis induced by T-bet deficient T cells

Although conflicting reports exist on the role of IL-17A in intestinal inflammation, several reports have demonstrated a pathogenic role for this cytokine in the development of colitis [227, 299, 323]. Since intestinal inflammation induced by transfer of *Tbx21*$^{-/-}$ T cells into *Rag1*$^{-/-}$ mice featured an exacerbated Th17 response we examined whether IL-17A has a functional role in mediating pathology in this setting. To test this we transferred cohorts of *Rag1*$^{-/-}$ recipients with *Tbx21*$^{-/-}$ T cells and administered either an IL-17A neutralizing mAb or isotype control mAb twice a week throughout the course of the experiment starting at the time of CD4^{+} T cell transfer. As expected *Rag1*$^{-/-}$ recipients of *Tbx21*$^{-/-}$ CD4^{+} T cells that were treated with isotype control mAb developed early onset wasting disease and colitis, although disease severity was less severe than typically observed (Figure 4.12A-B). By contrast, blockade of IL-17A resulted in significantly attenuated wasting disease and colitis in *Rag1*$^{-/-}$ recipients of T-bet deficient T cells. Interestingly, blockade of IL-17A resulted in a more marked reduction in accumulation of *Tbx21*$^{-/-}$ CD4^{+} T cells in the spleen than the colon, suggesting that neutralization of IL-17A has differential effects on systemic versus intestinal T cell accumulation (Figure 4.12C). Furthermore, neutralization of IL-17A, but not administration of control isotype mAb, prevented the accumulation of neutrophils in the spleen and colon of *Rag1*$^{-/-}$ mice transferred with *Tbx21*$^{-/-}$ CD4^{+} T cells demonstrating a role for IL-17A in promoting this accumulation of neutrophils (Figure 4.13A-B). Although preliminary, these results demonstrate that systemic wasting disease and colitis induced by transfer of T-bet deficient CD4^{+} T cells into *Rag1*$^{-/-}$ hosts is dependent on IL-17A.

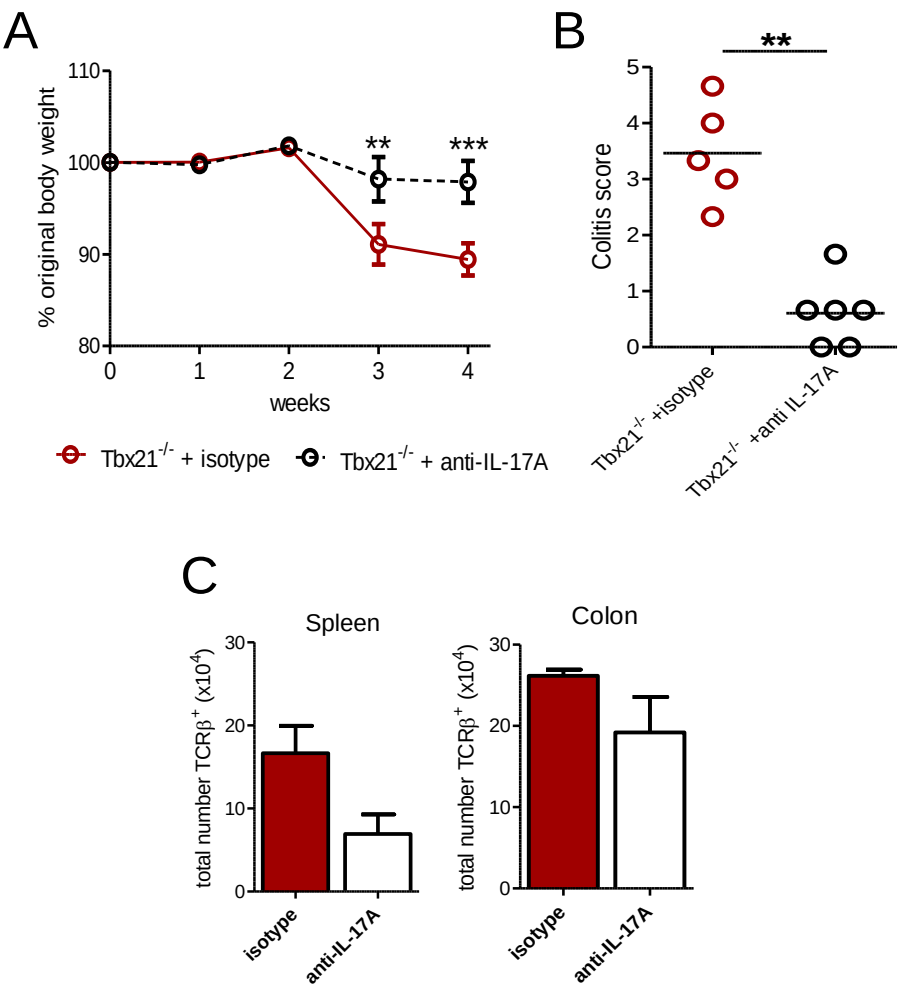

Figure 4.12. Wasting disease and colitis in recipients of T-bet deficient T cells is dependent on IL-17A

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 *Tbx21*$^{-/-}$ donors. Mice were injected with 1mg/ml of IL-17A blocking mAb or isotype control mAb twice a week throughout the course of the experiment. Mice were sacrificed at 4 weeks post-transfer.

(A) Weight loss during the course of the experiment shown as percentage of original weight at day 0. Points represent the mean, error bar represent SEM.

(B) Histological analysis of colonic inflammation. Bars represent the mean and each point represents an individual mouse.

(C) Total number of CD4$^+$ T cells in the colon. Bars represent the mean, error bars represent SEM.

Data represents results from a single experiment (n=5 mice for *Tbx21*$^{-/-}$ + isotype, n=6 mice for *Tbx21*$^{-/-}$ + anti-IL-17A). Statistical significance was determined using a repeated measure ANOVA followed by Bonferroni post-hoc test (A) and a Mann Whitney U Test (B-C). ** $p \leq 0.01$.

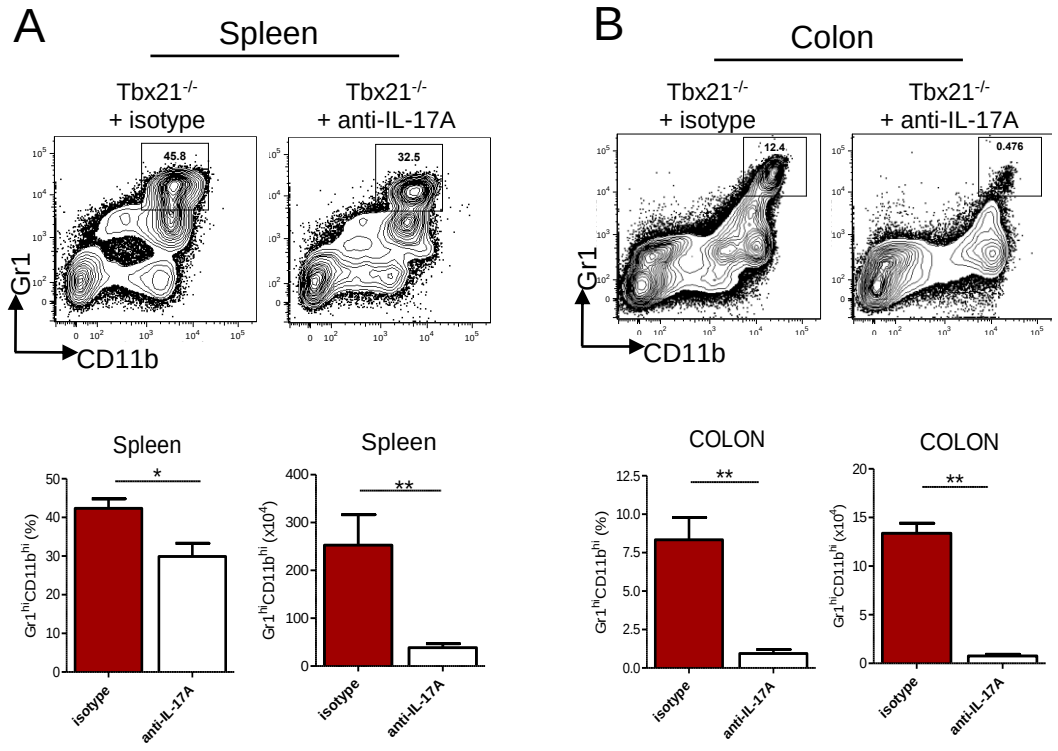

Figure 4.13. Blockade of IL-17A prevents the accumulation of neutrophils in recipients of T-bet deficient CD4$^+$ T cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells from C57BL/6 *Tbx21*$^{-/-}$ donors. Mice were injected with 1mg/ml of IL-17A blocking mAb or isotype control mAb twice a week for the duration of the experiment. Mice were sacrificed at 4 weeks post-transfer and cells were isolated from the spleen and colonic lamina propria and expression of CD11b and Gr1 was assessed by FACS.

(A) Representative FACS plots of viable cells in the spleen (top). Frequency and total number (bottom) of Gr1^{hi}CD11b^{hi} cells (neutrophils). Bars represent the mean, error bars represent SEM.

(B) Representative FACS plots of viable cells in the colonic lamina propria (top). Frequency and total number (bottom) of Gr1^{hi}CD11b^{hi} cells (neutrophils). Bars represent the mean, error bars represent SEM.

Data represents results from a single experiments (n=5 mice for *Tbx21*$^{-/-}$ + isotype , n=6 mice for *Tbx21*$^{-/-}$ + anti-IL-17A). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$, ** $p \leq 0.01$.

4.2.9 T-bet limits the ability of IL-23 to promote Th17 differentiation /accumulation

T cell transfer colitis induced by WT T cells is dependent on IL-23 [265, 330] . Although we did not determine whether this also applies to colitis induced by *Tbx21*$^{-/-}$ T cells, we performed *in vitro* cultures to determine the response of *Tbx21*$^{-/-}$ CD4^{+} T cells to IL-23. To this end we cultured naïve CD4^{+}CD62L^{+}CD44^{lo} T cells from WT or *Tbx21*$^{-/-}$ mice under Th0 conditions or Th17 polarizing conditions that are optimal for the induction of *Il23r* expression. We found that *Tbx21*$^{-/-}$ CD4^{+} T cells gave rise to a higher percentage of IL-17A-expressing cells than WT CD4^{+} T cells under Th0 conditions suggesting that T-bet deficient T cells have an intrinsic tendency to develop into Th17 cells (Figure 4.14A). By contrast, the frequency of IL-17A^{+} cells was comparable between WT and *Tbx21*$^{-/-}$ CD4^{+} T cells under minimal Th17 polarizing conditions and addition of IL-23 further enhanced differentiation of IL-17A-expressing cells in both populations of T cells. However, the increase in IL-17A-expressing cells in the presence of IL-23 was more pronounced in *Tbx21*$^{-/-}$ CD4^{+} T cells. Although these results are only preliminary at this stage, they suggest that T-bet limits the ability of IL-23 to promote Th17 differentiation /accumulation *in vitro*.

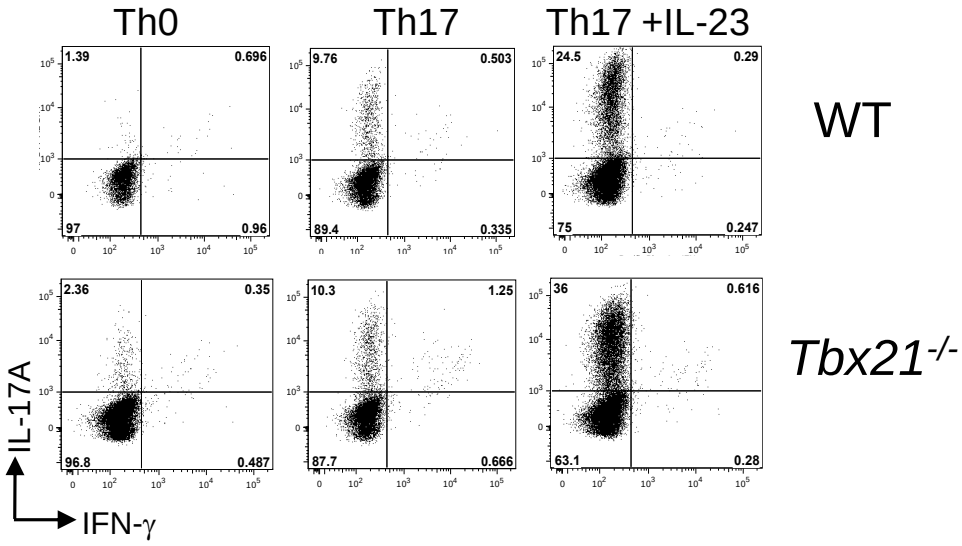

Figure 4.14. T-bet limits the ability of IL-23 to promote Th17 differentiation /accumulation *in vitro*

$CD4^+CD62L^+CD44^-$ naive T cells were FACS sorted and $2x10^5$ naive T cells were stimulated with plate-bound anti-CD3 (5μg/ml) and anti-CD28 (5μg/ml) and cultured for 3 days in the presence of anti-IFN-γ and anti-IL-4 (Th0) or TGFβ1 (200pg/ml), IL-6 (20ng/ml) and IL-1β (20ng/ml) without (Th17) or with (Th17 + IL-23) IL-23 (20ng/ml) in the presence of blocking antibody to IFN-γ and IL-4. Cultured cells were restimulated with PMA and ionomycin in the presence of monensin and were assessed by intracellular FACS. Data represent a single experiment (n=1).

4.3 Discussion

$CD4^+$ T cell lineage commitment and plasticity have received much attention in recent times and it has now become clear that $CD4^+$ T cell subsets exhibit functional flexibility and can adopt alternative fates [253]. For example, analyses of the stability of Th17 cells *in vivo* suggest that IL-$17A^+$IFN-γ^+ $CD4^+$ T cells represent an intermediate phenotype in the transition from Th17 cells into Th1-like cells [393]. The transcription factors T-bet and STAT4, which are required for Th1 differentiation, have been shown to mediate the transition of Th17 cells into IFN-γ-producing cells *in vitro* through repression of the Th17 program and remodelling of the *Ifng* locus [132, 263]. Recently, elegant fate mapping experiments of IL-17A-producing cells during chronic inflammation of the CNS have shown that the majority of IL-$17A^+$IFN-γ^+ and IL-$17A^-$IFN-γ^+ effector cells arise from Th17 cell progeny [393]. This transition of Th17 cells into IFN-γ-producing "ex"Th17 cells required IL-23 and correlated with increased expression of T-bet. Our finding that IL-$17A^+$IFN-γ^+ T cells co-express RORγ and T-bet lends further support to the notion that these cells represent an intermediate Th1/Th17 phenotype. Interestingly, levels of T-bet and RORγ expression among IL-$17A^+$IFN-γ^+ T cells were similar to those observed in Th1 and Th17 cells respectively. Co-expression of "lineage-defining" transcription factors by $CD4^+$ T cells has been described by several groups. For example, $ROR\gamma^+Foxp3^+$ T cells are highly enriched within the intestine and it has been shown that this population remains stable during acute inflammatory responses and may represent a regulatory counterpart of Th17 cells [130, 256]. In addition, infection with the lymphocytic choriomeningitis virus (LCMV) was shown to reprogram committed GATA-3^+ Th2 cells into $GATA3^+$T-bet^+ T cells, which co-expressed IL-4 and IFN-γ, remained stable *in vivo* and induced less immunopathology [270]. Furthermore, T-bet

expression by Foxp3$^+$ Treg cells is required for the appropriate control of Th1-mediated immunity [225]. Thus co-expression of lineage-defining transcription factors by CD4$^+$ T cells is not always associated with a transitional state and could also represent functional adaptation. Therefore, it remains to be determined whether the intestinal RORγ^+T-bet$^+$ T cells described here represent a stable poly-functional effector T cell population or transitional Th17 cells on the way to becoming Th1-like.

The high levels of RORγ and T-bet expression found in intestinal IL-17A$^+$IFN-γ^+ T cells implies the presence of signals in the inflamed environment of the colon that support sustained co-expression of both transcription factors. We previously showed that IL-23 promotes the emergence of IL-17A$^+$IFN-γ^+ cells through cell intrinsic effects on T cells [391]. In addition, IL-23 has been shown to drive the acquisition of IFN-γ production by Th17 cells in a T-bet dependent manner [132] and can promote RORγ expression in naïve CD4$^+$ T cells transduced with IL-23R [125]. Surprisingly, direct IL-23 signals into T cells did not contribute to induction and/or maintenance of T-bet or RORγ expression, as *Il23r*$^{-/-}$ and WT T cells expressed similar levels of both transcription factors in the inflamed colon of chimaeric mice. This finding contradicts a recent report by Hirota *et al* which found that IL-23 signalling was required for T-bet expression in Th17 cells in the EAE model [393]. However, contrary to our study, these authors did not analyze T-bet expression in WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the same inflammatory environment, and we previously demonstrated that some of the defects in *Il-23r*$^{-/-}$ T cells can be overcome by presence of WT T cells [391]. Therefore it is likely that IL-23 contributes to T-bet expression in Th17 cells through cell extrinsic effects. Despite normal levels of T-bet and RORγ expression in *Il23r*$^{-/-}$ CD4$^+$ T cells we cannot exclude that IL-23 modulates their transcriptional activity.

Binding of RORγ to transcriptional co-activators such as STAT3 or Runx1 enhances expression of Th17 cell associated cytokines [125, 135] . Similarly, T-bet co-operates with STAT4 in Th1 specification by promoting expression of the transcription factors Hlx, Runx3 and Etv5 [78, 82-84]. Although little is known about the contribution of IL-23 signals to the regulation of these transcription factors it has been shown that IL-23R mediates its signal mainly through STAT3 but also induces weak phosphorylation of STAT1, STAT4 and STAT5 [304]. Thus IL-23 could modulate $CD4^+$ T cell phenotype through previously unappreciated activation of additional transcription factors.

Two independent studies have shown that $CD4^+$ T cells deficient in T-bet or RORγ fail to induce colitis upon transfer into immunodeficient hosts [88, 326]. Neurath *et al* convincingly showed that adoptive transfer of *Tbx21*$^{-/-}$ $CD4^+$ T cells into SCID recipients failed to induce colitis and this correlated with reduced IFN-γ and increased IL-4 production [88]. Importantly, a follow-up study revealed that IL-4 plays a functional role in inhibiting the colitogenic potential of *Tbx21*$^{-/-}$ T cells, as recipients of $CD4^+$ T cells deficient in both T-bet and STAT6 developed severe colitis [86]. Moreover, the intestinal inflammation that developed in recipients of *Stat6*$^{-/-}$*Tbx21*$^{-/-}$ T cells could be blocked by administration of IL-17A neutralizing mAb suggesting that the potent inhibitory effect of IL-4/STAT6 signals on Th17 differentiation normally prevent colitis induced by *Tbx21*$^{-/-}$ T cells. Similar to the findings with *Tbx21*$^{-/-}$ T cells, it has been shown that RORγ-deficient T cells fail to induce colitis upon transfer into *Rag1*$^{-/-}$ recipients [326]. Simultaneous ablation of IL-17F in T cells and neutralization of IL-17A also abrogated intestinal inflammation suggesting that RORγ promotes disease through induction of these cytokines. Together, these

findings add further support to a possible pathogenic role of RORγ^{+}T-bet^{+} CD4 T cells chronic intestinal inflammation. Strikingly, our T cell transfer experiments revealed that RORγ but not T-bet expression by T cells was required for the development of colitis. T-bet deficient T cells induced severe early onset wasting disease and promoted the development of intestinal pathology comparable to that induced by transfer of WT T cells. This observation is in stark contrast to the published results with *Tbx21*$^{-/-}$ CD4^{+} T cells described above and various explanations could account for the discrepancy between our study and these earlier findings. Firstly, in contrast to the published report we used naïve *Tbx21*$^{-/-}$ CD4^{+} T cells from C57BL/6 mice instead of BALB/c mice. An important difference between *Tbx21*$^{-/-}$ CD4^{+} T cells from these genetic backgrounds appears to be their differential susceptibility to suppression by IL-4/STAT6 signals. We found that transfer of *Tbx21*$^{-/-}$ T cells induced IL-17A-dependent colitis despite increased frequencies of IL-4-expressing cells in the intestine. This discrepancy may be due the higher amount of IL-4 produced by recently activated CD4^{+} T cells from BALB/c versus C57BL/6 mice [114]. Secondly, differences in the composition of the intestinal microbiota between animal facilities can have a substantial role in skewing CD4^{+} T cells responses. In particular, the *Clostridium*-related segmented filamentous bacteria (SFB) have been shown to drive the emergence of IL-17 and IL-22 producing CD4^{+} T cells in the intestine [7]. The heightened Th17 response in SFB colonized mice contributes to resistance to infections with intestinal pathogens but also increases susceptibity to experimental autoimmune diseases including arthritis and EAE [7, 283, 284]. Importantly, the ability of naïve CD4^{+} T cells to induce colitis is dependent on the presence of intestinal bacteria, as germ-free mice do not develop pathology upon transfer of naïve T cells [285]. Colonization of germ-free mice with intestinal

microbiota containing SFB was found to be necessary to restore the development of colitis [285]. Since our *Rag1*$^{-/-}$ colony is SFB^{+} (data not shown), and the presence of SFB was not confirmed in the previous studies, it is possible that SFB contributed to the observed differences in disease induced by *Tbx21*$^{-/-}$ T cells. Thirdly, although our C57BL/6 *Tbx21*$^{-/-}$ colony has been backcrossed for at least 9 generations to C57BL/6 mice we cannot exclude that minor genetic mismatches between *Tbx21*$^{-/-}$ T cell donor and *Rag1*$^{-/-}$ recipient cause a graft-versus-host type pathology that contributes to the development of colitis. Finally, the original study was performed by transfer of CD4^{+}CD62L^{+}CD45RB^{hi} T cells rather than CD4^{+}CD25^{-}CD45RB^{hi} cells used in our study. Previous work from our lab has shown a differential requirement for IL-10 in the prevention of T cell transfer colitis by Tregs when defined as either CD4^{+}CD25^{+} or CD4^{+}CD45RB^{lo} T cells [407, 408], demonstrating that slight variations in the composition of purified subsets depending on the surface markers used for their isolation might reveal differential functions for a given gene. Therefore, we cannot exclude that CD4^{+} T cells with differences in the percentage which are truly naive have differing requirements for T-bet.

Despite the aforementioned differences between our study and published reports the transfer of RORγ and T-bet-deficient CD4^{+} T cells in parallel allowed us to delineate the contribution of these transcription factors to intestinal T cell differentiation. Our experiments confirmed the known requirement for RORγ and T-bet in the development of Th17 [49] and Th1 [79] responses, respectively. However, Th17 differentiation was not completely abrogated in RORγ-deficient T cells. This could relate to the activity of RORα, which has been shown contribute to Th17 differentiation [139]. Furthermore, T-bet-deficient T cells were impaired in their

ability to differentiate into Th1 cells but a substantial proportion of *Tbx21*$^{-/-}$ T cells retained the ability to express IFN-γ. The T-box transcription factor Eomes, a paralogue of T-bet, has been shown to compensate for IFN-γ production in T-bet deficient CD4^{+} T cells [85, 86]. In accordance with this, Eomes was highly expressed in *Tbx21*$^{-/-}$ T cells suggesting that IFN-γ expression by T-bet-deficient T cells may be driven by Eomes. In addition to their effect on IFN-γ production both Eomes and T-bet have been shown to suppress Th17 differentiation [151, 164]. However, we observed a significant increase in IL-17A-producing cells among *Tbx21*$^{-/-}$ T cells despite high expression of Eomes suggesting that Eomes is not sufficient to repress the Th17 program in the absence of T-bet. We demonstrated here that IL-17A^{+}IFN-γ^{+} T cells co-express RORγ and T-bet suggesting that these transcription factors contribute to the differentiation of this population. The frequency of IL-17A^{+}IFN-γ^{+} T cells was decreased in *Rorc*$^{-/-}$ T cells but increased in *Tbx21*$^{-/-}$ T cells as compared to WT CD4^{+} T cells. However, T-bet contributed to IFN-γ production by Th17 cells, as there was a reduced proportion of *Tbx21*$^{-/-}$ Th17 cells expressing IFN-γ relative to WT or *Rorc*$^{-/-}$ CD4^{+} Th17 cells. Interestingly, Eomes expression was mostly restricted to IL-17A^{-}IFN-γ^{+} cells in WT and *Tbx21*$^{-/-}$ T cells making it unlikely that Eomes contributes to IFN-γ expression by IL-17A-expressing *Tbx21*$^{-/-}$ T cells. This data suggest that T-bet contributes to IFN-γ expression by Th17 cells but is not necessary for the emergence of IL-17A^{+}IFN-γ^{+} T cells. This is in contrast to a recent study in EAE, which showed that IL-17A^{+}IFN-γ^{+} CD4^{+} T cells did not arise in the CNS of T-bet deficient mice immunized with MOG peptide plus CFA [164]. An important difference between this study and ours is the lymphopenic environment of the *Rag1*$^{-/-}$ host. The transfer of naïve CD4^{+} T cells into *Rag1*$^{-/-}$ recipients results in rapid

lymphopenia induced proliferation [386] and this environment has been shown to favour the conversion of Th17 cells into IFN-γ producing cells [409]. Thus, it is plausible that IFN-γ-expressing *Tbx21*$^{-/-}$ T cells represent a feature of the rapid proliferation of T cells under lymphopenic conditions. Regardless of how IL-17A^{+}IFN-γ^{+} T cell arise in the absence of T-bet the emergence of this population correlated with disease activity as the frequency and total number of these cells were reduced in recipients of *Rorc*$^{-/-}$ T cells as compared to recipients of WT and *Tbx21*$^{-/-}$ T cells.

Several studies have reported increased Th17 responses in T-bet deficient mice [161, 322, 404]. Recently, it was reported that T-bet suppresses differentiation of naïve T cells into Th17 cells by preventing Runx1-mediated induction of *Rorc* [164]. In addition to its inhibitory effects on developing Th17 cells, T-bet was also shown to be required for conversion of established Th17 cells into IFN-γ producing cells. In keeping with the negative effect of T-bet on Th17 differentiation we found that *Tbx21*$^{-/-}$ T cells gave rise to increased frequencies and numbers of IL-17A-expressing cells upon transfer into *Rag1*$^{-/-}$ hosts. This enhanced Th17 response could be caused by the intrinsic propensity of *Tbx21*$^{-/-}$ to differentiate into Th17 cells or the failure of Th17 cells to transition into IFN-γ-producing cells or a combination of both. Interestingly, WT and *Tbx21*$^{-/-}$ T cells gave rise to phenotypically distinct populations of Th17 cells. WT Th17 cells expressed IFN-γ, TNFα and GM-CSF and only low levels of IL-17F and IL-22. By contrast, *Tbx21*$^{-/-}$ Th17 cells expressed high levels IL-17F and IL-22 but expression of IFN-γ, TNFα and GM-CSF was significantly reduced as compared to WT Th17 cells. Thus, T-bet expression in CD4^{+} T cells not only restrains Th17 differentiation but also modulates cytokine secretion by Th17

cells. Due to the enhanced IL-17A response in *Tbx21*$^{-/-}$ we hypothesized that this cytokine might have role in driving disease. Indeed, blockade of IL-17A in recipients of *Tbx21*$^{-/-}$ T cells prevented the development of wasting disease and colitis. This contradicts published reports that CD4 T cell derived IL-17A is dispensable for induction of colitis in this model [326, 329, 330, 344]. However, we neutralized IL-17A responsiveness in all cell types rather than specifically in T cells. In addition it has been suggested that the level of cytokine production, or the cytokine milieu, contribute to protective versus pathogenic effects of Th17 associated cytokines. For example, transfer of CD4^{+}CD45RB^{hi} cells into *Rag1*$^{-/-}$ hosts revealed a protective role for T cell derived IL-22 in the development of colitis, possibly through its effects on epithelial cell proliferation and survival [331]. On the other hand transfer of Treg-depleted CD4^{+}CD45RB^{low} cells, which produced much higher levels of IL-22 than CD4^{+}CD45RB^{hi} cells, contributed to pathology by inducing epithelial hyperplasia [410]. Thus, the increased expression of IL-17A by *Tbx21*$^{-/-}$ T cells compared to WT T cells might be a prime factor in revealing the pathogenic potential of this cytokine in the context of intestinal inflammation and wasting disease. Interestingly, it has been shown that *Il17a*$^{-/-}$ and *Il17ra*$^{-/-}$ T cells induce more aggressive wasting disease compared to WT cells [344] suggesting that the relatively low levels of IL-17A expressed by WT T cells contain excessive systemic inflammation. A similar host protective function of IL-17A has also been described in the intestine during acute epithelial injury induced by DSS [342, 343]. In support of a more pro-inflammatory function of IL-17A we detected high levels of IL-17A target genes G-SCF, CXCL1 and CXCL2 in the inflamed colon of *Tbx21*$^{-/-}$ transferred recipients and this was accompanied by a marked accumulation of neutrophils. In contrast to previous reports [326] we found that IL-17F could not compensate for IL-17A, as blockade of IL-17A

was sufficient to block intestinal inflammation and reduce accumulation of neutrophils in the colon and the spleen. In addition, we also detected increased expression of anti-microbial peptides, which correlated with the high levels of IL-22 produced by *Tbx21*$^{-/-}$ CD4^{+} T cells; however, the contribution of IL-22 to disease was not established in this setting. It should be noted that the colitis induction by *Tbx21*$^{-/-}$ T cells treated with control isotype mAb was suboptimal compared to previous experiments for unidentified reasons, and thus, there may still be a contribution by other cytokines to the development of disease in *Tbx21*$^{-/-}$ T cell transfer colitis.

The recent focus on molecular studies of helper T cells has highlighted the concept of cooperativity between transcription factors classically defined as lineage specific [253]. However, one of the central features of the original Th1/Th2 model put forward by Mossman and Coffman [62, 65] was the cross regulation of one subset by soluble factors produced by the other. Early studies demonstrated that both Th1 and Th2 cytokines could suppress the differentiation of Th17 cells [161, 163]. Therefore, the reduced IFN-γ production in *Tbx21*$^{-/-}$ CD4^{+} T cells could facilitate the expansion/differentiation of Th17 cells independently of a cell autonomous role for T-bet-mediated suppression of the Th17 programme. Although a recent report demonstrated that T-bet deficiency promotes IL-17A production *in vitro* independently of IFN-γ these two hypotheses are not mutually exclusive [164]. It will also be important to assess whether or not colitis driven by *Tbx21*$^{-/-}$ T cells is dependent on IFN-γ, as is the case for WT CD4^{+} T cells [87], or whether T-bet expression drives intestinal inflammation with differential cytokine requirements. If *Tbx21*$^{-/-}$ CD4^{+} T cell driven colitis develops independently of IFN-γ it might suggest that detailed profiling of CD4^{+} T cell in IBD patients could help determine the most

appropriate treatment, with patients exhibiting low levels of T-bet/IFN-γ being treated with IL-17A blockade and patients with high level expression of this pathway with anti-IFN-γ.

It has been suggested that T-bet expression by Th17 cells is required for their pathogenicity [164, 269]. In EAE, an IL-23 dependent model of chronic autoimmune inflammation of the CNS, both RORγ and T-bet are required for the development of pathogenic CD4 T cell responses [49, 315]. In addition, T-bet is highly expressed in IL-23 stimulated Th17 cells and these cells exhibit increased pathogenic potential *in vivo* [154]. However, little is known about the mechanism by which T-bet regulates pathogenicity of Th17 cells. Recently it was demonstrated that T-bet represses IL-23R expression through inhibition of *Rorc*, and although *Tbx21*$^{-/-}$ CD4 T cells in the CNS expressed higher levels of IL-23R they failed to induce EAE [164]. This suggests that T-bet activation downstream of IL-23R may be required for pathogenic Th17 responses. By contrast another study found that T-bet enhances pathogenic Th17 responses by inducing IL-23R expression [411]. Our finding that *Tbx21*$^{-/-}$ T cells generated stronger Th17 responses in response to IL-23 stimulation *in vitro* and expressed higher levels of the IL-23 inducible cytokine IL-22 *in vivo* supports the notion that T-bet modulates the effects of IL-23 in CD4^{+} T cells, which could potentially be mediated through negative regulation of IL-23R expression. This could also explain why TGF-β promotes expression of the IL-23 receptor as it inhibits T-bet expression [412]. Alternatively, T-bet could modify the effector programme induced by IL-23 independently of effects on IL-23 signalling. Indeed T-bet expression in Th17 cells could represent a negative feedback loop in the intestine to limit excessively damaging Th17 responses induced by IL-23. This is not supported by our

findings as *Tbx21*$^{-/-}$ CD4^{+} T cells did not drive more severe intestinal inflammation than their WT counterparts but in this setting the Th1 response is also impaired by T-bet deficiency. The combination of WT levels of Th1 cells and the enhanced level of Th17 responses observed upon transfer of *Tbx21*$^{-/-}$ CD4^{+} T cells might drive even more severe intestinal inflammation. This hypothesis could be tested by the co-transfer of WT and *Tbx21*$^{-/-}$ CD4^{+} T cells into the same host. Together these findings point towards a role of IL-23 in *Tbx21*$^{-/-}$ CD4^{+} T cell colitis and suggest that IL-23 might drive pathogenic Th17 responses independently of T-bet. In support of this, it has been shown that experimental autoimmune myocarditis, which is dependent on IL-23, is exacerbated in T-bet deficient mice due to enhanced IL-23-driven IL-17A production [322]. In addition, T-bet deficient mice also developed IL-17-driven disease in a model of cardiac allograft rejection [404] and an experimental model of systemic autoimmune disease [403]. Collectively, these findings strongly suggest that Th17-mediated pathologies can develop in the absence of T-bet.

In addition to their role in T cells, T-bet and RORγ are also known to regulate the function of innate immune cells. T-bet-deficient *Rag2*$^{-/-}$ mice have been shown to develop spontaneous colitis due to increased TNF-α production by intestinal DCs [413]. Thus, T-bet expression in the innate compartment is likely to have a host protective role in the T cell transfer model of colitis. By contrast, we recently showed that IL-23 promotes innate colitis through induction of IL-17A and IFN-γ production by IL-7Rα^{+} Thy1^{hi} SCA-1^{+}CD4^{-} NKp46^{-} ILCs [171]. Importantly, RORγ had a functional role as *Rag1*$^{-/-}$*Rorc*$^{-/-}$ failed to develop innate colitis. Since *Rag1*$^{-/-}$ recipients of *Rorc*$^{-/-}$ T cells were not completely devoid of intestinal inflammation in our T cell transfer experiments it is possible that the residual inflammation observed

in these mice is driven by RORγ^{+} ILCs and the residual inflammatory activity of *Rorc*-/- CD4^{+} T cells. Thus, it will be important to transfer WT T cells into *Rag1*$^{-/-}$ *Rorc*$^{-/-}$ mice to assess the contribution of RORγ^{+} ILCs to T cell driven colitis.

In summary, we have shown here that IL-23 signals into T cells are not required for the induction/maintenance of RORγ and T-bet expression by intestinal CD4^{+} T cells. Unexpectedly, we found that RORγ but not T-bet expression by T cells was required for the induction of intestinal inflammation. RORγ was also necessary for the emergence of IL-17A^{+}IFN-γ^{+} T cells, and although T-bet contributed to IFN-γ production by these cells it was not required for their differentiation. Importantly, colitis induced by T-bet deficient T cells was dependent on IL-17A, and showed a unique inflammatory phenotype associated with increased neutrophil influx, demonstrating that pathogenic intestinal Th17 responses can develop independently of T-bet. These studies further highlight the potential importance of host genetics given the different responses seen between T-bet deficient CD4^{+} T cells on the BALB/c or C57BL/6 background. Finally, different human populations may also have differential requirements for T-bet expression and thus our findings support the idea of tailored therapies for treatment of IBD in humans.

Chapter 5: Identification and functional characterisation of IL-23 target genes in intestinal T cells

5.1 Introduction

IL-23 is known to play a pathogenic role in several models of autoimmune and chronic inflammatory diseases such as experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA) and intestinal inflammation [116, 264, 265, 291, 319, 323]. In addition to being required for bacterially triggered and adoptive $CD4^+$ T cell transfer models of intestinal inflammation [264, 265, 323], IL-23 expression is increased in patients with active inflammatory bowel disease [354, 360]. Furthermore, mutations in the *IL23R* gene in human populations have been linked to increased susceptibility to the development of IBD [348]. Collectively these data suggest an essential role for IL-23 in intestinal inflammation in both mouse and human. However, despite the requirement for IL-23 in intestinal inflammation we still lack detailed knowledge of the downstream mediators responsible for disease induction and maintenance in comparison to models such as EAE where the pathways have been better defined [117, 131, 154, 402].

Th17 cells, which require RORγt for their differentiation [49], express high levels of IL-23R [117], and although IL-23 is not required for their differentiation [116, 117, 122, 124], a role for IL-23 in exposing the pathogenic effector function of Th17 cells *in vivo* has been proposed [117, 131, 154, 402]. For example, exposure of Th17 cells to IL-23 was shown to promote expression of the cytokine GM-CSF, which is required for development of EAE [133, 134]. In addition Th17 cells generated in the presence of IL-23, IL-1β and IL-6 express high levels of the Th1 associated transcription factor T-bet and exhibit increased pathogenic potential in an adoptive

transfer model of EAE [154]. This correlates well with findings that T-bet expression by Th17 cells is required for their pathogenicity in that model [269].

Similar to the findings in EAE, IL-23 activity on $CD4^+$ T cells is essential for mediating intestinal inflammation following naïve $CD4^+$ T cell transfer [391]. However, as described in the previous chapter, T-bet deficient $CD4^+$ T cells retain their colitogenic potential demonstrating that, in contrast to findings obtained in EAE, induction of colitis did not require T-bet expression by $CD4^+$ T cells. In addition, T cell derived IL-17A [326, 329, 330], IFN-γ [325], IL-17F [326], IL-22 [326, 331] or GM-CSF [332] are not required for development of $CD4^+$ T cell transfer colitis, suggesting the existence of as yet unidentified pathogenic factors that contribute to IL-23 driven intestinal inflammation. Furthermore, IL-23 has also been shown to promote intestinal inflammation indirectly through inhibition of $Foxp3^+$ Treg cell differentiation [330]. Adoptive transfer of naïve $CD4^+$ T cells into IL-23 deficient *Rag1*$^{-/-}$ recipients led to increased $Foxp3^+$ Treg differentiation and this was later shown to be dependent on direct signalling of IL-23 into $CD4^+$ T cells [391]. By contrast, transfer of naïve $CD4^+$ T cells from Foxp3 deficient donors, whereby Treg development post transfer was prevented, allowed the development of intestinal inflammation in the absence of IL-23. However, the mechanism by which IL-23 blocked iTreg generation in the intestine remains enigmatic and is further complicated by the fact that this potent inhibitory effect of IL-23 on Treg induction could not be revealed *in vitro* [330], suggesting that IL-23 might inhibit iTreg conversion in response to unknown tissue specific factors not present in *in vitro* cultures. Thus, the aim of experiments described in this chapter was to further characterise IL-23R-driven effector responses.

5.2 Results

5.2.1 Identification of IL-23 regulated genes in colonic CD4$^+$ T cells.

It is thought that IL-23 is essential for the development of pathogenic Th17 responses [117, 131, 154, 269, 402] and we previously described that IL-23 restrains the differentiation of Foxp3$^+$ Treg cells [330, 391]. However, little is known about the transcriptional program induced in CD4$^+$ T cells by IL-23. Therefore, we decided to characterize IL-23-regulated genes in colonic CD4$^+$ T cells by comparing the gene expression profile of WT and *Il23r*$^{-/-}$ CD4$^+$ T cells. We have previously shown that *Il23r*$^{-/-}$ CD4$^+$ T cells fail to accumulate in the intestine and induce only mild colitis upon transfer into *Rag1*$^{-/-}$ recipients (Chapter 3). However, *Il23r*$^{-/-}$ CD4$^+$ T cells accumulate normally when transferred in parallel with WT CD4$^+$ T cells. Importantly, co-transfer of WT and *Il23r*$^{-/-}$ CD4$^+$ T cells also leads to the development of severe intestinal inflammation. Thus, the co-transfer approach is ideally suited for the characterisation of direct cell-intrinsic effects of IL-23 signalling into CD4$^+$ T cells, and further abrogates potential confounding factors such as the environment from which the cells are isolated, i.e. inflamed or non-inflamed. Therefore, we transferred *Rag1*$^{-/-}$ recipients with an equal number of WT (CD45.1$^+$) and *Il23r*$^{-/-}$ (CD45.1$^-$) or WT (CD45.1$^-$) naive CD4$^+$ T cells alone (Figure 5.1A). Upon development of clinical signs of inflammation we FACS-sorted viable TCRβ$^+$CD4$^+$CD45.1$^-$ WT or *Il23r*$^{-/-}$ T cells from the inflamed colon of individual mice and extracted total RNA from the isolated cells without further manipulation. This was followed by whole transcriptome gene expression analysis on the Illumina Bead Array platform. In total we found 168 transcripts whose expression was significantly different between *Il23r*$^{-/-}$ and WT CD4$^+$ T cells. Among these transcripts, 104 were upregulated, while 64 were downregulated in *Il23r*$^{-/-}$ CD4$^+$ T cells (Figure 5.1B-C). Interestingly, we found that

the Th2 signature cytokine *Il4* was 3-fold upregulated in *Il23r*$^{-/-}$ T cells and further analysis by RT-qPCR revealed that gene expression of *Il4*, *Il5*, *Il13*, *Gata3*, *maf* and *Il1rl1*(*Il33r*) was also increased in *Il23r*$^{-/-}$ CD4^{+} T cells (Figure 5.2), suggesting that IL-23 is a negative regulator of Th2-associated genes. The most upregulated gene in *Il23r*$^{-/-}$ CD4^{+} T cells was *Cdk5rap1* which is a negative regulator of CDK5, a kinase that has been shown to promote STAT3 phosphorylation (Figure 5.1C and 5.2) [414]. In the list of genes downregulated in *Il23r*$^{-/-}$ CD4^{+} T cells were the cell surface receptors *Ltb4r1* (receptor for the lipid chemoattractant leukotriene B_4) and *Tnfrsf1a* (the receptor for TNFα). The most downregulated gene was *Ide* (insulin degrading enzyme) but the relevance of this gene to CD4^{+} T cell function is not known. Importantly, we also confirmed that expression of *Il22*, which is a known target gene of IL-23, was significantly decreased in *Il23r*$^{-/-}$ CD4^{+} T cells (Figure 5.2). It is important to note that these differences in transcript abundance could be caused by true changes in the rate of gene transcription, mRNA stability and turnover, or simply reflect variations in the cellular composition between WT and *Il23r*$^{-/-}$ CD4^{+} T cells. For example, *Foxp3* was upregulated in *Il23r*$^{-/-}$ CD4^{+} T cells (Figure 5.1C) which probably reflects the increased frequency of Foxp3^{+} CD4^{+} T cells found in the absence of IL-23 signals into CD4^{+} T cells[391]. In summary, we have identified a core set of genes that is regulated by direct cell-intrinsic IL-23 signals into intestinal CD4^{+} T cells but the relevance of these genes to IL-23-driven inflammation, and whether IL-23 controls their transcriptional activity remains to be shown.

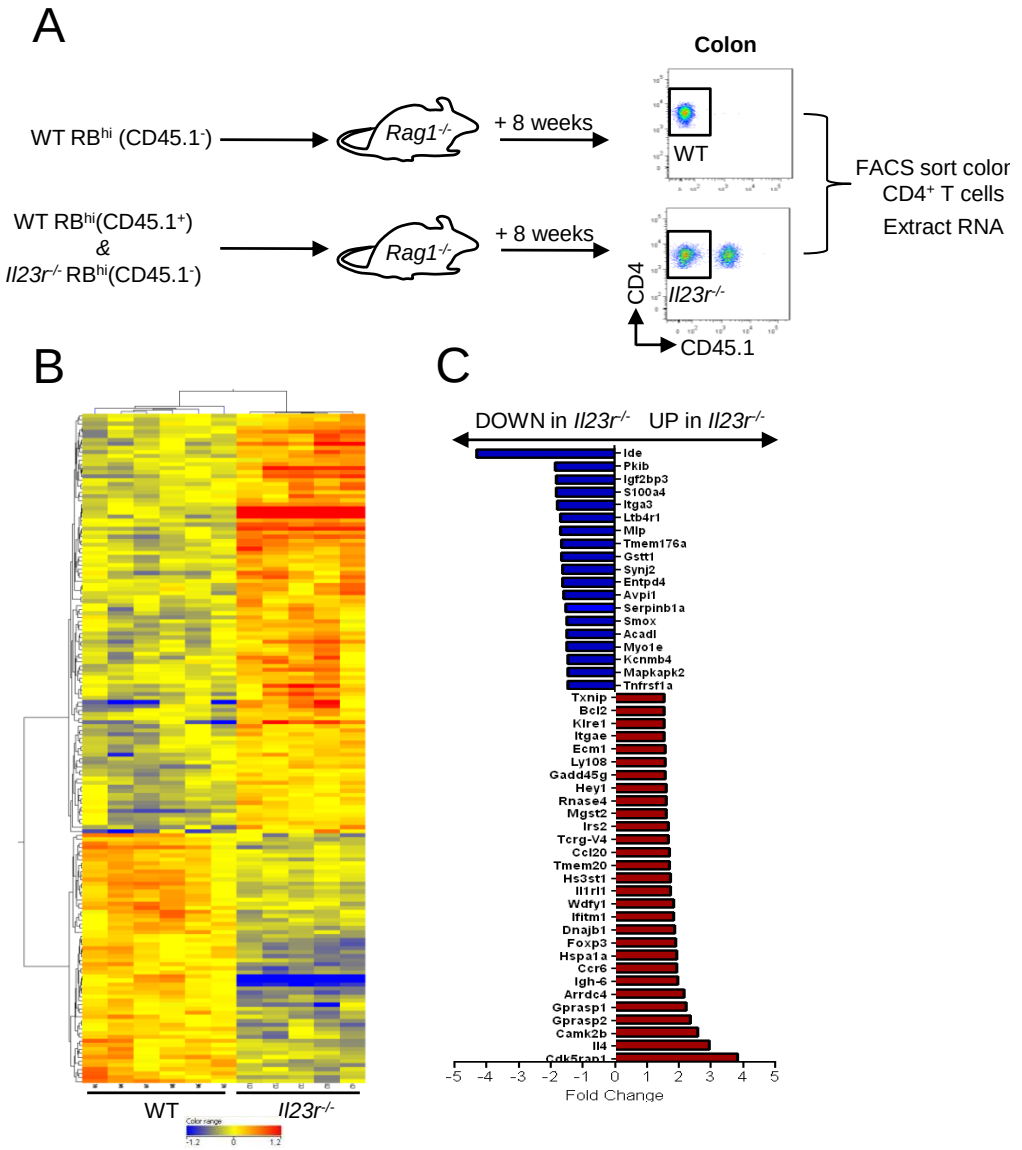

Figure 5.1. Identification of IL-23 target genes in intestinal CD4$^+$ T cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 WT (CD45.1$^-$) CD4$^+$CD25$^-$CD45RB^{hi} T cells or a 1:1 mixture of 2x10^5 WT (CD45.1$^+$) and 2x10^5 *Il23r*$^{-/-}$ (CD45.1$^-$) CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed upon development of clinical signs of inflammation (~8 weeks). Viable TCRβ$^+$CD4$^+$CD45.1$^-$ WT or *Il23r*$^{-/-}$ CD4$^+$ T cells were FACS sorted from the colon and RNA extracted without further manipulation. Whole transcriptome gene expression analysis was performed using the Illumina Bead Array platform.

(A) Schematic describing the experimental strategy for the identification of IL-23 target genes

(B) Two-dimensional cluster analysis (hierarchical, Pearson uncentered, average linkage, unsupervised) of the 168 transcripts whose expression was significantly affected in the comparison of *Il23r*$^{-/-}$ versus WT CD4$^+$ T cells. Each row corresponds to a gene and each column to a sample. The 168 transcripts represent 147 unique genes including genes with unknown function. Blue = low expression, red = high expression.

(C) Absolute fold change of all known genes greater than 1.5 fold differentially regulated in *Il23r*$^{-/-}$ versus WT colonic CD4$^+$ T cells. Blue bars represent genes that are down-regulated in *Il23r*$^{-/-}$ CD4$^+$ T cells and red bars represent genes that are up-regulated in *Il23r*$^{-/-}$ CD4$^+$ T cells. Bars represent the mean fold change.

Data represent results from a single experiment (n=6 mice for WT, n=5 mice for *Il23r*$^{-/-}$). 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.

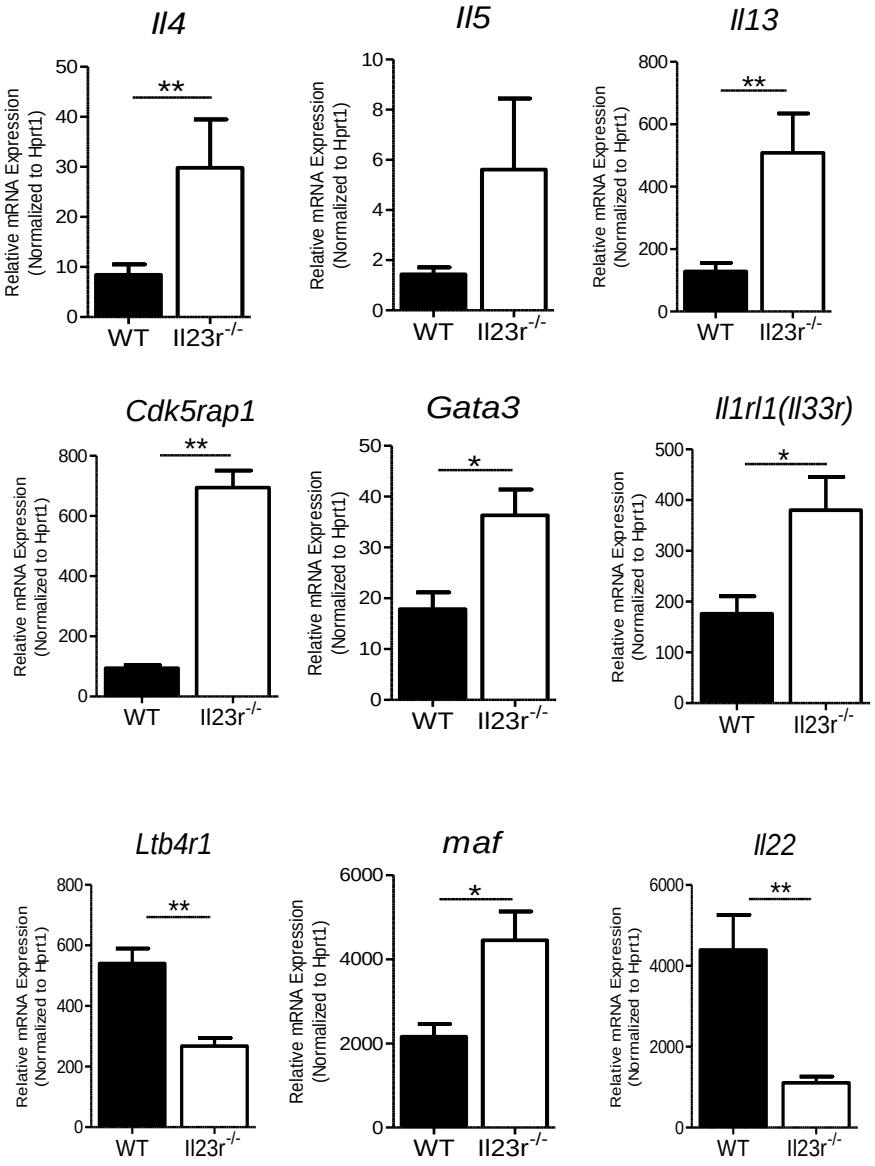

Figure 5.2. RT-qPCR validation of selected IL-23-regulated genes.

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 WT (CD45.1$^-$) CD4$^+$CD25$^-$CD45RB^{hi} T cells or a 1:1 mixture of 2x10^5 WT (CD45.1$^+$) and 2x10^5 *Il23r*$^{-/-}$ (CD45.1$^-$) CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed upon development of clinical signs of inflammation (~8 weeks). Viable TCRβ$^+$CD4$^+$CD45.1$^-$ WT or *Il23r*$^{-/-}$ T cells were FACS sorted from the colon and RNA extracted without further manipulation. These samples represent the same RNA as used for the microarray experiment in Figure 5.1. Expression levels of the indicated genes were assayed by RT-qPCR analysis of cDNA prepared from mRNA. Expression levels were normalised to *Hprt*. Bars represent the mean, error bars represent SEM.

Data represent results from a single experiment (n=6 mice for WT, n=5 mice for *Il23r*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$, ** $p \leq 0.01$.

5.2.2 GATA3 expression by CD4$^+$ T cell restrains their colitogenic potential

One of the most intriguing findings of the microarray analysis was the observation that IL-23 signals into CD4$^+$ T cells negatively regulated Th2 associated genes, including GATA3. To assess the contribution of GATA3 to development of intestinal inflammation we utilized GATA3$^{f/f}$-OX40Cre mice which are a useful tool for the deletion of GATA3 as activated CD4$^+$ T cells express high levels of OX40, and therefore CD4$^+$ T cells from these mice will delete GATA3 upon activation [415, 416]. Thus, we transferred naïve CD4$^+$ T cells from WT or GATA3$^{f/f}$-OX40Cre donors into *Rag1*$^{-/-}$ recipients. Strikingly, transfer of GATA3$^{f/f}$-OX40Cre CD4$^+$ T cells led to significantly greater weight loss (Figure 5.3A) and intestinal inflammation (Figure 5.3B) than transfer of WT CD4$^+$ T cells. The increased inflammatory response in recipients of GATA3$^{f/f}$-OX40Cre CD4$^+$ T cells correlated with enhanced accumulation of CD4$^+$ T cells in the colon (Figure 5.3C). The frequency of IL-17A and IFN-γ expressing cells was comparable but the frequency of IL-13$^+$ cells was significantly decreased among GATA3$^{f/f}$-OX40Cre compared to WT CD4$^+$ T cells confirming the essential role for GATA3 in driving IL-13 expression (Figure 5.3D) [417]. Recent reports have demonstrated a functional role for GATA3 expression in Foxp3$^+$ Treg cells [254, 415], suggesting that impairment in iTreg generation from the transferred naive CD4$^+$ T cell progeny rather than enhanced pathogenic activity of effector CD4+ T cells could account for the increased inflammation. However, Foxp3$^+$ cells were below the limit of detection in both groups and could not be quantified accurately, and therefore it is unlikely that GATA3 control of Treg function was responsible for the difference in disease outcome. Together these data show that GATA3 expression in CD4$^+$ T cells restrains their colitogenic potential and

further suggest that IL-23-mediated inhibition of GATA3 might contribute to the development of inflammation in the context of $CD4^+$ T cell transfer colitis.

5.2.3 IL-23 inhibits the emergence of IL-17A$^+$IL-13$^+$ CD4$^+$ T cells in T cell transfer colitis

Among the Th2 signature cytokines *Il13* appeared to be the most highly expressed cytokine in intestinal $CD4^+$ T cells, and its expression was significantly increased in *Il23r*$^{-/-}$ $CD4^+$ T cells. Recently, Th17 cells co-expressing GATA3 and RORγt and capable of IL-13 production have been described in the lung [418] and this prompted us to investigate whether intestinal Th17 cells are the source of increased IL-13 production in *Il23r*$^{-/-}$ $CD4^+$ T cells. Therefore we transferred naïve $CD4^+$ T cells from WT ($CD45.2^-$) and *Il23r*$^{-/-}$ ($CD45.2^+$) donors at a 1:1 ratio into *Rag1*$^{-/-}$ hosts (Figure 5.4A) which allowed us to identify the cellular source of IL-13 in WT and *Il23r*$^{-/-}$ $CD4^+$ T cells in the same inflammatory environment. Interestingly, WT $CD4^+$ T cells contained a sizeable proportion of IL-13-producing cells and a fraction of these cells co-expressed IL-17A (Figure 5.4B-C). As compared to WT $CD4^+$ T cells the frequency of IL-17A$^+$IL-13$^+$ cells was significantly increased in *Il23r*$^{-/-}$ $CD4^+$ T cells, whereas the frequency of IL-17A$^-$IL-13$^+$ cell did not change. In keeping with our previous results the frequency of IL-17A$^+$IFN-γ$^+$ $CD4^+$ T cells was significantly decreased among *Il23r*$^{-/-}$ $CD4^+$ T cells as compared to WT $CD4^+$ T cells (Figure 5.4B-C). These data show that IL-23 signals into $CD4^+$ T cells prevent the emergence of IL-17A$^+$IL-13$^+$ $CD4^+$ T cells and suggest that IL-23 might have differential effects on IL-13 and IFN-γ expression by Th17 cells.

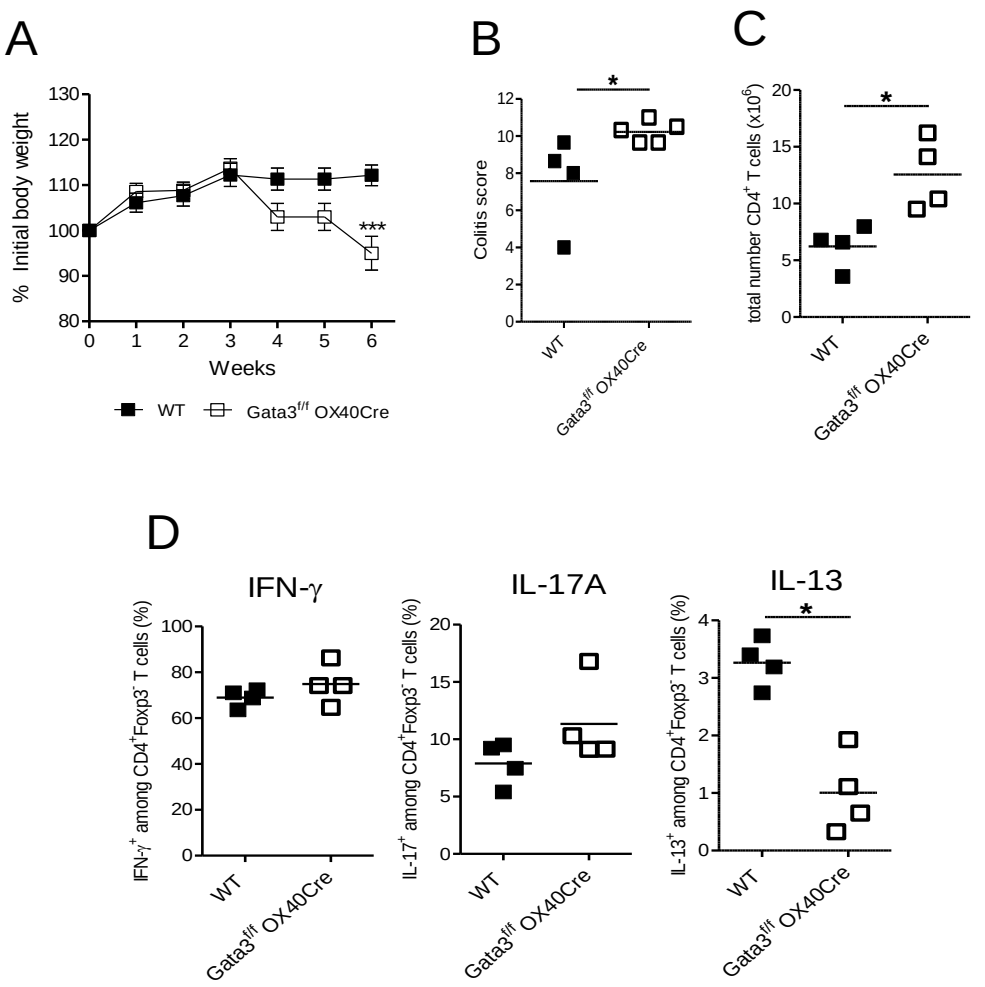

Figure 5.3. GATA3 expression by CD4$^+$ T cells restrains their colitogenic potential

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from C57BL/6 WT or C57BL/6 Gata3$^{f/f}$ OX40Cre donors. Mice were sacrificed when recipients of Gata3$^{f/f}$ OX40Cre CD4$^+$ T cells developed clinical signs of disease (~6 weeks). Single cell suspensions from the colonic lamina propria were re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours. Expression of cytokines was determined by intracellular FACS.

(A) Weight loss during the course of the experiment shown as percentage of original weight at day 0. Points represent the mean ±SEM.

(B) Histological analysis of colonic inflammation. Bars represent the mean and each point represents an individual mouse.

(C) Total number of CD4$^+$ T cells in the colon. Bars represent the mean and each point represents an individual mouse.

(D) Frequency of IFN-γ, IL-17A or IL-13 expressing CD4$^+$ T cells present in the colon. Bars represent the mean and each point represents an individual mouse.

Data represent a single experiment (n=4 mice). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$, *** $p \leq 0.001$.

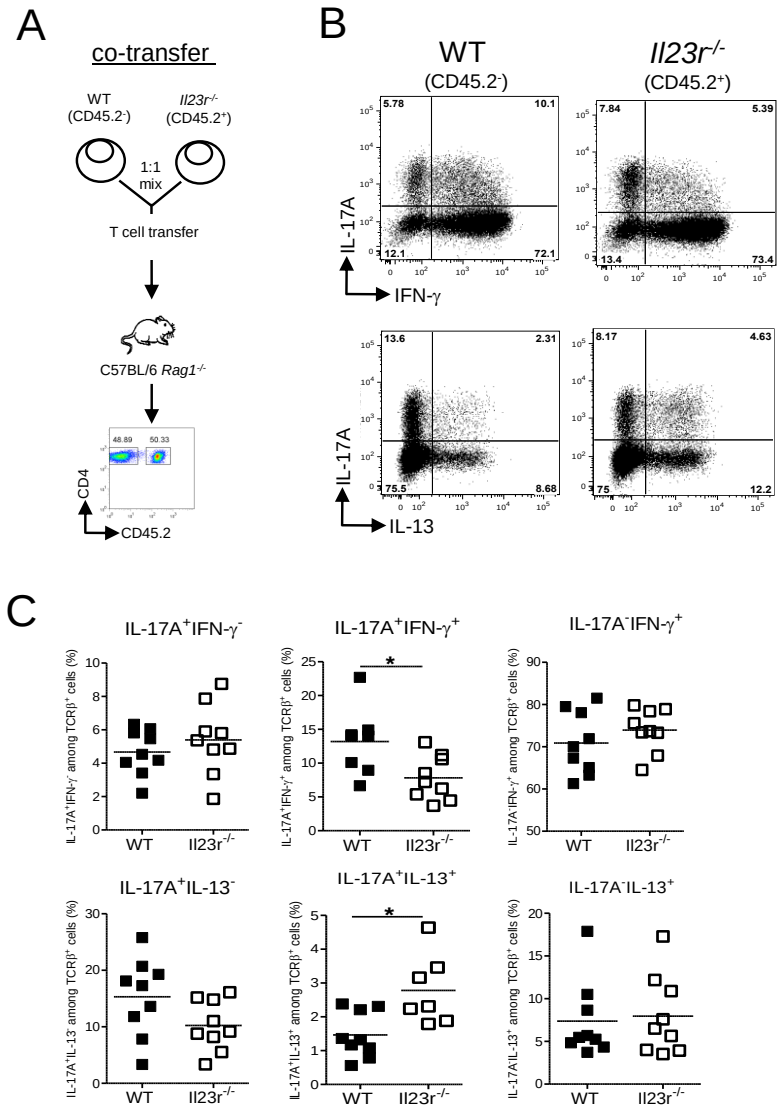

Figure 5.4. IL-23 inhibits the emergence of IL-17A$^+$IL-13$^+$ CD4$^+$ T cells in a cell intrinsic manner

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with a 1:1 mixture of 2x10^5 WT (CD45.2$^-$) and 2x10^5 *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed upon development of clinical signs of inflammation (~8 weeks). Single cell suspensions were re-stimulated with PMA and ionomycin in the presence of monensin for 4 hours and expression of cytokines determined by intracellular FACS.

(A) Schematic of the experimental design.

(B) Representative FACS plots of WT or *Il23r*$^{-/-}$ colonic lamina propria cells, gated on viable CD4$^+$ T cells showing IL-17A, IFN-γ and IL-13 expression

(C) Frequency of IL-17A$^+$ and/or IFN-γ$^+$ CD4$^+$ T cells in the colonic lamina propria gated on either WT (CD45.2$^-$) or *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$ T cells. Bars represent the mean and each point represents an individual mouse.

(D) Frequency of IL-17A$^+$ and/or IL-13$^+$ CD4$^+$ T cells in the colonic lamina propria gated on either WT (CD45.1$^+$) or *Il23r*$^{-/-}$ (CD45.1$^-$) CD4$^+$ T cells. Bars represent the mean and each point represents an individual mouse.

Data represent results from a single experiment (n=9 mice). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. * $p \leq 0.05$.

5.2.4 IL-23 limits IL-33R expression on Foxp3$^+$ Treg cells in inflamed lymphocyte-replete mice

Our microarray analysis revealed that IL-23 signalling into CD4$^+$ T cells modulates expression of *Il1rl1*, which encodes the receptor for the IL-1 family cytokine IL-33. Although IL-33 has mostly been studied in the context of Th2 driven immune responses [419], recent reports have described IL-33R expression on Foxp3$^+$ Tregs [420], and in addition, IL-33 has been shown to promote expansion of Foxp3$^+$ Treg cells *in vivo* [420, 421]. As we previously reported that IL-23 inhibits the emergence of Foxp3$^+$ Treg cells [330, 391] we hypothesized that IL-23 might regulate Treg cell responsiveness to IL-33 through regulation of the IL-33R. As detection of IL-33R expression on CD4$^+$Foxp3$^+$ T cells in the T cell transfer model proved challenging due to the low frequency of Foxp3$^+$ Treg cells that arise in this model [330] we decided to assess the effect of IL-23 on IL-33R expression in the *Helicobacter hepaticus*/anti-IL-10R model of colitis. To this end we generated mixed bone marrow chimaeras by reconstituting irradiated *Rag1*$^{-/-}$ mice with WT (CD45.2$^-$) and *Il23r*$^{-/-}$ (CD45.2$^+$) bone marrow cells at 1:1 ratio (Figure 5.5A). After 8 weeks, to allow for full reconstitution of the hematopoietic system, we induced intestinal inflammation by infection with *Helicobacter hepaticus* and administration of an IL-10R blocking antibody. This allowed us to investigate WT and *Il23r*$^{-/-}$ CD4$^+$ T cells in the same inflammatory environment. Interestingly, IL-33R expression on Foxp3$^+$ Treg cells was significantly increased among *Il23r*$^{-/-}$ compared to WT CD4$^+$ T cells from the colon, MLN and spleen (Figure 5.5B-C). In addition, IL-33R$^+$ Foxp3$^+$ Treg cells of WT and *Il23r*$^{-/-}$ origin were enriched in the colonic lamina propria as compared to the MLN and spleen. Together these data suggest that IL-23 limits IL-33R expression by Foxp3$^+$CD4$^+$ T cells in a cell intrinsic manner.

5.2.5 Kinetics of *Il23* and *Il33* mRNA expression during the course *Helicobacter hepaticus* driven intestinal inflammation.

The above experiment suggests that IL-23 controls responsiveness of $Foxp3^+$ Tregs to IL-33. However, little is known about the kinetics of IL-23 or IL-33 expression during the course of intestinal inflammation in this model. Therefore, we determined the expression of *Il23* and *Il33* by RT-qPCR in whole colon homogenates at various time points during development of intestinal inflammation induced by *Helicobacter hepaticus* and concomitant blockade of IL-10R signalling. Induction of *Il23* and *Il33* transcripts was detected as early as 3 days post treatment but the two cytokines followed different kinetics thereafter (Figure 5.6A-B). Expression of *Il23* peaked at day 3 and then declined progressively although it remained above steady state levels throughout the course of the experiment (Figure 5.6A). By contrast, *Il33* expression levels continued to rise until day 14 and then suddenly declined but, as with *Il23*, remained above steady state levels over the analyzed time course (Figure 5.6B). An overlay of the kinetics of *Il23* and *Il33* expression and colitis scores during the course of inflammation showed that both cytokines were induced prior to the peak of pathology at day 14 (Figure 5.6C). This indicates that expression of *Il23* and *Il33* is dynamically regulated over the course of *Helicobacter hepaticus*/anti-IL-10R colitis and suggest that IL-33 could have a role in modulating intestinal inflammation.

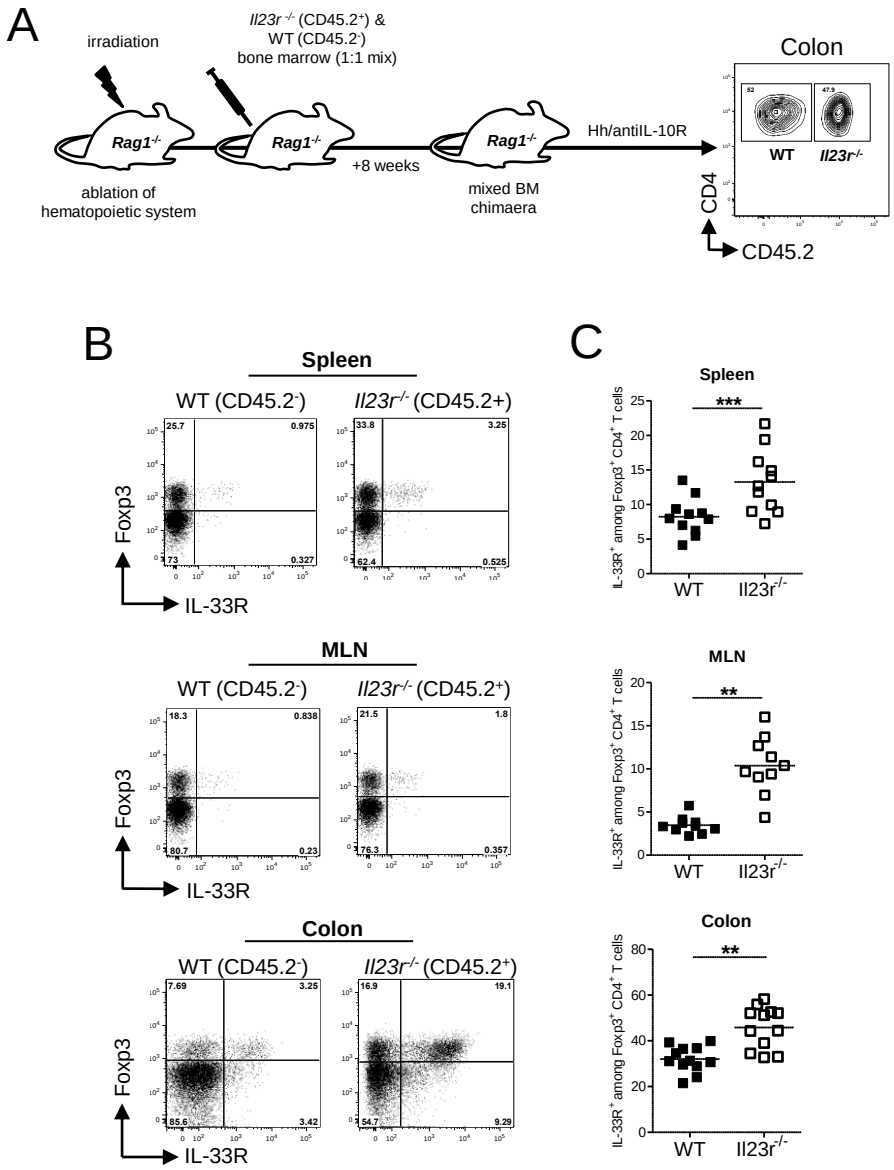

Figure 5.5. IL-23 limits IL-33R expression on Foxp3$^+$ Treg cells in inflamed mice.

Mixed bone marrow chimaeras were generated by lethal irradiation of C57BL/6 *Rag1*$^{-/-}$ mice followed by i.v. injection of 2.5x10^6 WT (CD45.2$^-$) and 2.5x10^6 *Il23r*$^{-/-}$ (CD45.2$^+$) bone marrow cells. Mice were rested for 8 weeks to allow re-constitution of the hematopoietic system. Re-constituted mice were infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* on three consecutive days with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at 2 weeks post infection and cells were isolated from the colonic lamina propria. Expression of indicated markers was determined by cell surface and intracellular FACS.

(A) Schematic of the experimental design.

(B) Representative FACS plots of WT or *Il23r*$^{-/-}$ Foxp3$^+$CD4$^+$ T cells in the indicated organs.

(C) Frequency of IL-33R expressing cells among Foxp3$^+$ cells in the colonic lamina propria gated on either WT (CD45.2$^-$) or *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$ T cells. Bars represent the mean and each point represents an individual mouse.

Data represent pooled results from two independent experiments (n=11 mice for spleen, n=10 mice for MLN, n=12 mice for colon). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. ** $p \leq 0.01$, *** $p \leq 0.001$.

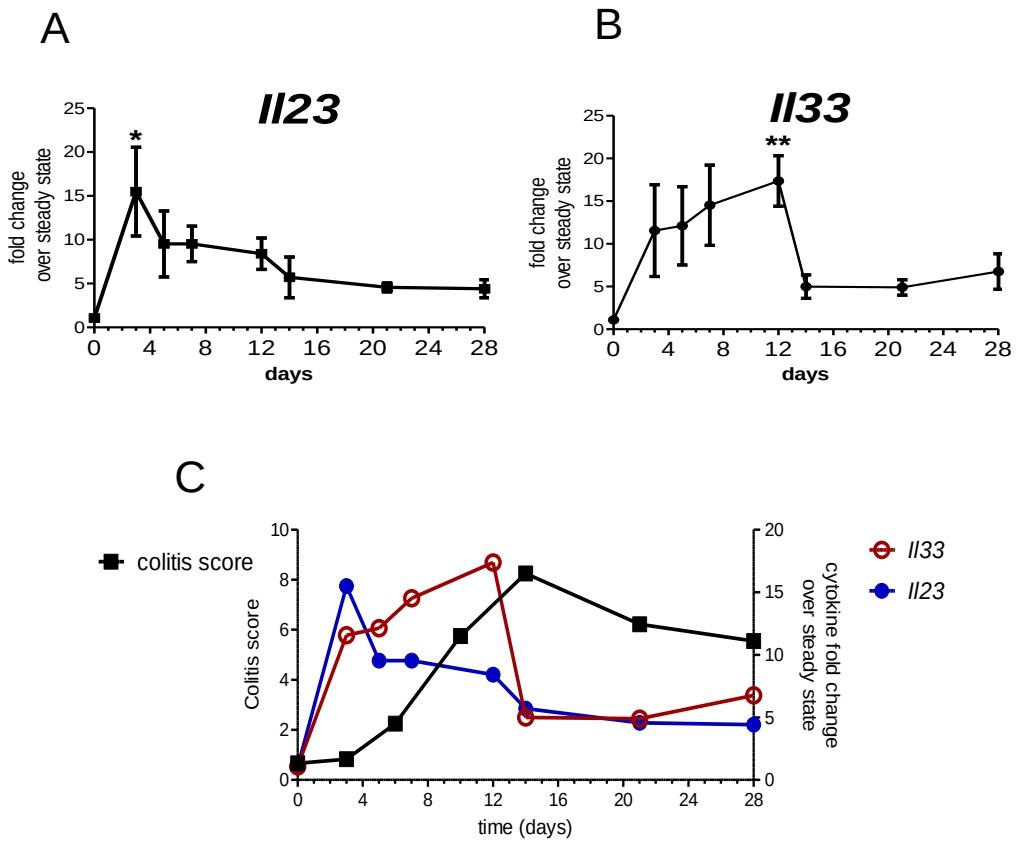

Figure 5.6. Kinetics of *Il23* and *Il33* mRNA expression in the colon during *Helicobacter hepaticus*/anti-IL-10R colitis

C57BL/6 mice were infected by oral gavage with ~10^8 cfu *Helicobacter hepaticus* on three consecutive days with concomitant *i.p*. injections of a blocking anti-IL-10R mAb (1mg/week) starting on the day of the first infection. Mice were sacrificed at indicated time points. At each time point, RNA was extracted from whole colon homogenates and intestinal inflammation was assessed by histological analysis.

(A&B) RT-qPCR analysis of *Il23 or Il33* gene expression from cDNA reverse transcribed from mRNA of whole colon homogenates at various time points. Gene expression upon induction of colitis is represented relative to transcript levels at steady state (day 0). At all time points expression levels were normalised to *Hprt*. Points represent the mean ±SEM.

(C) Overlay of histological analysis of colitis (left Y axis) and cytokine mRNA transcript levels from (A-B) (right Y axis) at the indicated time points (X axis).

Data represent results from a single experiment (n=4 mice for each time point). Statistical significance was determined using a two-way ANOVA followed by a Bonferroni post-hoc test. * $p \leq 0.05$, ** $p \leq 0.01$.

5.2.6 IL-33 promotes the generation of iTreg cells and inhibits the emergence of CD4$^+$ IL-17A$^+$ T cells in the absence of IL-23

We previously showed that IL-23 inhibits iTreg induction [330, 391]. Adoptive transfer of naive CD4$^+$ T cells into IL-23 deficient *Rag1*$^{-/-}$ recipients resulted in an increased proportion of Foxp3$^+$ Tregs cells in the intestine. Furthermore, Foxp3$^+$ Treg cells were found to have a functional role in restraining inflammation in that setting as transfer of Foxp3-deficient naïve CD4$^+$ T cells into *Il23a*$^{-/-}$*Rag1*$^{-/-}$ hosts induced colitis similar to that observed upon transfer into control IL-23 sufficient *Rag1*$^{-/-}$ recipients. However, this potent effect of IL-23 on Foxp3$^+$ Treg cells differentiation *in vivo* could not be repeated *in vitro* suggesting that IL-23 might inhibit Treg induction in response to tissue specific factors not present in *in vitro* cultures [330]. So far our experiments suggest that IL-23 regulates IL-33R expression on Foxp3$^+$ Tregs and this, together with published reports that IL-33 promotes Treg expansion [420, 421], led us to hypothesise that IL-33 might contribute to the emergence of Foxp3$^+$ Treg cells in the absence of IL-23. Thus we transferred WT or *Il33r*$^{-/-}$ deficient CD4$^+$ T cells into *Il23a*$^{-/-}$*Rag1*$^{-/-}$ hosts and compared induction of Foxp3 among these cells. The frequency of Foxp3$^+$ Tregs in *Il33r*$^{-/-}$ CD4$^+$ T cells was significantly reduced as compared to WT CD4$^+$ T cells (Figure 5.7A-B) suggesting that IL-33 promotes conversion of naïve CD4$^+$ T cells into Foxp3$^+$ CD4$^+$ T cells in the absence of IL-23. This decreased frequency of Foxp3$^+$ Treg cells among *Il33r*$^{-/-}$ CD4$^+$ T cells also correlated with significantly increased intestinal pathology and accumulation of CD4$^+$ T cells in recipients of *Il33r*$^{-/-}$ CD4$^+$ T cells compared to WT CD4$^+$ T cells in the absence of IL-23 (Figure 5.8A-C). Next, we assessed the differentiation of cytokine producing CD4$^+$ T cells in the colon. We found equal differentiation of IL-17A$^+$IL-13$^+$ and IL-17A$^+$IFN-γ^+ cells in recipients of WT and

Il33r$^{-/-}$ CD4^{+} T cells, whereas the frequency of CD4^{+} T cells expressing only IL-17A was significantly increased in recipients of *Il33r*$^{-/-}$ CD4^{+} T cells (Figure 5.9A-C). These data suggest that IL-33 signals into CD4^{+} T cells promote the generation of Foxp3^{+} iTreg cells and inhibit the emergence of CD4^{+} IL-17A^{+} T cells in the colon and demonstrate that IL-33 acts predominantly as an anti-inflammatory cytokine in the absence of IL-23 in the setting of intestinal inflammation.

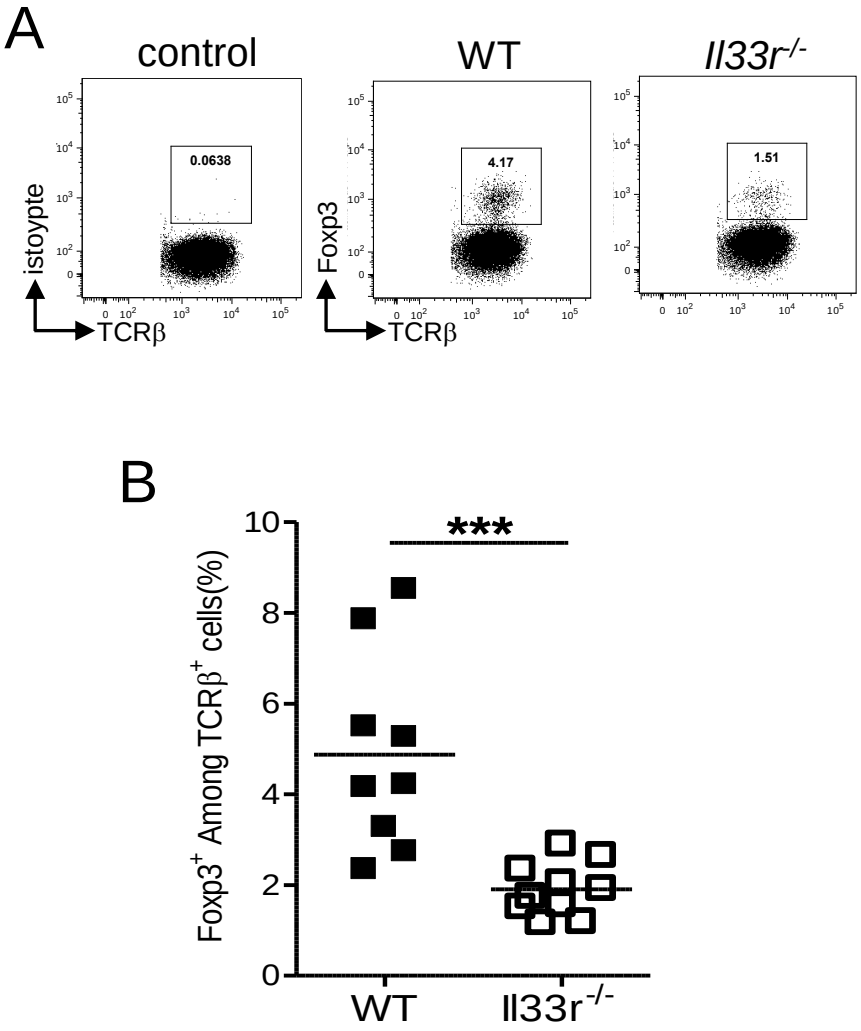

Figure 5.7. IL-33 promotes generation of Foxp3$^+$ Treg cells in the absence of IL-23

C57BL/6 *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from C57BL/6 WT or *Il33r*$^{-/-}$ donors. Mice were sacrificed when recipients of *Il33r*$^{-/-}$ T cells developed clinical signs of disease (6-8 weeks). Single cell suspensions from the colonic lamina propria were prepared and Foxp3 expression in CD4$^+$ T cells was assessed by intracellular FACS.

(A) Representative FACS plots showing Foxp3 expression of WT or *Il33r*$^{-/-}$ colonic lamina propria cells, gated on viable CD4$^+$ T cells.

(B) Frequency of Foxp3$^+$ cells among CD4$^+$ T cells in the colon. Bars represent the mean ±SEM.

Data represent pooled results from two independent experiments (n=9 mice for WT, n=10 mice for *Il33r*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. *** $p \leq 0.001$.

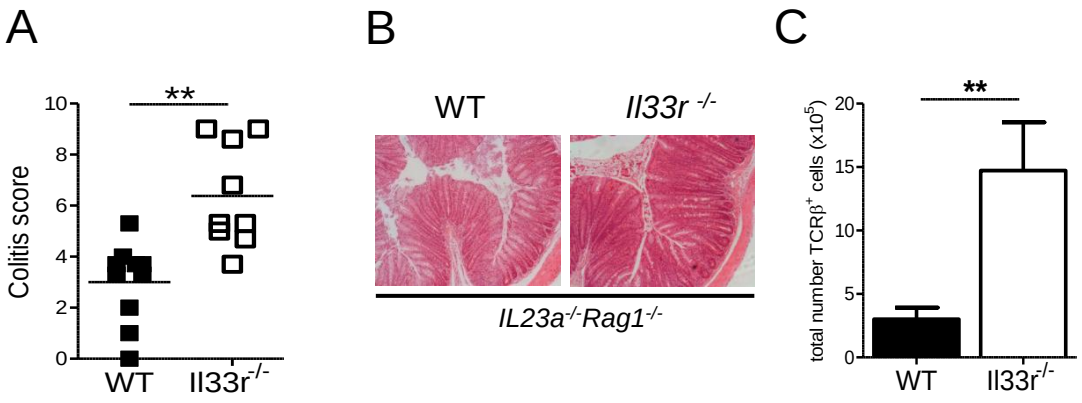

Figure 5.8. IL-33R deficient T cells induce colitis in the absence of IL-23

C57BL/6 *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from C57BL/6 WT or *Il33r*$^{-/-}$ donors. Mice were sacrificed when recipients of *Il33r*$^{-/-}$ T cells developed clinical signs of disease (6-8 weeks). Mice were assessed for intestinal inflammation and single cell suspensions from the colonic lamina propria were prepared.

(A) Histological analysis of colitis. Bars represent the mean, error bars represent SEM and each dot represents an individual mouse.

(B) Representative photomicrographs of mid colon sections (50X magnification).

(C) Total number of CD4$^+$ T cells in the colon. Bars represent the mean and each point represents an individual mouse.

Data represent pooled results from two independent experiments (n=10 for WT, n=9 for *Il33r*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. ** $p \leq 0.01$.

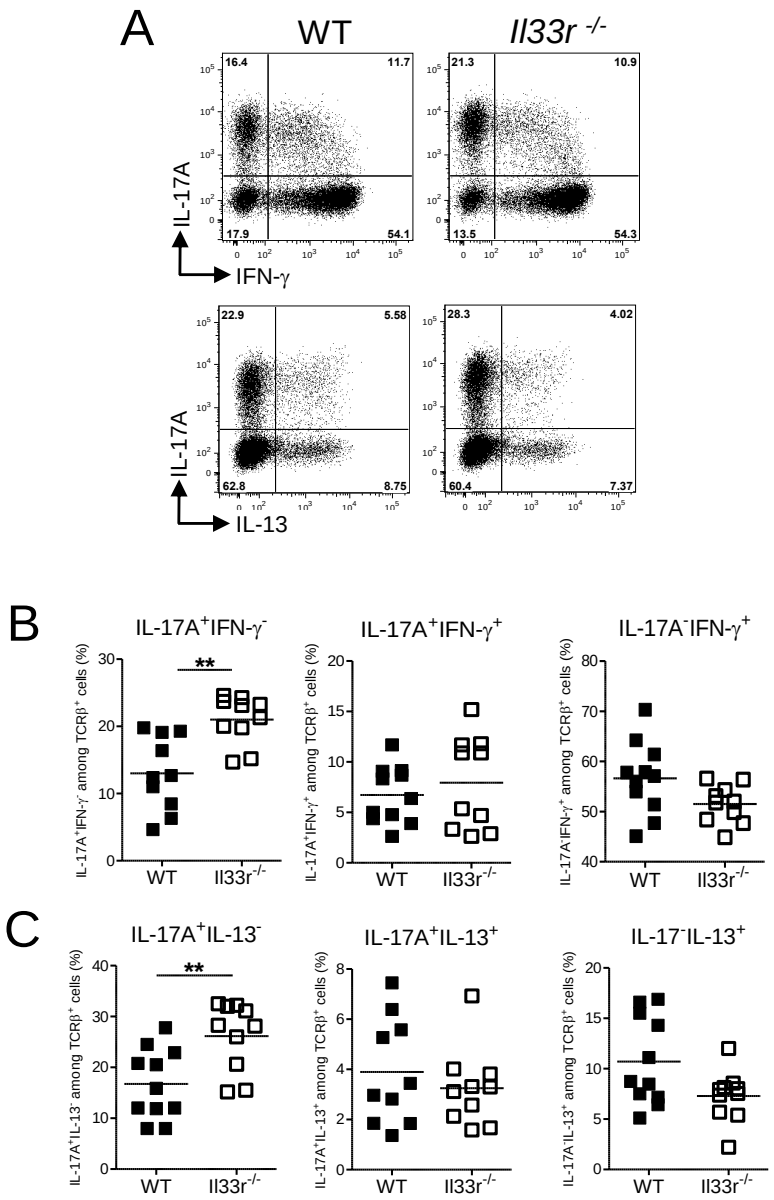

Figure 5.9. IL-33 does not drive IL-13 production by colonic Th17 cells in the absence of IL-23

C57BL/6 *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from C57BL/6 WT or *Il33r*$^{-/-}$ donors. Mice were sacrificed when recipients of *Il33r*$^{-/-}$ T cells developed clinical signs of disease (6-8 weeks). Single cell suspensions from the colonic lamina propria were restimulated with PMA and ionomycin in the presence of monensin for 4 hours and IL-17A, IFN-γ and IL-13 expression was assessed by intracellular FACS.

(A) Representative FACS plots of IL-17A, IFN-γ and IL-13 expression in WT or *Il33r*$^{-/-}$ colonic lamina propria cells, gated on viable CD4$^+$ T cells.

(B) Frequency of IL-17A$^+$ and/or IFN-γ$^+$ cells among CD4$^+$ T cells present in the colon. Bars represent the mean and each point represents an individual mouse.

(C) Frequency of IL-17A$^+$ and/or IL-13$^+$ cells among CD4$^+$ T cells present in the colon. Bars represent the mean and each point represents an individual mouse.

Data represent pooled results from two independent experiments (n=11 for WT, n=10 for *Il33r*$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. ** $p \leq 0.01$.

5.2.7 IL-33 enhances TGF-β1 driven Treg induction *in vitro* and this is inhibited by IL-23.

The above experiments suggest that IL-33 promotes the generation of iTreg cells in the absence of IL-23. This directly implies that IL-23 might inhibit IL-33 driven iTreg induction. To test this we cultured naïve T cells in the presence of TGF-β1 and assessed the effect of IL-33 and IL-23 on the generation of $Foxp3^+$ Tregs (Figure 5.10). Importantly, the concentration of TGF-β1 used in this experiment was shown to be suboptimal for Foxp3 induction but optimal for IL-23R mRNA expression [130]. We found that TGF-β1 mediated induction of Foxp3 could be enhanced by addition of IL-33 to the culture suggesting that IL-33 favours iTreg conversion. Strikingly, this activity of IL-33 was completely inhibited in the presence of IL-23, whereas addition of IL-23 did not inhibit or promote iTreg induction in the presence of TGF-β1 alone, confirming our previous reports that failed to detect a direct effect of IL-23 on Treg induction *in vitro* [330]. This preliminary experiment suggests that IL-23 regulates the ability of IL-33 to act as a co-factor in TGF-β1 mediated iTreg induction, however, the mechanism by which it achieves this remains to be determined.

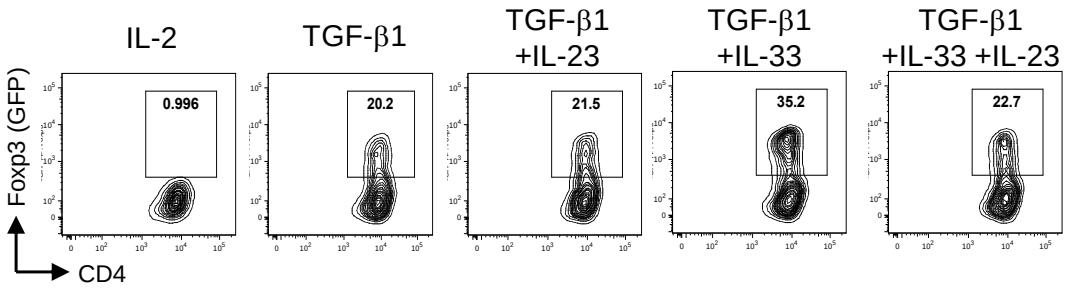

Figure 5.10. IL-33 enhances TGF-β1 driven Treg induction *in vitro* and this is inhibited by IL-23.

$CD4^+CD62L^+CD44^-$ $Foxp3^-$ naive T cells were FACS sorted from $Foxp3^{gfp}$ mice. $2x10^5$ naive T cells were stimulated with plate-bound anti-CD3 (5μg/ml) and soluble anti-CD28 (2μg/ml) in the presence of indicated cytokines (TGF-β1, 500pg; IL-2, 100U/ml, IL-23, 20ng/ml and IL-33, 20 ng/ml). After 3 days, Foxp3 expression was determined by FACS. Data represent a single experiment (n= 1 well per condition).

5.2.8 IL-33R is preferentially expressed by colonic Foxp3$^+$ Tregs in the steady state

So far our findings indicate that IL-33 could be involved in the generation of iTregs. This suggested to us that IL-33R$^+$ Treg cells might be enriched in the intestine, a major site of iTreg induction [38, 39, 422]. Therefore we characterised IL-33R expression on Foxp3$^+$ Treg cells in the colon, MLN and spleen of steady state mice. Remarkably, IL-33R-expressing Foxp3$^+$ Treg cells were highly enriched in colon compared to MLN and spleen and the majority of IL-33R$^+$CD4$^+$ T cells in the colon were Foxp3$^+$ Treg cells (Figure 5.11). Thus, the compartmentalisation of IL-33R expression on colonic Treg cells suggests a tissue specific role for IL-33 in the generation/function of these cells.

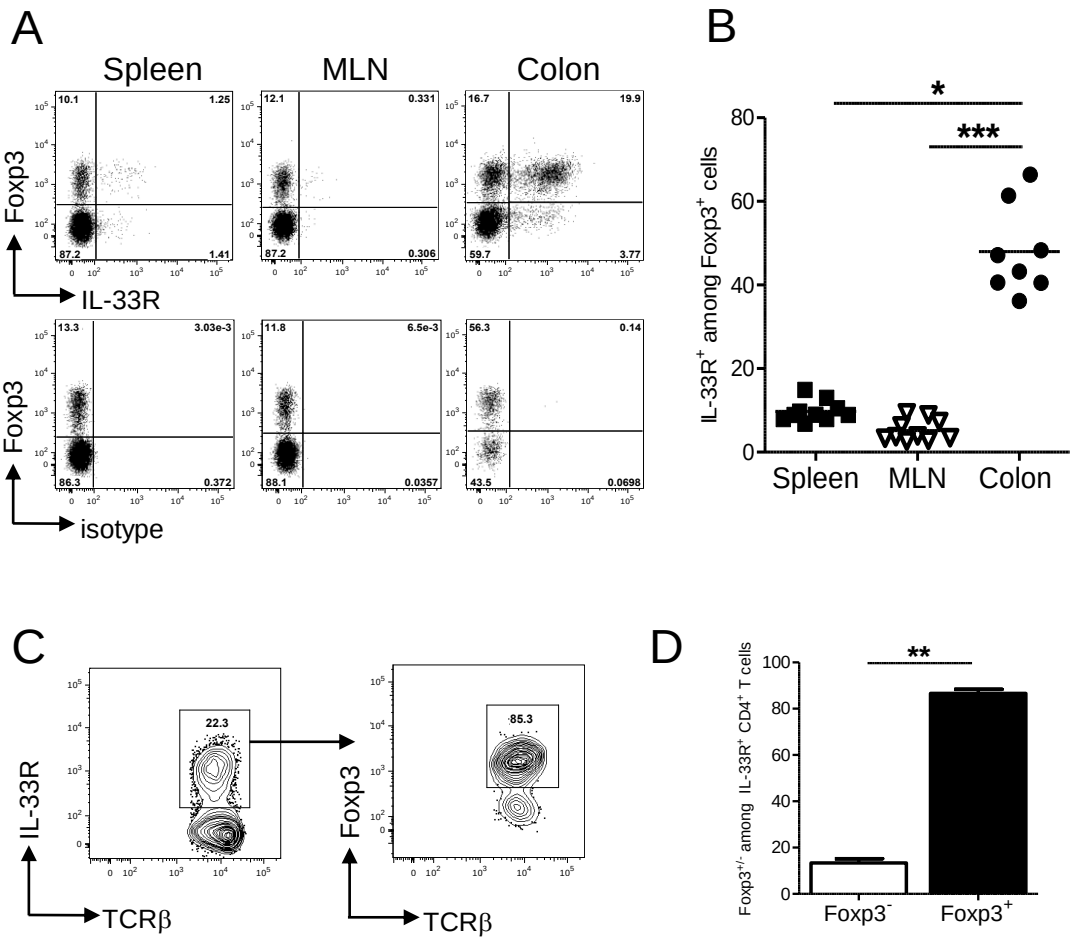

Figure 5.11. IL-33R is preferentially expressed by colonic Foxp3$^+$ Treg cells in the steady state

C57BL/6 mice (~8 weeks old) were sacrificed at steady state and cells were isolated from the indicated organs. Single cell suspensions were prepared from the indicated organs and cell surface and intracellular markers was determined by FACS.

(A) Representative FACS plots of cells from the spleen, MLN and colonic lamina propria, gated on viable TCRβ$^+$CD4$^+$ cells.

(B) Frequency of IL-33R expressing cells among CD4$^+$Foxp3$^+$ T cells in the indicated organs. Bars represent the mean and each point represents an individual mouse.

(C) Representative FACS plots of colonic lamina propria CD4$^+$ T cells.

(D) Frequency of Foxp3$^+$ and Foxp3$^-$ cells among IL-33R expressing CD4$^+$ T cells present in the colonic lamina propria. Bars represent the mean, error bars represent SEM.

Data represent pooled results from two independent experiments (n=10 mice for spleen, n=10 mice for MLN, n=8 mice for colon). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test (A) or a Mann Whitney U Test (D). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

5.2.9 IL-33R deficient Treg cells are functionally impaired

Next we sought to determine whether IL-33R expression on Tregs is important for their suppressive function *in vivo*, or whether the role for IL-33 is limited to their induction. As it was not feasible to utilize colonic IL-33R^{+}Foxp3^{+} T cells as a source of Treg cells, due to the low numbers of total cells that can be retrieved from the steady state lamina propria, we isolated CD4^{+}CD25^{+} Treg cells from the spleen of WT and *Il33r*$^{-/-}$ mice, and tested their ability to suppress colitis induced by transfer of WT naive CD4^{+}CD45RB^{hi} T cells (RB^{hi}). In addition, we used CD45.1^{-} mice as donors for WT and *Il33r*$^{-/-}$ CD4^{+}CD25^{+} Treg cells and CD45.1^{+} mice as the source for WT CD4^{+}CD45RB^{hi} T cells (Figure 5.12A). This allowed us to differentiate between the progeny of Treg cells and naive CD4^{+} T cells within the same recipient. As expected, transfer of CD4^{+}CD45RB^{hi} T cells into *Rag1*$^{-/-}$ recipients led to the development of colitis, whereas co-transfer of WT CD4^{+}CD25^{+} Treg cells with naive CD4^{+} T cells prevented the development of disease (Figure 5.12B). By contrast, *Il33r*$^{-/-}$ CD4^{+}CD25^{+} Treg cells were impaired in their capacity to prevent colonic inflammation induced by naive CD4^{+} T cells. Moreover, colitis induced by naive CD4^{+} T cell transfer alone was more severe than that observed in recipients co-transferred with naive CD4^{+} T cells and *Il33r*$^{-/-}$ Treg cells, suggesting that IL-33 deficient Treg cells still retain some of their suppressive capacity. WT Treg cells efficiently prevented cellular infiltration and accumulation of CD45.1^{+} progeny of naive CD4^{+} T cells in the colon and spleen, whereas *Il33r*$^{-/-}$ Tregs were impaired in doing so (Figure 5.12C-D). Furthermore, analysis of IL-33R expression in recipients of WT Treg cells and naive CD4^{+} T cells revealed that IL-33R expression was largely restricted to progeny of Treg cells and, in contrast to the steady state, the frequency of IL-33R^{+}Foxp3^{+} Treg cells was comparable in the colon, MLN and spleen (Figure

5.13A-B). Together, these preliminary results suggest that IL-33 signals into Treg cells contribute to their suppressive capacity in the colon as well as systemically.

5.2.10 Progeny of IL-33R-deficient Treg cells contain fewer Foxp3$^+$ cells and acquire the ability to produce IL-17A and IFN-γ

We reasoned that the impaired protective capacity of *Il33r*$^{-/-}$ Treg cells could be due to their failure to accumulate, maintain Foxp3 expression, or a combination of both. We found a significantly decreased frequency of CD45.1$^-$CD4$^+$Foxp3$^+$ T cells in the colon, MLN and spleen of mice co-transferred with *Il33r*$^{-/-}$ Treg cells compared to mice co-transferred with WT Treg cells (Figure 5.14A-B); however, the total number of CD45.1$^-$CD4$^+$Foxp3$^+$ T cells was comparable in both groups (Figure 5.14C). This suggests that although Foxp3$^+$ *Il33r*$^{-/-}$ Treg cells can accumulate in the inflamed colon they are not as competitive as their WT Treg counterparts. Next we determined Foxp3 expression in progeny of WT and *Il33r*$^{-/-}$ Treg cells. In recipients co-transferred with WT CD4$^+$CD25$^+$ Treg cells and naive CD4$^+$ T cells, the frequency of Treg cell progeny expressing Foxp3 was approximately 80%, indicating that most CD25$^+$ WT Treg cells had maintained Foxp3 expression (Figure 5.15A-B). Strikingly, progeny of IL-33R-deficient Treg cells contained a significantly lower proportion of Foxp3$^+$ compared to WT Treg cell progeny, suggesting they had lost Foxp3 expression or were outcompeted by Foxp3$^-$ T cells present in the CD25$^+$ T cell fraction. Importantly, prior to transfer into *Rag1*$^{-/-}$ hosts both WT and *Il33r*$^{-/-}$ CD4$^+$CD25$^+$ T cells contained an equal proportion of Foxp3$^+$ cells and Foxp3 expression was similar regardless of IL-33R expression (Figure 5.15C). Therefore differences in these populations prior to transfer are unlikely to account for the observed differences. In addition the Foxp3$^-$, but not Foxp3$^+$ progeny, of WT and *Il33r*$^{-/-}$ Tregs acquired expression of IL-17A and

IFN-γ (Figure 5.16A-B). However, the frequencies of IL-17A and IFN-γ expressing cells was significantly greater among progeny of *Il33r*$^{-/-}$ Treg cells, suggesting a role for IL-33 in regulation of pathogenic effector phenotype in addition to effects on Treg cells. Together, these data suggest that IL-33 signals into Treg cells contribute to the maintenance of the Treg cell phenotype.

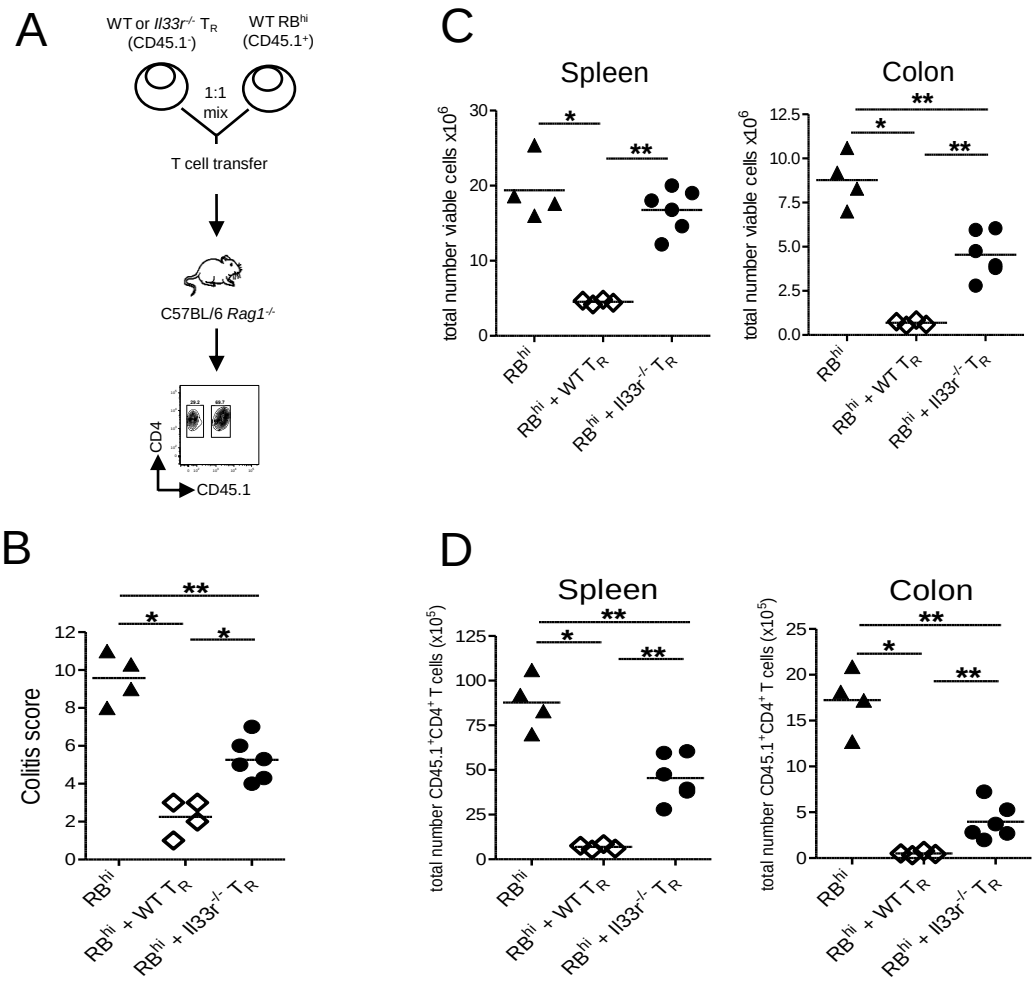

Figure 5.12. IL-33R deficient Treg cells are functionally impaired

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} CD45.1$^+$ naive T cells alone (RB^{hi}) or in combination with 2x10^5 WT or *Il33r*$^{-/-}$ CD4$^+$CD25$^+$ CD45.1$^-$ Treg cells (T_R). Mice were sacrificed when recipients of naive T cells developed clinical signs of disease (6-8 weeks). Single cell suspensions were prepared from the spleen and colonic lamina propria and mice were assessed for histological signs of intestinal inflammation.

(A) Schematic of the experimental design**.**

(B) Histological analysis of colonic inflammation. Bars represent the mean and each point represents an individual mouse.

(C) Total number of viable cells in the spleen and colon. Bars represent the mean and each point represents an individual mouse.

(D) Total number of viable CD45.1$^+$CD4$^+$ T cells in the spleen and colon. Bars represent the mean and each point represents an individual mouse.

Data represent a single experiments (n=4 mice for RB^{hi}, n=4 mice for WT T_R, n=6 mice for *Il33r*$^{-/-}$$T_R$). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. However this test appeared to be too stringent in this case and as standard deviations of the three groups clearly do not overlap we performed a Mann Whitney U test comparison between the different groups instead. * $p \leq 0.05$, ** $p \leq 0.01$.

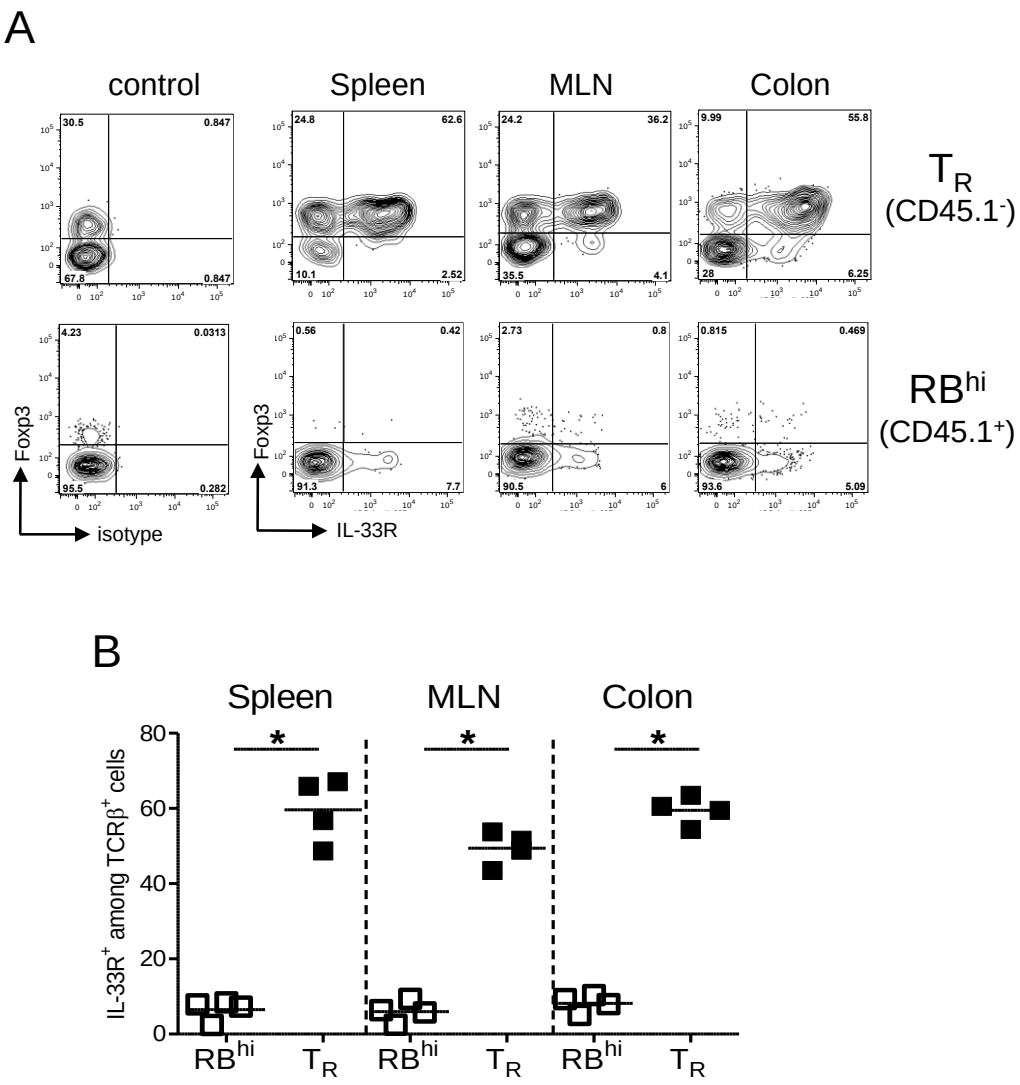

Figure 5.13. IL-33R is preferentially expressed on Treg cell progeny in protected mice.

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} CD45.1$^+$ naive T cells in combination with 2x10^5 WT CD4$^+$CD25$^+$ CD45.1$^-$ Treg cells (T_R). Mice were sacrificed at 6-8 weeks post transfer. Single cell suspensions were prepared from the indicated organs and expression of cell surface and intracellular markers was determined by FACS.

(A) Representative FACS plot of indicated organs gated on progeny of CD4$^+$CD45RB^{hi} cells (CD45.1$^+$) or WT T_R (CD45.1$^-$) cells.

(B) Frequency of IL-33R expressing cells among CD4$^+$ T cells in the indicated organs among the progeny of CD4$^+$CD45RB^{hi} cells (CD45.1$^+$) or WT T_R (CD45.1$^-$) cells as indicated. Bars represent the mean and each point represents an individual mouse.

Data represent a single experiments (n=4 mice). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. * $p \leq 0.05$.

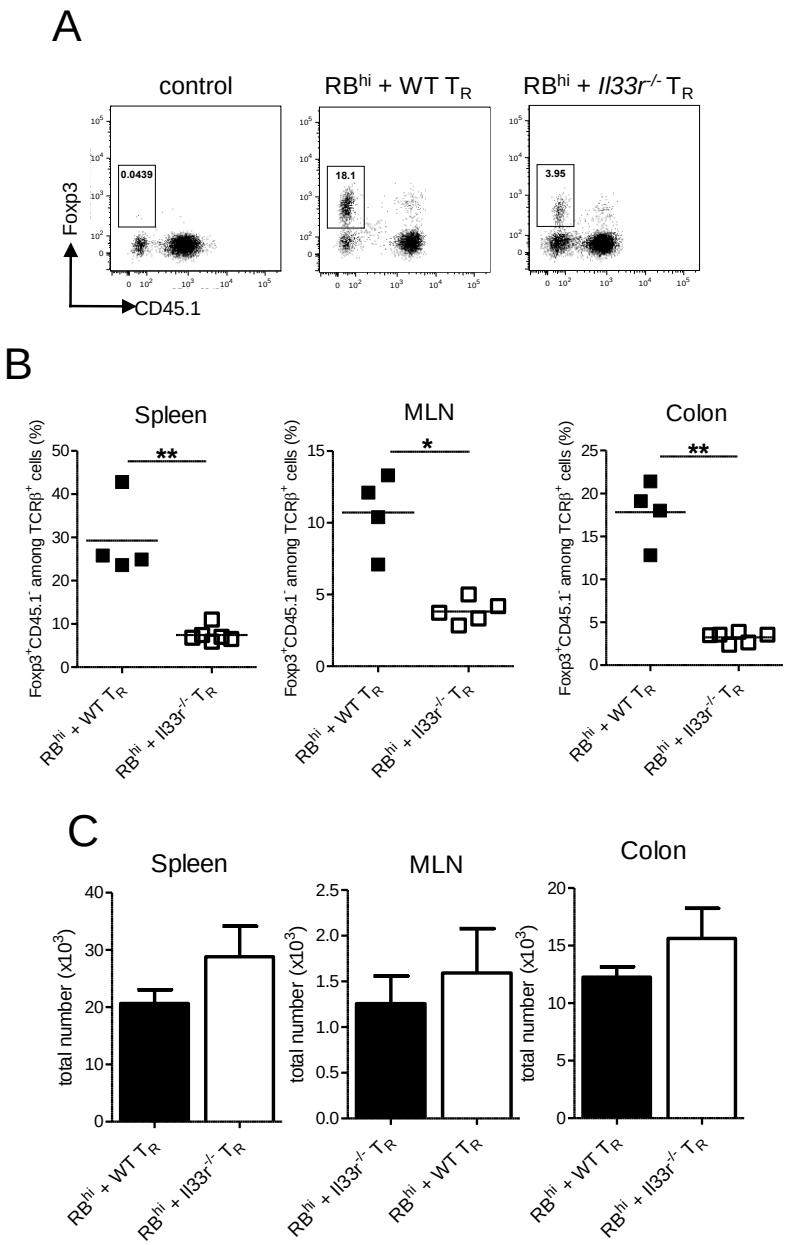

Figure 5.14. The frequency of Foxp3$^+$ T reg cell progeny is reduced in the absence of IL-33R signalling into Tregs

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} CD45.1$^+$ naive T cells in combination with 2x10^5 WT or *Il33r*$^{-/-}$ CD4$^+$CD25$^+$ CD45.1$^-$ Treg cells (T_R). Mice were sacrificed at 6-8 weeks post transfer. Single cell suspensions were prepared from the indicated organs and expression of cell surface and intracellular markers was determined by FACS.

(A) Representative FACS plot of viable CD4$^+$ T cells in the colonic lamina propria.

(B) Frequency of T_R derived Foxp3$^+$ cells among total CD4$^+$ T cells in the indicated organs. Bars represent the mean and each point represents an individual mouse.

(C) Total number of T_R derived Foxp3$^+$ cells among total CD4$^+$ T cells in the indicated organs. Bars represent the mean, error bars represent SEM.

Data represent a single experiments (n=4 mice for RB^{hi} + WT T_R, n=6 mice for RB^{hi} + *Il33r*$^{-/-}$ T_R). Statistical significance was determined using a Mann Whitney U Test. * $p \leq 0.05$, ** $p \leq 0.01$.

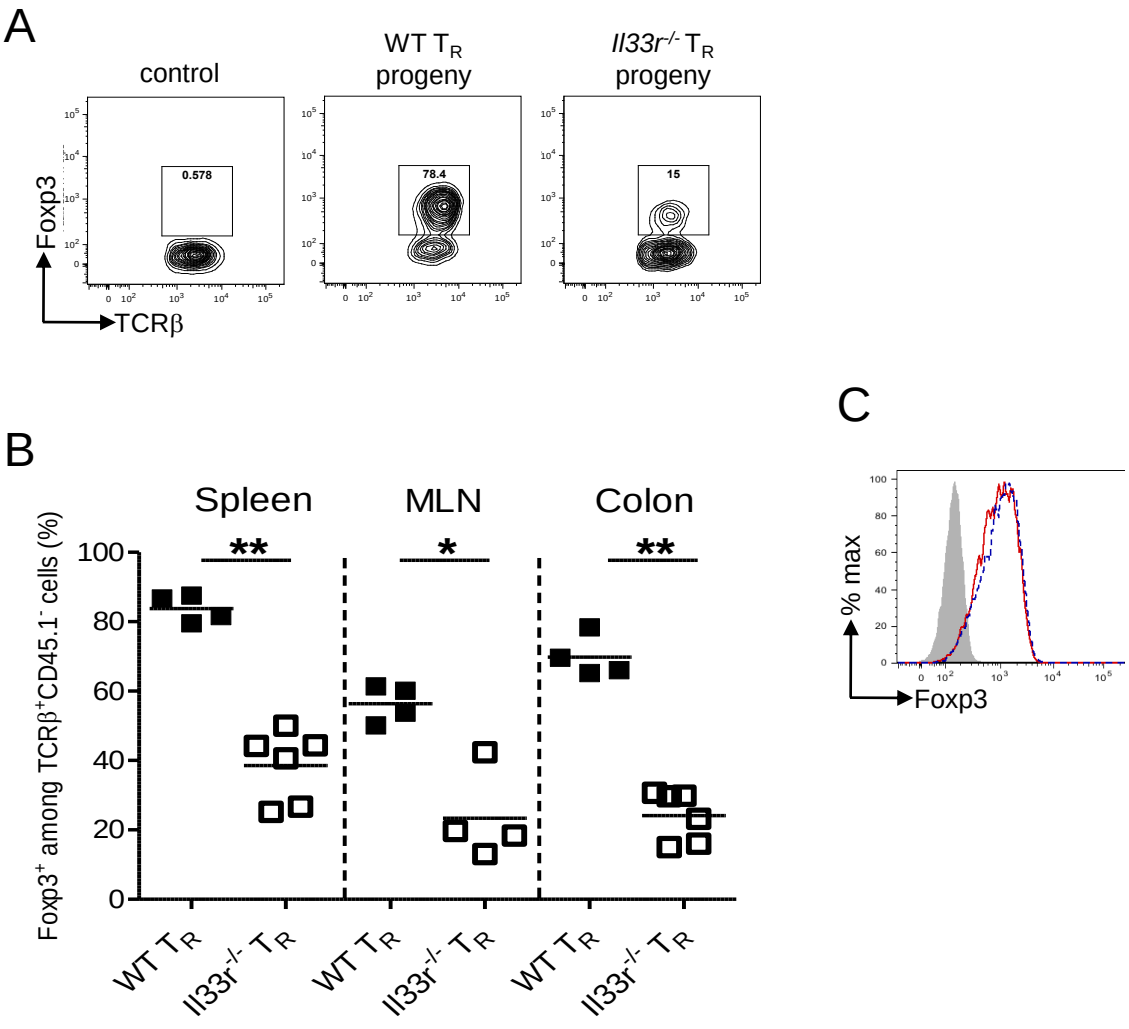

Figure 5.15. Progeny of IL-33R deficient Treg cells contain fewer Foxp3 expressing cells

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} CD45.1$^+$ naive T cells in combination with 2x10^5 WT or *Il33r*$^{-/-}$ CD4$^+$CD25$^+$ CD45.1$^-$ Treg cells (T$_R$). Mice were sacrificed at 6-8 weeks post transfer. Single cell suspensions were prepared from various organs and expression of cell surface and intracellular markers was determined by FACS.

(A) Representative FACS plots of viable CD4$^+$CD45.1$^-$ T cells in the colonic lamina propria.

(B) Frequency of Foxp3 expressing cells among CD45.1$^-$ progeny of WT or *Il33r*$^{-/-}$ T$_R$ cells in the indicated organs. Bars represent the mean and each point represents an individual mouse.

(C) Histogram overlay of Foxp3 expression by WT (red solid line) or *Il33r*$^{-/-}$ (blue dotted line) CD4$^+$CD25$^+$ T$_R$ cells prior to injection into C57BL/6 *Rag1*$^{-/-}$ mice.

Data represent a single experiments (n=4 mice for RB^{hi} + WT T$_R$, n=6 mice for RB^{hi} + *Il33r*$^{-/-}$ T$_R$). Statistical significance was determined using a Mann Whitney U Test. * p≤0.05, ** p ≤ 0.01.

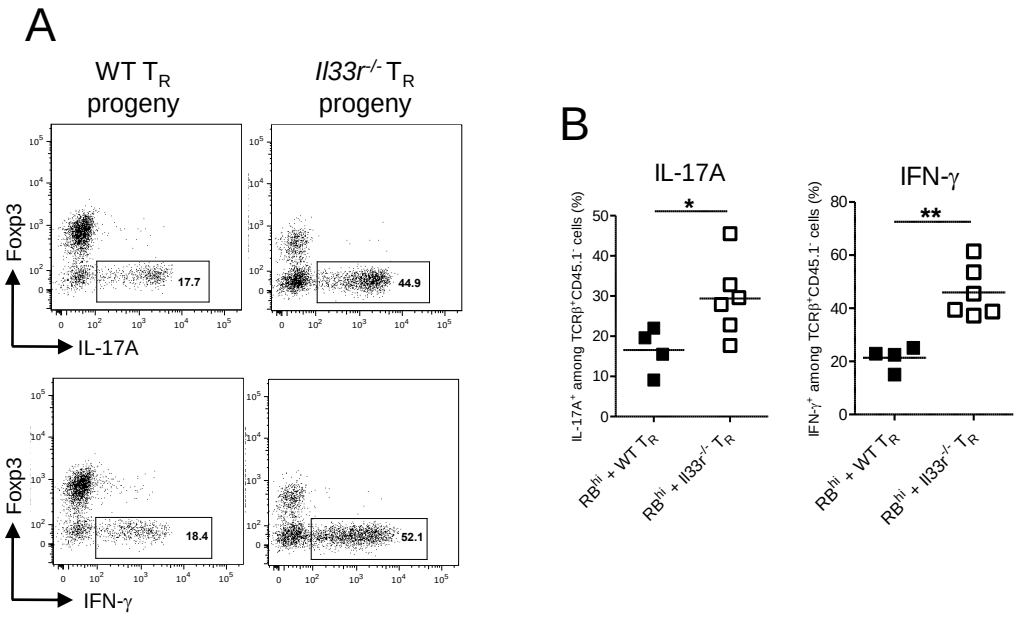

Figure 5.16. Foxp3$^-$ progeny of IL-33R deficient Treg cells produce IL-17A and IFN-γ

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} CD45.1$^+$ naive T cells in combination with 2x10^5 WT or *Il33r*$^{-/-}$ CD4$^+$CD25$^+$ CD45.1$^-$ Treg cells (T_R). Mice were sacrificed at 6-8 weeks post transfer. Single cell suspensions were prepared from various organs and expression of cell surface and intracellular markers was determined by FACS.

(A) Representative FACS plots of viable CD45.1$^-$ CD4$^+$ T cells in the colonic lamina propria.

(B) Frequency of IL-17A or IFN-γ producing CD45.1$^-$ CD4$^+$ T cells among the progeny of WT or *Il33r*$^{-/-}$ T_R cells in the colonic lamina propria. Bars represent the mean and each point represents an individual mouse.

Data represent a single experiments (n=4 mice for RB^{hi} + WT T_R, n=6 mice for RB^{hi} + *Il33r*$^{-/-}$ T_R). Statistical significance was determined using a Mann Whitney U Test. * p≤0.05, ** p ≤ 0.01.

5.3 Discussion

The mechanisms by which IL-23 promotes pathogenic $CD4^+$ T cell responses in the intestine are not well defined and little is known about the transcriptional targets of IL-23 in intestinal $CD4^+$ T cells. Here, we have used an experimental approach that allowed us to investigate direct cell intrinsic targets of IL-23 signalling into intestinal $CD4^+$ T cells primed *in vivo*. Our experiments have revealed a core set of genes that are regulated by IL-23 and have identified a previously unrecognised role for IL-23 as a negative regulator of Th2-type responses in intestinal inflammation.

The most upregulated gene in *Il23r*$^{-/-}$ $CD4^+$ T cells relative to WT $CD4^+$ T cells was *Cdk5rap1* and although no information currently exists on the role of this gene in $CD4^+$ T cells it could be a promising candidate to investigate. CDK5rap1 has been shown to inhibit the kinase activity of CDK5 [423], an unusual member of the cyclin dependent family of kinases as it is not regulated by cyclins and does not contribute to cell cycle progression [424]. The activity of CDK5 has been studied extensively in neuronal tissues, which highly express the CDK5 regulatory subunits p35 and p39 [425]. Association of CDK5 with one of these subunits is required for the activation of its kinase activity, and CDK5rap1 inhibits this interaction thereby preventing CDK5 activation [423]. Interestingly, CDK5 has been shown to phosphorylate STAT3 at serine 727 [414], and STAT3 serine phosphorylation is known to modulate its transcriptional activity [426]. Although a role for CDK5 in STAT3 serine phosphorylation has not been demonstrated in $CD4^+$ T cells it has been shown that CDK5 and its regulatory subunits are expressed in T cells upon TCR stimulation [427]. Importantly, CDK5 activity has been shown to be required for T cell activation and induction of EAE [427], an inflammatory disorder which requires STAT3 in

$CD4^+$ T cells for development of disease [428]. Thus, it is tempting to speculate that IL-23 signalling into $CD4^+$ T cells may promote CDK5 mediated S727 phosphorylation of STAT3 through its inhibitory effects on CDK5rap1. Thus, in addition to the known effects of IL-23 on STAT3 tyrosine phosporylation [304], further modulation of STAT3 transcriptional activity through serine phosphorylation might be a mechanism by which IL-23 affects gene transcription controlled by STAT3. Previous work from our laboratory demonstrated a requirement for STAT3 in $CD4^+$ T cells for development of $CD4^+$ T cell transfer colitis and additionally identified the genome-wide targets of tyrosine phosphorylated STAT3 in Th17 cells using ChIP-Seq [390]. It would be of interest to determine how targets of IL-23 activated STAT3 are affected in the absence of CDK5-mediated S727 phosphorylation. Moreover a recent study demonstrated a role for STAT3 in inhibition of iTreg generation in GvHD [429], again supporting a role for STAT3 inducing cytokines in the inhibition of Treg cells. Thus, STAT3 S727 phosphorylation could be a hallmark of pathogenic IL-23 signalling and different STAT3 modifications might explain how cytokines such as IL-23 and IL-10, which both signal via STAT3, have such divergent functions in intestinal inflammation [304, 430]. Therefore, it will be important to determine the expression of CDK5 and its regulatory subunits in intestinal $CD4^+$ T cells, establish whether stimulation of $CD4^+$ T cells with IL-23 leads to CDK5 dependent serine phosphorylation of STAT3 and interrogate the pathogenic function of CDK5 deficient $CD4^+$ T cells. Furthermore, generation of gene-targeted mice lacking particular sites in the STAT3 protein might reveal how particular residues in STAT3 modulate the balance between effector and regulatory $CD4^+$ T cells responses in response to cytokines such as IL-23. It should also be noted that CDKAL1, a close homologue of CDK5rap1, is a

susceptibility gene for Crohn's disease and UC as well as other inflammatory diseases [431-433]. Although the function of CDKAL1 is currently unknown, the homology of CDKAL1 and CDK5rap1 suggests that both proteins might be involved in the regulation of CDK5.

Among the list of genes that are decreased in the absence of IL-23 signalling were several cell surface receptors such as *Tnfrsf1a* (the receptor for TNF-α) and *Ltb4r1* (receptor for the lipid chemoattractant leukotriene B_4). Early studies identified TNF-α as a factor that promotes Th17 differentiation and IL-23 has been shown to promote TNF-α production by innate immune cells. This suggests a possible positive feedback loop by which IL-23 induces TNF-α by innate immune cells and increases responsiveness to TNF-α on $CD4^+$ T cells to promote Th17 differentiation. Leukotriene B_4 was originally described as a metabolite of arachidonic acid produced by neutrophils [434] and is a potent chemoattractant for many cell types including $CD4^+$ T cells [435, 436]. In particular, expression of LTB4R1 has been shown to be required for early effector $CD4^+$ T cell recruitment to the inflamed lung in a murine model of asthma [436]. In addition, early studies showed that LTB_4 can enhance IFN-γ and IL-4 production by Th1 and Th2 cells, respectively [437, 438]. Given that IL-23 controls responsiveness to TNF-α and LTB_4 it will be of interest to study their effects on intestinal $CD4^+$ T cell responses in more detail.

An important factor worth considering when interpreting the microarray data generated here is that only 6-8% of $CD4^+$ T cells express the IL-23R in the inflamed intestine of a reconstituted *Rag1*$^{-/-}$ mouse as determined with IL-23R$^{gfp/+}$ reporter mice (data not shown). Thus, the small set of core genes regulated by IL-23 might be an

underestimate as only a fraction of intestinal $CD4^+$ T cells were affected by the genetic ablation of IL-23R. The *Helicobacter hepaticus*/anti-IL-10R model of colitis might provide a more sensitive approach for the identification of IL-23 regulated genes as the frequency of IL-23R expressing cells is considerably greater in that model. As pointed out earlier, the differences in transcript abundance observed in our comparison of WT and *Il23r*$^{-/-}$ $CD4^+$ T cells could be caused by true changes in the rate of gene transcription and/or variations in the cellular composition between WT and *Il23r*$^{-/-}$ $CD4^+$ T cells. Stimulation of IL-$23R^+CD4^+$ T cells with IL-23 *in vitro* or the purification of IL-$23R^+$ $CD4^+$ T cells from the inflamed colon will be invaluable in determining whether IL-23 regulates the set of genes identified in this study at the transcriptional level. Moreover, we showed previously (Chapter 3) that IL-23R expressing $CD4^+$ T cells are abundant in the steady state colon and it would be of interest to determine whether IL-23 regulates the same set of genes in steady state $CD4^+$ T cells or whether these genes are only controlled by IL-23 in an inflammatory context. Such a comparison might highlight differences between steady state and inflammatory Th17 cells. In addition, it will be of interest to determine whether IL-23 regulates the same set of genes in other IL-23 responsive cell types such as innate lymphoid cells or γδ T cells.

One of the most intriguing findings of our microarray experiment was the increased expression of Th2 associated genes among *Il23r*$^{-/-}$ compared to WT $CD4^+$ T cells. We found that the transcripts for IL-4, IL-5, IL-13, GATA3, c-Maf and the IL-33R were significantly increased in *Il23r*$^{-/-}$ $CD4^+$ T cells suggesting that IL-23 negatively regulates the Th2 program during intestinal inflammation. Indeed transfer of $GATA3^{fl/fl}$OX40Cre $CD4^+$ T cells into *Rag1*$^{-/-}$ hosts resulted in accelerated wasting

disease and more pronounced intestinal inflammation compared to *Rag1*$^{-/-}$ recipients of WT CD4^{+} T cells. Although we did not confirm the degree of deletion in the transferred CD4^{+} T cells it is important to note that the frequency of IL-13 producing cells was significantly reduced in GATA3$^{fl/fl}$OX40Cre CD4^{+} T cells suggesting that GATA3 was efficiently deleted in a significant proportion of the transferred CD4^{+} T cells. Although these results are preliminary they indicate that GATA3 restrains the colitogenic potential of CD4^{+} T cells in this model. The mechanism by which GATA3 achieves this is not clear but it appears that IL-13, which requires GATA3 for its induction and maintenance of expression [95, 417], is more highly expressed than IL-4 or IL-5 in *Il23r*$^{-/-}$ CD4^{+} T cells. Similar to IL-4 [161], IL-13 has been shown to act directly on CD4^{+} T cells to suppress Th17 differentiation *in vitro* [160]. Wilson et al recently demonstrated that IL-13 attenuates colitis in *Il10*$^{-/-}$ mice through its inhibitory effects on Th1 and Th17 differentiation in the intestine [160]. By contrast, we found no difference in the frequency of IL-17A^{+} or IFN-γ^{+} intestinal CD4^{+} T cells suggesting that GATA3 deficiency did not lead to dysregulated Th1 or Th17 responses. A possible explanation for this could be that OX40-mediated deletion of GATA3 in transferred CD4^{+} T cells is incomplete and the residual IL-13 produced by GATA3$^{fl/fl}$OX40Cre T cells might be sufficient for inhibition of the Th17 response. Alternatively, IL-13 has been shown to block secretion of Th17 promoting cytokines IL-6, IL-1β and IL-23 from LPS stimulated DCs [439]. Therefore, the enhanced accumulation of GATA3$^{fl/fl}$OX40Cre CD4^{+} T cells in the colon could be a direct consequence of increased availability of STAT3 activating cytokines IL-23 and/or IL-6. However, this should also lead to increased Th17 differentiation, which we did not observe in this experiment. In addition to its role in Th2 differentiation GATA3 has recently been shown to control Foxp3 expression [254] and GATA3$^{fl/fl}$OX40Cre Treg

cells were shown to be functionally impaired and failed to protect from colitis induced by naïve $CD4^+$ T cell transfer [415]. However, we could not detect $Foxp3^+$ Treg cells in progeny of WT or $GATA3^{fl/fl}$OX40Cre $CD4^+$ T cells and thus it is unlikely that GATA3 deletion in $Foxp3^+$ Tregs caused the difference in disease outcome. Furthermore, our co-transfer experiments also showed that IL-23 specifically inhibits the emergence of IL-$17A^+$IL-13^+ $CD4^+$ T cell but promotes IL-$17A^+$IFN-γ^+ $CD4^+$ T cells in a cell intrinsic manner suggesting that IL-23 has differential effects on IL-13 and IFN-γ expression by Th17 cells. However, the relevance of this to the development of intestinal inflammation remains to be established. In any case the fact that several transcription factors such as c-maf, IRF4 and STAT3 are critical for the differentiation of both Th17 [129, 137, 440] and Th2 [98, 100, 103] cells suggests that coexpression of Th2 and Th17 signature cytokines might be more common than previously anticipated.

In addition to GATA3 and the Th2 cytokines described above IL-23 signalling into $CD4^+$ T cells also regulated the expression of the IL-33R also known as T1ST2 or ST2L. Regulation of the IL-33R has mostly been studied in the context of Th2 cells, which express high levels of IL-33R [419]. GATA3 and STAT5 activating cytokines such as IL-2, IL-7 or TSLP act synergistically to induce IL-33R expression on Th2 cells and stimulation of Th2 cells with IL-33 leads to production of IL-13 and IL-5 but not IL-4 [441, 442]. Recently, two independent studies demonstrated that administration of recombinant IL-33 led to the expansion of $Foxp3^+$ Treg cells and this correlated with improved cardiac allograft survival [420, 421]. Our mixed bone marrow chimaera experiments showed that IL-33R is highly expressed on intestinal $Foxp3^+$ Treg cells in inflamed mice and suggested that cell intrinsic IL-23 signals

limit IL-33R expression on Foxp3$^+$ Treg cells. Based on our previous finding that IL-23 signals into CD4$^+$ T cells restrain the emergence of Foxp3$^+$ Treg cells in a cell intrinsic fashion it is tempting to hypothesise that IL-23 might regulate Foxp3$^+$ Treg cell responsiveness to IL-33.

Although we did not test the contribution of IL-33 to Foxp3$^+$ Treg expansion in *Il23r*$^{-/-}$ mice we found that the transcripts for IL-23 and IL-33 in whole colon homogenates were dynamically regulated during the course of intestinal inflammation. Both IL-23 and IL-33 were induced early in the response, prior to onset of intestinal inflammation, suggesting that these two cytokines exert their effects at a similar stage of the immune response. It was previously shown that CD11cCre.IL-23p19$^{fl/fl}$ mice, which specifically lack IL-23 in CD11c$^+$ cells, are resistant to the development of intestinal inflammation in this model (Philip Ahern, DPhil Thesis) suggesting that DCs are an essential source of IL-23. Although we did not establish the cellular source of IL-33 it has been shown that IL-33 mRNA is expressed in myeloid cells as well as non-hematopoietic cells such as intestinal epithelial cells, endothelial cells and fibroblasts [441, 443]. As IL-33 is constitutively expressed in the nucleus of epithelial and endothelial cells it was originally thought of as an alarmin, similar to IL-1α [444] and HMGB1 [445], which is released by necrotic cells to alert the immune system to stress or tissue injury [441, 443, 446]. However, IL-33 expression has also been shown to be induced by TLR ligands or pro-inflammatory cytokines in non-necrotic cells demonstrating that it can also act as a more classic cytokine [447] [448]. The regulation of IL-33 bioactivity is complex. In contrast to IL-18 and IL-1β the secretion of IL-33 does not require cleavage by caspase 1, and several groups have independently shown that IL-33 cleavage by caspase 1 results in its inactivation [449-

451]. In addition, once released into the extracellular matrix IL-33 can be further cleaved into more bioactive forms by neutrophils elastase and cathepsin G [452]. Moreover, a soluble decoy receptor, termed sST2, can bind to and modulate the bioactivity of IL-33 [453]. Thus, more detailed analysis of IL-33 on the protein level is required to accurately assess its contribution to the development of *Helicobacter hepaticus/*anti-IL-10R driven colitis.

We previously described that IL-23 inhibits Treg induction [330, 391]. This was based on the observation that naïve $CD4^+$ T cells gave rise to greater frequencies of $Foxp3^+$ Treg cells when transferred into *Il23a*$^{-/-}$*Rag1*$^{-/-}$ recipients. Further analysis revealed that IL-23 inhibited iTreg induction through direct cell intrinsic effects on $CD4^+$ T cells but the mechanism by which IL-23 achieved this was not identified [391]. Here we have shown that transfer of IL-33R deficient $CD4^+$ T cells into *Il23a*$^{-/-}$ *Rag1*$^{-/-}$ hosts gave rise to significantly lower frequencies of $Foxp3^+$ Tregs than were observed upon transfer of WT $CD4^+$ T cells. This observation suggests that IL-33 signalling into T cells favours the differentiation of naïve $CD4^+$ T cells into $Foxp3^+$ Treg cells in the absence of IL-23. However, a confounding factor in the interpretation of these results is that *Il33r*$^{-/-}$ $CD4^+$ T cells induced more pronounced intestinal inflammation as compared to WT $CD4^+$ T cells. Thus, the reduced proportion of $Foxp3^+$ Treg cells among *Il33r*$^{-/-}$ $CD4^+$ T cells could also be a consequence of increased levels of pro-inflammatory cytokines that inhibit iTreg induction. Indeed, the frequency of IL-17A producing cells was increased in recipients of *Il33r*$^{-/-}$ $CD4^+$ T cells and Th17 cells are a source of IL-6 and IL-21, which negatively regulate Foxp3 expression [121, 125-127]. Thus, we performed *in vitro* Treg differentiation experiments to test the direct effect of IL-33 on TGF-β1

driven Treg induction. Strikingly, we found that IL-33 enhanced TGF-β1 mediated Treg conversion and this was inhibited in the presence of IL-23, while IL-23 had no affect on Treg induction driven by TGF-β1 alone. While the mechanism through which IL-33 promotes Treg induction remains to be identified, these results appear to mimic findings *in vivo* in the intestine and thus this system should allow for detailed interrogation of the mechanism by which IL-33 promotes, and IL-23 inhibits, Treg accumulation. Similar to the *in vivo* scenario discussed above IL-33 might promote TGF-β1-induced Foxp3 expression indirectly through downregulation of pro-inflammatory cytokines that interfere with Foxp3 induction, as has been shown for retinoic acid [454, 455]. Alternatively IL-33 could directly promote Foxp3 induction through control of its expression or modulate proliferation and/or survival of Treg cells. Interestingly, IL-33 has been shown to enhance IL-2 induced STAT5 phosphorylation in Th2 cells [442] and IL-2/STAT5 signals are also required for optimal TGF-β1 mediated induction of Foxp3 *in vitro* [456]. Furthermore, IL-33 in combination with IL-2 has been shown to induce GATA3 expression in Th2 cells [442]. Recent studies demonstrated that GATA3 controls Foxp3 expression and that GATA3 activity in Treg cells is essential for their suppressive function and maintenance of the Treg phenotype [254, 415]. Therefore it will be interesting to assess whether IL-33 can synergize with IL-2 in promoting Foxp3 expression through effects on GATA3. The fact that IL-23 has been previously demonstrated to suppress the expression of IL-2 [131] further supports the concept that this pathway is a target for inhibition by IL-23.

In order to determine whether IL-33R expression on Tregs is important for their suppressive function *in vivo* we transferred naïve $CD4^+$ T cells with WT or *Il33r*$^{-/-}$ $CD4^+CD25^+$ Treg cells into *Rag1*$^{-/-}$ recipients. In contrast to the low expression of IL-33R on splenic $Foxp3^+$ Treg cells, which were used as $CD4^+CD25^+$ Treg cell donors, we observed that IL-33R expression was highly expressed among progeny of WT $CD4^+CD25^+$ Treg cells in the colon, MLN and spleen suggesting that IL-33R is highly induced under lymphopenic conditions. Importantly, IL-33R deficient $CD4^+$ T cells were impaired in their ability to protect from naïve $CD4^+$ T cell driven colitis and systemic inflammation suggesting that IL-33 plays a role in nTreg function. Recently the stability of $Foxp3^+$ Treg cells has received much attention and reprogramming of $Foxp3^+$ Tregs into $Foxp3^-$ effector T cells has been described, especially in lymphopenic settings [41, 259, 261, 457]. We found a significantly lower proportion of $Foxp3^+$ cells among *Il33r*$^{-/-}$ Treg cell progeny as compared to WT Treg cell progeny suggesting that *Il33r*$^{-/-}$ Treg cells had lost Foxp3 expression or were outcompeted by $Foxp3^-$ T cells present in the $CD25^+$ T cell fraction. Although preliminary, these data suggest that IL-33 contributes to the maintenance of the Treg phenotype. In support of a role of IL-33 in Treg function Fukata et al found that MyD88$^{-/-}$ $CD4^+CD25^+$ Treg cells did not protect from wasting disease and colitis induced by naïve $CD4^+$ T cells [458]. Although the authors linked this to defective TLR signalling into Treg cells, it is equally likely that the loss of MyD88 impaired IL-33R signalling into these cells as MyD88 is also a downstream adaptor of IL-33 signalling. In addition, a recent study found that DC-derived IL-18 promoted iTreg induction *in vitro* and *in vivo* [459] demonstrating that IL-1 family members other than IL-33 can favour Treg differentiation. Our analysis also revealed a greater frequency of IL-17A and IFN-γ producing cells in $Foxp3^-$, but not $Foxp3^+$, progeny of

Il33r$^{-/-}$ Treg cells. By contrast two independent groups recently reported enhanced IL-17A production in GATA3 deficient Foxp3^{+} Treg cells [254, 415], which suggests that the effects of GATA3 and IL-33R deficiency on Treg cells are distinct and that IL-33 signalling into CD4^{+} T cells might not be required for GATA3 expression by Treg cells. Clearly, further work is required to better understand the role of IL-33 in Treg differentiation and function.

Here we have used transcriptional profiling to identify IL-23 target genes in intestinal CD4^{+} T cells. We have identified a previously unrecognised role for IL-23 in suppressing Th2 associated genes. Further analysis showed that GATA3 expression in CD4^{+} T cells limits their colitogenic potential suggesting that IL-23 might drive intestinal inflammation through inhibition of the GATA3 pathway. In support of this we found that direct IL-23 signals in CD4^{+} T cells suppressed the emergence of IL-17A^{+}IL-13^{+} CD4^{+} T cells and favoured the development of IL-17A^{+}IFN-γ^{+} CD4^{+} T cells. Furthermore we showed that IL-23 negatively regulates IL-33R expression on Foxp3^{+} Treg cells and that IL-33 signalling into Foxp3^{+} Treg cells is important for their suppressive function *in vivo*. We have also described that IL-33 signals into CD4^{+} T cells promote iTreg induction *in vitro* and *in vivo* and this effect of IL-33 was inhibited in the presence of IL-23. Together these data suggest that IL-23 interferes with immunoregulatory programs, at least in part, by suppressing the effects of IL-33 on Treg differentiation/function.

Chapter 6: General Discussion

6.1 Summary

In this thesis I have characterised the potential mechanisms by which IL-23 drives T cell dependent intestinal inflammation. Selective ablation of IL-23R in the innate or T cell compartment demonstrated that IL-23 signalling into $CD4^+$ T cells was required for their accumulation in the intestine and contributed to the emergence of IL-$17A^+$IFN-γ^+ T cell at this site. We further revealed that the IL-$17A^+$IFN-γ^+ population of T cells co-express RORγt and T-bet. Genetic ablation of these transcription factors in $CD4^+$ T cells showed that RORγ but not T-bet expression was required for the development of intestinal inflammation. In addition, using transcriptional profiling we have identified a core set of IL-23-regulated genes in colonic $CD4^+$ T cells. This approach revealed a previously unrecognised role for IL-23 as a negative regulator of Th2-type responses. Further experiments showed that GATA3 expression in $CD4^+$ T cells limited their colitogenic potential, and that IL-23 signals in $CD4^+$ T cells suppressed the emergence of IL-$17A^+$IL-13^+ T cells. Thus, IL-23 might promote intestinal inflammation through inhibition of Th2-type responses. In addition, we described a novel function for the Th2 associated cytokine IL-33 as a factor that promotes $Foxp3^+$ Treg differentiation and function. This activity of IL-33 was inhibited in the presence of IL-23, possibly through IL-23-mediated repression of the IL-33R on $CD4^+$ T cells, thus providing a mechanistic link for the known role of IL-23 in restraining iTreg generation. These findings are summarized in Figure 6.1 and some of the implications of these findings will be discussed in more detail here.

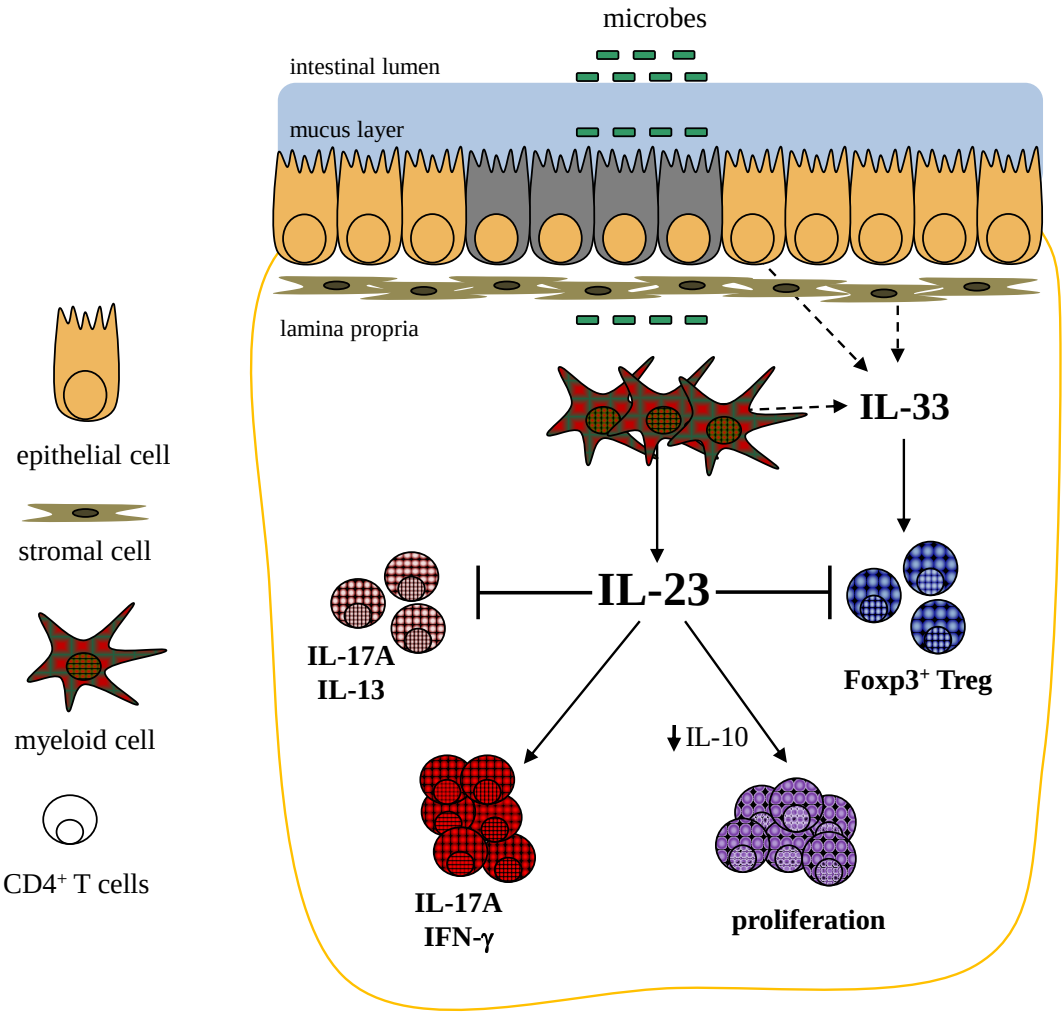

Figure 6.1 IL-23 signalling into CD4$^+$ T cells promotes intestinal inflammation through multiple mechanisms

Intestinal myeloid cells can be activated through exposure to microbial antigens. This leads to the production of IL-23 which can act directly on T cells to drive their proliferation and accumulation in the lamina propria, possibly through inhibition of T cell IL-10 production. IL-23 also modulates the Th17 cell phenotype by promoting expression of IFN-γ and inhibiting expression of IL-13. In addition, IL-23 signals directly into T cells limit their responsiveness to IL-33, which prevents IL-33 mediated accumulation of Tregs. Various cell types such as epithelial cells, stromal cells or myeloid cell might be a source of IL-33. Together, these events lead to the development of chronic intestinal inflammation.

6.2 IL-23 modulates Th17 pathogenicity

Our *in vivo* experiments revealed a cell intrinsic role for IL-23R signals in the emergence of IL-17A$^+$IFN-γ$^+$ T cells during intestinal inflammation. By contrast, the differentiation of IL-17A$^+$IFN-γ$^-$ and IL-17A$^-$IFN-γ$^+$ T cells was not affected in the absence of IL-23R signals into T cells, suggesting a specific role for IL-23 in driving the acquisition of IFN-γ expression by intestinal Th17 cells. Although we did not determine the origin of IL-17A$^+$IFN-γ$^+$ T cells in our experiments several lines of evidence suggest that this population derives from Th17 cells. First, epigenetic analyses have revealed that the loci for *Tbx21* and *Ifng* are associated with permissive histone modifications in Th17 cells, while the loci for *Rorc* and *Il17a-Il17f* are marked by repressive modifications, suggesting that IL-17A$^+$IFN-γ$^+$ T cells are more likely to arise from Th17 cells rather than Th1 cells [260, 263]. Second, *in vitro* differentiated Th17 cells have been shown to extinguish IL-17A expression and acquire the ability to produce IFN-γ upon adoptive transfer into lymphopenic hosts [132, 268, 460]. Lastly, fate mapping experiments using an IL-17A-eYFP reporter strain showed that the majority of IL-17A$^+$IFN-γ$^+$ as well as IL-17A$^-$IFN-γ$^+$ T cells that arise in the inflamed CNS during EAE were derived from CD4$^+$ T cells that had once expressed IL-17A [393]. Importantly this phenotype switch was dependent on IL-23. Thus, it is likely that intestinal IL-17A$^+$IFN-γ$^+$ T cells originate from Th17 cells in response to IL-23 signals. To formally test this hypothesis it will be important to generate IL-23R deficient IL-17A-eYFP reporter mice and follow the fate of IL-17A expressing cells after transfer into *Rag1*$^{-/-}$ recipients.

We showed that the IL-23 dependent emergence of IL-17A$^+$IFN-γ$^+$ T cells correlates with the severity of inflammation, which suggests a potential pathogenic role for this

population in the development of colitis. In support of this IL-17A$^+$IFN-γ$^+$ T cells have been found in inflamed intestinal lesions of CD patients [266, 361]. However, the assumption that the pathogenic potential of Th17 cells is restricted to the IFN-γ producing fraction may be an oversimplification. An important consideration is that IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells are thought to represent an intermediate phenotype in the transition from Th17 cells into Th1-like cells [132, 393], also referred to as 'ex-Th17' cells. It is therefore plausible that the decreased frequency of IL-17A$^+$IFN-γ$^+$ T cells in the absence of IL-23 reflects a reduced rate of Th17 to Th1 transition. Interestingly, IFN-γ expressing 'ex-Th17' cells that arise in the inflamed CNS of mice with EAE have been shown to retain expression of certain markers such as AHR and IL-1R1, which distinguish them from conventional IFN-γ producing Th1 cells [393]. Moreover, expression of other pro-inflammatory cytokines such as GM-CSF, IL-2 and TNF-α was shown to be restricted almost exclusively to 'ex-Th17 cells', suggesting that these cells are more pathogenic than Th1 cells [393]. Thus, it would be of interest to determine the relative contribution of Th1 and 'ex-Th17 cells' to the pool of IFN-γ producing cells among WT and IL-23R deficient T cells. Comparison of IL-1R1 and pro-inflammatory cytokine expression among the IL-17A$^-$IFN-γ$^+$ population in the presence or absence of IL-23 signals into T cells might reveal differences in the pathogenic potential of these cells.

Alternatively, IL-17A$^+$IFN-γ$^+$ T cells may represent a stable polyfunctional population of CD4$^+$ T cells capable of producing a variety of pro-inflammatory mediators. As neither IL-17A [326, 329, 330] nor IFN-γ [325] production by T cell is required for the development of T cell transfer colitis, it is likely that IL-17A$^+$IFN-γ$^+$ T cells express additional factors that render them pathogenic. Although it has not

been reported whether T cells deficient in both IFN-γ and IL-17A can induce intestinal inflammation, a recent report showed that *Il17a*$^{-/-}$*Ifng*$^{-/-}$ CD4^{+} T cells were not impaired in their ability to induce EAE [134]. Instead T cell derived GM-CSF was found to be essential for driving disease [133, 134]. However, we recently showed that GM-CSF production by T cells is not required for intestinal inflammation [332] suggesting that other mediators play a role in driving intestinal inflammation. Comparison of the transcriptional signatures of IL-17A^{+}IFN-γ^{-}, IL-17A^{+}IFN-γ^{+} and IL-17A^{-}IFN-γ^{+} T cells generated *in vivo* might reveal differential expression of specific pathogenic factors among these populations. For this purpose we have now obtained a mouse strain that reports IFN-γ and IL-17A expression [461]. This dual reporter strain will make it possible to isolate viable IL-17A^{+} and/or IFN-γ^{+} T cells from the inflamed intestine, and these different populations could then be transferred into *Rag1*$^{-/-}$ hosts to determine their stability and pathogenic potential *in vivo*. In addition, the requirement for IL-23 in the maintenance of IL17A^{+}IFN-γ^{+} T cells *in vivo* could be tested by transfer of this population into *Il23a*$^{-/-}$*Rag1*$^{-/-}$ mice.

Further analysis of IL17A^{+}IFN-γ^{+} T cells revealed that this population of cells expressed high levels of RORγt and T-bet. Based on published reports that have demonstrated that CD4^{+} T cells deficient in T-bet [88] or RORγ [326] fail to induce colitis in the T cell transfer model, we hypothesised that CD4^{+} T cells might require the activity of both T-bet and RORγ within individual cells for their pathogenic effector function. In support of this, T-bet expression by Th17 cells has been shown to be important in a T cell transfer model of EAE [269]. To test our hypothesis we wanted to utilise a co-transfer system where *Rorc*$^{-/-}$ and *Tbx21*$^{-/-}$ CD4^{+} T cells would be transferred together into the same host. If disease progression merely required the

development of a mixture of both Th1 and Th17 cells then the co-transfer would lead to disease as the *Rorc*$^{-/-}$ T cells would give rise to IFN-γ producing Th1 cells and the *Tbx21*$^{-/-}$ T cells would give rise to IL-17 producing Th17 cells. However, if there was a requirement for pathogenic T cells to co-express RORγ and T-bet then the co-transfer system would not result in intestinal inflammation. Although this was an attractive hypothesis we did not perform these experiments because unexpectedly, and contrary to published reports, we found that T cell transfer colitis was independent of T-bet expression by CD4^{+} T cells, and importantly, T-bet was not necessary for the differentiation of IL-17A^{+}IFN-γ^{+} T cells. We did, however, confirm the essential role for RORγ expression by T cells for the development of intestinal inflammation, as RORγ deficient T cells did not induce colitis. These observations are in contrast to *in vitro* studies that have shown that IL-23 driven acquisition of IFN-γ production by Th17 cells is dependent on T-bet [132]. Thus, during intestinal inflammation there appears to be a T-bet independent pathway for the generation of IL-17A^{+}IFN-γ^{+} T cells, and further investigation of the factors involved in this process will be important.

One of the most striking findings was that colitis induced by T-bet deficient T cells was dependent on IL-17A, and showed a unique inflammatory phenotype associated with high expression of IL-22, IL-17F, increased levels of antimicrobial peptides and marked neutrophil influx. The fact that WT as well as T-bet deficient T cells were able to induce colitis suggests the existence of T-bet dependent, as well as independent, modules for the generation of pathogenic Th17 cells. Therefore transcriptional profiling of pathogenic Th17 cells generated in presence of absence of T-bet in T cells might prove useful for the identification of distinct molecular

pathways associated with pathogenicity. In addition the increased levels of antimicrobial peptides such as S100A8, S100A9 and RegIIIγ found in *Rag1*$^{-/-}$ recipients of T-bet deficient T cell might lead to an imbalance in the composition of the intestinal microbiota that could further precipitate inflammation. Such colitogenic activity of altered microbiota has been described in mice deficient in certain components of the innate immune system [23, 353, 413], and dysbiosis of the microbiota is one of the features of human IBD [351, 352]. Whether the nature of the pathogenic adaptive immune response influences the colitogenic potential of the intestinal microbiota could be addressed by colonisation of *Rag1*$^{-/-}$ mice with faecal contents from inflamed *Rag1*$^{-/-}$ recipients of WT or *Tbx21*$^{-/-}$ T cells. Subsequent transfer of WT T cells into colonised *Rag1*$^{-/-}$ hosts might reveal differences in colitis severity or T cell cytokine production depending on the origin of the transferred microbiota.

6.3 IL-23 as a negative regulator of Th2 type responses

Evidence from experimental models and GWAS studies support an essential role for IL-23 as a mediator of chronic intestinal inflammation [12, 347]. However, the mechanisms by which IL-23 exerts its pathogenic effects have not been characterised in detail. In order to gain a better understanding of the cellular pathways that are regulated by IL-23, we performed transcriptional profiling of WT and IL-23R deficient CD4^{+} T cells isolated from the inflamed intestinal environment. Rather unexpectedly, this approach revealed that IL-23 is a negative regulator of Th2 type responses, as the expression of genes associated with this pathway such as *Il4*, *Il5*, *Il13*, *Gata3* and *maf* were increased in the absence of direct cell-intrinsic IL-23 signals into CD4^{+} T cells. Using the T cell transfer model of colitis we further established a functional role for GATA3 in restraining the colitogenic potential of

$CD4^+$ T cell. In addition, we showed that direct IL-23 signals in $CD4^+$ T cells suppressed the emergence of IL-$17A^+$IL-13^+ $CD4^+$ T cells and favoured the development of IL-$17A^+$IFN-γ^+ $CD4^+$ T cells. Collectively, these data suggest that IL-23 differentially regulates IL-13 and IFN-γ expression by intestinal Th17 cells, and raise the possibility that IL-23 might promote intestinal inflammation, at least in part, through inhibition of Th2 type responses.

Early studies documented that redirecting the T helper cell balance toward Th2 responses could prevent or ameliorate autoimmune diseases that were thought to be driven by Th1 cells [462-464]. These findings were in keeping with the original Th1/Th2 model put forward by Mossman and Coffman, which states that T helper subsets cross regulate one another through secretion of soluble factors [62, 65]. Accordingly, Th1 and Th2 associated cytokines are known to be potent inhibitors of Th17 differentiation *in vitro* [161-163]. More recent reports have highlighted a similar role for Th2 cytokines in the regulation of Th17 mediated inflammation *in vivo*. For example genetic ablation of IL-25 has been shown to lead to exacerbated Th17 driven pathology in EAE, an IL-23 driven disease, while administration of exogenous IL-25 ameliorated disease [439]. This protective effect of IL-25 was abrogated in IL-13 deficient mice, demonstrating that IL-25 suppressed Th17 responses through induction of IL-13. The authors went on to show that exposure of LPS stimulated DCs to IL-13 inhibited their secretion of IL-6, IL-1β and IL-23, indicating that IL-13 can regulate Th17 responses indirectly through effects on APCs. IL-13 signalling directly into T cells has also been shown to inhibit Th17 differentiation and secretion of IL-17A *in vitro* [159, 160]. In addition, IL-13 was shown to suppress the development of intestinal inflammation in IL-10 deficient mice

through inhibition of Th17 and Th1 responses [160]. Another report demonstrated that direct signalling of the Th2 cytokine TSLP into $CD4^+$ T cells resulted in reduced intestinal inflammation in the T cell transfer model of colitis [406]. Thus, Th2 type cytokines appear to be potent inhibitors of Th17/Th1 driven inflammatory responses, and this lends further support to the notion that inhibition of this axis may be a feature of IL-23 driven intestinal inflammation.

IL-23 might limit Th2 driven immune responses through inhibition of GATA3. Interestingly, IL-6 has been shown to promote expression of the transcription factor c-Maf under either Th2 [465] or Th17 differentiation conditions [440]. Expression of c-Maf is required for IL-4 production by Th2 cells [99, 100] while c-Maf activity in Th17 cells has been linked to the regulation of IL-10 [466] and IL-21 production [440]. Our microarray data indicate that IL-23 signalling into T cells limits expression of *Il4* as well as *maf* (which encodes c-Maf), suggesting that IL-6 and IL-23 have differential effects on the expression of these genes. Therefore, it is tempting to hypothesise that IL-23 signals into T cells limit IL-6-mediated induction of c-Maf, thereby preventing IL-4 expression and subsequent STAT6-mediated upregulation of GATA3. In support of this hypothesis, a recent study found that STAT3 was required for the ability of STAT6 to promote expression of *maf* and *Gata3* in Th2 cells [103]. Although this has not yet been demonstrated in Th17 cells, several independent reports have described the existence of $IL\text{-}17A^+IL\text{-}4^+$ and/or $GATA3^+ROR\gamma t^+$ T cells in mice and humans [418, 467, 468], suggesting that under certain conditions STAT3/STAT6 signalling events may also cooperate in the context of Th17 development. Moreover, McGeachy *et al* showed that IL-6 and IL-23 have opposing effects on IL-10 production by Th17 cells [402]. Stimulation of activated Th17 cells

in the presence of TGF-β1 and IL-6 maintained IL-10 expression whereas exposure to IL-23 did not. Importantly, Th17 cells stimulated with IL-23 were found to be more pathogenic in a T cell transfer model of EAE than Th17 cells exposed to TGF-β1 and IL-6. Interestingly, TGF-β1 and IL-6 have been shown to act in concert to induce expression of c-Maf, which can drive IL-10 production by Th17 [466]. Thus it is plausible that IL-23-mediated repression of c-Maf leads to the inhibition of regulatory modules driven by GATA3 and IL-10 that would normally act to restrain Th17 cell pathogenicity. Regardless of the mechanisms by which IL-23 suppress Th2 type responses, it will be important to establish whether GATA3 plays a functional role in restraining Th17 cell pathogenicity. This could be addressed by crossing *Gata3*$^{fl/fl}$ [95] mice with *Il17a*^{Cre} mice [393]. Such an approach would be advantageous over the use of GATA3$^{fl/fl}$-OX40Cre mice as it would lead to specific ablation of GATA3 in IL-17A-secreting cells rather than all activated CD4^{+} T cells. This would allow identification of GATA3 regulated pathways specifically in Th17 cells, and might provide novel insights into cross-regulation/cooperation between GATA3 and RORγt, which has not yet been addressed in detail. In addition, adoptive transfer of *maf* $^{-/-}$ or *Il23r* $^{-/-}$*maf* $^{-/-}$ T cells into *Rag1*$^{-/-}$ hosts will be important to determine the contribution of c-Maf to IL-23-driven T cell dependent colitis. Likewise, it will be of interest to establish the origin of the IL-17A^{+}IL-13^{+} population by crossing IL-23R deficient and IL-17A-eYFP reporter mice.

6.4 IL-33: a novel regulator of Treg differentiation and function

In addition to the Th2 associated genes described above our microarray analysis also revealed that IL-23 signalling into $CD4^+$ T cells limits the expression of *Il1rl1*, which encodes the receptor for IL-33. Interestingly, the IL-33R was highly expressed on $Foxp3^+$ Treg cells in the inflamed intestine and we showed that IL-23 signals directly into T cells regulate the expression of IL-33R on Treg cells. We also described that IL-33 signals into $CD4^+$ T cells promote iTreg induction *in vitro* and *in vivo*, and this effect of IL-33 was inhibited in the presence of IL-23. Thus, our previous finding that IL-23 inhibited iTreg induction [330, 391] probably reflects the ability of IL-23 to regulate T cell responsiveness to IL-33. Further interrogation of the mechanism by which IL-33 promotes, and IL-23 inhibits, iTreg generation will provide important insights into how these cytokines regulate the balance of regulatory responses in the intestine. Furthermore, we found that IL-33 activity was not limited to iTreg induction, as IL-33 signalling into $Foxp3^+$ Treg cells was also important for their suppressive function *in vivo*. Together these data suggest a novel function for IL-33 as an immunoregulatory cytokine that restrains chronic intestinal inflammation through direct effects on Treg differentiation and function. Importantly, GWAS studies have identified a genetic interval containing the gene coding for IL-33R as a susceptibility locus for Crohn's disease [349], suggesting that IL-33 might play a role in the pathogenesis of CD.

We found that IL-33R expressing $Foxp3^+$ Tregs were highly enriched in the colonic lamina propria of steady state mice, suggesting a tissue specific function for IL-33 in the control of Treg responses. Although it is currently not known what induces IL-33R expression in Treg cells it has been shown that IL-33R expression by Th2 cells is

dependent on GATA3 [442]. A recent study described the existence of $GATA3^{+}Foxp3^{+}$ Treg cells that were enriched at various barrier sites, including the small and large intestine and the dermis of the skin [415]. Given the essential role for GATA3 in IL-33R induction in Th2 cells [442], it is likely that $GATA3^{+}Foxp3^{+}$ Treg cells also express IL-33R. The compartmentalisation of IL-33R expression on colonic Treg cells is interesting because tissue resident cells such as intestinal epithelial cells, fibroblasts and macrophages have been shown to express IL-33 [441, 443]. Therefore it would be important to determine the cellular source of IL-33, and to assess whether IL-33 is expressed at higher levels in the intestine as compared to secondary lymphoid organs. Interestingly, it has been shown that administration of exogenous IL-33 can increase IL-33R expression among splenic $Foxp3^{+}$ Tregs [420], suggesting that local exposure to IL-33 in the intestine could account for the high frequency of $IL\text{-}33R^{+}$ Tregs at this site. Moreover, IL-33 has been shown to act as a chemoattractant for Th2 cells [469], and therefore might also promote accumulation of $IL\text{-}33R^{+}Foxp3^{+}$ Treg cells in the intestine. Given our finding that IL-33 enhances TGF-β1-mediated iTreg induction it would also be of interest to assess expression of IL-33 in $CD103^{+}$ DCs [38, 39], which are potent inducers of iTregs in the GALT. In addition, our observations that $IL\text{-}33R^{+}$ $Foxp3^{+}$ Treg cells were preferentially enriched in the colonic lamina propria of inflamed mice, and that IL-33R deficient Treg cells were functionally impaired, suggest that IL-33 might also have an important role in the maintenance of the Treg phenotype and function during intestinal inflammation. Clearly, more work is required to better understand the functional relevance of IL-33R expression by Tregs. For example, isolation of $IL\text{-}33R^{+}$ vs. $IL\text{-}33R^{-}$ Tregs from steady state or inflamed intestines and subsequent

comparison of their gene expression profiles and suppressive function might reveal functional differences between these populations.

Analysis of the kinetics of IL-33 mRNA expression in the colon revealed that IL-33 is dynamically regulated over the course of *Helicobacter hepaticus*/anti-IL-10R colitis. The mechanisms that regulate IL-33 production are not well understood. IL-33 is classically thought of as an alarmin, released by necrotic cells in response to tissue injury or stress [441]. However, recent findings indicate that IL-33 mRNA expression and protein production can be induced by TLR and non-TLR agonists in macrophages and fibroblasts *in vitro* [448]. In addition, a recent report demonstrated that Trefoil factor 2 (TFF2), an epithelial derived protein, is a potent inducer of IL-33 production by lung epithelial cells, macrophages and DCs in response to infection with the parasite *Nippostrongylus brasiliensis* [470]. Together, these studies indicate that IL-33 expression and secretion can be inducible. In this regard, it will be of interest to determine whether *Helicobacter hepaticus* can promote IL-33 expression and secretion.

Although we have described an anti-inflammatory role for IL-33 this cytokine was originally described as having potent pro-inflammatory properties. IL-33 has an important host protective role in defense against parasites [471-475]. The expression of IL-33R is highly enriched on Th2 cells [476, 477] and innate lymphoid cells such as nuocytes [473] and natural helper cells [475]. In response to IL-33 stimulation, these cells produce large amounts of IL-4, IL-5 and IL-13, which are important for the clearance of parasitic infections [89]. In addition, IL-33 activates eosinophils, basophils, mast cells and macrophages to produce a variety of pro-inflammatory

chemokines and cytokines [419]. Perhaps not surprisingly, these pro-inflammatory effects of IL-33 have also been shown to contribute to the pathogenesis of experimental models of airway inflammation and sepsis [478-480]. By contrast, several reports have described a protective role for IL-33 in atherosclerosis [481], cardiac graft rejection [420, 482], EAE [483], Con A-induced hepatitis [484] and models of intestinal epithelial injury [485, 486], demonstrating the complex immunological activities of this cytokine. The common theme that has emerged from these studies is that IL-33 exerts its protective effect by promoting expansion of Th2 cells and/or Treg cells. These studies are in accordance with our findings, which have provided further mechanistic insight by demonstrating that direct signalling of IL-33 into $CD4^+$ T cells was required for iTreg differentiation and nTreg function. However, we also found that IL-33 signalling into $CD4^+$ T cells was not required for the expression of IL-13 *in vivo*. Thus, the activity of IL-33 in T cell transfer colitis might be more restricted to its effects on Treg cells.

IL-33 has recently received much attention in the context of human IBD. Several independent groups have demonstrated increased expression of IL-33 in the inflamed mucosa of patients with active UC as compared to CD patients or healthy controls [487-490]. IL-33 expression in lesions of CD patients was mostly restricted to non-hematopoietic cells such as intestinal epithelial cells and myofibroblasts, but was also found in myeloid cells [488, 489, 491]. In addition, expression of the IL-33R was also elevated among myeloid cells and $CD4^+$ T cells in active UC [487, 489]. Given that UC is associated with increased levels of IL-13 and IL-5 [356, 357], which can both be induced by IL-33, these studies suggest that IL-33 might play a role in the pathogenesis of UC rather than CD. However, as mentioned previously, GWAS

studies have identified a genetic interval containing IL-33R as a susceptibility region for Crohn's disease [349]. Although this does not directly implicate IL-33R in the pathogenesis of CD it suggests that IL-33 might contribute to both types of IBD.

Collectively, our data suggest that IL-23 restrains Treg differentiation and function through regulation of IL-33 responsiveness. Given the involvement of both IL-23 and IL-33 in human IBD, a detailed understanding of how these cytokines cross-regulate one another will be important for effective therapeutic targeting of these pathways. We believe that the findings presented here raise some important issues for the potential use of IL-23 blocking reagents as a treatment for IBD. According to our results, blockade of IL-23 may lead to heightened activity of IL-33 and IL-13, which could act to dampen pro-inflammatory Th17/Th1 responses and ameliorate disease. However, IL-13 and IL-33 are also thought to play a pro-inflammatory role in UC. Thus, the protective vs. pathogenic activity of these cytokines in the intestine may be context dependent. This suggests that therapeutic blockade of IL-23 might also have adverse effects in some patients by redirecting the balance toward a Th2-type response, and highlights the importance of developing personalised treatments based on patient phenotypes. This possibility should be investigated in clinical trials targeting the IL-23 pathway.

Chapter 7: References

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