Studies of Intestinal Inflammation: The Roles of IL-23R, γδT-cells and IL-21

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A thesis submitted in partial fulfilment of the requirements for degree of Doctor of Philosophy, Trinity Term 2013

Acknowledgements

Firstly, I would like to acknowledge the patience, support and guidance of my supervisor Fiona Powrie, both throughout the period of time I spent undertaking the research described in this thesis, and during the seemingly endless period it took me to finally write up once I had returned to clinical medicine! She was a constant source of enthusiasm when things went well, and of wisdom when things appeared more challenging. The opportunity she gave me to work in such a dynamic and successful lab surrounded by high quality scientists was instrumental in the success of my DPhil studies, and has provided the inspiration and enthusiasm to continue to aspire to a career as an academic gastroenterologist. I will forever be grateful for the chance I had, as a clinician, to undertake my early research training under Fiona's leadership, and to see what 'real' science is. Equally, without the generous support of the Wellcome Trust, through the University of Oxford Doctoral Training Programme for Clinicians in Biomedical Science, none of this would have been possible.

This thesis would clearly also not have been possible without the help of a large number of fellow students and post-docs in the lab, the knowledge and ability of whom was often inspiring and intimidating in equal measure. Particular mention goes to Chris, Scott, Claire and Rose who often joined me at ungodly hours to perform some of the experiments, and took a constant interest in my results and always had useful thoughts. I am hugely grateful to Helen for her expertise and patience in attempting to comply with my often impossible sorting plans. However, the brilliant and inspiring atmosphere of the lab was a product of all those working within it, and I am grateful to everyone in the Powrie and Maloy labs during my time in Oxford. I will avoid naming individuals, for fear of causing offence by inadvertent omission, but I think each and every member provided me with advice, assistance, expertise or simply a laugh at some point. In this last regard it would be rude not to highlight the contribution of Thibault who was both a brilliant scientist and valuable source of all round entertainment throughout my time in Oxford. Equally, although not human, the many hours spent in the company of the LSRII flow-cytometer brought us closer together than most men and machines will ever achieve.

Finally, this period of research would have been impossible without the support of my wife, Libby. For three years, she lived with me leaving the house before dawn and coming back late, and talking about little but cytokines and colitic mice when I was at home. Somehow, we managed to both marry and start a family during this time, and she completed her own PhD thesis. Quite how we managed all this I remain unclear, but I have no doubt she was instrumental, and I thank her sincerely for that.

Abstract

The aetiology of the inflammatory bowel diseases ulcerative colitis and Crohn's disease remains uncertain. Genetic studies and model systems strongly implicate components of the IL-23/type-17 axis in the pathogenesis of disease, but the cellular and molecular mediators are uncertain. Using an IL-23R^{gfp} reporter mouse we analysed the cellular expression of IL-23R in homeostasis and disease. Whereas steady state expression in the intestine was dominated by a collection of unconventional lymphoid cells including γδT-cells, we found rapid accumulation of IL-23R^{+}CD4^{+} T-cells occurred in evolving colitis, and demonstrate an important role for IL-10 in the regulation of IL-23R specifically upon intestinal CD4^{+} T-cells. Examining the role of γδT-cells in a model of IL-23-dependent colitis, we demonstrate apparent redundancy of such cells for the development of the adaptive CD4^{+} Th17 response. Furthermore, treatment with FTY720 which is known to inhibit lymphocyte recirculation did not attenuate disease nor reduce intestinal Th17 cell accumulation, suggesting the mechanisms of accumulation of Th17 cells in the intestine may differ from other anatomical sites. Next, we addressed the role of IL-21, a cytokine implicated in the development and effector functions of the IL-23/Th17 axis. Remarkably, we found that although IL-21 was pathogenic in models of chronic colitis, its effects on effector T-cell subsets were model-specific and included Th17 and Th1 cells. However, increased regulatory T-cell populations and reduced *Ccl5* expression were common effects between models. Paradoxically, in a model of enteric infection, IL-21 was required for host defence, with IL-21R$^{-/-}$ mice developing increased bacterial colonisation and severe colitis, shown to be driven by increased Th1/IFN-γ responses. These studies provide novel insights into aspects of IL-23 driven cellular and molecular pathways in homeostasis and inflammation in the intestine, with implications for future therapeutic approaches to IBD.

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Abbreviations

AIEC	Adherent/invasive *E.coli*
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BSA	Bovine serum albumin
CD	Crohn's disease
CEACAM	Carcinoembryonic antigen-related cell adhesion molecules
CFU	Colony forming unit
CTLA-4	Cytotoxic T lymphocyte antigen 4
CNS	Central nervous system
DC	Dendritic cell
DETC	Dendritic epidermal T-cell
DN	Double negative
DSS	Dextran sodium sulphate
EAE	Experimental autoimmune encephalomyelitis
EPEC	Enteropathogenic *E.coli*
ER	Endoplasmic reticulum
FAE	Follicle associated epithelium
FGF	Fibroblast growth factor
Foxp3	Forkhead box P3
γ_C	Common γ-chain
GALT	Gut associated lymphoid tissue
GF	Germ free
GI	Gastrointestinal
GITR	Glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GVHD	Graft-versus-host disease
GWAS	Genome-wide association study
H&E	Haematoxylin and eosin
HPRT	Hypoxanthine phosphoribosyltransferase
IBD	Inflammatory bowel disease
ICOS	Inducible T-cell co-stimulator
IEC	Intestinal epithelial cell
IEL	Intra-epithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
ip	Intraperitoneal
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy X-linked
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motifs
iv	Intravenous
KGF	Keratinocyte growth factor
LCMV	Lymphocytic choriomeningitis virus
LP	Lamina propria
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer cell
MAIT	Mucosa associated invariant T cell
MAMP	Microbial associated molecular pattern

MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
mTEC	Medullary thymic epithelial cell
mTOR	Mammalian target of rapamycin
mRNA	Messenger ribonucleic acid
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor κB
NK	Natural killer
NKT	Natural killer T-cell
NLR	NOD-like receptor
NOD	Nucleotide oligomerisation domain
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PI3K	Phosphoinositol-3-kinase
pIgR	Polymeric immunoglobulin receptor
PP	Peyer's patch
PRR	Pattern recognition receptor
PSA	Polysaccharide A
Rag	Recombination activating gene
RORa	RAR-related orphan receptor alpha
RORγt	RAR-related orphan receptor gamma t
S1P	Sphingosine-1-phosphate
SED	Subepithelial dome
SCID	Severe combined immunodeficiency
SFB	Segmented filamentous bacteria
SI	Small intestine
SLAM	Signalling lymphocytic activation molecules
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
Tfh	Follicular helper T-cell
TGF	Transforming growth factor
Th	T-helper cell
TLR	Toll-like receptor
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
Treg	Regulatory T-cell
TSLP	Thymic stromal lymphopoetin
UC	Ulcerative colitis
WT	Wild-type

Chapter 1. Introduction

1.1 The Intestinal Immune System

The gastrointestinal (GI) tract faces the twin challenges of providing an environment to enable digestion and absorption of nutrients, whilst defending the host against ingested pathogens and responding appropriately to ingested foreign antigens. In addition, the healthy intestine is home to a diverse commensal microbial flora containing an estimated $>10^{14}$ bacteria, which is essential for digestive processes, and for normal immunological function, including beyond the gut itself.[1-6] In the face of such challenges, the GI immune system exhibits numerous unique anatomical and physiological adaptations to permit effective host defence whilst maintaining tolerance or ignorance to non-harmful or commensal stimuli.[1,4,7]

The GI tract is lined by a single layer of intestinal epithelial cells (IECs) which provide a physical barrier as well as giving rise to numerous specialised cell types such as Paneth cells which secrete antimicrobial factors and peptides, and Goblet cells which synthesise mucins to provide a mucus layer immediately above the epithelium, separating host cells from luminal contents, and trapping IgA derived from lamina propria B-cells to further defend against bacterial penetration.[8] IECs express pattern recognition receptors (PRR) including toll-like receptors (TLRs) and NOD-like receptor (NLRs), allowing direct interaction with microbial signals, via microbe associated molecular patterns (MAMPs), and indeed IEC expression of such receptors is essential for the normal development and function of the intestinal immune system.[8-10] A further specialised cell of the epithelial lineage, the M-cell,[11] is responsible for the uptake and delivery of luminal antigens through the follicle associated epithelium (FAE) to cells within Peyer's patches found in the small intestine (SI), although M-cells also occur away from such structures.[7] Peyer's patches (PP) are organised collections of myeloid cells and lymphocytes, and represent the prototypical inductive sites of the gut associated lymphoid tissue (GALT), which also includes the isolate lymphoid follicles (ILFs) and cryptopatches scattered throughout the GI tract, and the draining mesenteric lymph nodes (MLNs).[7,12,13]

In contrast, immune effector sites are characterised by the diffuse distribution of lymphocytes amongst non-immune cells (including epithelial and stromal cells) and collagen matrix. The major effector sites in the GI tract comprise the lamina propria (LP) and the intraepithelial lymphocyte (IEL) compartments.[7] Each site demonstrates significance bias towards specific cell types, such that the IEL is primarily occupied by $CD8^+$ T-cells with cytotoxic characteristics, whereas LP cells are more commonly $CD4^+$ T-cells of diverse function and B-cells.[14-16] However, numerous additional lymphoid cell populations, including innate lymphoid cells, are present throughout the healthy and inflamed intestine.[17]

Inductive and effector sites of the GI tract are also populated by multiple populations of myeloid antigen presenting cells which function to sample and present antigen to lymphoid cells, and provide signals influencing the subsequent immunological response, programming cells initially encountering antigen in the inductive sites of the intestine to express homing molecules specific for intestinal effector sites.[18,19]

Despite the significant challenges faced, the intestinal immune system is remarkably effective at combining host protection with tolerance of innocuous stimuli such as dietary and resident microbial antigens. However, dysfunction of this normal response may result in inappropriate and damaging activation of the immune response, as typified by the inflammatory bowel diseases, Crohn's disease and ulcerative colitis, as well as coeliac disease and the enterocolitides characterising a large number of rare immunological and genetic disorders.[1,20-22]

1.2 Intestinal Antigen Presenting Cells

Myeloid antigen presenting cells (APCs) of the intestine are diverse populations which demonstrates significant anatomical and functional heterogeneity, but primarily comprises subsets of dendritic cells (DCs) and macrophages, which can be distinguished on the basis of their expression of CD11b, CD11c, CX_3CR1 and CD103.[19] The complex lineage and developmental relationships of APCs in the murine and human intestine has been much clarified by significant

recent attention.[23-25] Although both strongly phagocytic, whereas macrophages primarily exhibit bactericidal activity, DCs are adapted to present antigen to cells of the adaptive immune system, and direct the character of the resulting immune response.[19,24,26] Importantly, intestinal DCs interact with naïve $CD4^+$ T-cells in the GALT and MLN to impart both functional characteristics such as cytokine production and the necessary molecular adaptations to permit homing back to the effector sites of the gut, including expression of α4β7 integrin and CCR9.[27-29]

Specific APC populations show anatomical restriction, such that DCs of the FAE and sub-epithelial dome (SED) of PP differ phenotypically, which further differ from cells of the interfollicular regions and from those resident in the MLN.[23,30,31] Within the LP, some APCs demonstrate the ability to sample luminal antigen by extension of transepithelial dendrites.[32,33] Although individual subpopulations may favour specific immune outcomes, the overall tone of intestine APC responses is skewed towards active tolerogenic responses, in contrast to the systemic immune system.[18,19,34] Specific locally derived factors have been demonstrated which contribute to this tissue conditioning of APCs, including TSLP, TGF-β1 and PGE2.[35-39] An important tolerogenic population of intestinal DCs has been described which express CD103 and contribute to intestinal homeostasis by favouring induced regulatory T-cell (iTreg) responses through the production of retinoic acid.[40,41] Similarly, a population of CX_3CR1^+ APCs in the intestine promotes LP Treg proliferation and maintenance of Foxp3 expression.[42] In contrast, other populations of intestinal DCs are potently pro-inflammatory, integrating PRR signals to drive production of significant quantities of cytokines including IL-6, IL-12 and IL-23.[43-45] A specific subset of $CD14^+CD33^+CD209^+$ APCs in the human intestine have recently been linked to the pathogenesis of Crohn's disease.[46] Even usually tolerogenic cells may be capable of driving inflammatory responses, as demonstrated by the finding that stimulation of $CD103^+$ DCs with bacterial flagellin within drives pro-inflammatory IL-23 production, favouring Th17 type responses.[47]

1.3 B-cells

B-cells are a component of the adaptive immune system specialised for the production of immunoglobulins (Ig), but which may also fulfil additional immunological roles including antigen presentation and cytokine secretion.[48,49] An almost limitless repertoire of immunoglobulin specificities can be generated through V(D)J recombination under the control of the recombinase activating genes, RAG1 and RAG2.[50,51] Immature B-cells express surface IgM or IgD, but upon antigen recognition undergo somatic hypermutation and class-switch recombination under the influence of T-cell derived cytokines, to generate IgA, IgE and IgG of increased affinity.[52] IgG subtypes include IgG1, IgG2, IgG3 and IgG4 in humans and IgG1, IgG2b and IgG3 in mice, with strain specific variation of the remaining subtype; IgG2a in BALB/c mice or IgG2c in C57BL/6 mice.[53] B-cell activation may occurs in the setting of antigen-presentation by follicular dendritic cells in lymphoid tissues, and through T-cell mediated CD40 signals.

B-cells are a significant constituent of the GALT,[7] where they undergo differentiation primarily to IgA producing cells. IgA class-switching principally occurs within PP, but may occur in ILFs and the MLN, and possibly in the LP.[48,54,55] The intestinal environment may favour IgA class switching through enrichment of factors including IL-10, TGF-β1, BAFF and APRIL.[48,54,56] Importantly, many of these factors are produced downstream of interaction between diverse intestinal cells and components of the microbial flora.[3,8,57] Reciprocally, IgA is important in regulation of components of the flora as well as pathogenic species.[58-61] Intestinal IgA is highly polyclonal and shows adaptation to evolution of the flora, with turnover of dominant plasma cell populations.[58] IgA is secreted by LP plasma cells in dimer form, linked by a J-chain, via a transepithelial transport process involving the polymeric Ig receptor (pIgR).[62] IgA exerts numerous effects including binding and trapping microbes in the mucus layer, neutralisation of bacterial toxins and LPS, and modification of the expression of pro-inflammatory epitopes by bacteria.[58,61,63,64]

1.4 Innate Lymphoid Cells

The existence of cells of lymphoid morphology with innate immune characteristics has long been recognised, typified by natural killer (NK) cells which are important for anti-viral and anti-tumour responses, through cytotoxicity and production of IFN-γ.[65,66] More recently, an array of related innate lymphoid cells (ILC) have been defined with diverse functional roles demonstrated in homeostatic and pathogenic immune and inflammatory responses, including in the intestine.[67-78] These cells are linked by their developmental dependence upon γ_c chain signals and expression of the transcriptional repressor Id2.[79,80] However, whereas conventional NK cells are IL-15 dependent, other ILC subsets may require IL-7 for their development.[81]

Accumulation of ILCs producing IFN-γ but distinct from conventional NK cells (ILC1) in the inflamed intestine has been reported in both murine[71] and human studies.[70,73] Similarly, ILCs producing Th2-type cytokines (IL-5, IL-9, IL-13) in response to IL-25, IL-33 or TSLP and dependent on GATA3 and RORα (ILC2) have been shown to mediate protection against intestinal parasitic and helminth infection.[75,76,82-86] A third subset of ILCs (type 3 ILCs), defined by their expression of RORγt and the Th17-type cytokines IL-17A and/or IL-22 appear particularly important in intestinal immunity.[67,69,72,74] The prototypical member of this subset, the $CD4^+$ lymphoid tissue inducer (LTi) cell, plays an essential role in the formation of intestinal lymphoid tissues, through integration of signals from the microbial flora.[78] Although a role for LTi cells in systemic innate immunity has been described,[77] further subsets (ILC3) appear functionally relevant in intestinal disease. These include IL-22^+ ILCs, which predominantly express NKp46 and are involved in protection from intestinal *Citrobacter rodentium* infection,[72,74] and IL-$17A^+IFN\gamma^+$ $Thy1^+Sca\text{-}1^+NKp46^-$ ILCs which mediate disease in an innate model of *Helicobacter hepaticus* induced murine colitis.[67] Similar subsets of IL-22^+ ILCs are present in humans, and notably IL-$17A^+$ ILCs accumulate in the inflamed intestine in Crohn's disease.[74,87,88] However, further work is required to fully define the biology of ILCs and in particular their specific contribution to intestinal immune responses in lymphocyte replete settings and in human disease.

1.5 Intestinal T-cells

Diverse populations of T-cells are distributed throughout the inductive and effector sites of the intestine, where they are implicated in a host of homeostatic and pathogenic functions (Figure 1.1).[14-16,89] Striking compartmentalisation of cells with specific phenotypes is evident within the intestine, with IEL T-cells predominantly expressing the TCRγδ or TCRβ in association with the CD8αα homodimer,[14] whereas LP T-cells are predominantly $CD4^+TCR\beta^+$ cells.[15] Whereas the majority of T-cells in all compartments display an activated, antigen experienced $CD44^+CD62L^-$ effector memory T-cell phenotype, it has recently been appreciated that a sizable population of naïve $CD62L^+$ T-cells exists within the LP, the function of which is unclear.[90] To contextualise further discussions of intestinal T-cell biology, an overview of their basic biology is required.

1.5.1 T-cell development

T-cells develop within the thymus from haematopoietic precursors characterised by a $Lin^-Thy^{lo}Sca\text{-}1^+c\text{-}kit^+CD127^+$ surface phenotype in a process regulated by the integration of multiple signals within the specialised thymic microenvironment.[91,92] Development proceeds through defined stages including an initial $CD4^-CD8^-$ double negative (DN) stage, which is further subdivided into DN1, DN2 and DN3 phases.[93,94] Transition from DN1 to DN2 occurs with the initiation of gene rearrangement at either the TCRβ or TCRγ and TCRδ loci, a process which is complete in DN3 cells.[95,96] The events controlling $TCR\beta^+$ versus TCRγδ fate are unresolved.[97-105] The expression of either a pre-TCRβ complex, composed of the TCRβ chain with a surrogate invariant TCRα chain (pre-Tα protein) or the γδTCR provides signals to rescue the developing thymocyte from apoptosis and drive proliferation,[95,106,107] although this process is more marked in TCRβ expressing cells.[95,108] Thereafter, $TCR\gamma\delta^+$ and $TCR\beta^+$ cell development differs, with $TCR\beta^+$ cells progressing through a $CD4^+CD8^+$ double positive (DP) stage and TCRα gene rearrangement to generate a functional TCRαβ which then undergoes MHC-dependent positive selection regulated by thymic cortical epithelial cells.[109-111] The resulting mature $CD4^+$ or $CD8^+$ cells, thereafter undergo negative selection against antigens expressed on medullary thymic epithelial

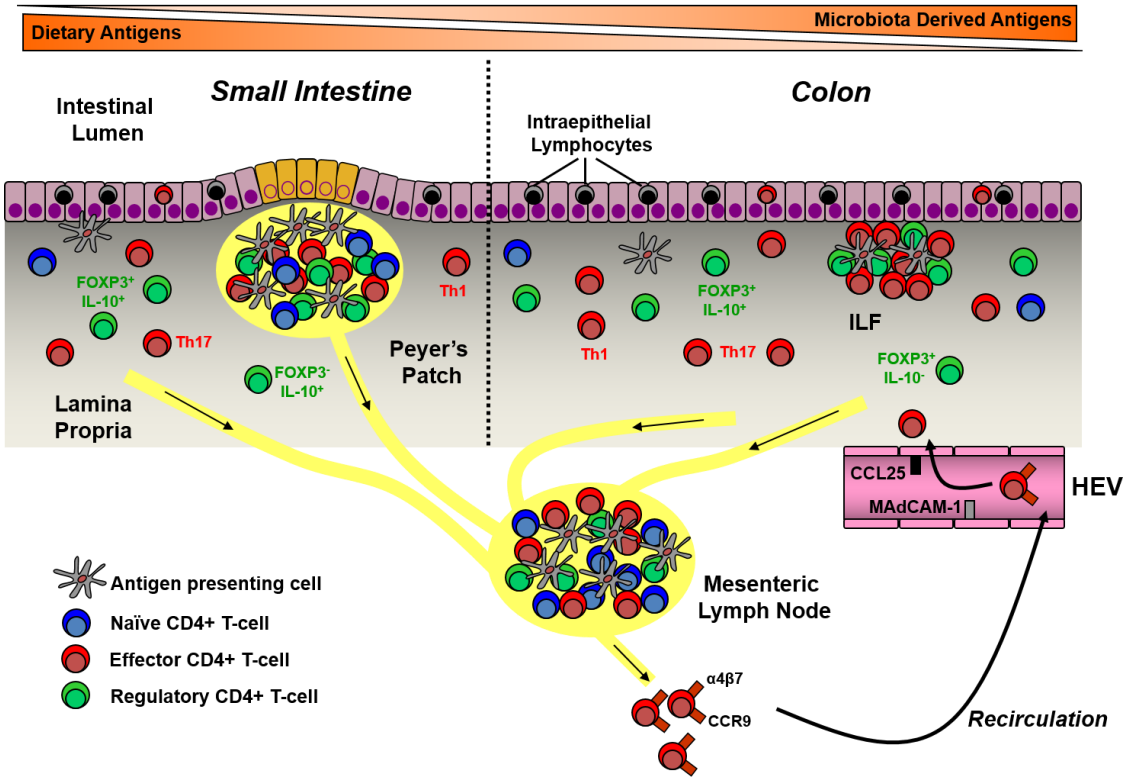

Figure 1.1 Intestinal T-cells demonstrate specific patterns of distribution throughout the gastrointestinal tract

Despite their presence throughout the entire GI tract, T-cells, particularly $CD4^+$ cells, demonstrate clear phenotypic variation between anatomical compartments. This is typified by the unique surface phenotype of IEL T-cells, and by the compartmentalised distribution of T-cells with regulatory function, including $Foxp3^+$ iTreg cells in the colon, and $Foxp3^-$ $IL\text{-}10^+$ Tr1 cells of the small intestine. Spatial differences may reflect different niches between proximal and distal GI tract, related to the prevailing antigenic challenges. T-cells distribute within the diffuse lamina propria and in lymphoid clusters, although the specific spatial aspects are poorly understood. T-cell accumulation in the intestine is regulated by the expression of adhesion molecule receptors upregulated upon priming in the inductive components of the GALT, and allow re-entry from the circulation.

cells (mTEC) and thymic DC before entering the circulation.[112,113] By contrast, TCRγδ$^+$ cells do not appear to undergo further selection events in the thymus,[98,108] nor do the majority of cells upregulate CD4 or CD8 prior to thymic egress, nor indeed later in development.[97] Extrathymic selection events have been reported to occur for γδT-cells but are not well characterised.[114] More detailed examination of the mechanisms controlling T-cell development and selection of TCRβ versus TCRγδ fate, and their inter-dependence, are beyond the scope of this thesis but have been recently reviewed in detail.[97-105,115-117]

1.5.2 T-cell Activation and Polarisation

After leaving the thymus, naïve T-cells require activation in secondary lymphoid tissues in order to acquire tissue specific homing characteristics and the ability to populate diffuse effector sites such as the LP.[118] T-cell activation occurs upon encounter of cognate antigen presented in the context of an appropriate MHC molecule, MHCI in the case of CD8$^+$ T-cells, MHCII for CD4$^+$ cells. In contrast, γδT-cells are do not require peptide-MHC presentation and have been shown to directly interact with intact molecules, particularly endogenous markers of cellular stress, lipids and components of microbial metabolic pathways, leading to their designation as unconventional T-cells.[119-123]

Ligation of the TCR results in phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) within the cytoplasmic regions of associated components of the TCR signalling apparatus, TCRζ and the CD3 complex (containing CD3γ, CD3δ, CD3ε subunits), by Lck and Fyn kinases. Phosphorylated ITAM motifs recruit adaptor molecules including ZAP-70, which after phosphorylation and activation by Lck, itself phosphorylates LAT, allowing binding of PLCγ1 and activation of downstream signalling through PCKθ and MAPK pathways, and Ca^{2+} release from the ER.[124-126]

Conventional T-cell activation requires a second co-stimulatory signal, which can be either stimulatory or inhibitory.[127] In the best characterised example, whereas interaction of CD80/CD86

with T-cell expressed CD28 provides positive signals,[128] interaction of this same molecule with CTLA-4 inhibits T-cell activation.[129,130] Additional molecules mediating this second signal include OX40, ICOS, PD-1, CD40, CD27, GITR, 4-IBB and RANKL/TRANCE.[127]

Activation of conventional αβT-cells in the presence of appropriate cytokine environments results in their polarisation to specific phenotypes based upon expression of characteristic transcription factors and effector molecules (Figure 1.2). By contrast, the effector phenotype of γδT-cells may be fixed during thymic development and without requirement for further peripheral education.

1.5.3 Th1 cells

Th1 cells are characterised by the expression of the transcription factor T-bet and production of the signature cytokine IFN-γ, and play a critical role in immunity against intracellular pathogens.[131,132]

Th1 cells develop through a molecular program involving STAT1 and STAT4 activation downstream of signals from IFN-γ and IL-12 respectively.[133-136] Initial STAT1 activation induces T-bet, upregulating the expression of IL-12Rβ2, which together with the constitutively expressed IL-12Rβ1 forms a functional IL-12 receptor.[134,135,137] IL-12Rβ1 expression is further upregulated by INF-γ through the IFN-γ inducible transcription factor, IRF1.[138] Further IL-12 signalling through STAT4 enhances T-bet expression and IFN-γ production, resulting in full Th1 commitment.[139] Additional signals from IL-2 drives STAT5 dependent T-bet and IL-12Rβ2 expression.[140] Upregulation of IL-18R in a STAT4 dependent manner enables IL-18 to synergise with IL-12 to drive TCR-independent IFN-γ production.[141,142]

T-bet inhibits expression of the Th2 associated transcription factor GATA3, to prevent Th2 differentiation,[143,144] and T-bet deficient mice develop Th2 mediated allergic disease.[145] However, T-bet deficient cells do not show entirely impaired Th1 differentiation due to the expression of the T-bet related transcription factor eomesodermin.[146] The development of Th17 cells is similarly inhibited through the interaction of T-bet with Runx1 to prevent upregulation of RORγt in developing T-cells.[147]

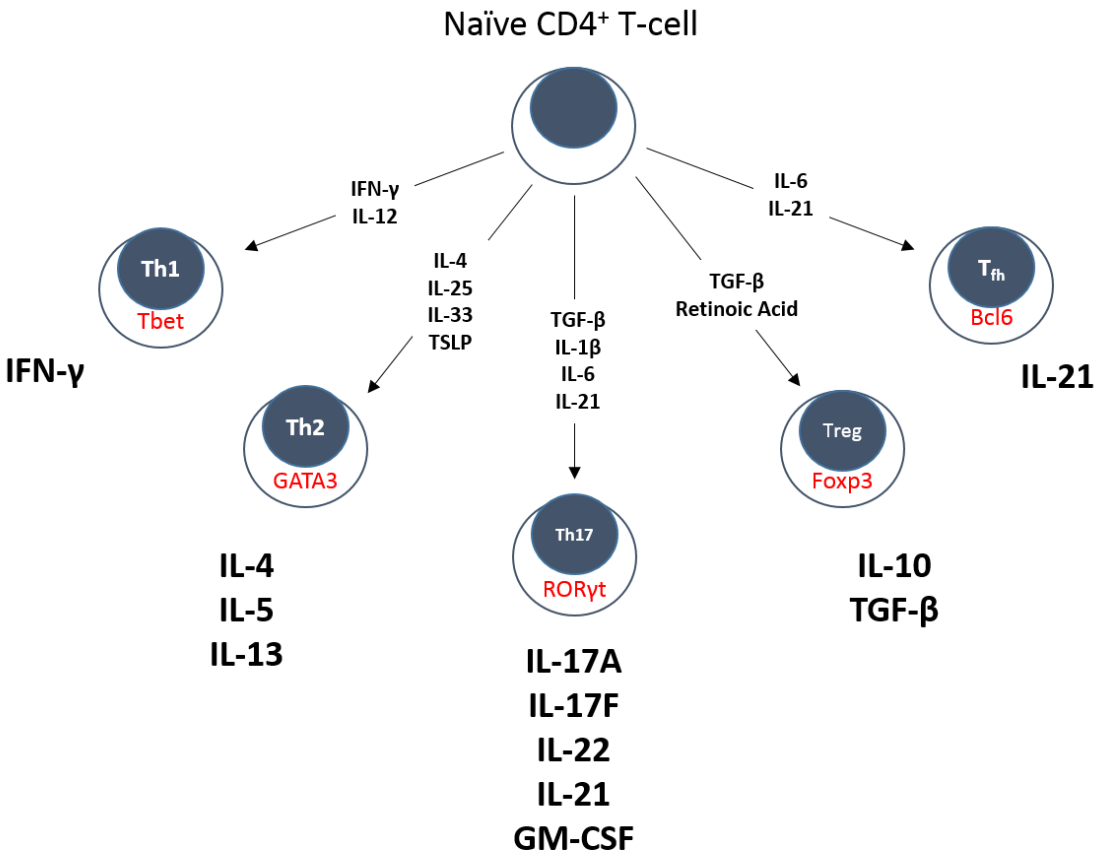

Figure 1.2 CD4⁺ T-cell Subsets

CD4⁺ T-cell subsets develop from naïve precursors under the influence of cytokines, to upregulate characteristic transcription factors and produce stereotyped cytokine profiles. Specific subsets often contribute to different aspects of immune and inflammatory responses.

1.5.4 Th2 cells

Th2 cells produce a variety of effector cytokines including IL-4, IL-5, IL-13, IL-9 and IL-25, and are important in host defence against extracellular parasites, as well as playing a role in allergic diseases including asthma.[148]

Differentiation results from the receipt of IL-4 signals at the time of initial TCR stimulation, resulting in STAT6 activation and induction of expression of the transcription factor GATA3, itself essential for Th2 function.[149-151] GATA3 drives transcription of Th2 associated genes including IL-4, and inhibits Th1 fate through suppression of STAT4 and IL-12Rβ2, and inhibition of IFN-γ production through interaction with Runx3.[151-154] IL-2/STAT5 signals contribute to Th2 differentiation through maintenance of IL-4Rα expression.[155] Additional transcription factors implicated in Th2 development include IRF4,[156] cMAF,[157,158] Gfi-1,[159,160] TCF-1[34] and members of the NFAT family.[161] Notably, STAT3 signals from IL-6 or IL-21 may co-operate with STAT6 to induce expression of Th2 associated molecular signatures.[162] IL-4 independent Th2 differentiation may occur under the control of TSLP, IL-25 and IL-33.[148,163]

1.5.5 Th17 cells

Defined by their production of IL-17A, but characterised by production of a panel of further mediators including IL-17F, IL-21, IL-22 and GM-CSF, Th17 cells are required for host immunity against both intracellular and extracellular infections, particularly at mucosal surfaces, and may be critical for responses to fungi.[164-168] Importantly, Th17 cells have received significant recent attention for their role in a range of auto-inflammatory diseases.

Murine Th17 cells differentiate under the influence of TGF-β1,[169-173] IL-1[169,174] and a STAT3 activating signal such as IL-6 or IL-21[175-179] although differing developmental requirements may exist in the steady state and inflammatory conditions. Human Th17 cells show broadly similar cytokine dependency. Importantly, beyond IL-17A expression, the functional characteristics of

Th17 cells developing in the absence of specific factors may differ considerably from conventional Th17 cells.

Activation of the Th17 program depends upon sustained expression of RORγt,[180,181] although a small residual population is seen in *Rorc*$^{-/-}$ mice, developing via through expression of the related RORα.[181,182] IL-6 or IL21 drive upregulation of IL-23R expression,[175] permitting the receipt of IL-23 signals required for survival and proliferation, and for the acquisition of further effector functions including IFN-γ or GM-CSF production.[183-186] In addition to RORγt and RORα, further transcriptional regulators implicated in Th17 development or function include Runx1,[187] BATF,[188] IRF4,[189,190] Aiolos,[191] components of NF-κB signalling including Rel,[192] IKKα[193] and IKBζ,[194] and the metabolic sensors HIF-1α[195,196] and Ahr.[191,197,198]

The activity of TGF-β1 in Th17 differentiation may relate to its ability to inhibit alternative cell fates, including inhibition of the Th1 associated eomesodermin which otherwise suppresses *Rorc* and *Il17a* expression.[199] Notably, GATA3 and T-bet deficient T-cells differentiate to Th17 cells in the absence of TGF-β1.[200] However, TGF-β1 may also directly promote Th17 activity, through ROCK2/IRF4 driven induction of RORγt.[201] Adding to the complexity of the situation, the absolute dependency on TGF-β1 for Th17 differentiation reported by early studies has recently been challenged,[202] implying other compensatory pathways may exists, although Th17 cells developing in the absence of TGF-β1 express greater levels of Th1 associated molecules, including T-bet and IL-18R.

Although Th17 cell development is inhibited by the expression of a number of Th1 and Th2 associated molecules including IFN-γ,[203] IL-2,[204] IL-4,[147,205] IL-13,[206,207] T-bet,[147] Gfi-1[208,209] and Ets-1,[210] the relationship between Treg and Th17 cells is particularly intriguing. T-cells developing in the presence of TGF-β1 express both RORγt and the Treg transcription factor Foxp3, with the alternate cell fates determined by the presence of additional inflammatory cytokines.[175,211-213] In the absence of such signals, Foxp3 directly inhibits RORγt, RORα and Runx1 to activate a Treg cell fate,[187] whereas the presence of STAT3 activating signals including IL-6 and IL-21 favours Th17 cells differentiation.[166,170,212,214]

1.5.6 Regulatory T-cells

The protection from inflammatory disease induced by transfer of naïve CD45RB^{hi} T-cells into immunodeficient mice by a further subset of CD4^{+} T-cells characterised by expression of CD25 (IL-2Rα) provided early evidence of the existence of regulatory T-cells.[215,216] Subsequent studies identified the product of the forkhead box P3 (Foxp3) gene as the critical transcription factor for the development and function of regulatory T-cells.[217-219] Deficiency of Foxp3 in mice leads to a fatal systemic inflammatory disease,[220] whilst human mutations in Foxp3 result in IPEX (immunodysregulation, polyendocrinopathy, enteropathy X-linked) syndrome, confirming the essential *in vivo* role of regulatory T-cells in restraining inflammatory responses, including within the intestine.[221,222]

The Foxp3^{+} regulatory T-cell pool contains 2 distinct populations. Natural Treg cells (nTreg) develop in the thymus under the effects of TCR stimulation and CD28 co-stimulation through MHCII signals[223] leading to activation of pathways including CARMA1,[224-226] PKCθ,[227,228] TAK1,[229,230] NFAT,[231] CREB,[232] Foxo1 and Foxo3,[233,234] the NF-κB components IKK[228,235-237] and c-Rel,[238-240] and suppression of the PI3K-Akt-mTOR pathway.[233,241-243] IL-2/IL-15 activates STAT5 to induce and stabilise Foxp3 expression.[223] In addition, non-thymically derived Foxp3^{+} developing peripherally from Foxp3^{-}CD4^{+} naïve T-cells (iTreg) represent an important population, particularly within the intestine.[244] Different transcriptional signatures have been demonstrated for nTreg and iTreg,[245-247] and segregation of each population has been reported based upon expression of the transcription factor Helios[248] or the surface marker neuropilin-1.[249,250] Defined on this basis, up to 45% of the colonic regulatory T-cell population in normal mice comprises iTregs.[244,249] iTreg development occurs under the influence of signals from TGF-β1 and IL-2, and is enhanced in the presence of retinoic acid.[40,41]

Mechanisms of regulatory T-cell activity include effects on effector T-cell differentiation, prevention of cell migration and homing and inhibition of effector function within the tissues.[251] The expression of cell surface receptors including GITR,[252,253] CTLA-4,[216,254,255] OX40[256] are important for this function, as are mechanisms including growth factor deprivation, and

suppression of DC maturation. In addition, regulatory T-cells produce an array of immunomodulatory cytokines including TGF-β1, IL-10 and IL-35.[257-261] The relative importance of any individual mechanisms varies by context, but important recent studies have highlighted the requirement for expression of Th1, Th2 or Th17 associated molecules by regulatory T-cells for full suppressive capacity of such target cells. $Foxp3^+$ T-cell expression of T-bet permits upregulation of CXCR3 and homing to sites of Th1 mediated inflammation.[262] Similarly, expression of IRF4[263] or STAT3[264] is required for the prevention of Th2 or Th17 mediated pathology.

In addition to $Foxp3^+$ regulatory T-cells, IL-10 or IL-35 producing $Foxp3^-$ $CD4^+$ T-cells with *in vivo* regulatory function have been described, and are particularly prominent in the small intestine, including the IEL.[265,266] The developmental origins of such Tr1 cells is unsettled, since IL-10 expression can be induced in all effector T-cell subsets, particularly related to chronic immune stimulation.[267] Therefore whether these cells develop from 'ex'-effector cells or represent a true separate T-cell subset remains to be defined.

1.5.7 Follicular Helper T-cells

Follicular helper T-cells (Tfh) are a specialised subset of T-cells present within lymphoid tissues, which contribute to germinal centre formation and the establishment of long lived serological memory.[268-270] Characterised by a $CXCR5^+PD\text{-}1^+ICOS^+CD40L^+$ surface phenotype, expression of the transcriptional repressor Bcl-6, and abundant production of IL-21, Tfh provide activation and survival signals to B-cells and drive class switching.

Development of Tfh cells requires both DC and B-cell signals,[271-275] and is highly dependent upon STAT3 activation, particularly by IL-6 and IL-21, although non-redundant roles exist for each of these cytokines.[276-281] As with other T-cell subsets, the Tfh program may inhibit alternative cell fates, with Bcl-6 over-expression reported to suppress the function of GATA3,[282] and to reduce expression of T-bet and RORγt, as well as the reciprocally expressed Blimp-1.[272,279,283] However,

Bcl-6 expression is insufficient to drive some features of key Tfh cells, including IL-21 production or CXCR5 expression,[283-285] and additional transcriptional regulators including c-MAF,[286] BATF[287,288] and IRF4[289] appear relevant to Tfh function. However, the full lineage relationships of Tfh to effector subsets remains debated, with Tfh development from apparently committed Th1,[290] Th2[275,291] or Th17[286,292] cells documented, and the Bcl-6 locus remaining open with permissive epigenetic marks in all effector subsets when differentiated *in vitro*.[293,294] Furthermore, transition through a Tfh phenotype is observed during initial development of Th1 cells, and a significant subset of $CXCR5^+PD\text{-}1^+CD4^+$ cells co-produce IL-21 and IFN-γ and express T-bet during antigen priming.[294,295]

1.5.8 $CD4^+$ T-cell plasticity

The concept of T-helper subsets as fixed, terminally differentiated cell lineages has been challenged in recent years, with the demonstration of significant functional plasticity amongst $CD4^+$ T-cells.[296,297] Indeed, the concept of master transcriptional regulators specific to each subset and responsible for the repression of alternative fates appears an oversimplification, since the co-expression of transcription factors associated with apparently opposing functional consequences is increasingly recognised, and may serve to shape the T-cell response throughout the immune response.

Th17 cells appear particularly unstable, and have been shown to adopt characteristics of Th1 cells, including T-bet and IFN-γ expression under the influence of IL-12 or IL-23.[183,298-302] Epigenetic analysis of Th17 cells has shown Th1 loci such as *Tbx21* (T-bet) and *Ifng* are flanked by permissive histone modifications.[303,304] Fate-mapping studies have suggest that in some inflammatory settings all Th1 IFN-γ^+ cells may arise from initially IL-$17A^+$ Th17 subsets.[298] The plasticity of Th17 to acquire T-bet expression and Th1 features has been strongly linked to their pathogenicity.[183,301,302,305] In the absence of inflammatory signals, Th17 cells may acquire IL-10

production.[306,307] Similarly, T-bet upregulation in GATA3$^+$ Th2 cells, through an IFN-γ and IL-12 dependent mechanism occurs in murine lymphocytic choriomeningitis virus (LCMV) infection, although this is associated with attenuated pathology and restraint of Th2 responses.[308,309] Plasticity of Foxp3$^+$ Treg cells appears to be central to their ability to regulate diverse effector responses, with co-expression of Foxp3 and factors associated with effector subsets including T-bet,[262,310] GATA-3,[311,312] RORγt,[313] IRF-4,[263] Bcl-6[314] and STAT3[264] fundamental to specific aspects of their suppressive effects in vivo. Furthermore, the acquisition of IL-17A production by Foxp3$^+$ cells has been described, although its significance remains unclear.[315] The loss of Foxp3$^+$ expression and development of various other effector phenotypes including IFN-γ$^+$ Th1 has been reported, linked to the development of inflammatory diseases, including experimental diabetes.[304,316,317] Loss of Foxp3[318] or RORγt[319] expression with transition to a Tfh phenotype may occur in murine Peyer's patches. Importantly, all CD4$^+$ effector subsets may acquire production of the regulatory cytokine IL-10 in appropriate settings.[267]

1.5.9 Non CD4$^+$ TCRβ$^+$ cells

A large number of non CD4$^+$ subsets of TCRβ$^+$ cells, including CD8$^+$, CD4$^-$CD8$^-$ and NK1.1$^+$ phenotypes have been described and studied in a range of immunological contexts, but a full discussion is beyond the scope of this thesis. In the intestine, a functional role has been described for IL-13 producing CD1d restricted NK1.1$^+$TCRβ$^+$ NKT-cells,[320] and the potential contribution of the related mucosa associated invariant T (MAIT)-cell continues to be explored.[321]

A large population of somewhat atypical TCRβ$^+$ cells resides within the intra-epithelial compartment, particularly of the small intestine,[14,322,323] and express either the CD8αβ heterodimer (type a IELs) or a CD8αα homodimer or are CD4$^-$CD8$^-$ (type b IELs). Type b IELs are non-MHC restricted, display limited TCR diversity, and are often self-reactive. These cells may play an important homeostatic role, and their absence is associated with increased susceptibility to intestinal inflammation which can be rescued by adoptive transfer of CD8αα$^+$TCRβ$^+$ IELs.[324]

1.5.10 γδT-cells

Accounting for <3-5% of circulating $CD3^+$ cells, γδT-cells are a minor, and functionally enigmatic population of T-cells, which demonstrate a specific enrichment at mucosal and epithelial surfaces.[14,325,326] Many γδT-cell subpopulations exhibit effector or surface profiles similar to subsets of conventional αβT-cells, but differ remarkably in their lack of MHC-restriction and ability to directly recognise and respond to endogenous antigens, with γδT-cell activation often TCR independent.

1.5.10.1 γδT-cell development

Murine γ-genes are encoded on chromosome 13, and consist of seven V and four C genes, one of the latter of which is a non-rearranging pseudogene in most murine strains.[327-330] δ-gene segments are located in chromosome 14, between the Vα and Jα clusters of the TCRα-chain locus, and encode a number of δ-chains demonstrating structural overlap with Vα chains.[329] Despite possible γδ-receptor combinations theoretically outnumbering those of the αβTCR, primarily driven by the presence of 2 D-regions in the δ-chain,[331] highly stereotyped recombination of Vγ/Cγ pairs and γ and δ-chain pairing result in limited receptor diversity.[329,332] Indeed, some γδT-cell populations are essentially oligoclonal, including those residing in the epidermis (dendritic epidermal T-cells; DTEC) which express an invariant Vγ5-Jγ1Cγ1/Vδ1-Dδ2-Jδ2Cδ combination,[333] and Vγ6-Jγ1Cγ1/Vδ1-Dδ2-Jδ2Cδ expressing cells within the epithelia of the reproductive tract, tongue and lung[334] (the nomenclature of γδT-cells is controversial and somewhat confusing; the scheme proposed by Heilig and Tonegawa is used throughout this thesis).[330] Even where cells demonstrate more diverse receptor combinations, their largely non-overlapping tissue distributions suggests specific Vγ-use is favoured at certain anatomical sites. Within the SI-IEL Vγ1+ and Vγ7+ cells predominate, whereas lymphoid tissue cells express Vγ1, Vγ2 or Vγ4.[334-339]

Marked differences exist in the stage of ontogeny at which specific Vγ-expressing subsets arise in the thymus, such that Vγ5+ cells are noted from 13 days of gestation after which they populate

the epidermis.[340] Vγ6+ cells demonstrate similar embryological development but delayed by 1-2 days.[341,342] The generation of such γδT-cells which lack TCR junctional diversity likely relates to the absence of the enzyme terminal deoxynucleotidyl transferase in the embryonic thymus.[343,344] Post natal γδT-cell development in the thymus is dominated by Vγg1, Vγ2 and Vγ4 populations with much greater, often extreme junctional diversity.[339,345] Importantly, the population of tissue niches by γδT-cell subsets appears to occur in functional waves from the embryonic thymus at specific stages of development.[333-335,339,340,345-348] Remarkably, γδT-cells can be detected in athymic mice, and the ability of the intestinal microenvironment to support extra-thymic development has been demonstrated, although its relevance remains unclear.[349-352] Human γδT-cells differ in genetic and structural aspects of their TCR, exhibiting generally greater levels of receptor diversity than the murine system.[329]

1.5.10.2 γδT-cell activation and effector function

The antigenic specificity of γδT-cells continues to be explored, but MHC-related structures,[353,354] stress-induced ligands expressed on infected or transformed cells,[355-358] protozoal[359,360] and bacterial derived molecules,[120,361,362] viral glycoproteins[363] and ATPase complexes[364] have all been shown to induce TCR activation of γδT-cells. In humans, the dominant circulating Vγ9Vδ2 population can be activated by phosphoantigen metabolites of the isoprenoid pathway used by micro-organisms.[120,121] Remarkably, whereas endogenous phosphoantigens are insufficient at basal levels to activate such γδT-cells, cellular stress may upregulate their expression to TCR triggering levels. γδT-cells may also be fully activated through TCR-independent pathways, including PRRs such as TLR2 and dectin-1,[365] NK-cell receptors including NKG2D,[366,367] and by cytokine signals including IL-1β, IL-12, IL-23 and type 1 interferons.[169,365,368-370]

γδT-cells exhibit many of the effector functions of other T-cell subsets, including perforin and granzyme mediated cytotoxicity via Fas and TRAIL pathways, and production of an array of Th1, Th2 and Th17 type cytokines, with effector activity in such subsets dependent upon the same

transcription factors as described for αβT-cells.[338,365,369,371,372] Although IL-10 producing γδT-cells with regulatory function are described,[373,374] and in vitro induction of Foxp3 expression reported,[375,376] the existence of a relevant functional regulatory γδT-cell subset *in vivo* is unproven.

The functions of γδT-cells in the immune response, separate from those of αβT-cells, continues to be explored, but in general γδT-cells exhibit innate characteristics, and contribute to the very earliest phases of host protection or disease development. Consistent with their early appearance in ontogeny, γδT-cells may be of specific importance for immune responses in young animals, prior to the development of a full αβT-cell repertoire.[377-380] Subsets of γδT-cells appear critical for the stress-surveillance response responsible for the detection and elimination of cells transformed by infection or neoplastic change.[119,366,367,381,382] The function of γδT-cells expressing RORγt and producing Th17-type cytokines has attracted significant recent interest for their role in early immune responses,[382-386] and in supporting the development of IL-23 dependent inflammation in the skin,[387,388] joints,[389] lungs,[390,391] and CNS.[365,369,392] In contrast, in other models of infective or inflammatory disease, γδT-cell deficiency results in exaggerated immune responses supporting a role in preventing the excessive activation or activity of other cells.[393-401]

Specific subsets of γδT-cells have been show to mediate tissue repair and wound healing through expression of factors acting upon epithelial cells, including keratinocyte growth factor (KGF)-1 (also termed fibroblast growth factor (FGF)-7), KGF-2, Insulin-like growth factor (IGF)-1 and fibropellin-1 (EGF-1).[402-405] In the intestine, subsets of γδT-cells may reinforce barrier function through production of anti-microbial peptides and induction of expression of epithelial cell tight junction proteins.[406,407]

Functional attributes often correlate with the expression of specific Vγ-Vδ combinations and tissue restriction, and suggest either ligand-TCR interactions in the thymus or the tissue microenvironment shape γδT-cell function. Although some subsets of γδT-cell demonstrate strict functional bias regardless of situation, others are able to adopt Th1, Th2 or Th17 phenotypes dependent upon the setting examined.[338] Importantly, little evidence exists for a similar degree of functional plasticity recently documented in $CD4^+$ T-cells. Molecular signals involved in thymic

determination of γδT-cell phenotype include signalling lymphocytic activation molecules (SLAM) family receptors,[408] the promyelocytic leukaemia zinc-finger (PLZF),[408] Skint,[409] CD27,[410] LIGHT/LTβR and NF-κB components RelA and RelB.[411]

1.6 The Intestinal Microbiota

The human intestinal microbiota comprises enormous numbers of organisms, including ~10^{14} bacteria as well as viral and fungal species, resulting in a metagenome >100 larger than the host genome.[412-414] Component species are increasingly recognised as essential for host digestive, metabolic and immunological processes, with dysbiosis implicated in the pathogenesis of numerous disease including asthma, type I diabetes, obesity, malnutrition, cardiac disease, thyroid disease, arthritis, susceptibility to infection and IBD.[5,415-418]

In the absence of a microbiota, germ free (GF) mice demonstrate abnormal development of the GALT and multiple immune compartments, including LP T-cell populations.[3,78,419,420] Differing microbial requirements for normal immune development are apparent between species. A number of innate cell populations, including some γδT-cell subsets develop in utero and appear independently of microbial colonisation.[181,335]

Conventionalisation of GF mice with SPF microbiota results in accumulation of $CD4^+$ T-cells and the expression of both pro-inflammatory and regulatory gene signatures.[421,422] In unmanipulated mice, both Th17 and Treg accumulation occurs with the development of a post-natal flora, although the largely non-overlapping anatomical distribution of each cell type suggests dependence upon differing component of the flora.[43,244,423,424] Studies using antibiotic treatment and mono-colonisation techniques have consistently reported the gram positive *Clostridium*-related species segmented filamentous bacteria (SFB), a species present within many SPF mouse colonies, to be necessary and sufficient to drive Th17 and possibly Treg accumulation in the steady state.[421,422,424] Mechanistically, it has been shown that SFB adheres to intestinal epithelial cells of the terminal ileum, and induces production of serum amyloid A, which drives intestinal

DC production of IL-6 and IL-23, favouring Th17 accumulation.[424] However, the basis for the more profound effects on additional T-cell subsets including Th1 and Treg cells reported by other groups is unclear.[422] Mice engineered to express human α-defensins display reduced colonisation by SFB, with reduction in Th17 but unaffected Th1 cells.[425] The SFB genome has recently been sequenced and may aid understanding of the basis for its role in intestinal T-cell homeostasis.[426,427] However, it remains unlikely that SFB is the only species capable of driving development of Th17 and other T-cell subsets. Importantly, in GF mice conventionalised with altered-Schaedlers flora, a defined cocktail of innocuous bacteria which does not include SFB, intestinal Th17 accumulation could occur when IL-10 signalling pathways were blocked.[428] Similarly, in GF mice, the rectal administration of ATP is reported to correct the Th17 deficit, although importantly SFB does not activate this pathway.[43] Indeed, the basis of sensing of SFB remains uncertain, since Th17 accumulation is unimpaired in MyD88$^{-/-}$Trif$^{-/-}$ and Rip2$^{-/-}$ mice which lack TLR and NOD signalling respectively.[43,421,424]

The role of the microbiota in regulatory T-cell accumulation may differ by site, with maintained populations reported in the SI, MLN and peripheral lymphoid tissues of GF mice, whereas colonic cells appear strictly dependent upon microbial colonisation.[244,429] Members of the clostridium family, particularly clusters IV and XIVa appear particularly important for colonic iTreg development. Colonisation of mice with a defined cocktail of *Clostridium* species led to the accumulation of CTLA-4^{hi}IL-10^{+}Helios^{-} Treg cells, a phenotype suggestive of an induced, extra-thymic origin.[244] Notably, patients with IBD have reduced intestinal representation of Clostridia, including clusters IV and XIVa.[430,431] As with Th17 cells, the relevant pathways by which Clostridia might drive iTreg development are not clear, with no defect apparent in MyD88, Rip2 or Card9 deficient strains.[244]

Induction of IL-10 production by intestinal CD4^{+} T-cells has been reported to underlie protection from *Helicobacter hepaticus* induced colitis occurring in mice colonised with *Bacteroides fragilis*, a component of the normal human intestinal flora.[432] Specifically, *B.fragilis* derived polysaccharide A (PSA) induced IL-10 production through a CD4^{+} T-cell intrinsic TLR2

dependent mechanism, whereas PSA deficient *B.fragilis* induced a Th17 cell response.[433,434] Remarkably, whereas PSA favours regulatory mucosal responses, it may promote Th1 responses in secondary lymphoid tissue resident cells.[435] Further bacteria have been implicated in specifically driving intestinal IL-10 production, including *Faecalibacterium prausnitzii*,[431] isolated from the flora of patients with Crohn's disease, suggesting this to be a functional characteristic conserved across multiple species.

1.7 Inflammatory Bowel Disease

The inflammatory bowel disease (IBD) ulcerative colitis (UC) and Crohn's disease (CD) are diseases of unknown aetiology characterised by chronic or relapsing and remitting inflammation of the gastrointestinal tract, with a combined prevalence of approximately 1:1000 in European and North American Caucasian populations.[20,436-438]

The peak of onset is in adolescents and young adults, with a further peak in the sixth decade. Clinically, patients with active IBD experience abdominal pain, diarrhoea, rectal bleeding, and anorexia and weight loss.[439,440] Although the aetiology of IBD remains to be fully defined, it is clear that both genetic and environmental factors are important.[441,442] The majority of patients with IBD experience periods of active disease interspersed with quiescent or inactive disease, however some patients experience continuous disease activity.[439,440] Effective therapeutic options for the treatment of active IBD and for the maintenance of remission have expanded in recent years, and now include corticosteroids, antibiotics, 5-aminosalicyates, thiopurine antimetabolites, methotrexate, and anti-TNF-α agents.[443-445] However, no treatment is curative, and up to 30% of UC patients and 75% of CD patients will require surgical management of their condition in the 20 years following diagnosis.[446-448] Whilst colectomy is curative in UC, many patients with CD require repeated operations each without the prospect of cure. There therefore remains a significant unmet need for more effective treatments for IBD.

1.7.1 Pathology of IBD

UC affects the colon only, and is characterised by continuous, relatively superficial mucosal inflammation, which uniformly involves the rectum but is highly variable in its proximal extent.[440] CD may occur anywhere along the length of the GI tract, often in a patchy or discontinuous fashion, and results in transmural inflammation which may penetrate the serosal surface to result in perforation and fistulation to adjacent intestinal segments and organs, and to the skin, particularly in the perianal region.[439] Alternatively, CD may result in scarring and structuring of the intestinal lumen. In a small proportion of patients (10-15%), the subtype of IBD may not be easily defined as UC or CD, and in such patients disease is described as IBD type unclassified (IBD-U).[449] Serological markers including perinuclear anticytoplasmic antibody (pANCA) and antibodies against the yeast *Saccharomyces Cerevisiae* (ASCA) may assist classification in this situation.[450-452]

Microscopically, active UC is characterised by goblet cell depletion, epithelial cell necrosis and neutrophilic infiltration of the crypts, resulting in cryptitis and crypt abcess formation.[453,454] With chronicity, abnormalities of the crypt architecture including branching and widened separation between crypts develop, along with a chronic plasma cell infiltrate and Paneth cell metaplasia. The defining histological feature of CD is focal inflammation, including crypt inflammation and aphthous ulceration in the absence of surrounding chronic inflammatory change.[453,454] Notably, aphthous ulcers predominantly develop in regions overlying lymphoid aggregates of the SI and colon.[455-457] Granulomata are a variable feature of CD, being observed in up to 70% of cases.[20] Formation of lymphoid aggregates in the submucosa and at the level of the myenteric plexus are further specific features of CD.[453,454]

Many of the histological features of both forms of IBD overlap considerably with other inflammatory conditions of the GI tract for which there are clear aetiological explanations.[439,452-454] Acute UC closely mimics infective colitis as commonly occurring due to *Salmonella*, *Camplyobacter*, *Shigella* or *E.coli*, whilst the granulomatous inflammation of CD bears striking resemblance to intestinal infection with *Mycobacterium tuberculosis*. Small intestinal aphthous

ulceration occurring secondary to non-steroidal anti-inflammatory drug (NSAID) use is an important mimic of SI CD, as are Behçets disease and chronic granulomatous disease (CGD).[458,459] Similarly, intestinal ischaemia may closely resemble IBD both macroscopically and histologically. This observation would suggest that intestinal inflammation develops in relatively limited patterns, with similar features, due to diverse initiating factors.

1.7.2 Genetics of IBD

A genetic contribution to IBD is suggested by twin-studies showing a 50-80% sibling risk in monozygotic twins of patients with CD although lower rates of disease concordance occur in UC (10-15%).[439,440,460] Recent GWAS studies have been remarkably successful in identifying single nucleotide polymorphisms (SNPs) associated with each subtype of IBD, with 163 loci independently associated with the risk of CD or UC now described.[461-463] Importantly, 110 of these loci are common to both conditions, and >70% are shared with extra-intestinal inflammatory diseases.[462,464] Whilst each loci individually imparts a modest effect, pathway analysis has demonstrated clustering to a number of fundamental processes, including cytokine production, lymphocyte activation, response to molecules of bacterial origin and the Jak-STAT pathway. Interestingly, significant enrichment for genes involved in primary immunodeficiencies is apparent, most notably for the condition Mendelian susceptibility to mycobacterial disease,[465-467] in which 7 of the 8 genes associated with this rare disorder also associate with IBD.[462] Furthermore, 7 of 8 loci implicated in a GWAS of the mycobacterial disease leprosy were identified by meta-analysis of IBD data.[468] These associations highlight the likely importance of host-bacterial interactions in the development of IBD.

Genes specifically associated with CD risk include mutations in the leucine rich repeat region of the intracellular PRR, NOD2, which imparts the greatest relative risk of any IBD gene described so far, but does not influence the risk of UC.[469,470] Autophagy has been highlighted as a potentially important process in CD (*ATG16L1*) and a functional interaction between autophagy and NOD2 signalling has been reported,[471,472] although recent analysis suggests the related *IRGM* gene may

also be associated with UC.[462] Multiple aspects of effector cytokine signalling have been implicated in both forms of IBD, including by IL-23/Th17 cells (*IL23R*, *IL12B*, *IL21*, *RORC*, *CCR6*, *STAT3*, *JAK2*, *TYK2*, *IL1R1*), and Th1 cells/IFN-γ (*IL12B*, *IFNG*, *IL18RAP*, *STAT1*, *STAT4*, *IL27*) as well as IL-10 responses (*IL10*).[461-463,473] Rare mutations in subunits of the IL-10R are associated with severe early onset Crohn's disease which can be cured by haematopoetic stem cell transplantation.[474]

The specific functional implications of most disease associated SNPs remain largely unknown, but are the subject of intense study.

1.7.3 The Intestinal Microbiota in IBD

A role for the flora in driving intestinal inflammation was revealed in early clinical observations that diversion of the faecal stream could ameliorate active IBD, which recurred upon restoration of intestinal continuity.[475] Similarly, antibiotics show some efficacy in the treatment of CD,[476,477] and both probiotics and faecal transplants have been reported to be effective treatment for UC.[477-479]

However, attempts to identify individual causative agents in IBD have been unrewarding. Multiple groups have failed to find evidence of mycobacterial infection as an underlying aetiological factor, a hypothesis based upon the occurrence of Johne's disease in *M.paratuberculosis* infected cattle.[480,481] More recently, studies using high throughput 16S rRNA sequencing and metagenomic sequencing techniques have demonstrated the association of each IBD subtype with a specific composition of the flora.[413,482] However, it remains uncertain to what extent such patterns underlie or result from the intestinal inflammation. Studies in mice have demonstrated the clear effects inflammation has on the composition of the intestinal flora. Patients with CD have significantly reduced bacterial diversity, with reduced representation of *Fermicutes*, *Bacteroidetes*, *Bifidobacter* and *Lachnopiraceae*.[430,482-484] Specific components of the *Clostridium* family, including clusters IV and XIV are reduced in CD, as are levels of the IL-10

inducing bacterium *Faecalibacterium prausnitzii*, whereas proteobacteria including *E.coli*, *Ruminococcus* and *Enterococcus* species may be over-represented.[430,431,483,484] However, compositional change may not be the only relevant factor in host-microbe interaction in IBD, and spatial distribution may be important. Increased populations of adhesive/invasive *E.coli* (AIEC) in close association with the terminal ileal epithelium have been reported in CD, due to binding to aberrantly expressed CEACAM-6.[485,486] Studies in mice demonstrate more severe experimental colitis in animals in which CEACAM-6 is artificially over-expressed.[486,487] However, there is currently limited understanding of the overall spatial distribution of components of the microbiota.

The role of host genetics, particularly disease associated genes, in determining the resident microbiota is rightly an area of intense research interest.[483,488,489] Notably, patients with ileal pouches following surgery for UC have a differing microflora to those receiving pouches for non-IBD indications, even in the absence of inflammation, further supporting the existence of a host specific propensity to develop a colitogenic flora.[490,491]

1.8 Models of Intestinal Inflammation

A large number of models systems to permit study of intestinal inflammation have been described. Although spontaneous IBD-like conditions occur in primates (cot-top tamarin)[492,493] as well as dogs and cats,[494,495] practical and ethical considerations have led to the development of numerous rodent models which permit detailed mechanistic study of intestinal immunity and inflammation.[496-500] Models of both acute and chronic disease have been described, occurring in the presence or absence of adaptive immunity. Although a small number occur otherwise spontaneously in the setting of genetic manipulation, most require a distinct initiating event such as exposure to a microbial or chemical challenge. Whilst no single model recapitulates all features of human IBD, the considered use of complimentary models permits dissection of biological

processes active in intestinal inflammation. Models used in this thesis are discussed in detail below, however detailed discussion of other models is available elsewhere.[496-500]

1.8.1 *H.Hepaticus*/anti-IL10R

Infection of mice with the microaerophilic gram negative bacillus *Helicobacter hepaticus* results in colitis and systemic disease in a strain dependent manner.[501,502] Named for its original isolation from the liver of A/JCr mice,[503,504] *H.hepaticus* causes typhlocolitis in multiple IL-10$^{-/-}$ strains and in *Rag*$^{-/-}$ mice,[505,506] most strikingly 129Rag$^{-/-}$ mice, whereas oral infection of C57BL/6 mice results in persistent colonisation of the caecum and colon and microbial dysbiosis, but does not cause inflammation.[501,507,508] *H.hepaticus* establishes infection of the colonic and caecal crypts,[503] and demonstrates a number of adaptations which appear to subvert host immune activation, including suppression of TLR4 signalling in IECs[509,510] and failure of its associated flagellin to activate TLR5,[511] as well as a Type VI secretion system which may downregulate host inflammatory gene signatures.[509]

In wild-type mice, infection promotes the emergence of IL-10^{+}CD4^{+} T-cells which are able to prevent symptomatic disease upon transfer of into 129Rag$^{-/-}$ animals, demonstrating the important functional role of IL-10 in this setting.[512] Similarly, the occurrence of apparently spontaneous colitis in IL-10$^{-/-}$ mice has been linked to the presence of *H.hepaticus*, but importantly monocolonisation of germ free IL-10$^{-/-}$ mice does not induce intestinal inflammation, demonstrating further components of the flora are required for disease.[513,514] Accordingly, infection of C57BL/6 mice with *H.hepaticus* and concurrent blockade of IL-10 signals with a monoclonal antibody against IL-10R results in marked chronic intestinal inflammation, not seen with antibody treatment alone.[508] Inflammation in this model is dependent upon IL-23 but not IL-12, and is characterised by increased mucosal expression of IL-17A and IFN-γ.[508,515]

Importantly, this model occurs in otherwise immunologically intact mice with normal compliments of innate and adaptive cells, allowing assessment of mechanisms potentially active

through multiple cellular components, or populations not represented in other models such as B-cells or γδT-cells. However, this also presents clear difficulties in dissecting the role of specific cells. The increasing availability of cell conditional genetic knockouts using the *cre-loxP* system will likely increase the utility of this model in the future.

1.8.2 T-cell Transfer

Early studies of T-cell subsets identified specific populations able to induce or prevent intestinal inflammation upon transfer into lymphocyte deficient hosts, leading to the development of one of the most widely used models of experimental colitis.[215,516-518] Transfer of $CD4^+CD45RB^{hi}$ naïve T-cells into $Rag^{-/-}$ (which lack native B and T-cells) or SCID mice results in systemic and intestinal disease, with marked wasting, splenomegaly and severe pancolitis, with accumulation of APCs and T-cells in the lamina propria, loss of goblet cells and epithelial hyperplasia.[215,516-518] Elevated levels of cytokines including IL17A, IFN-γ and TNF-α occur, and disease can be prevented by inhibition of IFN-γ or TNF-α.[519] Although the role of IL-17 is less clear,[520-523] Th17 cells appear important in this model, since transfer of $ROR\gamma t^{-/-}$ T-cells is reported to result in attenuated disease.[523] Notably, T-cell transfer colitis is dependent upon components of the intestinal flora and does not occur in germ free animals.[524,525]

Initial observations demonstrated that co-transfer of $CD4^+CD45RB^{lo}$ cells could prevent both colitis and systemic disease, and further definition of the responsible component revealed $CD4^+CD25^+$ Treg cells to be critical.[526,527] Further studies have shown transfer $CD4^+CD25^+$ cells can effect cure of mice with established disease.[528] The protective effects of $CD4^+CD25^+$ cells are mediated in part via the cytokines IL-10 and TGF-β1[258,529] and expression of surface molecules including CTLA-4,[216,527] OX40,[256] CCR4,[530] and CCR6.[531]

A particularly powerful aspect of the T-cell transfer model is the ability to restrict the expression of genes of interest in either the innate (host) component, through crossing the genetic

manipulation of interest onto the $Rag^{-/-}$ background, or to the T-cell component by transfer of cells from animals carrying specific genetic abnormalities.

Important limitations of this model of disease include its development in the complete absence of B-cells and non-$CD4^+$ T-cell subsets including $\gamma\delta TCR^+$, NKT, $CD8^+$ cells and Tregs, and abnormalities of the IEL and inductive immune structures of the gut in $Rag^{-/-}$ hosts. In addition, disease develops due to lymphopaenia induced proliferation of transferred cells in the 'empty' host, in the absence of Tregs, meaning that requirements for the induction of pathogenic T-cell responses may be less stringent than occur in a lymphocyte replete setting.

1.8.3 *Citrobacter rodentium* Infection

Infection of immunocompetent mice with the gram negative bacteria *Citrobacter rodentium* results in a self-limiting moderate typhlocolitis characterised by marked epithelial hyperplasia and goblet cell depletion, and serves as a useful model of human infection with enteropathogenic *E.coli* (EPEC).[532] Initially colonising the caecum followed by the entire colon,[533] *C.rodentium* adheres to the surface of epithelial cells by formation of an attaching/effacing (A/E) lesion, mediated through a Type III secretory system which delivers bacterial proteins encoded within the locus for enterocyte effacement (LEE) pathogenicity island.[534-536] Disease occurs in 3 distinct phases, with a number of host factors influencing each stage. Initial epithelial colonisation and A/E complex formation is influenced by the sex, strain, age and existing microbial flora of the mouse,[537-540] as well as the production of antimicrobial peptides, including C-type lectins,[541] cathelicidin[542] and defensins.[543] Defective signalling from upstream molecules including IL-23,[170,541] IL-22,[541] and IL-17C,[544] or absence of critical cellular sources including $CD4^+$ T-cells,[545] LTI-like cells[546,547] or $NKp46^+$ ILCs[72] is associated with increased, often fatal hypercolonisation and systemic infection, which contrasts with the lack of invasive disease seen in normal mice. In the second distinct phase in which typhlocolitis develops, the severity of disease is closely related to host factors, and is not clearly determined by colonisation levels. Mice lacking PRRs or adaptor

molecules required for their function such as NOD1 and NOD2,[548,549] NLRP3,[550] TLR2[551] or MyD88[552,553] develop severe colitis, although notably TLR4$^{-/-}$ mice are relatively protected in this phase.[554] DC activity appears crucial, with DC depleted animals[555] and CCR2$^{-/-}$ mice developing exacerbated disease,[549] as do GM-CSF$^{-/-}$ which fail to recruit and activate DCs.[555] During this phase, Th1 and Th17 CD4+ T-cells accumulate in the LP, whereas other T-cell subsets are redundant for survival.[556,557] However, the role of the Th1 cytokine IFN-γ is unclear, with both host protective and pathogenic effects reported.[543,556-559] Although Th17 cells appear critical to host protection, the critical downstream mediator is unclear, with IL-22 redundant beyond the initial phase of disease,[545,546] and IL-17A and IL-17F being variously described as protective, pathogenic or redundant.[541,560] Indeed, a non-T-cell source of IL-17F may be important.[560] Finally, bacterial clearance and recovery from disease occurs through a mechanism requiring B-cells, CD40L, and CD4+ T-cell dependent production of systemic IgG.[556,561-563]

C.rodentium therefore provides a valuable model to investigate host pathogen interactions, although caution is required in its use, particularly since it is so dependent upon inadequately defined aspects of the host microbial flora.

1.9 Innate Immune Pathways in Intestinal Inflammation

Whereas adaptive immune responses may contribute to the perpetuation and amplification of intestinal inflammation in IBD and experimental models, accumulating evidence points to innate immune pathways as critical regulators of homeostasis and inflammation, through interactions with the microbiota.[4,8,462] The development of a more severe disease phenotype in the DSS model in mice with disrupted bacterial sensing, including through TLR2, TLR4, TLR5, TLR9 or MyD88 demonstrates the critical importance of appropriate host-microbiota interactions as a regulator of disease susceptibility.[564] In murine models, the ability of the epithelium to sense and respond to the commensal flora has been shown to be essential for the prevention of severe inflammatory disease,[10] through elaboration of antimicrobial factors (AMP) regulating the microbial flora.[8]

Furthermore, IEC intrinsic NF-κB activation, a downstream pathway of PRR signalling, is required for the prevention of severe colitis in a Th2-model through cytokine conditioning of intestinal leukocytes.[35] Equally, IEC intrinsic NF-κB signals are required to promote cellular homeostasis and preservation of barrier function, breakdown of which induces severe spontaneous microflora driven intestinal inflammation.[9] Amongst specialised epithelial cells, Paneth cells appear of specific important to the regulation of intestinal immunity,[425,565] and Paneth cell intrinsic MyD88-signalling drives elaboration of AMPs critical for the regulation of the microbiota.[566] Beyond TLRs, the role of NLRs in intestinal immunity has attracted intense interest driven by the association of *NOD2* mutations with CD, which remains the strongest disease-susceptibility gene identified to date.[469,470] The mechanisms by which NOD2, an intracellular sensor of bacterial cell wall peptidoglycan[567] and viral ssRNA,[568] contributes to disease risk remains uncertain, but modifying interactions with TLR pathways have been reported,[569] with consequent functional implications for the resulting T-cell response.[570] Notably, Paneth cell expression of NOD2 drives expression of α-defensins required for regulation of bacteria and host defence, an effect lost with CD associated mutations.[571] NOD2 may also function to initiate autophagy,[572] a further innate pathway strongly associated with CD in GWAS studies,[462] through interaction with ATG16L1, with NOD2 mutations resulting in defective autophagy and impaired bacterial processing. Importantly, Atg16l1 hypomorphic (HM) mice demonstrate marked structural and functional Paneth cell abnormalities,[573] demonstrating the potential intersection of multiple implicated innate pathways in disease. Significantly, whereas Atg16l1^{HM} mice do not develop spontaneous disease, increased susceptibility to DSS colitis was shown to be dependent upon murine norovirus infection driving the Paneth cell abnormalities in these mice.[574] Recently, the unfolded-protein response (UPR) and ER stress have been implicated as central processes in intestinal inflammation. Genetic components of this pathway, including XBP1, AGR2 and ORMDL3 are associated with both CD and UC, and activation of the UPR via XBP-1 is required for mucosal protection in DSS disease, with increased Paneth cell apoptosis occurring in *Xbp1* deficient mice.[575] Similarly, *Winnie* and *Eeyore* mice, which produce a mutated MUC2 mucin, develop a UC-like disease associated with increased ER stress in Goblet cells, recapitulating a

phenotype seen in UC tissues.[576] Notably, ER-stress is an important activation signal for autophagy, highlighting the likely intersection of these pathways in IBD and intestinal immune homeostasis.[577]

1.10 Effector Pathways in Intestinal Inflammation

Early murine studies reported the presence of $CD4^+$ Th1 and Th2 cell within the intestine in the steady state and during inflammation.[15] Subsequently, studies in human IBD reported Th1/IFN-γ^+ cell accumulation in CD, whereas UC was characterised by elevated IL-5 and IL-13 mRNA levels but reduced IL-4^+ Th2 cells.[578] The latter finding was later attributed to the presence of an increased population of IL-5 and IL-13 expressing CD1d restricted NKT-cells.[320] More recent studies have implicated Th17 cells in the pathogenesis of murine models of colitis and both UC and CD.[183,301,520,523] In reality, it is likely multiple pathways are operative in any single disease setting, but that each assumes prominence at specific stages of disease, and that greater understanding of specific temporal requirements may have translational relevance.

1.10.1 Th1/IFN-γ

Th1/IFN-γ pathways are central to a number of murine models of colitis, including the T-cell transfer model and *H.hepaticus* driven inflammation in IL-$10^{-/-}$ hosts. Neutralisation of IFN-γ can prevent the development of disease in both these models, but importantly requires treatment from the initiation of disease for efficacy.[519,579,580] In contrast, IL-12p40 inhibition may ameliorate established disease.[580,581] T-cell expression of IFN-γ is dispensable for T-cell transfer disease,[582] but T-bet[583] or STAT4[582] deficient T-cells are reported not to induce disease upon transfer in to $Rag^{-/-}$ hosts, suggesting that either IFN-γ independent Th1 effects contribute to disease, or that alternative pathways are active in their absence. In CD, T-bet^+ and IFN-γ^+ T-cells accumulate,[583,584] and levels of pSTAT4 and expression of T-bet and IL-12Rβ2 are increased.[583,585] Inhibition of IL-12p40 is of some efficacy in active CD,[586] associated with

reduced mucosal Th1 cells accumulation, although the degree to which this also reflected anti-IL-23 activity is uncertain.[587,588] Unfortunately, clinical trials of anti-IFN-γ in CD have proven disappointing, and the pathophysiological relevance and therapeutic potential of the Th1/IFN-γ axis in established IBD remains unproven.

1.10.2 IL-23/Th17

The essential role of IL-23 in numerous models of intestinal inflammation is now well demonstrated. IL-23 drives disease in IL-10$^{-/-}$ mice[508] and in the *H.hepaticus*/anti-IL-10R model,[515] as well as T-cell transfer disease, where it may prevent or ameliorate established disease.[183,589] Additional support for the pathophysiological relevance of this pathway in IBD is provided by genetic studies implicating SNPs in a large numbers of IL-23/Th17 genes.[461-463] However, it is also clear that the IL-23/Th17 axis has important homeostatic and host protective functions,[541,545] and that the intestine is both an important site of Th17 differentiation, but also contains a large population of Th17 cells in the absence of detectable inflammation.[181,307] However, transfer of RORγt$^{-/-}$ naïve T-cells into immunodeficient hosts fails to induce significance colitis, confirming the importance of downstream effects for intestinal inflammation.[590] Therefore interest has focussed upon defining the specific characteristics which might impart pathogenicity upon cells of this pathway.

Although by definition Th17 cells produce IL-17A, it is likely that additional cytokines or effector molecules are responsible for the contribution of this pathway to disease, since IL-17A appears protective or redundant in models of colitis, including DSS[591,592] and T-cell transfer disease.[521,590,593] Similarly, antibody blockade of IL-17A in CD was associated with inferiority to placebo, and adverse effects including exacerbation of disease and intestinal fungal infection.[594] These findings notwithstanding, IL-17 does appear able to drive intestinal inflammation in specific scenarios, including Treg cell specific STAT3 deficiency[264] and in T-cell transfer using T-bet$^{-/-}$ deficient cells (C Schiering, unpublished observations). IL-17F appears able to compensate for the activity of IL-17A in T-cell transfer disease, with blockade of both mediators required to ameliorate disease.[590] This corresponds with the existence of a shared receptor for IL-

17A and IL-17F, composed to IL-17RA and IL-17RC subunits.[595] In *C.rodentium* disease, IL-17A and IL-17F exert similar but distinct protective roles,[560] whereas in DSS colitis a pathogenic role for IL-17F has been reported,[591] contrasting with the protective function of IL-17A reported by some[591,592] but not all[596] investigators. Importantly, IL-17A and IL-17F demonstrate shared and differential molecular aspects of regulation.[597,598]

IL-22 is a highly IL-23 dependent Th17 product which exerts significant effects upon IECs,[599] and has been reported to play a largely host protective role in intestinal immunity,[69,72,600] being essential for protection from *C.rodentium* infection through stimulation of antimicrobial peptide production.[541] In DSS colitis,[601] $CD45RB^{hi}$ T-cell transfer disease,[601] and the spontaneous colitis occurring in $TCR\alpha^{-/-}$ mice,[602] IL-22 is protective. In contrast, disease induced by Treg depleted $CD45RB^{lo}$ memory T-cell transfer is in part driven by IL-22,[603] as are both innate and T-cell dependent models of *H.hepaticus* infection (R Szebady, unpublished observations). In active UC, disease has been associated with a paucity of $IL\text{-}17A^{+}IL\text{-}22^{+}$ cells, with re-appearance of IL-22 producing Th17 cells noted in remission.[604] However, other groups report increased serum IL-22 levels in active IBD, which correlated with disease-associated *IL23R* polymorphisms.[605,606]

Similar uncertainty exists as to the critical Th17 mediator in neurological inflammation, but recent studies have reported GM-CSF to be essential for pathogenicity in EAE, produced by Th1 and Th17 cells under the control of IL-1β and IL-23.[185,186] In intestinal inflammation, neutralisation of GM-CSF ameliorates disease,[607] yet T-cell independent sources appear relevant since GM-CSF-deficient T-cells are unimpaired in their ability to cause disease.[608]

Whereas steady state Th17 cells express IL-17A but not IFN-γ,[421] co-expression of this latter cytokine has been linked to the pathogenic potential of Th17 cells in experimental models of colitis and IBD, as well as models of extra-intestinal inflammatory disease, with development of this Th1/Th17 subset dependent upon IL-23 signals.[183,301,302]

The role of the Th17 associated cytokine IL-21 in intestinal inflammation is less well understood. Studies in DSS and TNBS models,[609,610] and T-cell transfer colitis using $IL\text{-}21^{-/-}$ naïve T-cells[611,612]

suggest a pathogenic role, although the specific mechanisms remain undefined. Importantly, IL-21 is both a product and differentiation factor in Th17 biology adding complexity to interpretation of its function.[613]

In addition to uncertainty as to the relative role of mediators of the IL-23/Th17 axis, it is important to note the increasing number of innate and unconventional lymphoid cells reported to mediate 'type-17' responses in addition to $CD4^+$ Th17 cells in a number of extra-intestinal disease settings.[169,368,383,389,390,399,614,615] In innate models of colitis including anti-CD40 treatment and infection of $Rag^{-/-}$ mice with *H.hepaticus*, IL-23 drives disease through $ROR\gamma t^+$ innate lymphoid cells.[67,515,616] The full contribution of such diverse cellular and downstream molecular mediators of this axis in intestinal immunity and inflammation is incompletely described. Similarly, the role of IL-23 responsive γδT-cells, critical to disease in models of extra-intestinal inflammation,[365,368,387,392,614] and known to be present in the healthy colon,[421,617] is unknown and awaits definition.

1.10.3 Regulatory pathways

The presence of large numbers of potentially pathogenic effector T-cells in the intestine without detectable inflammation points to the existence of dominant regulatory mechanisms in the steady state. Indeed, the healthy intestine is enriched for regulatory T-cells, with 30-40% of colonic $CD4^+$ T-cells producing IL-10, the majority of which are $Foxp3^+$ cells Treg populations.[258] The healthy intestine appears to be a site favouring iTreg cell accumulation, under the influence of the microbiota.[244,249] Intestinal Treg cells demonstrate TCR repertoires enriched for reactivity against components of the flora, in contrast to cells within lymphoid tissues.[618,619] Interestingly whereas such TCR specificities show little overlap with steady state effector T-cells, in colitis $CD4^+$ effector T-cells may demonstrate TCRs usually associated with Treg function.[618] However, iTreg and nTreg populations appear to fulfil non-redundant functions in intestinal immune homeostasis[240] and prevention of colitis in $Foxp3^{-/-}$ mice and in disease induced by transfer of

naïve T-cells.[245,620] Furthermore, mice lacking the conserved non-coding sequence CNS1 in the Foxp3 locus have maintained nTreg cells but are unable to generate iTreg, and develop a Th2-type immunopathology in the lung and intestine, demonstrating a specific role for iTregs in controlling mucosal Th2 responses.[240,621]

Relevant pathways mediating Treg cell function in the intestine include TGF-β1, IL-10, IL-35, CTLA-4 and OX40.[255-258,260] Difference in the mechanism of IL-10 dependent control of intestinal inflammation are apparent between effector subsets. Th1 cells express low levels of IL-10R and are controlled in a cell-extrinsic fashion through effects on APCs. In contrast, Th17 cells express high levels of IL-10R and are susceptible to direct regulatory activity.[622] IL-10 prevents emergence of IL-17A^{+} and IL-17A^{+}IFN-γ^{+} cells in a T-cell intrinsic manner, and effector T-cell expression of IL-10R is essential for suppression of disease in experimental colitis.[622] Interestingly, the ability of Treg cells themselves to respond to IL-10 appears critical for their protective functions, since Foxp3^{+} cells deficient in either IL-10R$^{-/-}$ or the downstream mediator STAT3 are unable to prevent Th17 driven colitis.[264,622,623] Validation of the critical role of IL-10 in intestinal inflammation is provided by the association of IL-10R mutations and early-onset refractory IBD.[474,624]

TGF-β1 is a critical regulatory mediator in the intestine, and despite the abundance of other sources, production by CD4^{+} T-cells may be essential to protection from spontaneous colitis.[172] Naïve T-cells unable to respond to TGF-β1 signals are refractory to Treg control and induce severe intestinal inflammation.[259,625,626] Similarly, over-expression of SMAD7, which inhibits TGF-β1 signalling renders effector T-cells refractory to Treg control.[627] Importantly, upregulated T-cell SMAD7 expression has been reported in IBD.[628] IL-35 has recently emerged as a potential mediator of Treg function, and has been reported to be required for full suppressive function in T-cell transfer colitis, including in the cure model of disease.[260,261]

Treg cell activity in the intestine may be regulated by factors involved in effector T-cell function. IL-23 inhibits iTreg cell development whilst promoting Th17 responses.[183,521] IL-21 and IL-27 are both reported to inhibit TGF-β1 mediated Treg development,[611,629,630] favouring effector T-cell activity, whilst IL-12 may drive the emergence of IFN-γ^{+}Foxp3^{+} cells in the inflamed intestine, modifying the balance of effector/Treg cell activity.[631] OX40, a co-stimulatory molecule of the TNF superfamily promotes colitogenic Th1 responses in the colon, but is additionally required for suppressive Treg cell activity.[256] Notably, such activities may be context related, since in contrast to its activity in other inflammatory disease models, IL-27 has recently been shown to paradoxically promote intestinal Treg cell responses during *Toxoplasma gondii* infection.[310]

1.11 Interleukin-23

IL-23 is a heterodimeric cytokine of the IL-12 family which has in recent years assumed prominence for its pathogenic role in a number of experimental models of inflammatory disease, including experimental autoimmune encephalomyelitis (EAE),[164,184,632] and models of autoimmune myocarditis,[633] arthritis,[615,634] psoriasis,[635] and intestinal inflammation.[183,508,515,616] Importantly, IL-23 exhibits tissue specific effects on mucosal inflammation, in contrast to IL-12 which drives systemic immune responses, and therefore represents an attractive therapeutic target.[616]

Comprising a p40 subunit shared with IL-12, and a unique p19 component,[636] IL-23 is produced by DCs and macrophages in response to TLR and NLR signals, and CD40-CD40L interactions.[47,570,614,637] Distal intestinal DCs demonstrate constitutive IL-23 expression,[638] which is highly upregulated in inflammation.[515,616] Specific patterns of PRR ligation favouring IL-23 responses include dectin-1 and combined TLR2/NOD2 stimulation,[570] whereas others such as TLR4 stimulation instead promote IL-12 production.[639] IL-23 expression is also preferentially induced by signals including ATP from the intestinal microbiota,[43] PGE2[640] and downstream of

pathways induced by ER stress,[641] a mechanism increasingly thought to be of importance in the development of IBD.[20,575] The ER stress induced transcription factor C/EBP homologous protein (CHOP) directly targets the IL-23p19 gene.[641] Cytokines including IL-17A and GM-CSF may also enhance expression of IL-23 by APCs.[185,302]

IL-23 signals through a receptor combining the shared IL-12Rβ1 subunit and a specific IL23R, to predominantly activate STAT3, although weak activation of STAT1, STAT4 and STAT5 also occurs.[642] Although initially linked to Th17 cells,[184,643] expression of a functional IL-23 receptor has been reported on a host of lymphoid cells,[67,72,384,392,614,615,617] as well as macrophages and DCs,[541,617] although the relative contribution of each cell type to mediating IL-23 dependent effects is unknown. Regulation of IL-23R expression is incompletely described. IL-12Rβ1 is expressed downstream of IRF-1,[138] whereas IL-23R requires RORγt expression,[175] through STAT3 activating signals including IL-6 and IL-21, and is regulated by TGF-β1 in a dose-dependent manner.[175,211] The microRNA Let-7f has been shown to regulate IL-23R expression in the human system.[644] The functional implications of IBD associated *IL23R* polymorphisms continue to be explored, but the protective R381Q mutation has been shown to reduce cell surface expression of IL-23R and consequent ability to activate STAT3.[645-647] Alternatively, another IBD associated *IL23R* variant has been reported to result in a mutation of the 3'-untranslated region, rendering the gene refractory to microRNA regulation, increasing IL23R expression.[648]

1.12 Interleukin-21

IL-21 was initially described in 2000, following identification of the IL-21R receptor in a screen of cDNA from human lymphoid tissues cells seeking to identify type 1 cytokine receptors.[649,650] Cloning and sequencing revealed a 131-residue four helix-bundle cytokine structurally related to IL-2 and IL-4, with closest similarity to IL-15.[649,650] The human *IL21* gene maps to 4q26-q27, a

region in close proximity to *IL2* and *IL15* genes.[649] Identification and cloning of the murine orthologue of IL-21 revealed a 122-amino acid protein with 57% sequence homology to the human cytokine. Importantly, a number of residues at sites believed important for signal transduction by IL-21R are conserved between the human and murine molecules.[649]

1.12.1 Expression of IL-21

IL-21 protein expression is restricted to lymphoid cell populations in mice and humans, although low-levels of *Il21* mRNA have been reported in macrophages and DCs.[649,651,652] Although NKT and multiple subsets of CD4+ T-cells secrete IL-21, it is not produced by $CD8^+$ T-cells or B-cells.[649] Although naïve human T-cells are capable of *in vitro* IL-21 production,[295] *in vivo* IL-21 production is reported to exclusively occur in $CD4^+CD45RO^+$ memory T-cells,[653-655] whereas murine naïve T-cells may express *Il21* directly *ex vivo*.[656] Initially described as a product of Th2 cells,[657] subsequent studies have documented significant production of IL-21 by Th1,[294,654,658] Th17[176,654] and Tfh cells[276,659] suggesting IL-21 is not regulated as a classic lineage specific cytokine. Indeed, Th17 and Tfh cells appear dominant sources of IL-21 in murine, particularly *in vitro* studies,[176,659] whereas studies of human tissue resident cells report Th1 and Tfh subsets represent the major IL-21 expressing populations.[295,658]

Transcription and secretion of IL-21 by T-cells occurs rapidly following activation via the TCR and is augmented by co-stimulatory signals from CD28 and critically ICOS.[276,280,286,649] Cytokines including IL-6, IL-7, IL-12, IL-15, IL-23, IL-27 and IL-21 itself augment TCR-driven IL-21 production.[176,177,265,276,295,653,654,656,660] Notably, IL-12 is a critical stimulus for IL-21 production by murine and human CD4+ T-cells through activation of STAT3 or STAT4,[294,295] whereas in murine studies, IL-12 may not stimulate IL-21 production.[276,654,656] Furthermore, whereas IL-6 is essential for initial expression of IL-21 by murine CD4+ T-cells no such requirement is evident in humans.[295,656] In human and murine studies, significant functional overlap appears to exist between IL-21 and IL-6, related to their common activation of STAT3 signalling, and in many

settings IL-21 appears to mediate signals downstream of IL-6.[175-177,279,654,656] IL-21 is produced downstream of IL-6 and IL-23 through a STAT3 dependent mechanism, however T-cells carrying STAT3 mutations associated with Job's syndrome are unimpaired in their production of IL-21, demonstrating the *in vivo* activity of non-STAT3 pathways, particularly in the human system.[295,661] Notably, despite characterisation as a Th17 cytokine, transcription of *Il21* is RORγt independent, unlike other signature cytokines of this lineage.[190,279,280] Instead, dependence upon the transcription factor cMaf is apparent, with the IL-21 promoter contains MAF responsive elements, suggesting direct interaction occurs.[265,286,662]

1.12.2 IL-21 Signalling Pathways

IL-21 signals through a heterodimeric receptor comprising IL-21R and the common gamma chain (γ_c) which it shares with the receptors for IL-2, IL-4, IL-7, IL-15, IL-9, IL-15.[649,663] Activation of Jak1 and Jak3 results primarily in tyrosine phosphorylation of STAT3, but may also signal via STAT4, as well as STAT1, STAT5 and STAT6 pathways to a lesser extent.[663-666] IL-21R is expressed on lymphoid cells including T- and B-cells as well as NK cells and APCs.[649,650,667-669] Expression on epithelial and stromal cells has been reported but is less consistently observed, and may occur only in inflammatory or diseased states.[670]

Cell specific differences in the downstream signalling effects of IL-21 have been reported, with STAT3 the only pathway activated in human NK-cells, whereas in T-cells, STAT1, STAT3 and STAT5 activation occurs.[663,664,671] However, other groups have failed to demonstrate STAT5 activation by IL-21, possibly related to differences in the examined T-cell subsets.[653] Similarly, IL-21 induces expression of the transcription factor IRF4 in T-cells but not B-cells.[190] Blimp-1 and the reciprocally regulated Bcl6 have emerged as important targets of IL-21 in T- and B-cells.[289,672,673]

1.12.3 Biological Effects of IL-21

Diverse effects of IL-21 have been reported across multiple lymphoid cell types, including on cellular activation, survival and proliferation. IL-21 signals drive maturation of NK-cells and acquisition of cytotoxicity,[649] and promote IFN-γ production,[649,674,675] but may also limit IL-15 dependent expansion of NK-cells to terminate the innate phase of immunity.[674] In T-cells, IL-21 may enhance TCR-stimulated proliferation in some settings, leading to $CD8^+$ T-cell proliferation, but may have more limited effects on $CD4^+$ T-cell turnover, particularly of mature memory T-cells. IL-$21^{-/-}$/IL$21R^{-/-}$ mice are documented as harbouring normal steady state lymphoid tissue T-cell populations.[674,676] Whereas impaired homeostatic proliferation of IL-$21R^{-/-}$ cells has been reported, antigen driven proliferation was unimpaired.[177,677-679] IL-21 may promote cellular survival via the PI3K pathway and induction of the anti-apoptotic protein Bcl-2,[680,681] although inhibition of expression this latter molecule has also been recorded, with activation of the pro-apoptotic molecules Bim and Bid also noted, consistent with observed pro-apoptotic effects of IL-21 on T- and B-cells.[673,682-684]

IL-21 has consistently been reported as factor promoting Th17 and Tfh differentiation, but effects on Th1 and Th2 subsets are more inconsistent, and seemingly paradoxical. IL-21 was originally reported as a Th2 autocrine factor inhibiting Th1 development,[657] although subsequent molecular analysis suggested specific inhibition of IFN-γ production was occurring through direct repression of eomesodermin, with intact T-bet and IL-12Rβ2 expression.[685] Paradoxically, global promotion of Th1 responses including upregulation of key Th1 genes including IL-12Rβ2 and enhanced IFN-γ production in synergy with IL-15 and IL-18 has been reported in other experimental settings.[664,675] In *Toxoplasma gondii* infection, in which Th1/IFN-γ responses are required for survival of the acute phase of disease, IL-$21R^{-/-}$ mice have unimpaired IFN-γ responses and display similar survival to WT controls.[676,686] In models of graft-versus host disease (GvHD), IL-$21R^{-/-}$ T-cells show impaired Th1 and Th2 development, but comparable Th17 and increased $Foxp3^+$ Treg populations.[687] In other studies, addition of IL-21 to $CD4^+$ splenocytes differentiating *in vitro* had no effect on resulting Th1 (IFN-γ) or Th2 (IL-4) responses.[676]

The activity of IL-21 as a STAT3 signal driving Th17 cell development and *Il23r* expression is well documented.[654] *In vitro* differentiation studies report defective induction of *Il23r* in response to stimulation with TGF-β1 + IL-6 in IL-21$^{-/-}$ or IL-21R$^{-/-}$ CD4^{+} T-cells, supporting an essential role for IL-21 in Th17 biology.[175] Dose-dependent inhibition of TGF-β1 dependent upregulation of Foxp3 by IL-21 has been reported *in vitro*, but may not occur at physiological levels of TGF-β1, or is compensated for by other mediators in vivo, since IL-21$^{-/-}$ demonstrate normal Treg development.[176,611] Importantly, activation of effector T-cells in the presence of IL-21 releases them from Treg control.[177,678,688] A role in the development of IL-10^{+}Foxp3^{-} Tr1 cells has been reported, downstream of IL-27, with a 90% reduction in such cells recorded in IL-21$^{-/-}$ mice.[265]

IL-21 synergises with IL-15 to regulate CD8^{+} T-cell function, but exerts specific, non-overlapping effects to maintain CD28 and CD62L expression on effector cells,[689] and although IL-21 alone prevents CD44 expression, in the presence of IL-15 it enhances accumulation of CD44hi populations.[690] Similar synergy is apparent for effector function, since IL-21 alone may not affect IFN-γ production, but can augment IL-15 driven secretion.[649,674,675] IL-21 is critical for anti-viral CD8+ T-cell responses, with impaired antigen-specific proliferation and functional maturation apparent in deficient animals, and a rapid loss of function and immune exhaustion in the setting of chronic viral infection in the absence of T-cell expression of IL-21R.[651,691,692] IL-21 regulated transcriptional programs have been explored in some detail in CD8^{+} T-cells, and reveal co-operation in the regulation of gene expression by STAT3 and IRF4.[289] Importantly, IL-21 may be critical for memory CD8^{+} T-cell responses, through a STAT3 and IL-10 dependent pathway.[693]

IL-21 may drive pathogenic effects via DCs, driving maturation and enhancing antigen presentation and expression of co-stimulatory molecules,[668] and favouring Th17 responses.[669] In contrast, in some systems, IL-21 may favour alternative activation of macrophages.[694]

In B-cells, IL-21 augments proliferation induced by stimulation through CD40, but is inhibitory where the stimulus is IgM or IL-4, although it has no direct effects on proliferation by itself.[649] Class switching and production of IgG1, IgG2b and IgG3 antibodies may be impaired by IL-21/IL-21R deficiency, although in other scenarios variable effects on IgG subtypes have been noted, excepting IgG1 which is consistently reduced in a range of experimental settings.[656,667,676,695,696] The differing effects of IL-21 may reflect subtle differences in the composition of the cellular populations studied. However, $IgG1^+$B-cells appear to be highly sensitive to the absence of IL-21 signals and specifically show reduced survival compared to those expressing other isotypes.[697,698] Importantly, IgE levels are higher in the blood of unmanipulated $IL\text{-}21^{-/-}$/$IL\text{-}21R^{-/-}$ mice, and IgE responses to immunisation are increased,[667,676] in an Id2 and IL-4 dependent fashion,[699] and notably polymorphisms in human *IL21R* (T-83C) are associated with increased serum IgE levels.[700,701] Stimulation of WT and $IL\text{-}21R^{-/-}$ B-cells *in vitro* with anti-CD40 + IL-4 or LPS + IL-4 revealed no defect in IgG1 and IgE responses in the absence of IL-21R, but increased IgG1 responses to all stimuli on addition of IL-21, showing a cell intrinsic requirement for IL-21 for normal IgG1 production.[676] Differentiation of B-cells to mature plasma cells occurs under the influence of IL-21, with down-regulation of Bcl6 and up-regulation of Blimp-1.[673,702] Germinal centre formation is unimpaired in the absence of IL-21, but constituent B-cell numbers may be reduced, and follicles reduced in size.[280,667,695,696,703]

1.12.4 Role of IL-21 in Inflammatory and Neoplastic Disease

Predictably, ablation or stimulation of IL-21 signalling has multiple effects in diverse disease settings. IL-21 is elevated in psoriatic skin, and in experimental models drives skin thickening through accumulation of $IL\text{-}17A^+$ and IFN-γ^+ $CD4^+$ T-cells.[704,705] Whereas unmanipulated $IL\text{-}21^{-/-}$ mice display elevated IgE levels,[676] IL-21 treated mice are resistant to allergy and anaphylaxis in some settings,[699,706] In EAE, $IL\text{-}21R^{-/-}$ mice display a non-protected or even exacerbated phenotype,[677,707-709] although recombinant IL-21 administered at the initiation of disease also increases disease severity, whereas no effect is apparent with treatment later in disease.[677] In

GVHD, IL-21 favours effector over regulatory responses,[687] driving pathogenic IFN-γ^+ T-cell responses at the expense of Treg cells.[710] IL-21 has been implicated in the pathogenesis of SLE, rheumatoid arthritis and Sjogrens syndrome, and in models of these diseases, IL-21 drives pathogenic Tfh cell development and antibody responses.[711-718] However, paradoxical effects of blocking IL-21 related to the timing of treatment have been reported in some models. In models of infection, IL-21 may exert dominant protective or pathogenic effects, although IL-21 has consistently been shown to be required for chronic anti-viral responses.[651,691,692] IL-21 has been reported to exert profound anti-neoplastic effects in multiple murine systems through upregulation of NK and $CD8^+$ T-cell cytotoxicity,[719-722] and recombinant IL-21 is now in clinical trials for the treatment of malignant melanoma.[723,724] A SNP in the IL-2/IL-21 gene cluster is associated with type I diabetes mellitus,[725,726] and IL-$21^{-/-}$/IL-$21R^{-/-}$ mice are protected in the NOD model of disease.[668,727,728] IBD is also associated with a SNP in the IL-2/IL-21 locus,[729,730] with IL-21 expression increased in areas of active CD,[658,670,731] and IL-21 ablation protective in several models of disease, although the mechanisms are largely unexplored.[609,611,612]

1.13 Aims of Thesis

Accumulating evidence supports a critical role for IL-23 in animal models of intestinal inflammation and defence from infection, and in human IBD, yet aspects of the cellular and molecular mediators of this axis in homeostasis and disease are uncertain.

The aims of this thesis were therefore:

i) To characterise the *in vivo* cellular distribution and regulation of IL-23R expression in homeostasis and in intestinal inflammation

ii) To explore the role of γδT-cells, including IL-23R expressing populations, in chronic intestinal inflammation

iii) To define the contribution of the IL-23/Th17 associated cytokine IL-21 to host defence and inflammatory disease in the intestine, and the molecular mechanisms underlying its effects

Chapter 2. Materials and Methods

2.1 Mice

Wild-type (WT) C57BL/6 (B6), B6.IL-21R$^{-/-}$, B6.TCRδ$^{-/-}$, B6.IL-23R$^{gfp/gfp}$, B6.IL-23R$^{gfp/+}$, B6.Rag1$^{-/-}$, and B6.SJL-CD45.1 mice were bred and maintained in individually vented cages under specific pathogen free (SPF) conditions within accredited facilities at the John Radcliffe Hospital, University of Oxford. B6.IL-21R$^{-/-}$ mice were originally obtained from Zymogenetics (USA) and subsequently used under licence from NovoNordisk (Denmark). B6.IL-23R$^{gfp/gfp}$ mice were provided by Vijay Kuchroo and Mohamed Oukka (Harvard Medical School, USA). B6.TCRδ$^{-/-}$ were purchased from Jackson Laboratories (Maine, USA). Mice were routinely screened for *Helicobacter spp.* and were 6-12 weeks old when used unless otherwise specified. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

2.2 Isolation of Cells

2.2.1 Lamina Propria Leukocytes

Intestines were dissected free from rectum to ileocaecal valve and cleaned of adherent mesentery, before being opened longitudinally. Following removal of the luminal contents by washing, intestines were dissected into 0.5cm pieces and placed on ice in 10ml PBS/0.1%BSA (both PAA, Yeovil, UK) in a 50ml Falcon tube (BD Biosciences, Oxford, UK). Further processing commenced with transfer to 10ml pre-warmed (37°C) RPMI 1640 (Sigma, Poole, UK) supplemented with 5% FCS (Sigma) and 5mM EDTA (VWR International UK, Lutterworth, UK). Samples were incubated and shaken (200rpm) for 30 minutes at 37°C prior to aspiration of the supernatant and further incubation for 30 minutes in fresh RPMI/FCS/EDTA. Supernatants, containing epithelial cells and IELs, were reserved for processing as required. Tissue pieces were incubated for 10 minutes at room temperature in 10ml RPMI 1640 supplemented with 5% FCS and 15mM Hepes (PAA) to neutralise EDTA activity. After aspiration of the supernatant, 10ml

of RPMI/5%FCS/15nM Hepes containing 0.4-0.6mg/ml type VIII Collagenase (Sigma) was added to each tube, followed by incubation for 1 hour at 37°C with shaking (200rpm). Supernatants containing liberated LP cells were collected by filtration through a 70µm filter into 20ml RPMI/5%FCS/EDTA, prior to washing in ice cold PBS/0.1%BSA. Cells were collected by centrifugation at 1500rpm for 5 minutes, and resuspended in 3ml of 30% Percoll (GE Healthcare) in PBS (P30), then layered over pre-prepared gradients of 3ml 75% Percoll in PBS (P75) and 4ml 40% Percoll in RPMI (P40) in 15ml tubes (Falcon, BD Biosciences). Gradients were centrifuged at 1800rpm for 20 minutes without brake, and leukocytes enriched for lymphocytes were collected from the P40/P75 interface. Cells were twice washed in ice cold PBS/0.1%BSA to remove Percoll, and stored on ice prior to further processing. Small intestinal samples were processed in an identical way, except FCS was used at 10% in all appropriate solutions, and a lower concentration of EDTA (2mM) was used. Cell numbers were determined using a haemocytometer.

2.2.2 Intraepithelial Lymphocytes

Supernatants isolated after the EDTA incubation step in the above protocol for the isolation of LPLs were filtered through 70µm filters into ice cold PBS/0.1%BSA. Following washing in PBS/0.1%BSA cells were centrifuged for 5 minutes at 1500rpm and resuspended in 6ml 40% Percoll in RPMI (P40) and layered above 4ml 75% Percoll in PBS, in a 15ml tube. Following centrifugation at 1800rpm for 20 minutes, cells at the P40/P75 interface were collected and washed three times in ice cold PBS/0.1%BSA before immediate analysis. Cell numbers were determined using a haemocytometer.

2.2.3 Spleen and Lymph Nodes

Single cell suspensions were prepared by disruption of individual tissue samples through a 70µm filter using an inverted syringe plunger and collected into ice cold PBS/0.1%BSA. Samples were

pelleted by centrifugation at 1500rpm for 5 minutes, after which spleen samples underwent red cell lysis using 1ml ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2-7.4) for 3 minutes, followed by washing in PBS/0.1%BSA. Cells were resuspended in PBS/0.1%BSA and stored on ice prior to analysis. Cell numbers were determined using a haemocytometer.

2.3 Flow Cytometry

Stained cells were acquired on either an LSRII or FACS Fortessa flow-cytometer using DIVA software (all BD Biosciences), and analysed using FlowJo software (Treestar Inc). Reagents are described in Table 2.1

Marker	Clone	Conjugate Dye	Manufacturer	Conc.
Surface Staining				
CD16/32 (Fc block)	93	N/A	eBioscience	5 µg/ml
Fixable viability Dye	n/a	Aqua (Pacific orange), e780	Invitrogen or eBioscience	n/a
B220	RA3-6B2	FITC	Biolegend	1µg/ml
CD4	GK1.5	PerCP-Cy5.5,PE-Cy7,QDot605	Biolegend	1µg/ml
CD8a	53-6.7	FITC	Biolegend	1µg/ml
CD8b	YTS156.7.7	e450	Biolegend	1µg/ml
CD11b	M1/70	PE	eBioscience	1µg/ml
CD11c	N418	PE-Cy7	Biolegend	1µg/ml
CD25	7D4	FITC, PE	eBioscience	1µg/ml
CD27	L6.3A10	APC, PE	Biolegend	1µg/ml
CD44	IM7	APC,PE	eBioscience	1µg/ml
CD45	30-F11	PerCP-Cy5.5	Biolegend	0.5µg/ml
CD45.1	A20	PE	eBioscience	1µg/ml
CD45.2	104	PerCP-Cy5.5	eBioscience	1µg/ml
CD45RB	C363-16A	PE	eBioscience	1µg/ml
CD62L	MEL-14	APC,PE	eBioscience	2µg/ml
D69	H1.2F3	FITC, PE	Biolegend	2µg/ml
CD127	A7R34	PE-Cy7	BD Biosciences	2µg/ml
CXCR5	26B	PE	BD Biosciences	2µg/ml
F4/80	BM8	APC	Biolegend	2µg/ml
Gr1	RB6-8C5	FITC	eBioscience	0.5µg/ml
MHCII	M5/114.15.2	e450	eBioscience	0.5µg/ml
NK1.1	PK136	FITC	Biolegend	1µg/ml
NKp46	29A1.4	e450	Biolegend	1µg/ml
Thy1.2	30-H12	PE-Cy7	eBioscience	0.5µg/ml
Sca-1	E13-161.77	AF700	eBioscience	1µg/ml

Vγ1	2.11	PE, DyeLight647	P.Pereira, Institute Pastuer	2µg/ml
Vγ4	UC3-10A6	PE, DyeLight647	P.Pereira, Institute Pastuer	2µg/ml
Vγ7	F2.67	PE, DyeLight647	P.Pereira, Institute Pastuer	2µg/ml
Vγ5	536	APC	Biolegend	2µg/ml
TCRβ	H57-597	APC	Biolegend	2µg/ml
TCRγδ	GL3	FITC,PE,APC,e450	Biolegend	2µg/ml
Intracellular Staining				
IL-5	TRFK5	PE, APC	eBioscience	2µg/ml
IL-17A	eBio1787	PE,FITC	eBioscience	2µg/ml
IL-17F	9D3.1C8	APC	Biolegend	2µg/ml
IL-22	1H8PWSR	PE	eBioscience	2µg/ml
IFN-γ	XMG1.2	e450, PE-Cy7, APC	eBioscience	2µg/ml
Foxp3	FJK-165	e450	eBioscience	2µg/ml
GM-CSF	MP-122E9	PE	BD Biosciences	2µg/ml
Lineage Markers				
CD3	17A2	PerCP-Cy5.5	eBioscience	1µg/ml
B220	RA3-6B2	PerCP-Cy5.5	eBioscience	1µg/ml
CD11b	M1/70	PerCP-Cy5.5	eBioscience	1µg/ml
CD11c	N418	PerCP-Cy5.5	eBioscience	1µg/ml
Gr1	RB6-8C5	PerCP-Cy5.5	eBioscience	1µg/ml
Isotype Controls				
IgG1	R3-34	FITC, PE, APC	BD Biosciences or eBioscience	2µg/ml
IgG2a	R35-95	FITC, PE, APC	eBioscience	2µg/ml
IgG2b	A95-1	FITC, PE-Cy7	eBioscience	2µg/ml
CD4 Enrichment				
CD4	YTA 3.1	N/A	In-house. Hybridoma courtesy of H.Waldmann, University of Oxford.[732]	~10 µg/ml
CD4	GK1.5	N/A	In-house. Hybridoma courtesy of DNAX, USA.[733]	~10 µg/ml
CD8	YTS169	N/A	In-house. Hybridoma courtesy of H.Waldmann, University of Oxford.[732]	~10 µg/ml
MHCII	TIB120	N/A	In-house. Hybridoma from ATCC, USA.[7343]	~10 µg/ml
Mac-1 (CD11b)	M1/70	N/A	In-house. Hybridoma from ATCC, USA.[735]	~10 µg/ml
B220	RA36B2	N/A	In-house. Originally from DNAX, USA.[736]	~10 µg/ml
***In Vivo* Use**				
IL-10R (blocking)	1B1.2	N/A	In-house. Hybridoma originally from DNAX, USA.[737]	1mg/week ip
IFN-γ (blocking)	AN18	N/A	In-house. Hybridoma courtesy of J.Langhorne, NIMR.[738]	1mg3x/wk ip

Table 2.1 Antibodies used for in vitro and in vivo studies

2.3.1 Surface Staining

Cells were plated at $1x10^6$ cells/well in 96-well round bottom plates and resuspended in 100µl PBS containing fixable viability dye (see table) and anti-CD16/32 (Fc receptor block) (eBioscience) for 20 minutes at room temperature. Following washing with PBS, cells were incubated for 15 minutes on ice in the dark, with 50µl of antibody mix in PBS/0.1%BSA (Table 2.1), followed by multiple washing steps using PBS/0.1%BSA. Cells were resuspended in 200µl PBS and analysed immediately or fixed using 100µl PBS/2%PFA for 10 minutes on ice, followed by washing with PBS/0.1%BSA and analysis within 48 hours.

2.3.2 In vitro restimulation and intracellular cytokine staining

Cells were plated at $1x10^6$ cells/well in 96-well round bottom plates (Corning) in 200µl complete IMDM medium (Gibco) (supplemented with 10% FCS, 100U penicillin/streptomycin (PAA) and 0.05mM 2-mercaptoethanol (Sigma)), containing 0.1µg/ml PMA (phorbol myristate acetate) and 1µg/ml ionomycin (both Sigma), with 1µl/ml Brefeldin A (eBioscience). Following 4 hours incubation at 37°, cells were washed three times with PBS/0.1%BSA, with centrifugation at 1500rpm for 5 minutes. Cells were immediately stained for viability and surface markers, prior to fixation in 50µl fixation buffer (eBioscience) and resuspension in 100µl permeabilisation buffer (eBioscience) for 30 minutes in the dark on ice. Cells were pelleted by centrifugation at 1700rpm for 3 minutes, and resuspended in 50µl permeabilisation buffer containing relevant antibodies for 30 minutes in the dark on ice. Following 3 washing steps with permeabilisation buffer and centrifugation at 1700rpm for 3 minutes, cells were suspended in 200µl PBS/0.1%BSA and analysed within 48 hours. For intracellular staining where preservation of the gfp signal was required, fixation/permeabilisation steps were perfomed using buffer diluted 50:50 in PBS, and incubation was reduced to 10 minutes on ice.

2.4 Cell Sorting

Single cell suspensions were prepared as described above, and sorted on the basis of surface markers or gfp expression as appropriate using a FACS Aria (BD Biosciences).

For the isolation of $CD4^+$ T-cells for adoptive transfer, MLN and spleen cells were pre-enriched for $CD4^+$ cells by incubating in PBS/0.1%BSA containing a mixture of IgG antibodies to CD8, MHCII, CD11b and B220 for 20 minutes on ice, followed by resuspension at $1x10^8$ cells/ml in PBS/0.1%BSA containing $1x10^8$ beads/ml anti-IgG coated magnetic Dynal beads (Invitrogen), and incubation at 4° for 20 minutes on a rotary mixer. Thereafter, a magnet was applied to the mixture for 30-60 seconds, and the CD4 enriched cell suspension collected, filtered (70µm) and washed with PBS/0.1%BSA. Cells were suspended in PBS/0.1%BSA at $2.5x10^7$ cells/ml for sorting.

Cells sorted for in vitro analysis or adoptive transfer were collected into 200µl 100% FCS in polystyrene tubes on ice. For RNA analysis, cells were collected directly into RLT lysis buffer (Qiagen) and homogenised by vortex mixing for 2 minutes.

2.5 Gene Expression Analysis

2.5.1 RNA extraction

RNA was extracted from whole intestinal tissue samples snap frozen in liquid nitrogen and stored at -80° until analysis, by homogenisation using TRI Reagent (Ambion) in Lysing Matrix D tubes (MP Biomedical) at 6.5m/s for 2x15 seconds with a 30 second interval between steps, in a FastPrep-24 homogeniser (MP Biomedical). RNA was isolated using a RiboPure Kit (Ambion) according to the manufacturer's instructions, and quantified by Nanodrop (Thermo Scientific) analysis. RNA extraction from sorted single cell suspensions was performed using an RNeasy mini kit according to the manufacturer's suggested protocol, and quantified by Nanodrop analysis. Extracted RNA was processed on ice, and snap frozen on dry ice prior to storage at -80°C.

2.5.2 Real time quantitative PCR analysis

cDNA was synthesised from 1-5µg total RNA using Superscript III reverse transcriptase and oligo dTs (Invitrogen) according to manufacturers instructions. qRT-PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Glasgow, UK) according to the manufacturer's instructions, and assayed in triplicate in a total reaction volume of 20µl using a BioRad CFX96 machine. RNA processed without the addition of reverse transcriptase (-RT) was included as a negative control, and gene expression for individual samples normalised to expression of the housekeeping gene *Hprt1*. Expression was determined using the $2^{-\Delta Ct}$ method (ΔCt=Ct (gene of interest) – Ct (Hprt)), and relative expression by the $2^{-\Delta\Delta Ct}$ method as indicated.

2.5.3 RT-PCR for Vγ Profile

cDNA was synthesised as above from sorted γδT-cells, followed by amplification of 2-5µg by TaqMan Universal Mastermix (Invitrogen) and primers as detailed in Table 2.2 in a total reaction volume of 25µl, using a Techne thermal cycler (Bibby Scientific, UK). Denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute was performed for 40 cycles. Products were visualised on a 1.5% agarose gel containing GelRed (Cambridge Bioscience, UK).

Forward Primer	**Forward Sequence (5'-3')**	**Reverse Primer**	**Reverse Sequence (5'-3')**
Vγ1	CCGGCAAAAAGCAAAAAAGT	Cγ4	AAGGAGACAAAGGTAGGTCCCAGC
Vγ2	TTGGTACCGGCAAAAAACAAATCA	Cγ2	CAATACACCCTTATGACATCG
Vγ4	CTTGCAACCCCTACCCATAT	Cγ1	CCACCAC~TCGTTTCTTTAGG
Vγ5	GAGGATCCCGCTTGGAAATGGATGAGA		
Vγ6	GATCCAAGAGGAAAGGAAAGACGGC		
Vγ7	GATCCAACTTCGTCAGTTCCACAAC		
Positive Control			
B-actin F	GCTTCTTTGCAGCTCCTTCGTTG	B-actin R	TTCTCCATGTCGTCCCAGTTGG

Table 2.2 Primer sequences for studies of Vγ Profile (Originally from Ref[732])

2.6 Induction of Intestinal Inflammation

2.6.1 *Helicobacter Hepaticus*/anti-IL-10R model

H.hepaticus NCI-Frederick Isolate 1A (strain 51449 American Type Culture Collection) was grown at 37°C on blood agar plates containing TVP (trimethoprim (5µg/ml), vancomycin (10µg/ml) and polymixin B (25IU/ml)) (Oxoid, Basingstoke, UK) under microaerophilic conditions (10% CO_2, 10% H_2, balance N_2) in vented jars (Oxoid), before transfer to liquid culture in tryptone soya both (TSB, PAA) containing 10% FCS and TVP in a vented Erlenmeyer Flask (Corning) and ongoing culture at 37°C in microaerophilic conditions with shaking at 180rpm. Cultures were re-inoculated daily at OD_{600} 0.05. Bacterial viability was confirmed using BacLight live/dead fluorescent assay (Invitrogen), prior to harvesting of bacteria by ultracentrifugation at 7500rpm for 20 minutes.

Mice were gavaged with $1x10^8$ CFU *H.hepaticus* on 3 consecutive days, and treated with weekly intraperitoneal injection of 1mg anti-IL10R antibody (clone 1B1.2) affinity purified from the supernatants of hybridomas maintained in house, and shown to contain <1U/mg endotoxin (Lonza, Basel, Switzerland), beginning on the day of initial infection.

Mice were sacrificed at the indicated time points, and blood and tissues (colon, caecum, spleen, MLN) harvested for analysis. Caecal contents were snap frozen for assessment of *H.hepaticus* colonisation, and ~0.5cm samples from caecum and colon (proximal, middle and distal) were collected into 3.6% (w/v) formaldehyde (GPR Rectapur) in saline solution for histological assessment. Caecal tissue and pooled samples (~0.3cm) from proximal, middle and distal colon were snap frozen in liquid nitrogen for RNA extraction.

Where indicated, mice were treated by daily gavage of 1.0mg/kg FTY720 (Cambridge Biosciences, UK) or diluent (PBS).

2.6.2 T-cell Transfer Disease

Live splenic $CD4^{+}CD25^{-}CD45RB^{hi}$ naïve T-cells were isolated by cell sorting as described in section 2.4. Colitis was induced by intraperitoneal injection of $4x10^5$ cells in to $B6.Rag^{-/-}$ recipients. In co-transfer experiments, $4x10^5$ total cells of a 1:1 mixture of the indicated genotypes were used. Recipient mice were monitored by weighing and for signs of diarrhoea. Experiments were terminated at the development of disease in the majority of animals, usually occurring after 6-8 weeks. Samples were collected at sacrifice as detailed in section 2.6.1.

2.6.3 *Citrobacter rodentium* Disease

C.rodentium (strain ICC168, provided by Gad Frankel, Imperial College London) was grown by overnight culture in LB Broth (Sigma) supplemented with 50µg/ml Nalidixic Acid (Sigma-Aldrich) on a shaker at 150rpm and 37°C. Bacteria were concentrated 10-fold by centrifugation and resuspension in PBS before gavage into mice co-housed for >4/weeks prior to use. Bacterial dose administered was confirmed by plating of serial dilutions on LB agar plates containing 50µg/ml Nalidixic Acid, and was routinely $1\text{-}3x10^9$ CFU/mouse. Mice were monitored by daily weighing. Stool was collected at indicated time points and at termination tissues were collected for assessment of colonisation levels. Samples were homogenised using Lysing Matrix D tubes (MP Biomedical) at 6.0m/s for 2x20 seconds with a 30 second interval between steps, in a FastPrep-24 homogeniser (MP Biomedical). Homogenates were serially diluted and plated onto Nalidixic Acid containing LB agar plates, with colonies quantified after overnight incubation. Tissue from caecum and distal colon were snap frozen for RNA analysis, and similar samples collected into 3.6% (v/w) formaldehyde solution for histological assessment of intestinal inflammation.

Where indicated, mice were treated with blocking anti-IFN-γ (clone AN18), 1mg ip 3x/week or isotype control.

2.7 Generation of Mixed Bone Marrow Chimeras

B6.Rag$^{-/-}$ mice were twice irradiated with 550 RAD in a ^{60}Co source irradiator within a 4 hour interval, before intravenous injection with 5x10^6 cells from a 1:1 mixture of bone marrow cells harvested from CD45.1$^+$ mice and CD45.2$^+$ WT or CD45.2$^+$ IL-21R$^{-/-}$ mice. Single cell suspensions of bone marrow were prepared by flushing the cleaned femurs and tibias of mice with ice cold HBSS/0.1%BSA, and filtering (100µm) of the resulting cells, followed by ACK lysis (see above) and washing. Mice were allowed to reconstitute for 12 weeks prior to experimental use.

2.8 Histological Assessment of Colitis

Samples for histological analysis were fixed in 3.6% (v/w) formaldehyde solution in PBS and cut to 4µm sections using a microtome, followed by staining with haematoxylin and eosin. Severity of colitis was determined in a blinded fashion by 2 independent observers, assessed in sections from proximal, middle and distal colon in *H.hepaticus*/anti-IL-10R and T-cell transfer disease, and distal colon alone in *C.rodentium* infection (Table 2.3). The average of the scores from each section was calculated as an overall colitis score, and the mean of these values from each assessor used for statistical analysis. Caecal inflammation was examined separately using the same scoring system. Scores in *H.hepaticus*/anti-IL-10R and T-cell transfer disease are up to 12, for *C.rodentium* up to 15.

Where indicated, severity of colitis was also assessed by endoscopic examination, using a Coloview Miniendoscope HD system (Karl Storz, Tuttlingen, Germany). Immediately upon sacrifice, the endoscope was inserted into the rectum and advanced for up to 4cm under direct vision with air insufflation. Colitis was assessed using the validated murine endoscopic index of colitis severity.[733]

	Score			
Criteria	0	1	2	3
Epithelial Hyperplasia and/or Goblet Cell Depletion	None /None	Mild (1.5x) /Mild (25%)	Moderate (2-3x) / Moderate (25-50%)	Severe (>3x) / Substantial (>50%)
Lamina Propria Inflammation	None – few leukocytes	Mild – some increase in leukocytes at tips of crypts **OR** many lymphoid follicles	Moderate – marked infiltrate (notable broadening of crypt)	Severe – dense infiltration throughout
Area Affected (% of section)	None	<25%	25-50%	>50%
Markers of Severe Inflammation	None	Submucosal inflammation **OR** few crypt abscesses (<5)	Submucosal inflammation **AND** few crypt abscesses (<5) Many crypt abscesses (>5) **OR** extensive submucosal inflammation **OR** crypt branching	Many crypt abscesses (>5) **AND** extensive submucosal inflammation **OR** Ulceration **OR** Extensive fibrosis
Additionally for *C.rodentium* only:				
Submucosal Inflammation	None	Mild	Moderate (marked oedema **OR** marked infiltration)	Severe (marked oedema **AND** marked infiltration)

Table 2.3 Scoring criteria for histological assessment of colitis severity

2.9 Quantification of *H.hepaticus* colonisation

DNA was extracted from caecal contents using the Stool DNA Isolation Kit (Qiagen, Crawley, UK) according to the manufacturer's suggested protocol. *H.hepaticus* colonisation was quantified by quantitative real time PCR using primers specific for the *cdtB* gene (Forward CCGCAAATTGCAGCAATACTT, Reverse TCGTCCAAAATGCACAGGTG, Probe

AATATACGCGCACACCTCTCATCTGACCAT (Sigma-Aldrich)) using a BioRad CF96 machine, with reference to a standard curve generated by serial dilution of *H.hepaticus* DNA derived from pure culture using a DNeasy kit (Qiagen) according to the manufacturer's instructions. 20ng of total caecal DNA was added to each reaction in a final volume of 25µl.

2.10 *C.rodentium* Specific Immunoglobulin Quantification

NUNC Maxisorp 96-well flat bottom ELISA plates (eBioscience) were coated for 12 hours at room temperature with 200µl/well heat-killed homogenised *C.rodentium* grown by overnight culture as described in section 2.6.3 followed by washing and blocking of plates with 10% BSA. Blood was collected at sacrifice by cardiac puncture and centrifuged in a serum separator tube (BD Biosciences), with collected serum frozen at -20°C until analysis. Supernatants were added to wells in triplicate at consecutive 10-fold dilutions (in PBS) from neat to $1:1x10^7$ and incubated for 1 hour at room temperature. After repeated washing (ELISA Wash Buffer, R&D Systems, Abingdon, UK), HRP-conjugated antibodies specific for IgG1, IgG2b, IgG2c, IgG3 and IgM (Southern Biotech, Alabama, USA) were added at pre-optimised concentrations and incubated at room temperature for 1 hour. Unbound antibody was removed by further washing steps, before the addition of Substrate Reagent (R&D Systems) and further incubation at room temperature for 30 minutes. The reaction was stopped by addition of 2N H_2SO_4 stop solution (R&D Systems) and plates immediately read at 450nm using an LumiStar Optima plate reader (BMG Labtech, Offenberg, Germany).

For the measurement of total stool IgA, samples were homogenised in PBS as described in section 2.6.3 and solid material discarded. Samples were analysed by sandwich ELISA, using an ELISA development kit from R&D Systems with an HRP-conjugated anti-IgA detection antibody (Southern Biotech) and quantitation by reference to a 4-point fitted standard curve created using Prism Software.

2.11 Antibiotic Treatment of IL-23R$^{gfp/+}$ mice

Breeding pairs of C57BL/6 female and B6.IL-23R$^{gfp/gfp}$ male mice were created at 8 weeks of age and treatment commenced with antibiotic supplemented water, containing ampicillin (1mg/ml), neomycin (1mg/ml), metronidazole (1mg/ml), gentamicin (1mg/ml) and vancomycin (0.5mg/ml) (all Sigma-Aldrich). Water was changed every 3 days, and mice were treated for up to 24 weeks. At weaning, resulting IL-23R$^{gfp/+}$ offspring were maintained on antibiotic containing water, until sacrifice at ~8 weeks of age. Successful ablation of the flora was confirmed at sacrifice by characteristic massive caecal enlargement and by gram staining of caecal contents to reveal massively depleted live bacterial numbers.

2.12 Statistics

Due to the non-normally distributed data generated from *in vivo* studies, non-parametric statistical tests were used for all analyses except for weight loss and kinetic studies where ANOVA with a Bonferroni correction was applied. All analyses were performed using Prism 6.0 (Graphpad Software, California, USA). Data is depicted as mean+/- SD or +-/SEM as appropriate and as indicated in figure legends. Results were considered statistically significant where $p \leq 0.05$.

Chapter 3. Characterisation of IL-23R Expression in Intestinal Homeostasis and Inflammation

3.1 Introduction

A critical role for IL-23 in mediating immune and inflammatory reactions in the intestine has emerged in recent years. Genetic ablation or pharmacological blockade of IL-23 is protective in a number of murine models of inflammatory bowel disease,[67,183,508,515,520,521,741] whilst genome-wide association studies in patients with IBD have reported robust associations between susceptibility to IBD and single nucleotide polymorphisms (SNPs) within genes involved in IL-23 signalling.[461-463,473] Furthermore, such studies have implicated SNPs in *IL23R* in both Crohn's disease and ulcerative colitis, strengthening the association of this pathway with intestinal inflammation.

Significant attention has focused upon defining the role of IL-23 in Th17 biology, however accumulating evidence supports a role for other cellular mediators of IL-23 signals. Critically, the development of IL-23 dependent models of acute or chronic colitis in *Rag*$^{-/-}$ mice lacking T-cells demonstrates an *in vivo* functional role for non-Th17 cell targets of IL-23.[67,515,742] Furthermore, a large and diverse range of cells have been reported to express *Il23r* or to respond to IL-23 stimulation in a variety of experimental settings, including γδT-cells,[392,614,617] mucosa associated invariant T (MAIT)-cells,[321] NKT-cells,[743] NKp46^{+} cells,[72] lymphoid tissue inducer (LTi) and LTi-like cells,[546,744] Thy1^{+}Sca-1^{+} innate lymphoid cells,[67] and myeloid cells including macrophages and CD11c^{+} dendritic cells.[541,617] However, the *in vivo* targets of IL-23 in homeostatic and inflammatory settings in the intestine remain unclear.

Many of these IL-23 responsive populations described share molecular characteristics with Th17 cells, including expression of RORγt and the elaboration of signature cytokines including IL-17A, IL-21, IL-22 and GM-CSF.[384,392,614,745] However, studies suggest significant differences in the

regulation of their development, and many of the more innate-like populations may be relatively limited and stereotyped in their cytokine responses, in contrast to the apparent plasticity and potential polyfunctionality of conventional Th17 populations.[409,410,746,747]

Furthermore, it is increasingly evident that not all Th17 cells are pathogenic, and aspects of the IL-23/Th17-type pathway are critically required for host defence. Genetic deficiency or the development of endogenous neutralising antibodies against IL-17A or IL-17F is associated with chronic mucocutaneous candidiasis.[748-749] In mice, IL-23 dependent production of IL-22 from LTi-like cells is required to survive infection with the *E.coli* like organism *Citrobacter rodentium,*[541] and deficiency of IL-17A is associated with impaired recovery from intestinal epithelial injury induced by dextran sodium sulphate (DSS).[592] However, the contribution to these homeostatic or protective effects from IL-23 responsive non-Th17 cell populations is poorly defined.

IL-23 driven inflammatory pathways represent attractive therapeutic targets in IBD, and IL-12/23p40 inhibitors have shown promise in initial trials for the treatment of Crohn's disease,[751] although the extent to which this reflects blockade of IL-23 or IL-12 pathways is unclear.[587] However, a recent trial of anti-IL-17A therapy in Crohn's disease reported significant inferiority to placebo in control of disease activity, and an increased susceptibility to intestinal infection in patients receiving the active drug.[594] Therefore, it is clear that the development of more efficacious and specific therapeutics targeting IL-23 driven responses in IBD will require a better understanding of the cellular and molecular mediators of this pathway in host defence and inflammatory settings.

We therefore undertook studies to define the *in vivo* cellular distribution and regulation of IL-23R expression in healthy and inflamed murine intestine

3.2 Results

3.2.1 Validation of IL-23R$^{gfp/+}$ reporter mice for studies of IL-23R expression

The identity, distribution, and function of cellular targets of IL-23 *in vivo* are poorly defined, in part reflecting a lack of reliable reagents to detect the IL-23R at the cellular level. We therefore took advantage of a recently described mouse strain in which the insertion of an IRES-GFP (internal ribosomal entry site-green fluorescent protein) cassette between exons 8 and 10 of the *Il23r* locus permits the identification of IL-23R expressing cells (Fig 3.1A,B).[617] Whilst homozygous mice (hereafter referred to as IL-23R$^{gfp/gfp}$) are functional knockouts, when bred as heterozygotes (IL-23R$^{gfp/+}$) these mice are described to report IL-23R expression whilst remaining responsive to IL-23 stimulation. Initial validation of this system using a cell sorting and quantitative RT-PCR approach confirmed *Il23r* mRNA was only detected within the gfp^{+} fraction (Fig 3.1C). Furthermore, following overnight *in vitro* stimulation with IL-23, only gfp expressing cells responded with the production of the highly IL-23 dependent cytokine, IL-22 (Fig 3.1D). These results therefore support the utility of this reporter strain for identifying IL-23R expressing cells in mice which remain responsive to IL-23.

Although cells from IL-23R$^{gfp/+}$ mice demonstrated responsiveness to IL-23 *in vitro*, we next asked whether the presence of one wild-type *Il23r* allele was sufficient to preserve wild-type responses *in vivo*. We infected C57BL/6 wild-type, IL-23R$^{gfp/+}$ and IL-23R$^{gfp/gfp}$ mice with *H.hepaticus* and concomitantly treated with anti-IL-10R blocking antibody to induce an IL-23 dependent model of colitis. As expected, IL-23R$^{gfp/gfp}$ mice were highly resistant to disease, whereas histological markers of disease severity were indistinguishable between wild-type and IL-23R$^{gfp/+}$ animals (Fig 3.1E). Therefore IL-23R$^{gfp/+}$ mice display intact *in vivo* responses to IL-23, validating their use for studies of IL-23R expression in intestinal inflammation.

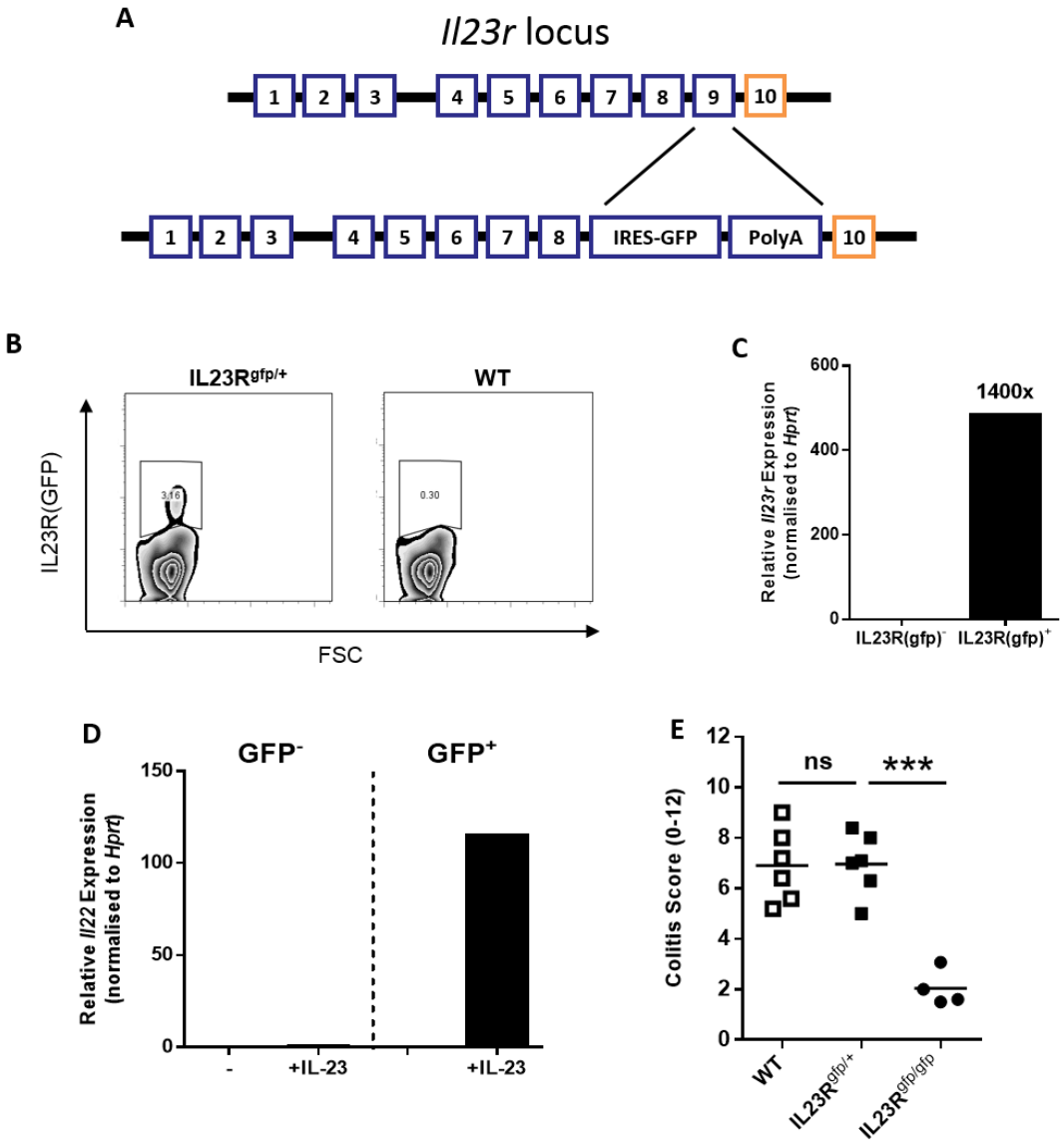

Figure 3.1 Validation of IL-23Rgfp reporter mice

(A) IL-23Rgfp reporter mice carry an IRES-GFP insertion between exon 8 and 10 of *Il23r* permitting identification of IL-23R+ cells. **(B)** Representative plots of IL-23R(gfp) expression, gated on CD45+ live colonic lamina propria cells. WT; wild-type C57BL/6 **(C)** *Il23r* mRNA expression in splenic CD45+ live cells FACS sorted on the basis of gfp expression, measured by qPCR and normalised to *Hprt*. (n=1) **(D)** *Il22* expression by FACS sorted CD45+ live splenocytes ($5x10^4$) from IL-23R$^{gfp/+}$ mice incubated overnight with or without 20ng/ml IL23. Il22 mRNA assessed by qPCR and normalised to *Hprt*. **(E)** C57BL/6 (WT), IL-23R$^{gfp/+}$ and IL-23R$^{gfp/gfp}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* and treated weekly with anti-IL10 antibody (1mg/week). Mice were sacrificed after 28 days and colonic histology assessed. Representative of single experiment n=4-6 each group. Points represent individual mice, bars indicate mean. Statistical significance determined by Mann-Whitney test. *** $p<0.001$, ns = not significant.

3.2.2 IL-23R expression is enriched in the steady state intestinal lamina propria

Using flow-cytometry, we next characterised IL-23R(gfp) expression in the steady state mouse, within multiple intestinal and systemic immune compartments including the lamina propria and IEL of the small and large intestine, mesenteric and peripheral lymph nodes, liver, and spleen. In all locations examined, IL-23R(gfp) was exclusively expressed within the $CD45^+$ fraction of cells of haematopoetic origin (Fig 3.2A and data not shown). Marked variation was seen in the proportion of IL-23R$(gfp)^+$ cells present within each anatomical location (Fig 3.2A&B), with significant enrichment of the intestine for IL-23R(gfp) expressing cells. Notably, IL-23R$(gfp)^+$ cells were confined to the lamina propria in all intestinal segments examined, with no significant expression observed within the IEL compartment (Fig 3.2A). Furthermore, clear differences were noted between the small and large intestinal lamina propria, with approximately 4-8% of all colonic lamina propria $CD45^+$ cells expressing IL-23R(gfp), compared to somewhat lower proportions in the caecum and small intestine (Fig 3.2B). By contrast, the proportion of IL-23R$(gfp)^+$ cells within various lymphoid compartments was significantly lower at just 0.1-0.5%.

Since expression of IL-23 itself may be highly compartmentalised, we speculated it might be contributing to the spatial distribution of IL-23R$(gfp)^+$ cells observed. However, examination of IL-$23R^{gfp/gfp}$ mice revealed similar proportions of IL-23R$(gfp)^+$ cells within the intestines and lymphoid tissues to those seen in IL-$23R^{gfp/+}$ animals, indicating that IL-23 itself is redundant for the establishment of normal patterns of IL-23R distribution in the steady state (Fig 3.2C).

3.2.3 IL-23R expressing cell populations demonstrate significant variation between anatomical compartments

IL-23R expression, assessed either at the mRNA level or by responsiveness to direct IL-23 stimulation, has been reported for a variety of leukocytes, including $CD4^+TCR\beta^+$ cells,[175,184,643] MAIT cells,[321] γδT-cells,[617] NK and NKT-cells,[72,743] innate lymphoid cells (including LTi-like cells, $NKp46^+$ cells and $Lin^-Sac\text{-}1^+Thy1^+$ cells),[67,72,546,744] and myeloid cells including

macrophages and dendritic cells.[541,617] We therefore further analysed steady state IL-23R(gfp)$^+$ populations using flow-cytometry and a panel of surface markers.

Initially studying the colonic lamina propria, we found expression of IL-23R(gfp) was largely confined to cells with morphological characteristics of lymphoid cells, with no significant expression observed upon myeloid cells, defined by CD11b$^+$, CD11c$^+$ or MHC-II$^+$ markers (data not shown). Instead, γδT-cells were the predominant IL-23R(gfp)$^+$ population in the steady state, accounting for more than 50% of all IL-23R(gfp)$^+$ cells (Fig 3.3A&B). TCRβ$^+$ cells represented the next largest population of IL-23R(gfp)$^+$ cells, however only a minority expressed the CD4 co-receptor identifying them as conventional Th17 cells (Fig 3.3A). CD8α was not expressed by IL-23R(gfp)$^+$ cells, thus many such cells had a somewhat unconventional CD4$^-$CD8$^-$ double-negative phenotype. Approximately 30% of IL-23R(gfp)$^+$ cells were negative for both CD3, and for other lineage markers (CD11b, CD11c, B220, NK1.1), compatible with the phenotype of innate lymphoid cells (ILC). Amongst such cells, up to 20% were CD4$^+$ suggestive of LTi-like cells (Fig 3.3A). Despite the description of *Il23r* expression by NKp46$^+$ ILCs in the intestine,[72] we could not detect IL-23R(gfp)$^+$NKp46$^+$ cells using this reporter system (data not shown).

Similar IL-23R(gfp)$^+$ cell types were found in other parts of the intestine and within lymphoid tissues, however, striking variation was seen between anatomical locations in the composition of total IL-23R(gfp)$^+$ populations (Fig 3.3B&C). Whereas γδT-cells were the predominant colonic IL-23R(gfp) population, within the small intestine these cells were a minor fraction, and CD4$^+$ T-cells were the predominant cell (Fig 3.3C). Interestingly, studies in which the small intestine was further divided into proximal, middle and distal segments revealed a clear gradient in the composition of the total IL-23R(gfp) pool (Fig 3.3C). Although IL-23R(gfp)$^+$ cells within the spleen and mesenteric lymph nodes were almost entirely CD4$^+$TCRβ$^+$ cells, the composition of

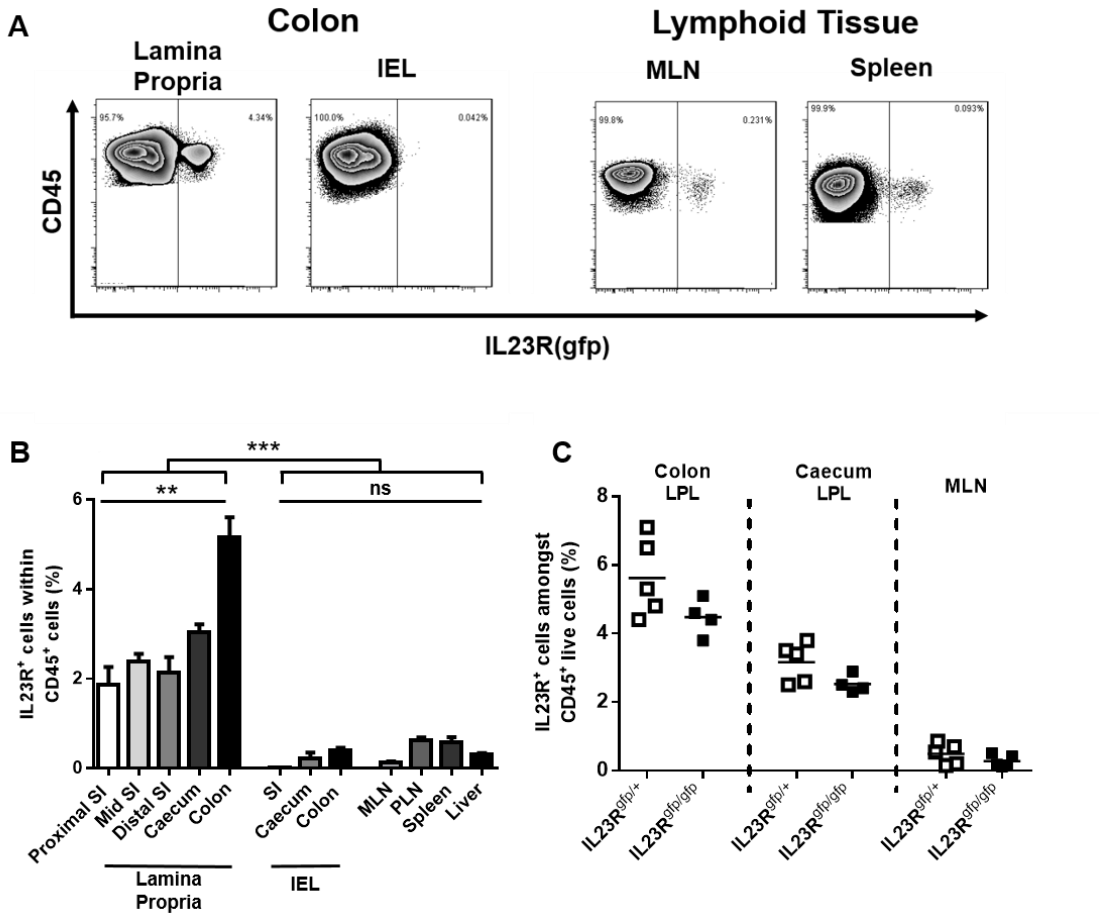

Figure 3.2 IL-23R expression is enriched in the steady state intestinal lamina propria

8-14 week old steady state SPF IL-23R$^{gfp/+}$ mice were sacrificed and organs harvested for cell isolation. IL-23R(gfp)+ fraction was determined using wild-type cells as negative control. **(A)** Representative flow cytometry plots of IL-23R(gfp) expression within indicated anatomical compartments. Gated on CD45+ live cells. **(B)** Proportion of CD45+ live cells expressing IL-23R(gfp) within indicated anatomical compartment. SI; Small intestine, M/PLN; mesenteric/peripheral lymph nodes, IEL; intraepithelial lymphocytes. **(C)** IL-23R(gfp) expression among CD45+ live cells within indicated anatomical compartment in IL-23R$^{gfp/+}$ and IL-23R$^{gfp/gfp}$ mice. Points represent individual mice, bars indicate mean +/- SD. LPL; lamina propria leukocytes. Results representative of 4 independent experiments, n=5-8 per experiment (A&B), or single experiment (n=4-5 per group) (C). Statistical significance analysed using ANOVA. ** p<0.01, *** p<0.001.

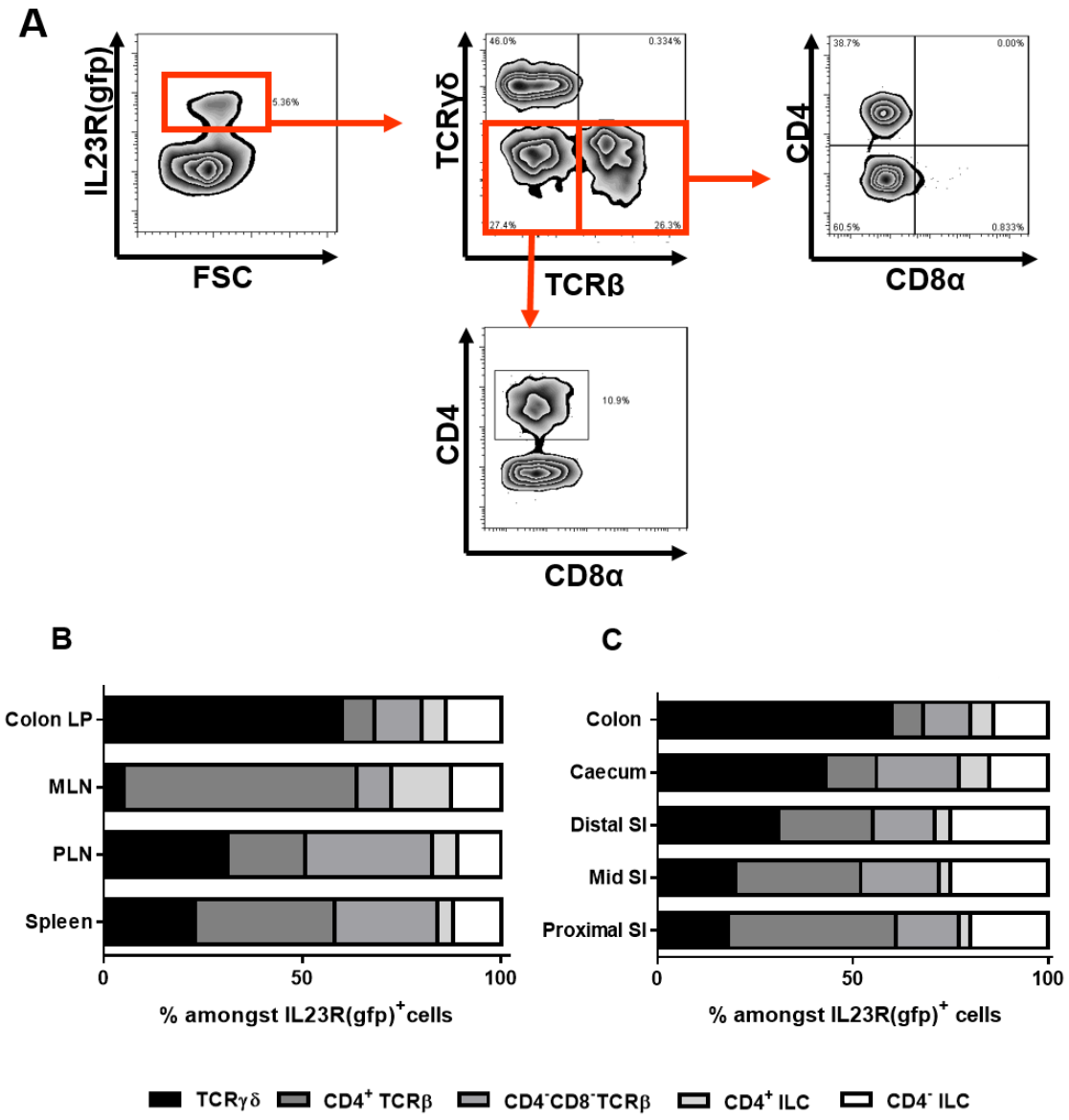

Figure 3.3 IL-23R expressing cell populations demonstrate marked variation between anatomical compartments

8-14 week old steady state SPF IL-23R$^{gfp/+}$ mice were sacrificed and organs harvested for cell isolation. IL-23R(gfp)+ fraction was determined using wild-type cells as negative control. **(A)** Representative flow cytometry plots showing gating strategy and surface marker expression upon IL-23R(gfp)+ cells. **(B)** Composition of IL-23R(gfp)+ cell populations in indicated anatomical compartments determined using gating strategy depicted in (A). M/PLN; mesenteric/peripheral lymph nodes. **(C)** Composition of IL-23R(gfp)+ population within multiple intestinal segments. Small intestine (SI) was divided at sacrifice into thirds for processing and analysis. Results representative of 4 (A-B) or 2 (C) independent experiments, n=5-8 per experiment.

peripheral lymph node IL-23R(gfp) populations was markedly different, containing large populations of γδT and $CD4^{-}CD8^{-}TCR\beta^{+}$ cells (Fig 3.3B).

This striking variation and complexity is compatible with different roles for IL-23 responsive cells within various immune compartments. Furthermore, it also suggests that accumulation of $IL\text{-}23R^{+}$ populations may be differentially regulated in the steady state.

3.2.4 IL-23R expression marks diverse IL-17A producing cell populations in the steady state

IL-23R expression has been closely linked to IL-17 producing cells, including Th17 cells, γδT-cells and a variety of innate lymphoid cells, however the role of IL-23/IL-23R in the biology of such cells remains to be fully determined. $IL\text{-}23^{-/-}$ mice may harbour reduced numbers of $CD4^{+}IL\text{-}17^{+}$ cells, suggesting a role for IL-23 in regulating this population, through effects on proliferation or survival.[752] However, further studies have suggested that IL-23 may instead act to drive acquisition of additional pathogenic features by Th17 cells, including TGF-β3 or IFN-γ production, whereas in its absence, such cells co-produce IL-10.[183,306,307,753]

We therefore attempted to define the functional correlates of IL-23R expression in the steady state intestine. *Ex vivo* re-stimulation of colonic lamina propria cells and intracellular cytokine staining revealed that amongst both $TCR\beta^{+}$ and $TCR\gamma\delta^{+}$ cells, the majority of $IL\text{-}23R(gfp)^{+}$ cells made IL-17A, but did not produce IFN-γ either as single positive $IL\text{-}17A^{-}IFN\text{-}\gamma^{+}$ or double positive $IL\text{-}17A^{+}IFN\text{-}\gamma^{+}$ cells (Fig 3.4). Establishing a similar relationship for non T-cells was not feasible due to the very low numbers recovered from the un-inflamed colon. Amongst both γδT and $TCR\beta^{+}$ cells, the vast majority of $IL\text{-}17A^{+}$ cells were $IL\text{-}23R(gfp)^{+}$. Importantly, fixation and permeabilisation for intracellular staining consistently resulted in an approximate 10-fold

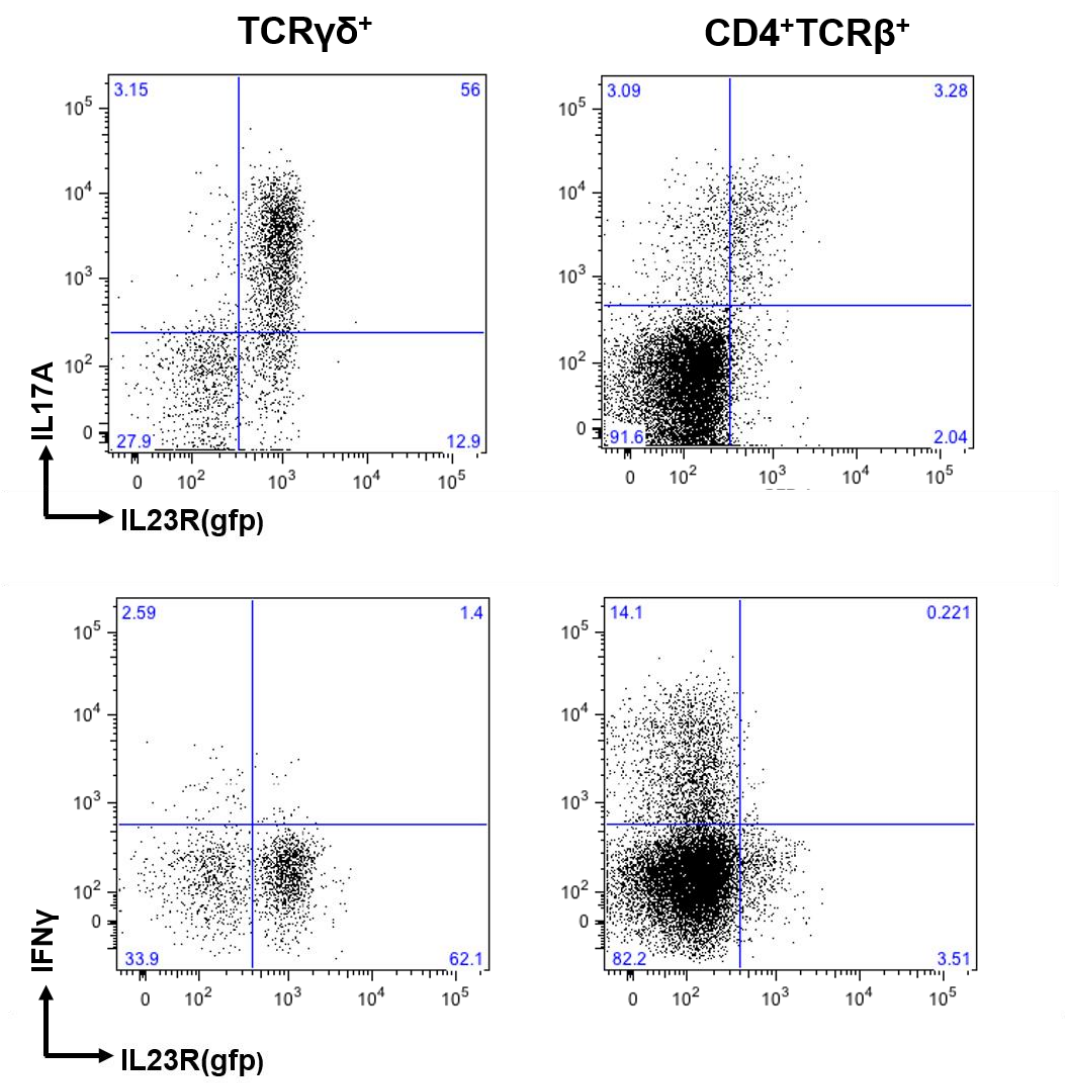

Figure 3.4 IL-23R expressing cells from the steady state intestine produce IL-17A but not IFN-γ

Colonic lamina propria lymphocytes were isolated from 8-14 week old steady state SPF IL-23R$^{gfp/+}$ mice and restimulated *in vitro* with PMA/ionomycin in the presence of Brefeldin A for 4 hours, followed by surface and intracellular staining. Representative flow cytometry plots gated on TCRγδ+ or CD4+TCRβ+ cells shown from single experiment n=8 mice.

reduction in the fluorescent intensity of the gfp signal and the apparent presence of IL-17A^{+}IL-23R^{-} cells could be artefactual rather than a true population.

Therefore, IL-23R(gfp) expression marks a heterogeneous subset of cells poised to make IL-17A, but which appear to lack production of IFN-γ in the steady state.

3.2.5 Colonic IL23R^{+}CD4^{+} T-cells display a uniform memory phenotype and a broad Vβ repertoire

The intestinal lamina propria has been consistently reported to be enriched for Th17 cells in the steady-state, but further characteristics of such cells are poorly defined.[175,177,181,307,752] To understand more about the nature of steady state IL-23R expressing CD4^{+} T-cells in the intestine, we further analysed their phenotype by flow-cytometry. Consistent with previous reports, IL-23R was not expressed on the small proportion of naïve CD4^{+} T-cells (CD44^{-}CD62L^{hi}) present within the colon,[175] and instead IL-23R^{+}CD4^{+}TCRβ^{+} cells uniformly exhibited the CD44^{hi}CD62L^{lo} phenotype of effector memory T-cells (Fig 3.5A).

Functional subsets of T-cells may preferentially express distinct TCR specificities, such that intestinal effector and regulatory subsets show little overlap in their TCR repertories.[618,619] Similarly, the nature of the cognate antigen may determine the effector phenotype. Whilst fine mapping of a specific TCR is complex, a broad overview of TCR use within a population can be determined by analysis of TCR Vβ repertoires. By this approach a number of unconventional IL-23R expressing T-cells, including MAIT cells,[321] thymic derived natural Th17-cells[754] and NKT-cells[755] have been shown to preferentially use specific TCRVβ-chains. However, the Vβ repertoire of intestinal Th17 cells is undefined. We therefore analysed Vβ expression by colonic T-cells isolated from steady-state IL-23R$^{gfp/+}$ mice using a panel of antibodies against common Vβ chains. This revealed IL-23R expressing cells display a diverse repertoire of Vβ use, however compared to IL-23R^{-} cells, a significantly lower proportion of IL-23R^{+} cells utilised TCRVβ4, 8.1, 12 or 14

(Fig 3.5B). Furthermore, whereas the Vβ chain could be identified on 90% of all IL-23R- cells, in the case of IL-23R$^+$ cells, approximately 40% of cells were not identified using available Vβ reagents (Fig 3.5C), suggesting steady state IL-23R$^+$CD4$^+$ T-cells may use a TCR repertoire which differs markedly from that of IL-23R$^-$ cells.

These studies show steady state intestinal IL-23R$^+$CD4$^+$ T-cells are a diverse and polyclonal population of effector memory T-cells.

3.2.6 Microbial signals differentially regulate IL-23R expressing populations in the healthy colon

Quantitative and qualitative differences in systemic and intestinal leukocyte populations occur in the absence of a normal microbial flora. Intestinal IL-17 producing CD4$^+$ T-cells[43,421] and some populations of RORγt$^+$ innate lymphoid cells[72,756] appear particularly dependent upon signals from the microbiota for their development and accumulation in the intestine, whereas other IL-17$^+$ populations are less affected in germ-free mice.[78,421,757] However, these studies have generally concerned small intestinal rather than colonic populations. Furthermore, some groups have reported paradoxical effects in the colon, where the microbiota may instead restrain Th17 cells via an IL-25 dependent mechanism.[758] Microbial factors are required for constitutive expression of the p40 subunit of IL-23 in the distal small bowel and colon,[638] therefore it is unclear whether alterations in IL-17 producing populations in germ-free animals reflect a lack of IL-23 signals needed for accumulation and proliferation, or a requirement for the acquisition of functional characteristics such as IL-17 production by such cells within the intestine. We therefore treated IL-23R$^{gfp/+}$ mice with broad spectrum antibiotics from birth to prevent the development of a normal intestinal flora. Analysed in adulthood, these animals harboured similar proportions and numbers of total CD45$^+$ cells expressing IL-23R(gfp) cells to untreated mice with a normal SPF flora, showing that signals from the normal live flora are not required *per se* for the generation,

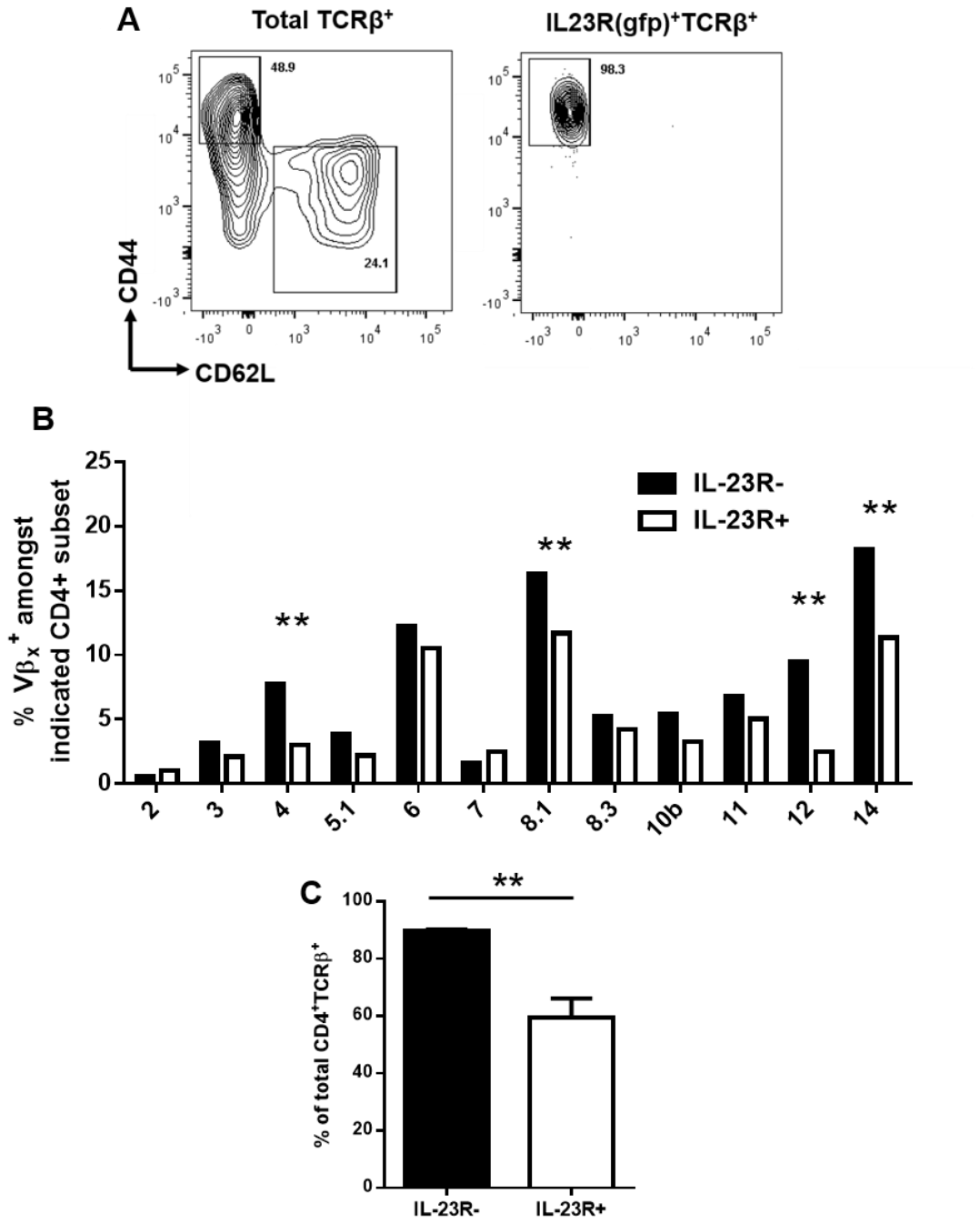

Figure 3.5 Colonic IL-23R+CD4+ T-cells are effector memory cells with a broad Vβ repertoire

8-14 week old steady state SPF IL-23R$^{gfp/+}$ mice were sacrificed and colonic lamina propria cells isolated and surface stained for analysis by flow cytometry. **(A)** Representative plots of CD44 and CD62L expression on total CD4+ T-cells (left panel) and IL-23R(gfp)+ CD4+ T-cells isolated from the colonic lamina propria. Gated on live cells and markers indicated. **(B)** Proportion of cells expressing indicated Vβ-chain according to IL-23R(gfp) status. Gated on live CD4+ T-cells. **(C)** Proportion of colonic CD4+ T-cells for which Vβ-chain expression could be determined using panel of markers indicted in (B), according to IL-23R(gfp) status. Results pooled from 2 independent experiments, n=32 each experiment, analysed in pools of 4. Columns indicate mean +/- SD where shown. Statistical significance determined by Mann-Whitney test. ** $p<0.01$.

accumulation or survival of IL-23R expressing cells in the non-inflamed intestine (Fig 3.6A). However, the composition of the IL-23R(gfp)$^+$ population was markedly changed by antibiotic treatment, with a >50% reduction in the proportion of TCRβ$^+$ cells, and a smaller reduction in γδT-cells, but a corresponding increase in the proportion of IL-23R(gfp)$^+$ cells not expressing either T-cell receptor (Fig 3.6B&C).

Within the IL-23R(gfp)$^+$ TCRβ$^+$ pool, CD4$^+$ T-cells were significantly reduced by antibiotic treatment, with expansion of DN T-cells, and the notable emergence of IL-23R(gfp)$^+$CD8$^+$ T-cells, a population not observed in normal adult mice (Fig 3.6C). Interestingly, we had previously observed IL23R(gfp)$^+$CD8$^+$ T-cells in the intestines of very young mice (5-7 days old), suggesting that antibiotic treatment could be resulting in the persistence of an immature immune phenotype. Alternatively, CD8$^+$ cells might be populating an empty niche in the absence of CD4$^+$ Th17 cells. More strikingly, when we restimulated lamina propria cells *ex vivo*, IL-17A$^+$ expression was virtually absent amongst CD4$^+$ T-cells from mice treated with antibiotics, an effect highly specific for this subset, since expression of this cytokine by CD4$^-$ T-cells was correspondingly increased, and a large population of IL-17A$^+$ γδT-cells remained (Fig 3.7).

In total, these experiments show that signals from the intestinal flora influence the development of a normal intestinal compliment of IL-23R expressing cells, but that significant differences exist between cell types in their requirements for such signals.

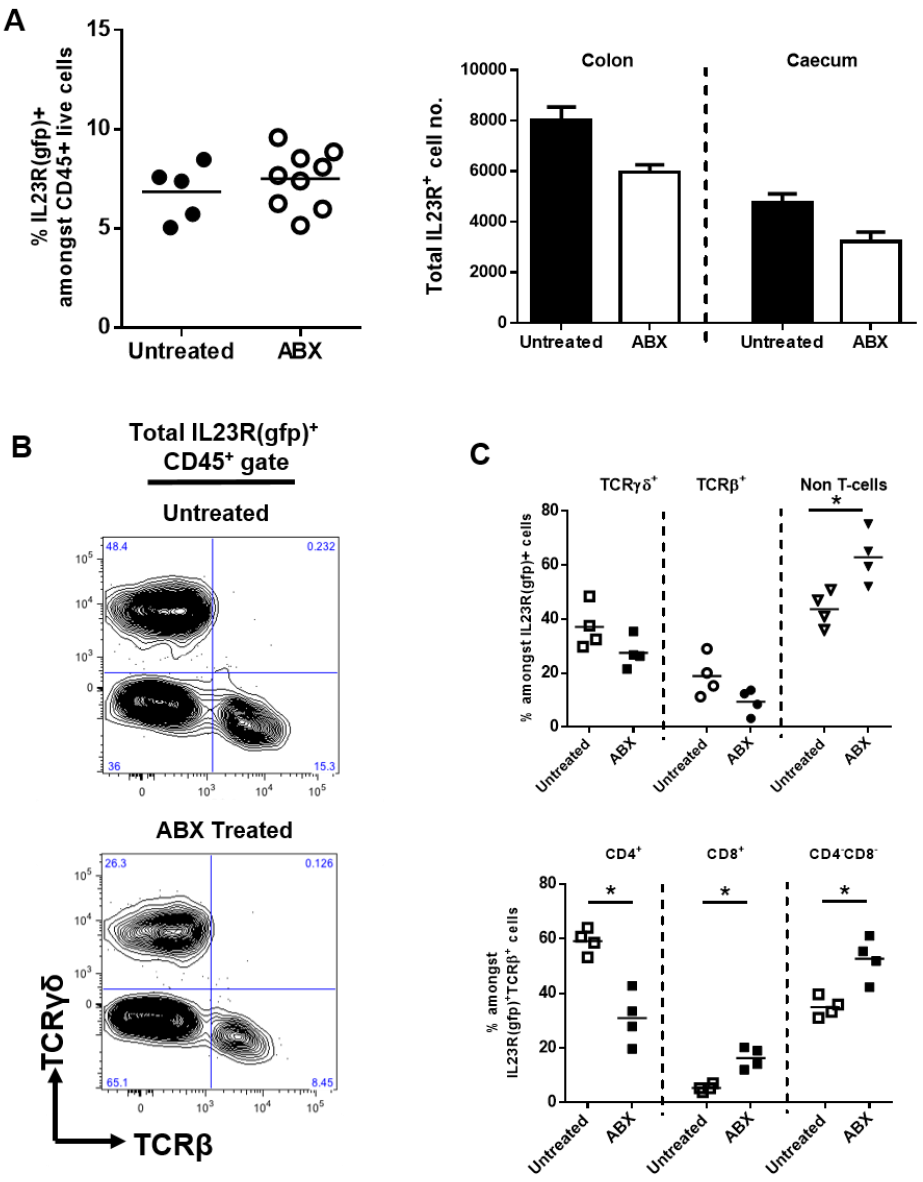

Figure 3.6 Microbial signals differentially influence IL-23R expressing cell accumulation in the healthy intestine

8-10 week old IL-23R$^{gfp/+}$ mice born to antibiotic treated breeding pairs (female C57BL/6 x male IL-23R$^{gfp/gfp}$), and maintained from birth on broad spectrum antibiotic treatment, and age and sex matched SPF IL-23R$^{gfp/+}$ controls were sacrificed and colonic lamina propria cells isolated for analysis. **(A)** IL-23R expression amongst colonic CD45+ live cells by antibiotic treatment status; points represent individual mice, bars indicate mean. (left panel) total IL-23R(gfp)+ cell numbers in colon and caecum by antibiotic treatment status; bars show mean+/-SEM (right panel). **(B)** Representative flow cytometry plots gated on IL-23R(gfp)+CD45+ live cells. **(C)** Composition of colonic lamina propria total IL-23R(gfp)+ population (top panel) and IL-23R(gfp)+TCRβ+ cells (bottom panel) determined by surface markers using gating strategy as shown in (B) and Fig 3.3A. Non T-cells defined as TCRγδ-TCRβ- cells. ABX; antibiotics. Results representative of 3 independent experiments n=6-9 each group per experiment. Points indicate individual mice, bars show mean. Statistical significance determined by Mann Whitney test. *p<0.05.

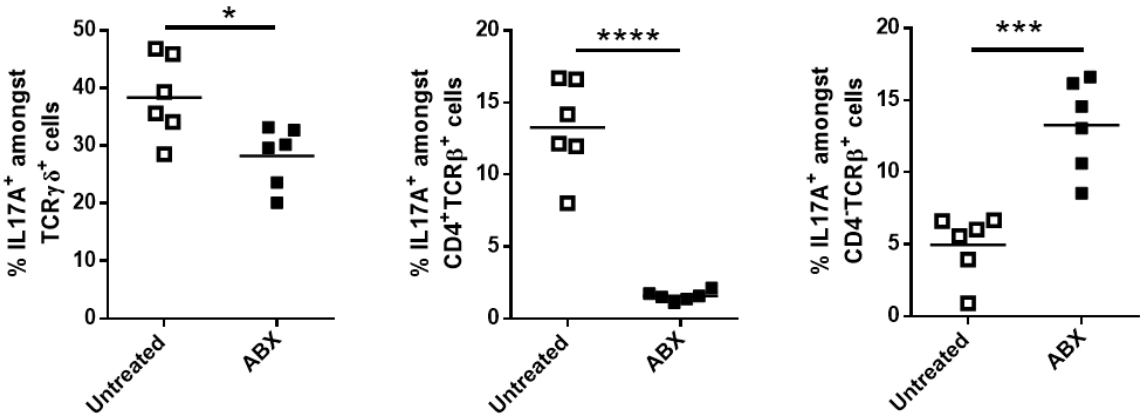

Figure 3.7 Microbial signals differentially influence IL-17A producing cells in the intestine

8-10 week old IL-23R$^{gfp/+}$ mice born to antibiotic treated breeding pairs (female C57BL/6 x male IL-23R$^{gfp/gfp}$), and maintained from birth on broad spectrum antibiotic treatment, and age and sex matched SPF IL-23R$^{gfp/+}$ controls were sacrificed and colonic lamina propria cells isolated. Cells were restimulated with PMA/ionomycin in the presence of Brefeldin A for 4 hours, followed by surface and intracellular staining and flow cytometry analysis. Points show individual mice, bars indicate mean. Data representative of 2 independent experiments, n=6-9 per group each experiment. Statistical significance analysed using Mann Whitney Test. *p<0.05; ***p<0.001; ****p<0.0001.

3.2.7 IL-23R expressing CD4$^+$T-cells accumulate in chronic intestinal inflammation

Despite the essential role of IL-23 in a number of models of colitis, the critical cellular targets mediating downstream effects are unknown, with both CD4$^+$ T-cells and RORγt$^+$ ILCs implicated in specific settings.[67,183,521] The finding of such diversity of cells expressing the IL-23R in the steady-state intestine led us to question whether all IL-23R cells might contribute to disease, or whether specific subsets become dominant during inflammation. We therefore studied changes in IL-23R expression in chronic colitis using the well characterised model of IL-23 dependent disease induced by infection with *H. hepaticus* combined with anti-IL10R treatment.

Remarkably, the proportion of CD45$^+$ colonic lamina propria cells expressing IL-23R(gfp) (~5-8%) was similar in established colitis to that seen in the steady state, implying balanced accumulation of IL-23R$^+$ and IL-23R$^-$ populations overall (Fig 3.8A&B). However, the composition of such cells in the inflamed colon revealed striking differences to the steady-state (Fig 3.9A), with CD4$^+$TCRβ^+ cells the majority cell type, accounting for ~80-90% of all IL-23R(gfp)$^+$ cells present (Fig 3.9B). Analysis of cell numbers suggested that this resulted from accumulation of IL-23R$^+$CD4$^+$TCRβ^+ cells, with numbers of other IL-23R$^+$ populations similar to the steady-state (Fig 3.9C). The accumulation of IL-23R(gfp)$^+$CD4$^+$TCRβ^+ cells resulted in an increased proportion of CD4$^+$ T-cells

expressing IL-23R from approximately 10% in the steady state to 25-40% in colitis (Fig 3.10A&B). In marked contrast, the proportion of lamina propria $\gamma\delta$T-cells expressing IL-23R(gfp) fell from >50% in the steady state to <20%. As in the steady state, no significant IL-23R(gfp) expression was seen on myeloid cells, however in inflammation a small population of IL-23R(gfp)$^+$CD4$^+$TCRβ^+ cells were recovered from the IEL (data not shown).

In total, these results demonstrate that in chronic intestinal inflammation, conventional CD4$^+$TCRβ^+ cells become the dominant IL-23 responsive population, with limited representation

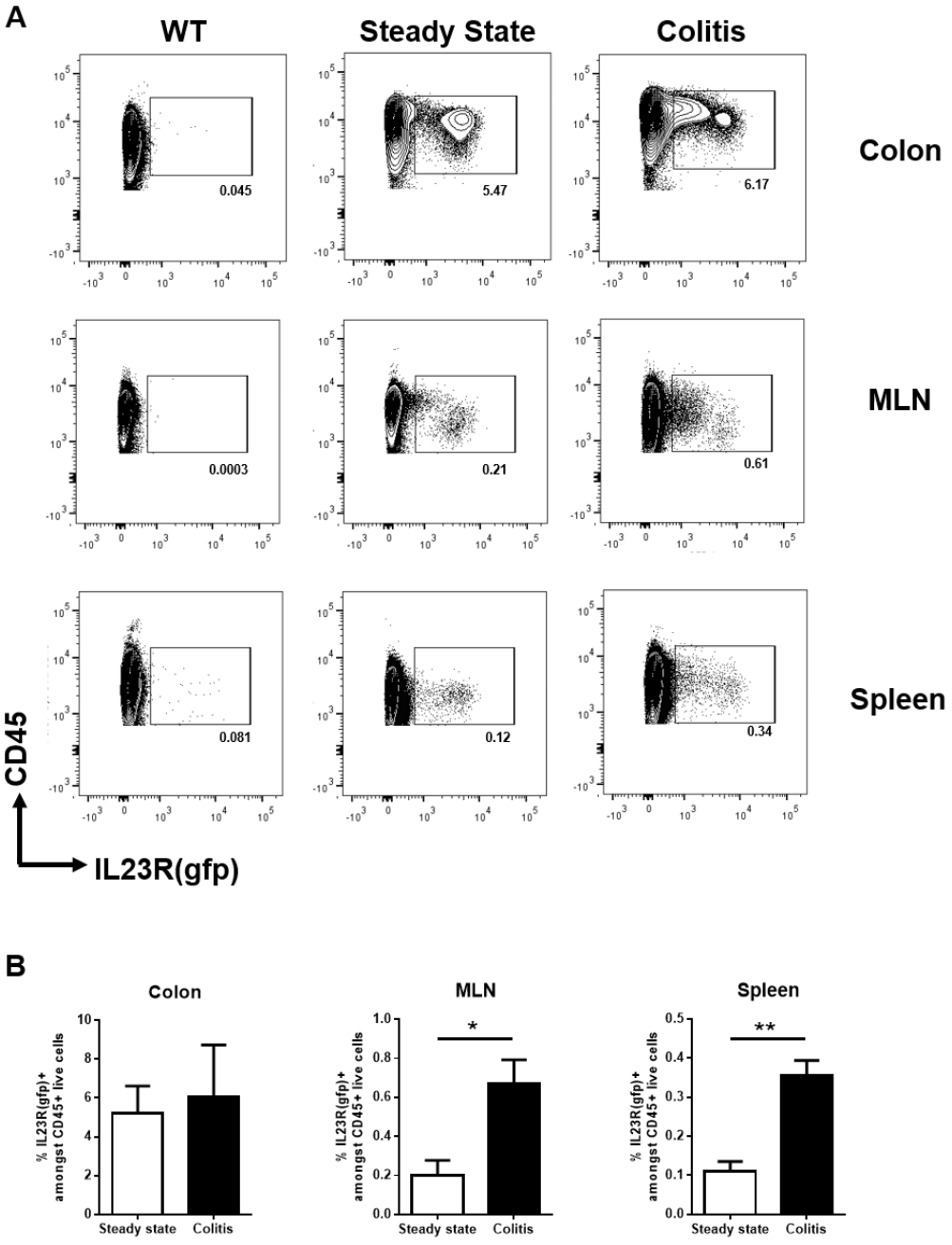

Figure 3.8 Overall IL-23R expression is not increased in the inflamed intestine

IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) for 4 weeks to induce colitis. Mice were sacrificed 28 days after initial infection along with age/sex matched steady state IL-23R$^{gfp/+}$ and C57BL/6 controls and organs harvested for analysis. **(A)** Representative plots of IL-23R(gfp) expression on total CD45+ live cells by anatomical compartment. **(B)** Proportion of CD45+ live cells expressing IL-23R(gfp) within each compartment in steady state and colitis. Data representative of 3 independent experiments, n=6-8 each group per experiment. Bars represent mean +/-SD. Statistical significance analysed using Mann Whitney test. *p<0.05; **p<0.01.

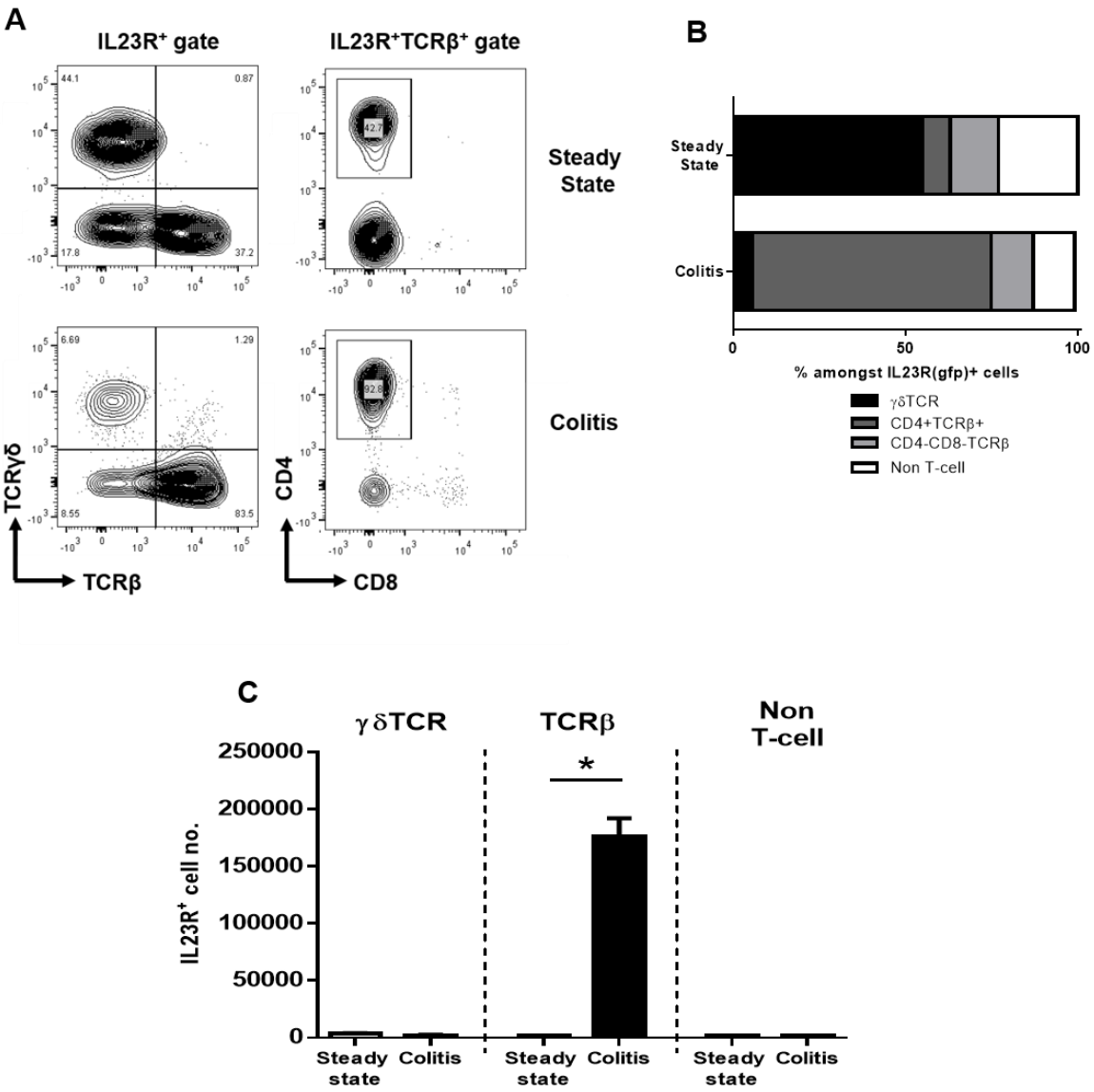

Figure 3.9 CD4+ T-cells are the dominant intestinal IL-23R+ population in chronic colitis

IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) for 4 weeks to induce colitis. Mice were sacrificed 28 days after initial infection along with age/sex matched steady state IL-23R$^{gfp/+}$ and C57BL/6 controls and organs harvested for analysis. **(A)** Representative plots of surface marker expression in steady state and colitis, gated on total IL-23R(gfp)+ (left) or IL-23R(gfp)TCRβ+ cells (right). **(B)** Composition of colonic lamina propria total IL-23R(gfp) populations in steady state and chronic colitis. **(C)** Numbers of colonic IL-23R(gfp)+ cells expressing surface marker indicated. Data pooled from 2 independent experiments (n=16 each group). Bars represent mean +/-SEM. Statistical significance analysed using Mann Whitney test. *p<0.05.

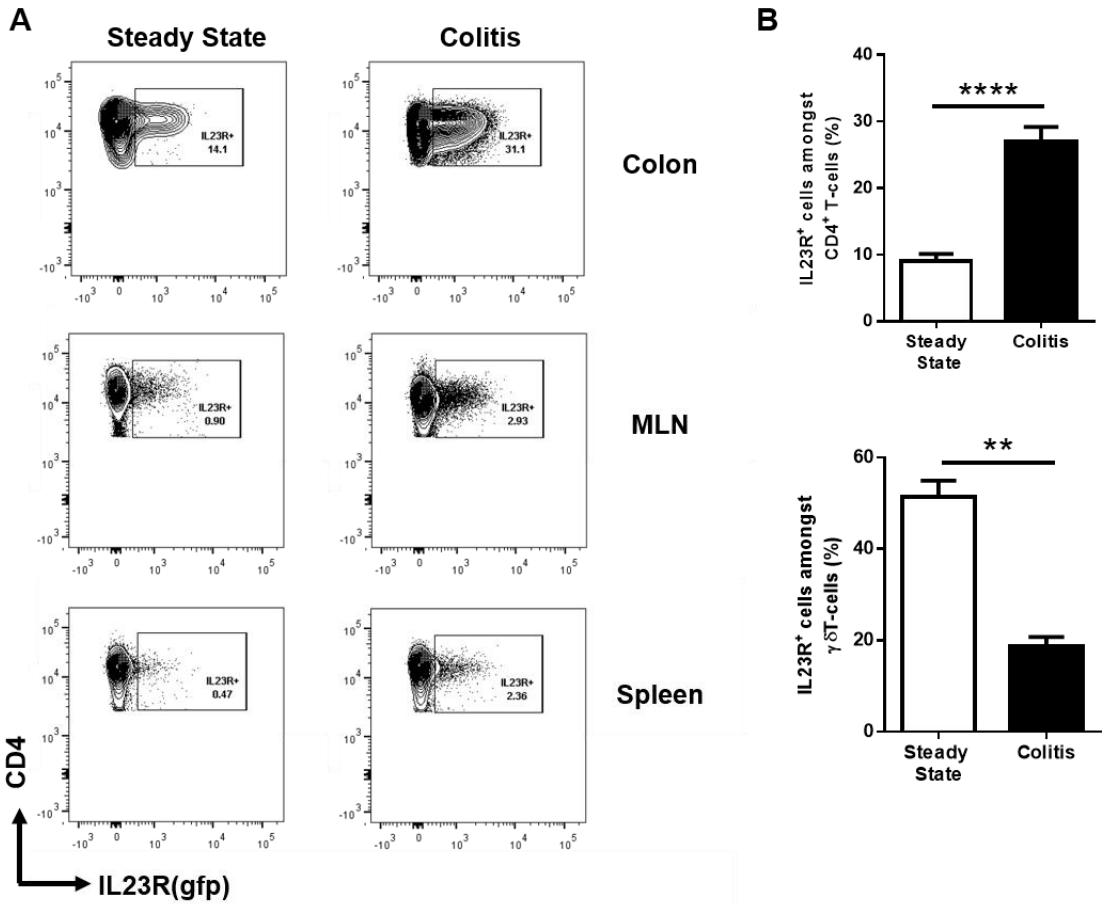

Figure 3.10 Increased proportion of CD4+ T-cells expressing IL-23R in chronic colitis

IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) for 4 weeks to induce colitis. Mice were sacrificed 28 days after initial infection along with age/sex matched steady state IL-23R$^{gfp/+}$ and C57BL/6 controls and organs harvested for analysis. **(A)** Representative plots of IL-23R(gfp) expression on CD4+ T-cells in steady state and colitis. gfp+ gate determined using wild-type control. **(B)** IL-23R(gfp) expression on colonic CD4+ T-cells (top panel) and TCRγδ+ cells in colitis. Data pooled from 3 independent experiments (n=24 each group). Bars represent mean +/-SEM. Statistical significance analysed using Mann Whitney test. ** $p<0.01$, **** $p<0.0001$.

of steady state IL-23R$^+$ populations in established disease. This suggests that the accumulation of IL-23R$^+$ populations in colitis may be differentially controlled, either at the level of trafficking or through cell-specific regulation of IL-23R expression.

3.2.8 Differential kinetics of IL-23R expressing populations in the evolution of colitis

γδT-cells and innate lymphoid cells display characteristics of innate cells, and may respond to stimulation in a more rapid manner than conventional T-cells. IL-17 producing γδT-cells have been reported to be the dominant responding population in pulmonary inflammation,[759,760] and such cells may support the early development of Th17 driven disease in models of joint, skin and neurological inflammation.[387,389,392] We therefore considered that our finding of CD4$^+$ T-cells as the dominant IL-23R(gfp)$^+$ population in colitis might simply reflect the time point examined, in established chronic disease. We therefore undertook kinetic analysis of IL-23R expression within the colonic LP during the development of disease (Fig 3.11A-C). Surprisingly, despite modest expansion of other IL-23R(gfp)$^+$ populations including γδT-cells, CD4$^-$CD8$^-$TCRβ$^+$ cells and non-T-cells, within 7 days of disease induction CD4$^+$ T-cells already accounted for >85% of all IL-23R$^+$ cells present in the colonic LP (Fig 3.11A), and continued to further accumulate until day 14-21. Importantly, the kinetics of all IL-23R(gfp)$^+$ populations were similar, without selective expansion of γδT-cells or non-T-cell numbers at the very earliest stages of disease development (Fig 3.11B&C). The early expansion of IL23R(gfp)$^+$CD4$^+$TCRβ$^+$ cells we noted was paralleled functionally by CD4$^+$ T-cells becoming the dominant IL-17A$^+$ population in the intestine within the first 7 days of disease (Fig 3.12A). In addition, whereas steady state CD4$^+$IL-23R$^+$ T-cells which produced IL-17A were uniformly IFN-γ$^-$, a significant IL-17A$^+$IFN-γ$^+$ population had already emerged at this early time point (Fig 3.12B). Overall, these results question the contribution of non-CD4$^+$ T-cell IL-23R$^+$ populations present in the steady state intestine to the development of colitis.

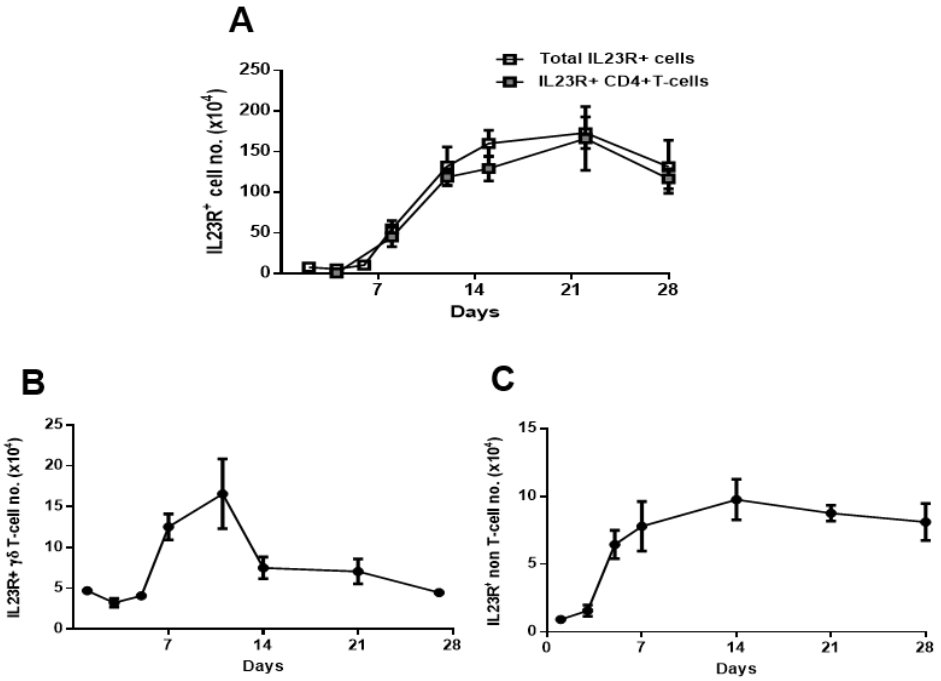

Figure 3.11 Kinetics of colonic IL-23R expressing populations in the evolution of chronic intestinal inflammation

IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) weekly for up to 4 weeks. Mice were sacrificed at indicated time points until 28 days after initial infection and colonic cells isolated for analysis. **(A)** Kinetics of colonic accumulation of total IL-23R(gfp)+ (open symbols) and IL-23R(gfp)+CD4+ T-cells (closed symbols) in evolving colitis. **(B)** Kinetics of colonic IL-23R(gfp)+ γδTCR+ cells in evolving colitis. **(C)** Kinetics of colonic IL-23R(gfp)+ TCRγδ-TCRβ- cells in evolving colitis. Points represent mean at each time point +/- SEM. Data pooled from 2 independent experiments, n=4-8 mice at each time point.

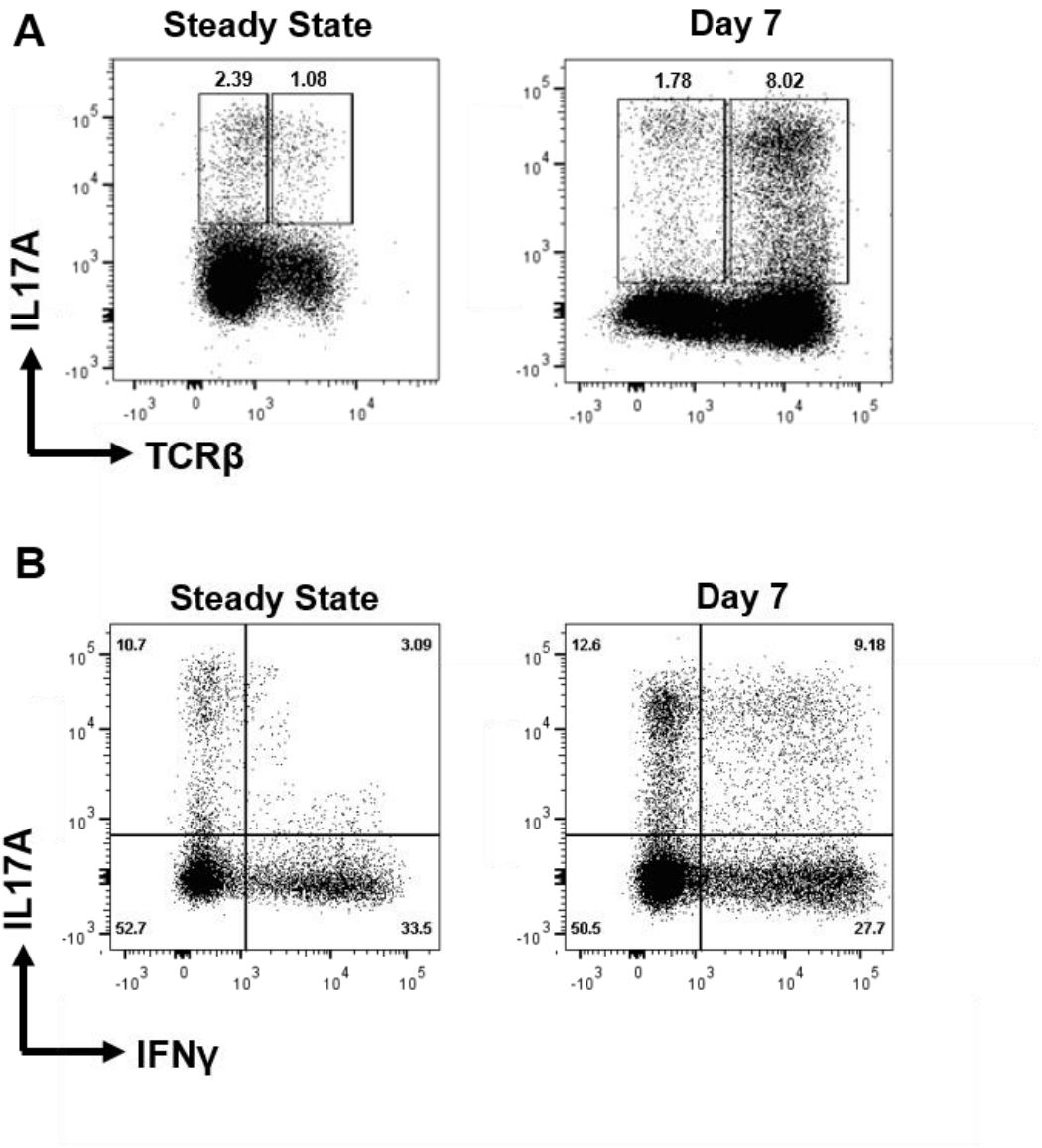

Figure 3.12 CD4+ T-cells become the dominant colonic source of IL-17A early in evolving colitis and acquire additional effector functions

IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) and sacrificed 7 days after initial infection. Colonic cells were isolated and restimulated in vitro with PMA/ionomycin in the presence of Brefeldin A for 4 hours, followed by surface and intracellular staining. **(A)** Representative plots of IL-17A producing cells isolated from steady state and early colitic lamina propria. Numbers shown represent overall percentage for population within total live CD45+ gate. **(B)** Representative plots of restimulated colonic lamina propria CD4+ T-cells isolated at indicate time points. Gated on CD4+ T-cells Data representative of results from a single experiment, n=12.

3.2.9 IL-10 regulates expression of IL-23R by intestinal $CD4^+$ T-cells in a cell-specific manner

In view of the above findings we were interested to understand the mechanisms controlling accumulation of IL-$23R^+CD4^+$ T-cells in the intestine in colitis. Our earlier studies showed that in contrast to tissues such as the skin and CNS in which recruitment of T-cells from the circulation is absolutely required for disease, the intestine is already enriched for IL-23R expressing $CD4^+$ T-cells in the steady state. We considered that early expansion of colonic IL-23R$(gfp)^+$ cells might occur due to upregulation of the receptor on T-cells already resident within the steady state lamina propria.

IL-10 has previously been reported to directly control Th17 function, and to inhibit the development of pathogenic IL-$17A^+$IFN-γ^+ T-cells.[622] Since the emergence of this latter subset is known to be dependent upon IL-23,[183] we speculated that IL-10 might be regulating IL-23R expression on T-cells. Therefore, we treated IL-$23R^{gfp/+}$ mice with blocking anti-IL-10R antibody, with and without infection with *H.hepaticus*, and analysed IL-23R(gfp) expression at 7 days. Remarkably, we found that inhibition of IL-10 signalling alone was sufficient to increase the proportion of colonic $CD4^+$ T-cells expressing IL-23R(gfp) to levels observed in colitic mice (Fig 3.13A). Importantly, total $CD4^+$ T-cell numbers in the colon were similar (Fig 3.13B), strongly suggesting that the increase in IL-23R$(gfp)^+$ cells was due to expression on previously negative cells rather than due to selective proliferation of an existing population, or to homing from outside the intestine.

Anti-IL-10R treatment alone had no effect on γδT-cells, suggesting IL-10 exerts a $CD4^+$ T-cell specific role in regulating IL-23R (Fig 3.13A). Analysis of sorted cells from the steady state colon confirmed that whereas IL-23R$(gfp)^+CD4^+TCR\beta^+$ cells demonstrated abundant mRNA for *Il10r*, very low levels of expression were detected in IL-23R(gfp)+ γδT-cells, compatible with this being a direct effect (Fig 3.13C). Studies using IL-$23R^{gfp/gfp}$ mice showed that receptor upregulation was unaffected by the absence of IL-23 signals, confirming it does not contribute significantly to this phenomenon (Fig 3.13D).

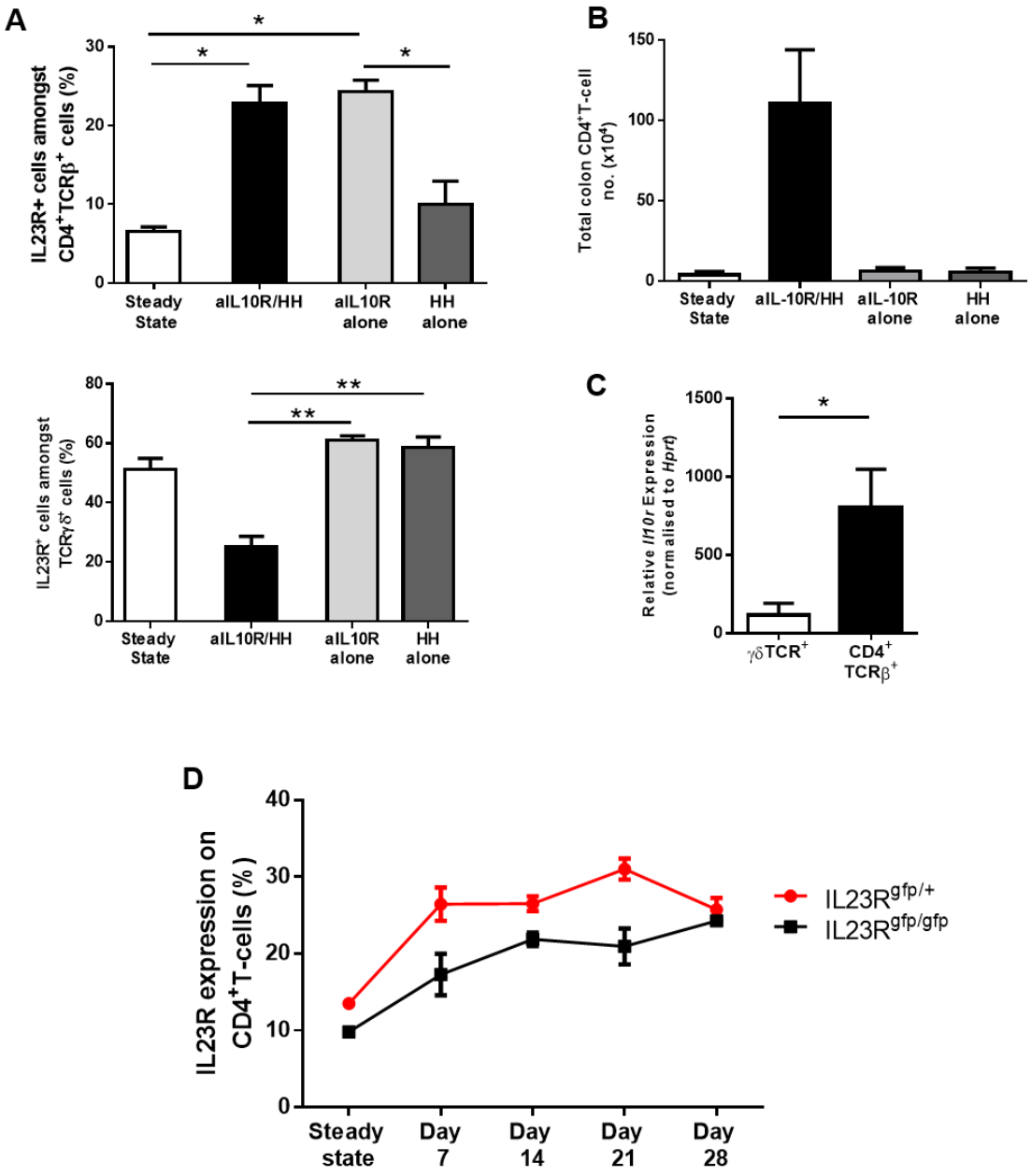

Figure 3.13 IL-10 regulates IL-23R expression on intestinal CD4+ T-cells

(A-C) IL-23R$^{gfp/+}$ mice were administered 1mg/week i.p. anti-IL-10R antibody with or without concurrent infection with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage, or received *H.hepaticus* alone. Mice were sacrificed at 7 days after initial treatment and colonic cells isolated for analysis. (D) Co-housed age and sex matched IL-23R$^{gfp/+}$ and IL-23R$^{gfp/gfp}$ mice were treated with weekly anti-IL01R 1mg/week i.p. and concomitant *H.hepaticus* infection, and sacrificed at the indicated time points. **(A)** Expression of IL-23R(gfp) on colonic CD4+ T-cells (top panel) and γδTCR+ cells (bottom panel) **(B)** Numbers of total colonic CD4+ T-cells **(C)** Expression of *Il23r* mRNA in FACS sorted IL-23R(gfp)+ CD4+ T-cells and γδTCR+ cells, determined by qPCR and normalised to *Hprt*. **(D)** Kinetics of expression of IL-23R(gfp) on colonic lamina propria CD4+ T-cells from IL-23R$^{gfp/+}$ and IL-23R$^{gfp/gfp}$ mice. Results from single experiments, n=6 each treatment group (A-B) and n=4 each genotype at each time point (D). Sorted cells (C) obtained from single experiment n=4 replicates each pooled from 2 mice. Statistical significance analysed using Mann Whitney test. *p<0.05, **p<0.001.

3.2.10 Accumulation of IL-23R$^+$ CD4$^+$ T-cells in colitis is not prevented by blocking lymphocyte recirculation

The early increase in CD4$^+$ T-cell expression of IL-23R we noted led us to question the functional contribution of existing steady state T-cell populations to the development of chronic colitis. IL-23 is both a potent proliferative signal for T-cells and drives acquisition of functional characteristics such as IFN-γ production.[183,184,299,753] We therefore examined IL-23 expression in the colon during the evolution of disease, and found that although modestly elevated levels occur throughout disease, the peak was seen within the initial 3 days (Fig 3.14A). Resident T-cells would thus be well positioned to respond to such an early signal. Alternatively, it could drive the accumulation of existing IL-23R(gfp)$^+$ cells from the circulation. Since the steady state MLN and spleen contain numerically sizable populations of IL-23R(gfp)$^+$ CD4$^+$ T-cells, we examined the kinetics of these cells after the induction of disease. Remarkably, although the total number of IL-23R(gfp)$^+$ CD4$^+$ T-cells within each site increased rapidly, the peak cell number in both occurred within 5 days, before declining to a plateau level thereafter maintained throughout the development of chronic colitis (Fig 3.14B).

To better understand the potential contribution of lymphoid tissue IL-23R$^+$CD4$^+$ T-cells to the accumulation we noted in the inflamed colon, we next sought to analyse the proliferation of steady state lamina propria resident cells in evolving disease. However, techniques to analyse *in situ* cellular proliferation, such as Ki67 or BrdU staining require cellular permeablisation which would extinguish the IL-23R(gfp) signal. We therefore utilised the alternative approach of blocking lymphocyte re-circulation, by treatment with FTY720, an inhibitor of sphingosine-1-phosphate which reversibly sequesters cells in lymphoid tissues.[761-763]

IL-23R$^{gfp/+}$ mice were continuously treated with FTY720 and colitis was induced using the *H.hepaticus*/anti-IL-10R model. Animals were analysed after 14 days, the expected peak of

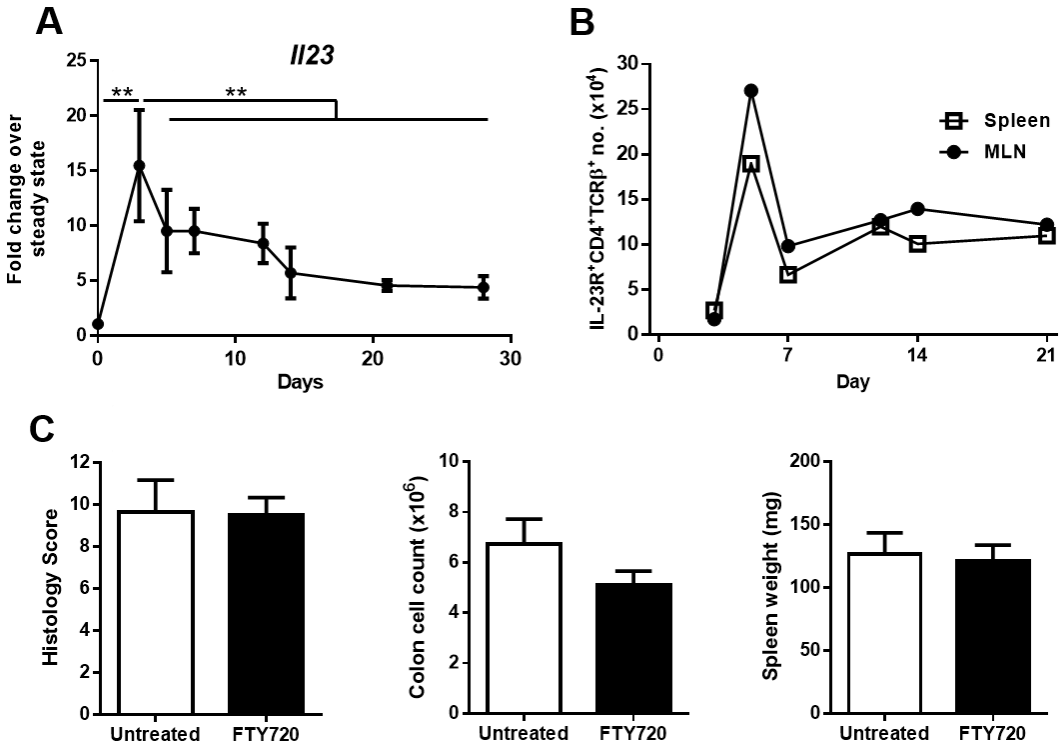

Figure 3.14 Chronic IL-23 dependent colitis is not prevented by FTY720 treatment

C57BL/6 and IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) for up to 28 days weeks to induce colitis. Where indicated mice received FTY720 (1mg/kg/day) or PBS by oral gavage as indicated and were sacrificed at day 14 (C). Mice were sacrificed at the indicated time points and tissues collected for analysis. **(A)** *Il23* mRNA expression in colonic tissue, determined by qPCR. Results normalised to *Hprt* and expressed as fold-change over steady state. **(B)** Kinetics of IL-23R(gfp)+ CD4+T-cell numbers in lymphoid tissues **(C)** Colonic histology score (0-12), total colonic lamina propria cell counts and spleen weight at day 14 in mice treated with FTY720 or placebo. Data from single experiment (n=4-6 each time point) (A) or pooled from 2 independent experiments (n=6-8 each group) (B&C). Bars represent mean +/-SEM. Statistical significance analysed using ANOVA test. ** $p<0.01$ for comparison day 3 vs. day 0, and day 3 vs. all later points.

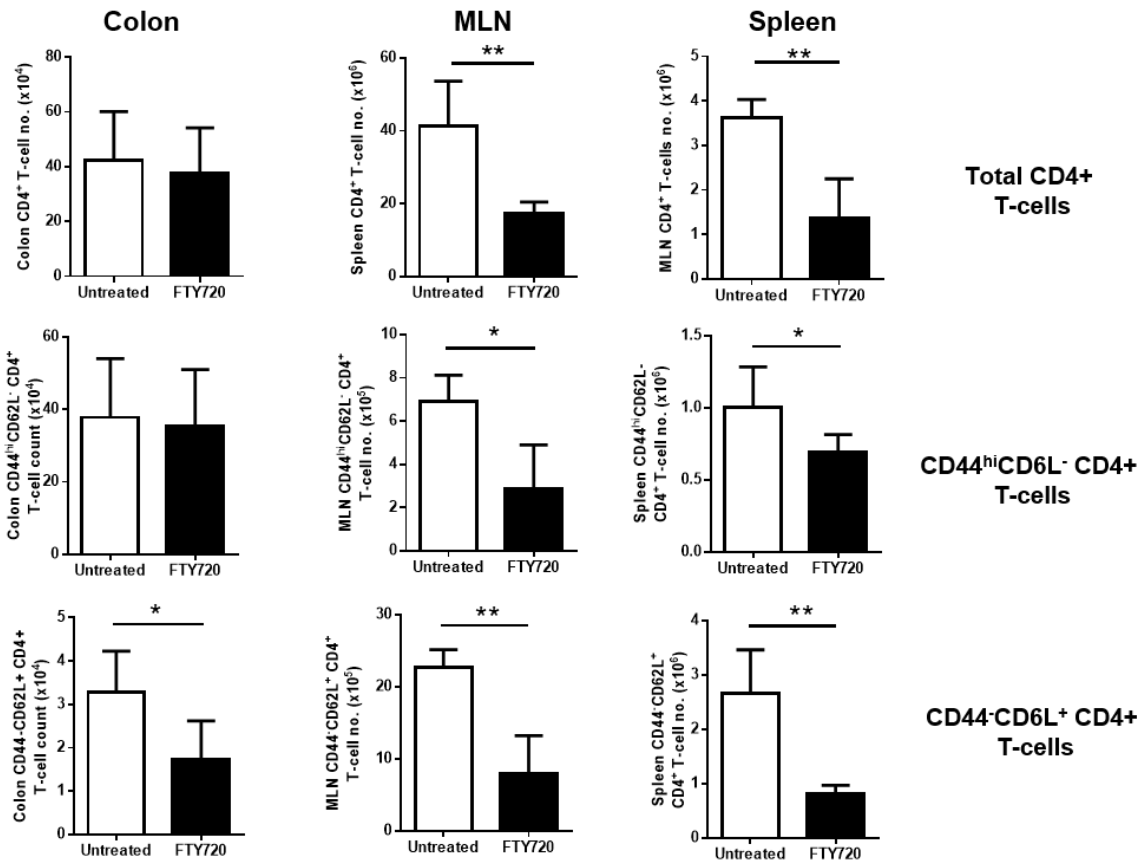

Figure 3.15 FTY720 treatment exerts tissue specific effects on CD4$^+$ T-cell populations

C57BL/6 or IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) to induce colitis. Groups received FTY720 (1mg/kg/day) or PBS by oral gavage as indicated. Mice were sacrificed 14 days after initial infection and organs harvested for analysis. Numbers shown are of T-cells of indicated phenotype within each anatomical compartment in mice treated with FTY720 or placebo (PBS). Data pooled from 2 independent experiments (n=6-8 each group). Bars represent mean +/-SD. Statistical significance analysed using Mann Whitney test. *p<0.05. **p<0.01.

lymphocyte accumulation in the colon, but remarkably FTY720 treatment did not result in attenuation of disease, with similar histological scores, total colon cell counts and splenomegally to untreated controls (Fig 3.14C). Colonic $CD4^+$ T-cell numbers were similar in both groups, despite the significantly reduced circulating lymphocyte counts induced by FTY720 treatment, which confirmed the *in vivo* activity of the drug and dosing regimen used (Fig 3.15).

Expression of IL-23R on $CD4^+$ T-cells within the colon was similar, both in proportion and absolute numbers regardless of FTY720 treatment (Fig 3.16A&B). Furthermore, intracellular cytokine staining of T-cells recovered from the lamina propria did not show any significant differences from untreated controls (Fig 3.16C). The only significant difference observed in the colons of treated mice was an approximate 50% reduction in the number of cells displaying a $CD44^-CD62L^+$ naïve t-cell phenotype (Fig 3.15).

Overall, these studies show that despite the induction of marked peripheral lymphopenia and prevention of lymphocyte recirculation, FTY720 could not attenuate colitis, nor prevent the accumulation of IL-23R$(gfp)^+CD4^+$ T-cells in the colon. This suggests that in the *H.hepaticus*/anti-IL-10R model of colitis the steady state tissue resident $CD4^+$ T-cell pool is sufficient to initiate colitis, and that the increased population of IL-23R expressing T-cells accumulating in disease is not dependent upon recruitment of recirculating lymphocytes, at least at the time point examined.

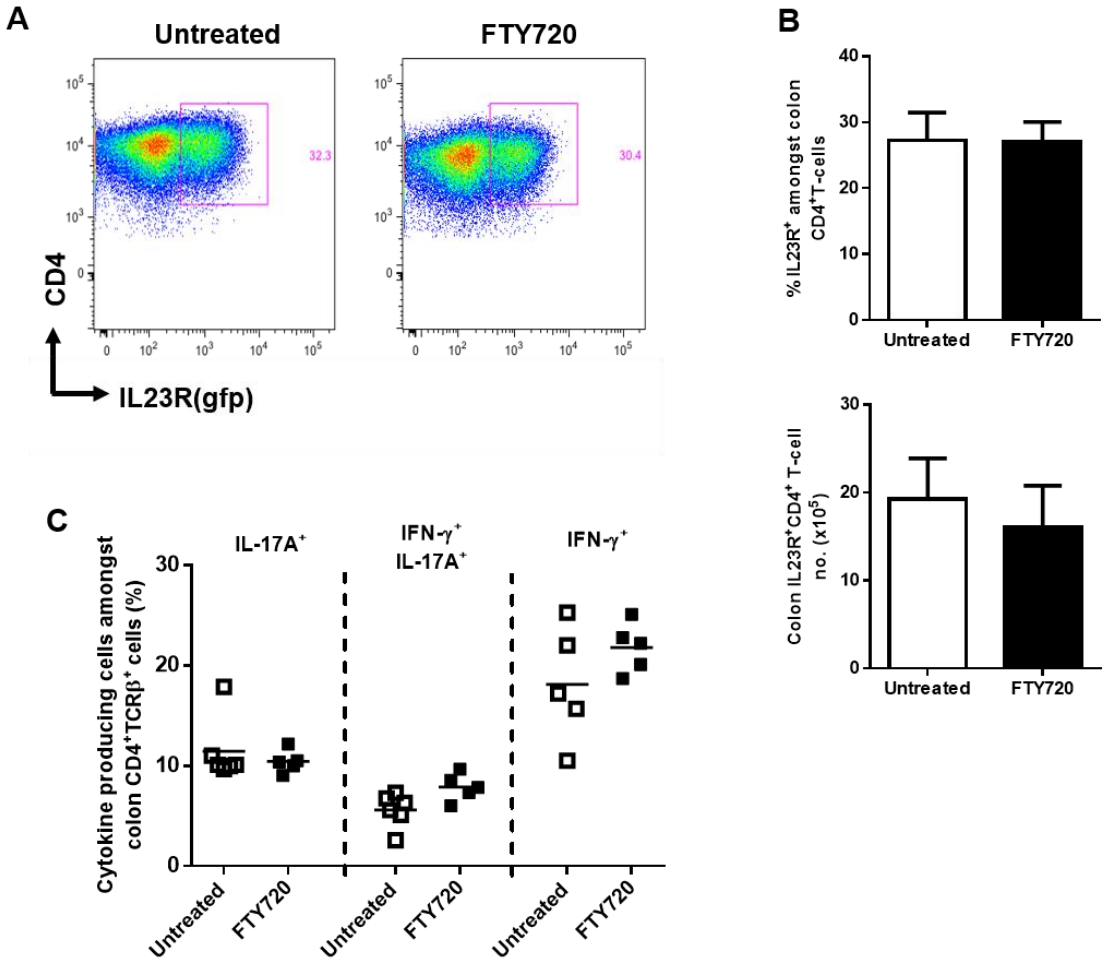

Figure 3.16 FTY720 treatment does not influence colonic CD4+ T-cell expression of IL-23R

IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) to induce colitis. Groups received FTY720 (1mg/kg/day) or PBS by oral gavage as indicated. Mice were sacrificed 14 days after initial infection and organs harvested for analysis. Where indicated, cells were restimulated in vitro with PMA/ionomycin in the presence of Brefeldin A for 4 hours, followed by surface and intracellular staining. **(A)** Representative plots of IL-23R(gfp) expression, gated on colonic live CD4+ T-cells in mice treated with FTY720 or placebo **(B)** Proportion and number of IL-23R(gfp)+ CD4+ T-cells within colonic lamina propria of mice treated with FTY720 or placebo **(C)** Cytokine producing phenotype of colonic CD4+ T-cells flowing in vitro restimulation. Data from single experiment (n=6 each group). Bars represent mean +/-SD. Points represent individual mice. Statistical significance analysed using Mann Whitney test.

3.3 Discussion

The critical role of IL-23 signalling in human IBD and in experimental models of disease is well demonstrated. However, significant uncertainties remain regarding the relative contribution of the various cellular and molecular mediators of IL-23 driven signals to intestinal inflammation. An array of IL-23 responsive cells have been reported and characterised in multiple experimental settings, however data regarding their physiological and pathophysiological roles in the intestine in immunologically intact animals remain poorly defined. We therefore undertook studies to define the distribution and regulation of IL-23R in the healthy and inflamed murine intestine using IL-23R(gfp) reporter mice.

A notable initial finding was the sizable population of IL-23R expressing cells present within the steady state intestine in the absence of inflammation, reflecting significant enrichment compared to lymphoid organs. Although implicated in intestinal immune responses and inflammation in the gut, the role of IL-23 in the steady state is not well understood. Expression of the IL-12/23p40 subunit can be detected in the steady state, with increasing levels seen in the more distal portions of the small intestine and the colon, linked to the presence of the microbiota.[638] Less is known about expression of the IL-23p19, however even in the absence of inflammation, it could be detected at the mRNA level within the colon, although at significantly lower levels than in colitis. Any function for such basal expression remains unclear, however the absence of spontaneous disease in IL-23p19$^{-/-}$ or IL-23R-/- mice argues against an essential contribution to homeostasis. Notably, the presence of an essentially normal complement of IL-23R^{+} cells in IL-23R$^{gfp/gfp}$ mice shows that IL-23 is dispensable for the accumulation or proliferation of steady state IL-23R^{+} cells, in contrast to its essential role in driving accumulation in colitis, where it exerts a cell-extrinsic T-cell dependent effect.[183] It is therefore likely that IL-23R marks specific subsets of cells which show preferential accumulation in the uninflamed intestine through alternative mechanisms. The expression of homing markers such as CCR6 appears conserved across cells displaying other characteristic features of a Th17-type programme, including CD4^{+} T-cells and γδT-cells and is

one such potential contributing mechanism.[365,614] Studies of IL-17 producing γδT-cells, a population which largely overlaps with the IL-23R$^+$ cells studies here, suggests that such cells are fixed in their function, based upon signals received during thymic development.[409,747,764] Therefore IL-23R expression, particularly amongst the more 'innate' types of lymphoid cell present in the non-inflamed intestine, may be a hardwired characteristic rather than being subject to further regulatory signals as it appears to be on CD4$^+$ T-cells.

Despite the description of IL-23R expression amongst myeloid cells including macrophages and DCs,[617] we were unable to reliably detect such populations within the intestine. Small proportions of both these subsets have been reported within lymphoid tissue using the same reporter mouse utilised here, however we struggled to reproducibly replicate this finding in our facility. The combination of the relatively weak gfp signal generated in this system, and the high autofluorescence of these structurally more complex cells both contributed to difficulty in the reliable detection of a positive population. It remains possible that a minor fraction of the myeloid cells in the intestine could express very low levels of IL-23R, however any function for this is unknown. Notably, in T-cell transfer disease, IL-23 signals into innate cells alone are insufficient to drive the development of colitis (C.Schiering, DPhil Thesis). More surprising was the absence of detectable IL-23R(gfp)$^+$NKp46$^+$ cells, since much interest has focussed upon the function of RORgt$^+$NKp46$^+$ cells in IL-23 mediated aspects of intestinal immunity.[72,765] The basis of this is not immediately clear, but replicates observations made by others using this reporter mouse (M.Oukka, personal communication). Importantly, attempts to define the spatial distribution of IL-23R$^+$ cells within the lamina propria by immunofluorescence were unsuccessful, despite using amplification techniques to try and boost the relatively weak gfp signal, therefore we were unable to confirm how representative the cellular populations we observed were of all subsites within the intestine. Localisation of RORγt$^+$ ILCs to lymphoid structures within the intestine has been noted previously,[67] and it is possible that our failure to detect previously reported cell types could be the result of differences in the digestion of such compartments within the intestine, failing to liberate

all cells from complex lymphoid structures, but enriching for those cells diffusely distributed throughout the lamina propria. The successful recovery of large numbers of IL-23R(gfp)$^-$ cells expressing markers such as NKp46 makes this unlikely. Alternatively, whilst it remains possible that this could reflect such low levels of IL-23R expression by cells that they were misclassified as negative, it could also conceivably reflect differences in the colonising flora between facilities. In the steady state we did observe the presence of a number of other ILC subsets expressing IL-23R, including CD4$^+$ LTi-like cells and Thy1$^+$Sca1$^+$ cells, although others remain to be fully characterised. The inter-relatedness of these ILC subsets is currently the subject of much interest,[766-769] and it is possible that there exists a defined niche for IL-23R$^+$ or RORγt$^+$ ILCs within the intestine, with the specific occupying population characteristics determined by modifying factors such as the intestinal microbiota.[765]

The significant enrichment of IL-23R$^+$ cells within the intestine suggests the presence of specific factors promoting their accumulation. The most obvious unique feature of the intestine which might contribute is its resident microbiota, which similarly to IL-23R$^+$ populations shows significant differences by anatomical location. In order to examine this effect in detail, we treated breeding mice with broad spectrum antibiotics, in order to produce offspring in which a normal flora was never established. We used this approach as we were concerned that any effects of the flora on more innate-type IL-23R$^+$ cells, such as ILCs and γδT-cells might be specifically active at an early stage in post-natal development, and that such cells might not display later microbial dependence for their continued presence. Interestingly, we observed a similar total proportion of cells expressing IL-23R(gfp), but marked differences from normal SPF mice in the composition of this pool, implying the existence of a niche for IL-23R$^+$ cells, with the specific occupying subsets determined by as yet undefined factors. The most notable effect of antibiotic treatment was to greatly reduce the IL-23R(gfp)$^+$CD4$^+$ T-cell population in the colonic lamina propria, an observation largely mirroring the striking microbial dependence previously reported for IL-17A

producing CD4+ T-cells, although in replicating this latter finding we found effects on IL-23R expression were less marked than those seen for IL-17A production.[421]

Whether IL-23R expression on T-cells is reduced in antibiotic treated animals due to failure of these cells to ever populate the intestine, or due to a failure to maintain this population is not clear. Similarly, whether IL-23R is downregulated in the absence of the flora, leaving 'ex-IL-23R$^+$' cells, or whether such cells undergo cell death in the absence of important survival signals remains to be determined. The more pronounced changes in IL-17A$^+$ cells resulting from antibiotic treatment implies that different or additional microbial influences mediate this effect. Previous studies have implicated ATP derived from the microbiota,[43] or serum amyloid A protein[424] as mediators of intestinal Th17 development, defined by IL-17A production, although a more recent study highlighted a role for IL-1β.[770] It is likely that a range of factors are involved, all present in the normally colonised intestine. However, our findings contrast with a single study which reported expanded colonic IL-17A$^+$CD4$^+$ T-cell numbers in germ-free mice, attributed to loss of IL-25 mediated suppression of the IL-23/Th17 axis.[758] It is important to note the significant differences between antibiotic treatment and germ-free conditions, since antibiotic treated mice still harbour a flora, albeit a significantly diminished one. Furthermore, the presence of dead bacteria in the intestines of antibiotic treated animals will result in effective ligation of PRRs within the intestine, influencing the prevailing cytokine environment.

Importantly, these studies strongly suggested differences may exist in aspects of the regulation of the diverse IL-23R expressing cells found in the steady state lamina propria. Whereas IL-23R expression on T-cells is linked to RORγt, and downstream of signals including TGF-β and the STAT3 activating cytokines IL-6 and IL-21,[175,211] its regulation on non T-cells is incompletely defined. Furthermore, the functional relevance of IL-23R expression on non T-cells is similarly uncertain. Although IL-23R marks populations of cells highly enriched for IL-17A production, it

is clear that this function is independent of IL-23 signals, at least in $CD4^+$ and γδT-cells. In $CD4^+$ T-cells IL-23 has been reported to drive the progression from IL-$17A^+$ cells to an IL-$17A^+$IFN-γ^+ and ultimately IL-$17A^-$IFN-γ^+ phenotype, at the expense of IL-10 production.[183,298,299,306] However, little evidence exists for such plasticity amongst cells other than $CD4^+$ T-cells. Extensive studies undertaken to better define the role of intestinal γδT-cells in intestinal immunity are described later (chapter 4), but the function of some of the other unconventional IL-$23R^+$ populations described here, such as ILCs and $CD4^-CD8^-$ T-cells remains to be determined, particularly in lymphocyte replete hosts. Importantly, a functional role for these latter cells has been demonstrated in an IL-23 dependent model of intraperitoneal *Listeria monocytogenes* infection,[384] in pulmonary infection with *Francisella tularensis*, and in a model of enthesitis.[615,771]

In an attempt to understand how various IL-$23R^+$ cells might be contributing to intestinal inflammation, we studied the kinetics of each of the major subpopulations during evolution of disease. Remarkably, although all IL-$23R^+$ populations increased in number in colitis, $CD4^+$ T-cells very rapidly became the numerically dominant subset, and were the major source of IL-17A from very early after initiation of disease. Moreover, the increases in IL-$23R^+$ γδT-cell and ILC numbers occurred with similar kinetics to $CD4^+$ T-cell accumulation, somewhat arguing against the hypothesis that these cells might be poised to mediate early IL-23 driven immunity, awaiting the development of the adaptive T-cell response. This is in notable contrast to observations in models of inflammatory diseases in the skin, lung, and central nervous system, wherein large numbers of IL-$23R^+$ or IL-$17A^+$ γδT-cells accumulate rapidly and are the dominant early IL-17A producing population until the later appearance of $CD4^+$ Th17 cells.[383,387-390,392,614] This phenomenon has been proposed to amplify and augment the subsequent Th17 response. We hypothesised that the basis for this difference between anatomical sites might relate to the large pre-existing population of $CD4^+$ T-cells in the intestine, which is not present in other sites studied.

However, notable differences exist between the IL-$23R^+CD4^+$ T-cells we observed in steady state mice and those accumulating in inflammation. This is perhaps unsurprising, since the presence of

large numbers of such cells does not by itself result in spontaneous intestinal disease, indeed expansion of small intestinal Th17 cells in conditions of extra-intestinal infection, without obvious negative effects on the gut further supports this.[307] Whereas steady state IL-23R$^+$CD4$^+$ T-cells were largely IL-17A$^+$ without additional effector cytokine production, in the inflamed intestine sizable populations of IL-17A$^+$IFN-γ$^+$ cells emerged. The presence of such 'double-positive' cells is widely reported in diseases thought to be driven by Th17 cells, including murine models of colitis[183,302] and human Crohn's disease.[301] The emergence of this subset has been strongly linked to IL-23 signals,[183,299] and has been proposed as a mechanism mediating its pathogenic effects in inflammatory diseases. However, the relationship of such inflammatory Th17 cells to those present in the steady state intestine is currently unclear. Previous studies *in vitro* and *in vivo* in non-intestinal diseases have defined differentiation conditions or factors which increase or attenuate Th17 pathogenicity, including exposure to TGF-β1 or IL-23 and the expression of T-bet.[202,298,306] Importantly, it has been reported that T-cells differentiated to IL-17A producing Th17 cells without exposure to IL-23 lack pathogenicity in an adoptive transfer model of EAE, linked to a requirement for IL-23 to drive the production of TGF-β3 and the acquisition of a specific transcriptional profile.[753] Whether this effect of IL-23 stimulation is only active during initial differentiation or could occur in existing effector memory T-cells, as we observed in the healthy intestine, has not been reported. Further studies of the transcriptional differences between steady state Th17 cells and those accumulating in disease will be informative. Similarly, studies of the molecular pathways downstream of IL-23 signalling into steady state IL-23R$^+$ cells might yield important insights. Finally, analysis of the epigenetic status of genes implicated in the pathogenicity of 'inflammatory' Th17 cells in steady state cells may be enlightening

We were interested to understand the rapid accumulation of IL-23R$^+$CD4$^+$ T-cells occurring in colitis, and considered a number of potential underlying mechanisms. Components of the existing IL-23R$^+$ cell population might be expanding through proliferation, or previously IL-23R$^-$ cells

might be upregulating receptor expression. Alternatively, existing IL-23R^{+} cells might be specifically recruited from the circulation, deriving from lymphoid tissues, or newly differentiated Th17 cells might be homing back to the gut; however, the accumulation seemed to begin rather too early for the latter explanation. It was therefore notable that simply inhibiting IL-10R signalling by antibody treatment resulted in a significant increase in the proportion of CD4^{+} T-cells that expressed IL-23R. This effect was unique to CD4^{+} T-cells, and may relate to the high levels of IL-10R expression reported on Th17 cells.[622] Studies using CD4 specific dominant-negative IL-10R expression have reported IL-10 signals directly inhibit the emergence of IL-17A^{+} and IL-17A^{+}IFN-γ^{+} populations,[622] and it seems likely that our findings at least partially underlie such observations. The rapid increase in the proportion of colonic T-cells expressing the IL-23R following treatment with anti-IL10R antibody, without significant increases in total cell numbers, would support receptor upregulation on previously IL-23R^{-} cells as the underlying mechanism. Importantly, we did not undertake further functional assessment of these cells, so it cannot be determined whether the increase in IL-23R(gfp) expression was paralleled by an increase in IL-17A^{+} populations. Our studies in antibiotic treated animals demonstrated the imperfect correlation between IL-23R expression and IL-17 production by CD4+ T-cells. Similarly, it cannot be determined what proportion of 'newly' IL-23R^{+} cells were truly negative previously. In contrast to the clear separation of IL-23R(gfp)$^{+}$ and $^{-}$ populations amongst γδT-cells and CD4^{-}CD8^{-} T-cells using this reported system, expression on CD4^{+} T-cells was more of a continuum between highly positive and clearly negative. Therefore, the increase in IL-23R^{+} cells may have resulted from upregulation on cells which were previously not clearly gfp^{+}, representing low but not zero expression. However, the rapid increase in numbers of IL-23R^{+}CD4^{+} T-cells in colitis demonstrates that such an effect is not operating in isolation. IL-23 is itself a potent proliferative signal for T-cells, therefore cells upregulating receptor expression within the IL-23 rich inflammatory environment may subsequently proliferate. Previous studies of IL-23R$^{-/-}$:WT bone marrow chimeras in this model of disease, examined after 4 weeks, have shown that IL-23 drives proliferation through a cell extrinsic effect, however this does not exclude a non-essential contribution from such signals to early accumulation.[183]

Finally, considering the presence of existing IL-23R^{+}CD4^{+} cells in the uninflamed lamina propria, and the relative paucity of such cells within the circulation or lymphoid tissues, we questioned what effect blocking lymphocyte circulation would have on their accumulation in disease. Importantly, treatment with FTY720 as used here has been shown to prevent disease in EAE,[772,773] in which expansion of Th17 cells in the lymph nodes followed by accumulation in the CNS is a critical event.[392] Although FTY720 has previously been shown to prevent intestinal disease in both naïve and CD44^{hi} T-cell transfer models,[774-776] its potential effect in lymphocyte replete models of chronic colitis are less clear.[777-779] This is an important issue, since colitogenic T-cells have been reported to reside outside of the intestine,[780-782] predicting that recirculation may be a critical step in disease development. However, despite successfully achieving adequate drug levels to induce a number of expected changes, including peripheral lymphopenia,[761-763] no effect on colitis was seen. In addition, no reduction in the proportion of lamina propria CD4^{+} T-cells expressing IL-23R occurred in FTY720 treated animals, nor was there any apparent effect on the effector phenotypes of accumulating cells. These experiments therefore suggest that the steady state intestinal T-cell pool contains cells which are able to initiate and drive colitis in this model, and that trafficking of IL-23R^{+}CD4^{+}T-cells from lymphoid tissue is not required for the development of disease.

However, these studies do require a number of cautions in their interpretation. Firstly, inflammation was assessed at an early stage, and whether chronic colitis would be similarly unaffected was not studied. Secondly, despite being in clinical use, the mechanism of action of FTY720 in treating inflammatory disease remains debated. Whilst clear effects on lymphocyte recirculation are reported in both rodents and humans, additional effects on the function of subsets such as regulatory[774,779,783,784] or effector memory T-cells[762] have been noted in specific settings. Similarly, non-immunological effects have been proposed by some groups to underlie the efficacy of FTY720 in neuroinflammation.[785,786] Furthermore, effects on naïve T-cell circulation may be more pronounced than occur for mature effector populations.[761,763,787] Although initially FTY720

treatment increases lymphoid tissue cell counts due to sequestration, more prolonged administration (>14 days) is associated with reduced cellularity, and depleted total body lymphocyte counts, as observed in our mice.[788] The mechanism underlying this is unknown, although FTY720 may induce apoptosis at supratherapeutic doses.[789-791] Therefore it is difficult to quantify how effective treatment was at preventing effector cell re-circulation. Finally, FTY720 reduces egress from small intestinal Peyer's patches, and could theoretically exert a similar effect on organised lymphoid structures in the colon.[792-794] Therefore, FTY720 might be paradoxically 'locking' cells in the intestine, overcoming normal regulatory mechanisms. Studies inhibiting other key pathways involved in cell trafficking to sites of inflammation, such as integrins and adhesion molecules could clarify and enhance the significance of these findings.

However, beyond these issues, it is highly relevant to note the simple lack of efficacy of a treatment for colitis which is a potent inhibitor of disease in neurological inflammation. This finding highlights the potential differences in the underlying mechanisms active in initiating and sustaining inflammation between these 2 anatomical sites. From a translational viewpoint this is important as clinical trials of S1P-inhibitors including FTY720 (Fingolimod) are in progress in human IBD. The results of our studies suggest this may be a less effective approach in IBD than has been noted in trials in multiple sclerosis.[795,796]

In conclusion, our studies highlight the complexity of the underlying cellular basis of IL-23 dependent immune and inflammatory pathways in the intestine. We show that remarkable changes occur in the cellular targets of IL-23 in inflammatory states, firmly implicating $CD4^+$ T-cells as the most relevant population to target in intestinal disease. Furthermore, we demonstrate specific mechanisms regulating IL-23R expression on $CD4^+$ T-cells, and regulating their accumulation in disease, which should now be explored in human IBD.

Chapter 4. The Role of γδT-cells in Chronic Intestinal Inflammation

4.1 Introduction

Shortly after the initial description of a population of T-cells characterised by their use of the TCRγδ, it became clear that such cells were remarkably enriched at mucosal surfaces, including the intestines, reproductive tract and skin.[325,334,345] Numerous subsequent studies have described in intricate detail aspects of the ontogeny and molecular programmes of these cells,[329,797,798] as well as their contributions to homeostasis and to aspects of infectious, inflammatory and neoplastic diseases.[119,338,798,799] Importantly, γδT-cells share a number of overlapping features with conventional αβT-cells, being capable of cytokine production and cytotoxicity,[365,372,390,392,399,401,614,800] as well as the regulation of other cell types during immune responses.[400,801-803] However, in contrast to conventional T-cells, γδT-cells display a limited TCR repertoire with tissue-specific patterns of expression, with many cells appearing to recognise self-antigens including those induced by cellular stress.[354,355,358,367,804,805] Uniquely, γδT-cells may be activated without TCR ligation, and the role of the TCR itself on such cells remains uncertain. Therefore, γδT-cells are often characterised to lie between true innate lymphoid cells and adaptive αβT-cells in their immunological function.[797]

However, it is increasingly clear that some subsets of γδT-cells may exert more specialised functions not generally observed amongst αβT-cells, particularly relating to the resolution of inflammation,[399,802] tissue repair and wound healing.[402,404,405] Subsets of γδT-cells present within the intestinal intraepithelial lymphocyte (IEL) compartment may interact with luminal contents to exert a direct regulatory effect over the composition of the microbiota through elaboration of antimicrobial peptides and fortification of the epithelial barrier.[403,406,407,806,807] An important functional role for this has recently been shown in the maintenance of intestinal homeostasis and susceptibility to inflammation.[198,407] Within the broader context of intestinal inflammation, γδT-

cells have been ascribed both protective and pathogenic roles in diverse models of disease. In acute colitis induced by dextran sodium sulphate (DSS) administration, mice deficient in γδT-cells develop more severe disease, linked to impairment of barrier function and epithelial recovery.[403,808] Conversely, in TCRα$^{-/-}$ mice which develop a spontaneous disease resembling ulcerative colitis, γδT-cells accumulate and directly promote disease, with TCRα$^{-/-}$TCRγ$^{-/-}$ animals largely protected.[809,810] Furthermore, when transferred into Rag$^{-/-}$ mice, γδT-cells appear able to drive a disease similar to that seen with naive αβT-cell transfer, and when co-transferred with naïve CD4^{+} αβT-cells, result in more severe disease.[811,812] Mice lacking γδT-cells may be more susceptible to specific enteric infections, but this appears a highly age related effect, disappearing with the full development of conventional αβT-cells.[813] Therefore, significant uncertainties remain as to the true significance of γδT-cells in intestinal immunity.

Following the recent description of CD4^{+} Th17 cells, it became clear that a corresponding phenotype of γδT-cells exists which is capable of rapid IL-17A production, without need for TCR stimulation.[365,614] Important roles for such cells have now been demonstrated in a number of IL-23 or Th17 dependent disease models, including skin, joint, lung and neurological inflammation, in which such cells provide an early source of IL-17A and other 'Th17' type cytokines, including IL-21 and IL-22, and may suppress regulatory T-cell activity to allow Th17 responses to develop.[365,387,389,614,814,815] However, in contrast to conventional T-cells, such cells appear developmentally fixed in their phenotype and display little of the functional plasticity which is a notable feature of αβT-cells.[410,816,817]

In our previously described studies of IL-23R expression, we noted sizable populations of IL-23R^{+} and IL-23R^{-} γδT-cells within the lamina propria, the functions of which were not clear. We hypothesised that such cells might be providing an important contribution to intestinal homeostasis, immunity and inflammation. A better understanding of intestinal γδT-cells may

become increasingly relevant as therapies directed at these cells become increasingly available. We therefore undertook studies to better define the role of intestinal γδT-cells in chronic intestinal inflammation.

4.2 Results

4.2.1 Intestinal lamina propria γδT-cells are a phenotypically distinct population

During studies of IL-23R expressing cells in the steady state intestine, we identified a significant population of γδT-cells in the colonic lamina propria, of which >50% were IL-23R^{+} (Chapter 3). Whilst undertaking phenotyping studies of these cells, it became apparent that whereas γδT-cells in the intestine have been extensively studied in the context of the large population of such cells resident in the small intestinal IEL compartment,[335,337,818] very little information is available as to the distribution, phenotype and function of γδT-cells in other anatomical locations such as the lamina propria or the colonic IEL. Therefore, using flow-cytometry, we analysed γδT-cells present in the IEL and the lamina propria of small intestine and colon, and from lymphoid tissue including peripheral lymph nodes, mesenteric lymph nodes and the spleen.

Initially, confirming previous reports, we found γδT-cells to comprise 50-80% of small intestinal IELs,[335,337,818] amongst which >80% of cells expressed the CD8αα co-receptor (Fig 4.1A). Within colonic and caecal IELs, a somewhat smaller fraction (12-20%) of cells expressed the γδTCR, and amongst these a similarly smaller proportion expressed the CD8αα homodimer (20-40%) (Fig 4.1A). In contrast, γδT-cells were a minor population amongst total lymphocytes within the lamina propria and lymphoid organs, although the colonic lamina propria was relatively enriched for these cells (Fig 4.1B). Cells in locations other than the IEL demonstrated comparatively low rates of CD8α expression, although the small intestinal lamina propria was a notable exception.

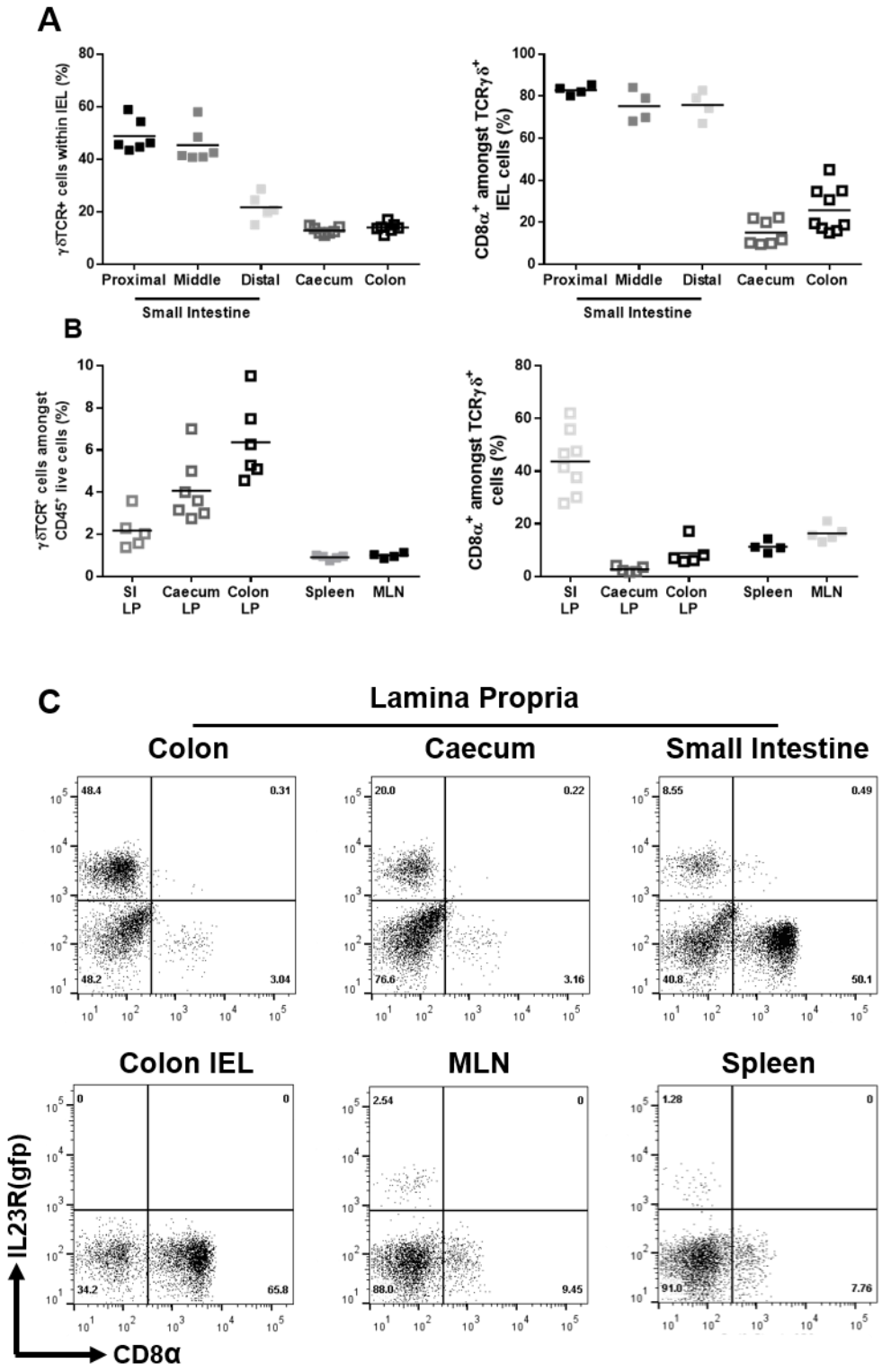

Figure 4.1 Variation in the surface phenotype of γδT-cells in diverse anatomical sites

Unmanipulated 8-12 week old C57BL/6 (A&B) or IL-23Rgfp/+ (C) mice were sacrificed and cells isolated from indicated tissues. Cell phenotype was determined by surface staining and flow cytometry analysis. **(A)** & **(B)** Proportion of total CD45+ live cells expressing TCRγδ (left panel) and proportion of TCRγδ+ cells expressing CD8α (right panel) by indicated location.**(C)** Representative plots of IL-23R(gfp) and CD8α expression by location. Gated on live TCRγδ+ cells. Results representative of >3 independent experiments, (n=5-8).

Within the distal intestine, IL-23R+ cells constituted major populations within the total γδT-cell pool, although a clear increasing gradient from ileum to caecum and colon was apparent (Fig 4.1B). Minimal proportions of lymphoid γδT-cells expressed either IL-23R or CD8α, and indeed no co-expression of these markers was observed in any compartment (Fig 4.1B).

γδT-cells isolated from specific anatomical sites are reported to display limited and stereotyped Vγ chain usage. We therefore studied Vγ expression using flow-cytometry within multiple anatomical compartments (Fig 4.2A). Confirming published reports,[335] Vγ1$^+$ cells, often implicated as a pro-inflammatory population, predominated in lymphoid tissues and were highly represented within the IEL, whereas Vγ7$^+$ cells were the major component of the IEL but uncommon elsewhere. Vγ4$^+$ cells were a minor population within all tissues examined. Importantly, whereas we could identify the Vγ expression of 70-90% of cells at other sites, our results only identified the Vγ profile of ~40% of lamina propria γδT-cells (Fig 4.2B). Further studies confirmed the absence of cells expressing Vγ5, the receptor associated with dendritic epidermal T-cells (DETC) within the skin (data not shown), however commercial reagents against remaining Vγ chains are not available.

γδT-cells within the colonic lamina propria displayed a near universal (>90%) antigen-experience, activated phenotype, based upon patterns of expression of CD44, CD62L and CD69 (Fig 4.2B). This contrasted with lymphoid tissues, in which large proportions of cells expressed CD62L, and only minor fractions (<10%) displayed the activation marker CD69. Further differences between γδT-cells present within the intestinal lamina propria and lymphoid tissues were apparent for expression of the cytokine receptors CD25 and CD127 (Fig 4.2C). As expected in view of the critical role IL-7 as a survival factor for γδT-cells,[819-821] high levels of CD127 expression were seen in all compartments, yet CD25 was only observed on significant proportions of cells within the lamina propria.

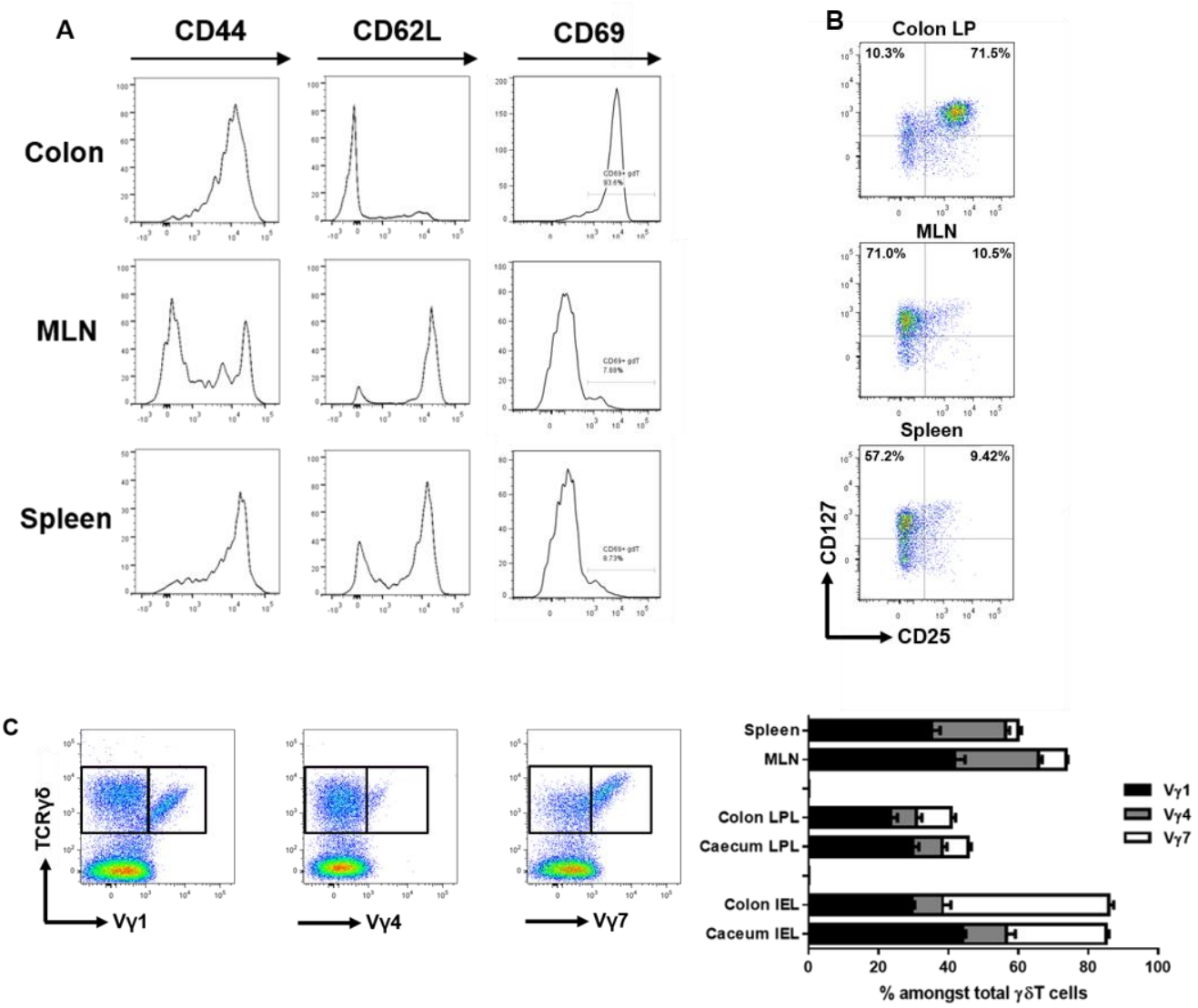

Figure 4.2 Variation in activation status and TCR use by γδT-cells in diverse anatomical sites

Unmanipulated 8-12 week old C57BL/6 mice were sacrificed and cells isolated from indicated tissues. Cell phenotype was determined by surface staining and flow cytometry analysis. **(A)** & **(B)** Expression of indicated surface markers by tissue site. Gated on live TCRγδ+ cells **(C)** Representative plots of TCR staining for indicated Vγ on colonic cells, gated on live CD45+ cells (left), and composition of total γδTCR+ by location. Results each representative of 1 experiment analysing 6 replicates of cells pooled from 4 mice.

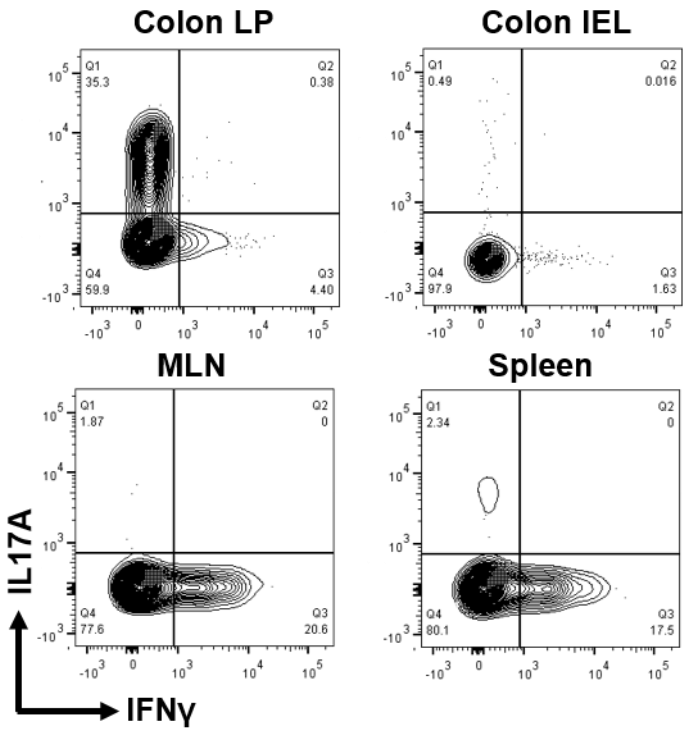

Figure 4.3 Variation in cytokine producing phenotype of γδT-cells in diverse anatomical sites

Unmanipulated 8-12 week old C57BL/6 mice were sacrificed and cells isolated from indicated tissues. Cells were restimulated in vitro using PMA/ionomycin for 4 hours in the presence of Brefeldin A, followed by surface and intracellular staining. Plots shown representative of results from 2 independent experiments (n=6-8 each).

Finally, we examined the function of γδT-cells within the intestine by *ex vivo* restimulation and intracellular cytokine staining. Whereas a significant proportion of colonic lamina propria cells produced IL-17A (Fig 4.3), with a smaller fraction producing IFN-γ, no production of these cytokines could be detected amongst IEL cells, confirming previous reports.[372,822] Within lymphoid tissues, although IFN-γ producing cells were readily detected, IL-17A$^+$ cells were virtually absent.

In total these results demonstrate lamina propria γδT-cells populations to be markedly different from those present within the IEL and lymphoid tissues, suggesting they may be occupying a specific cellular niche within the intestine.

4.2.2 Intestinal IL-23R$^+$ γδT-cells exhibit tissue-specific characteristics

IL-23R$^+$γδT-cells are functionally important early in the development of murine models of intra-peritoneal infection[384] and neuroinflammation.[392] Similarly, IL-17A$^+$γδT-cells, which are enriched for expression of the IL-23R, are reported to support early immune responses in the skin, lung and joints. Noting both the enrichment of IL-23R$^+$γδT-cells within the intestine, particularly at more distal sites, and the significant differences between overall peripheral and intestinal γδT-cells, we therefore questioned whether intestinal IL-23R$^+$γδT-cells were similarly site specific, or represented a more conserved population.

Studying colonic IL-23R$^+$γδT-cells, we noted a CD27^{lo}CCR6$^+$NK1.1$^-$ phenotype, similar to that reported for extra-intestinal populations[392,614,617] (Fig 4.4A). Previous characterisation at other anatomical sites has shown such cells are enriched for Vγ1 and to a greater extent Vγ4 expression, with circumstantial evidence suggesting some may use Vγ6.[392,614,617] In our previous studies of unsorted γδT-cells we had been unable to identify the Vγ use of the majority of colonic cells.

Repeating these studies using IL-23R(gfp) mice confirmed that <5% of colonic IL-23R$^+$ γδT-cells utilised either Vγ1 or Vγ4 (Fig 4.4B). We therefore sorted γδT-cells from the colons of un-manipulated IL-23R$^{gfp/+}$ mice, and examined the expression of Vγ sequences by RT-PCR. Whilst these studies confirmed low levels of expression of Vγ1 and Vγ4, they did not reveal significant expression of any other Vγ analysed (Fig 4.4C). We were particularly interested to assess Vγ6 expression, since lung IL-17A$^+$ γδT-cells have been reported to use this receptor chain,[823] however we struggled to consistently detect Vγ6 amplification within the lymphoid cells isolated from lung tissue used as a positive control, despite the use of published sequences. Therefore, our failure to detect its expression within intestinal γδT-cells might reflect true lack of expression or technical limitations, and the Vγ repertoire of intestinal IL-23R$^+$γδT-cells remains to be fully defined.

Using sorted γδT-cells from steady-state IL-23R$^{gfp/+}$ colons, we next analysed gene expression by quantitative RT-PCR (Fig 4.4D). This revealed similar expression profiles to those previously reported for both CD4$^+$ Th17 cells and for extra-intestinal IL-23R$^+$γδT-cells, including significant enrichment for *Rorc* and *Il1r*, and very low expression of *Il12r*. Interestingly, expression of *Ahr* was not apparently enriched in unstimulated steady-state lamina propria IL23R$^+$γδT-cells, in contrast to the transcriptional profile reported for the lymphoid resident IL-17A$^+$γδT-cells implicated in driving neuroinflammation.[365]

Finally, we assessed the effect of IL-23 stimulation on *in vitro* proliferation, an effect previously demonstrated for lymphoid populations.[814] However, despite replicating the latter finding, we were unable to induce intestinal IL-23R(gfp)$^+$γδT-cells to proliferate at the concentrations of IL-23 used (data not shown).

In total, these studies show that intestinal IL-23R$^+$γδT-cells demonstrate both conserved features of extra-intestinal cells and tissue specific characteristics.

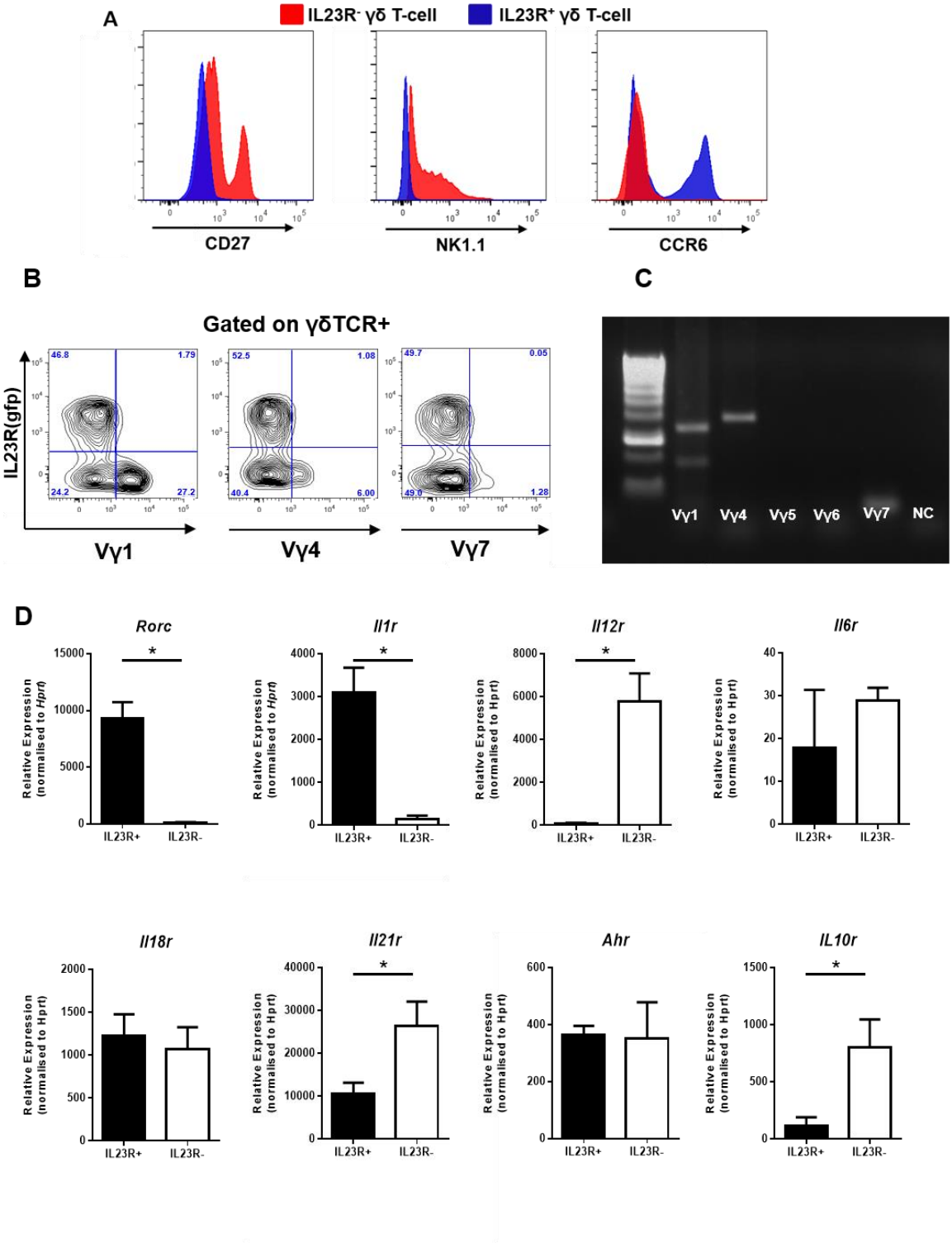

Figure 4.4 Intestinal IL-23R+ γδT-cells display conserved and tissue specific characteristics

Unmanipulated 8-12 week old IL-23Rgfp/+ mice were sacrificed and colonic cells isolated. **(A)** Expression of indicated surface marker on γδT-cells by IL-23R status **(B)** Representative plots of Vγ staining on colonic cells, gated on live γδT+ cells. **(C)** RT-PCR for Vγ expression within colonic IL-23R(gfp)+ γδT+ cells isolated by flow cytometric sorting. **(D)** Expression of indicated genes in colonic γδT+ cells sorted on the basis of IL-23R(gfp) status. mRNA levels determined by qPCR and normalised to *Hprt*. Representative results from 2 independent experiments (n=6-8) (A&B), or single experiment (C&D) (n=24 analysed as 6 pools of 4). Columns show mean + SD. Statistical significance determined by Mann Whitney Test. *$p<0.05$.

4.2.3 γδT-cells accumulate in the lamina propria and IEL compartments in chronic intestinal inflammation

Noting the enrichment of γδT-cells within the healthy colon, we next questioned their potential contribution to intestinal inflammation. γδT-cell infiltration in the colon has been reported in several models of colitis, however the majority of reports fail to differentiate IEL from lamina propria populations (either in their preparation or reporting), or have utilised models in which mice lack either conventional αβT-cell function[810,815] or are lymphopoenic prior to receiving adoptively transferred cells including the γδT-cells.[811,812] The pathophysiological relevance of these studies is unclear, particularly where transferred γδT-cells are derived from lymphoid tissues. Therefore, we studied the changes occurring amongst intestinal γδT-cell populations in a more physiologically relevant model.

Using *H.hepaticus* infection combined with anti-IL-10R blockade, we induced disease in wild-type C57BL/6 and IL-23R$^{gfp/+}$ mice, and followed the kinetics of lamina propria and IEL populations during the evolution of chronic colitis (Fig 4.5). Total IEL numbers increased 3-5 fold in established colitis, although the proportion of colonic IELs expressing TCRγδ was unchanged (Fig 4.5A). Notably, a significant increase in the proportion of CD8α+ cells occurred, with such cells representing >70% of total γδT-cells in this compartment during inflammation. IL-23R(gfp)$^{+}$γδT-cells could not be detected within the IEL during colitis (data not shown). More marked changes were noted within the lamina propria, as total γδT-cell populations expanded significantly within 7 days of induction of disease, peaking during the second week before undergoing a contraction up to day 28, kinetics which were remarkably similar to the total colonic leukocyte infiltrate, resulting in γδT-cells accounting for ~2-5% of total CD45^{+} cells in the lamina propria at all time points (Fig 4.5B). At the peak, this represented an 8-10 fold increase in total γδT-cells numbers compared to the steady state population. Strikingly, this expansion of the total γδT-cell population was almost exclusively driven by accumulation of IL-23R(gfp) negative cells, with the major steady state IL-23R(gfp)$^{+}$ population never more than trebling in numbers, even

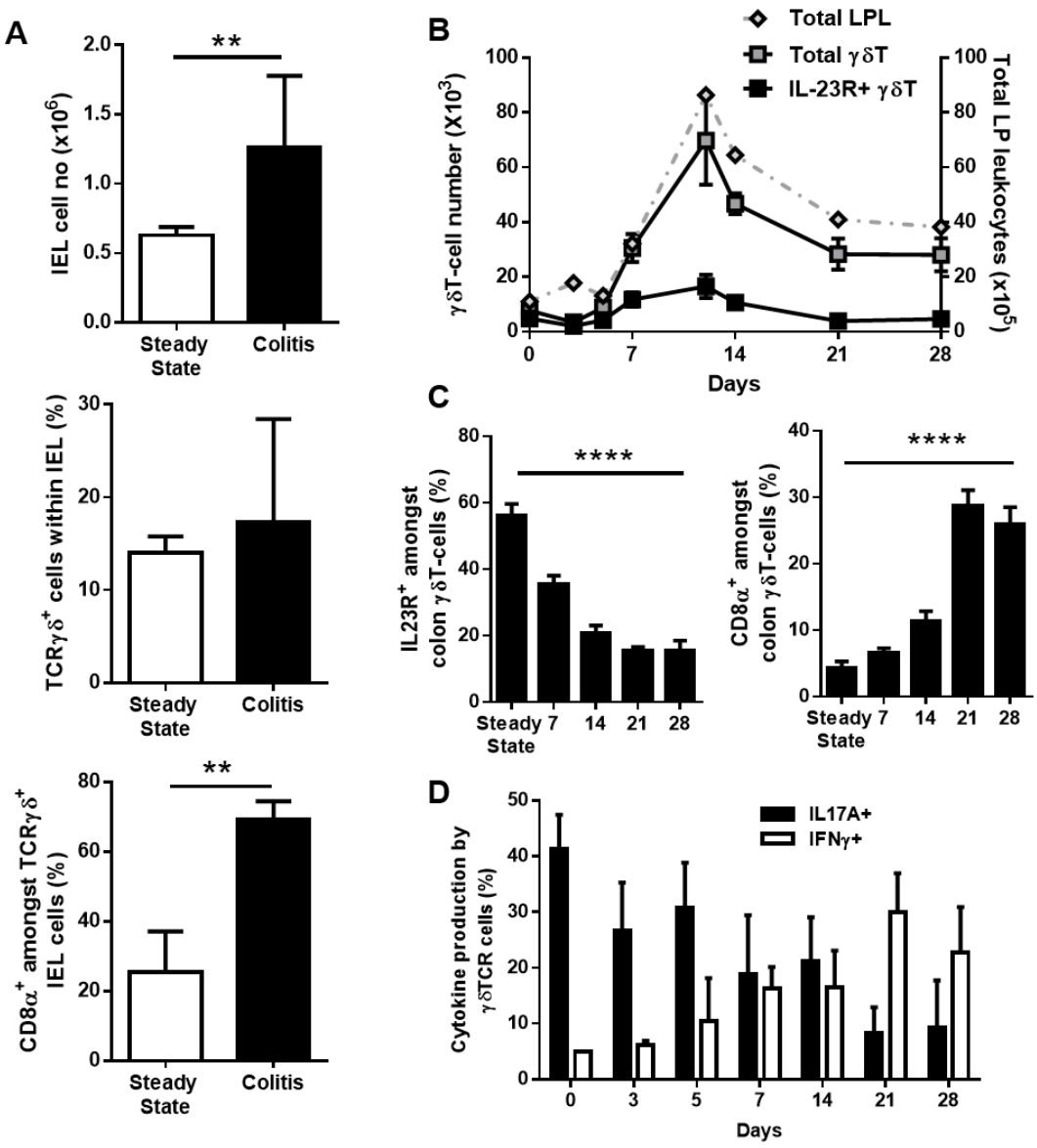

Figure 4.5 γδT-cells accumulating in chronic colitis exhibit phenotypic differences to steady state populations

IL-23R$^{gfp/+}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) weekly for up to 4 weeks. Mice were sacrificed at indicated time points until 28 days after initial infection and colonic cells were isolated for analysis. Where indicated cells were restimulated in vitro using PMA/ionomycin for 4 hours in the presence of Brefeldin A, followed by surface and intracellular staining. **(A)** Number and phenotype of colonic IEL γδT-cells in colitis. **(B)** Kinetics of γδT-cell accumulation in the colonic lamina propria throughout evolving colitis. **(C & D)** Kinetics of subpopulations of colonic TCRγδ+ cells in evolving colitis by surface phenotype (top) and function (bottom). Points and bars represent mean at each time point +/-SEM. Data pooled from 2 independent experiments, n=8-16 mice at each time point. Statistical significance analysed using Mann Whitney test. ***p*<0.01, *****p*<0.0001.

during the very earliest stages of disease (Fig 4.5B & C). The expanded γδT-cell population exhibited marked functional differences from steady state populations, with accumulation of IFN-γ producing cells, and reduction in the proportion of cells producing IL-17A, although as seen for IL-23R expression, numbers of such cells were maintained (Fig 4.5D).

To assess whether these patterns of change were simply related to our choice of model, or were typical of the colonic lamina propria γδT-cell response to inflammatory stimuli, we repeated these experiments using the *Citrobacter rodentium* model of acute infective colitis. These studies demonstrated that acute colitis was also associated with accumulation of γδT-cells, although the overall proportion amongst colonic $CD45^+$ cells was again little changed from the steady state (Fig 4.6). As in chronic disease, accumulating cells were primarily IL-$23R^-$γδT-cells, with preservation but limited expansion of the IL-$23R^+$ populations.

In total, these findings suggest that in contrast to the rapid accumulation seen in infection or inflammation models within other anatomical compartments, colonic IEL and lamina propria γδT-cells are relatively slow to expand in evolving inflammation, and do so due to the appearance of populations which differ phenotypically and functionally from steady state cells.

4.2.4 Deficiency of γδT-cells results in increased severity of disease in *H.hepaticus* induced colitis

We were interested to understand more regarding the function of γδT-cells in the intestine and their potential contribution to chronic colitis. The consequences of γδT-cell deficiency have previously been reported in a number of models of acute intestinal inflammation with conflicting results, appearing to be mainly protective in models where epithelial injury is prominent, but pathogenic in other settings.

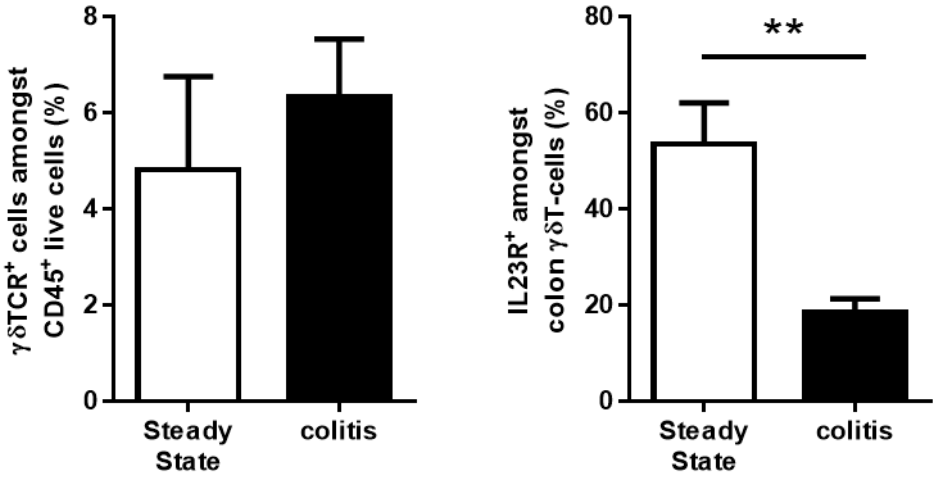

Figure 4.6 γδT-cells accumulating in *C.rodentium* induced acute colitis exhibit phenotypic differences to steady state populations

IL-23R$^{gfp/+}$ mice were infected with a single dose of ~10^9 CFU *C.rodentium* by oral gavage, and sacrificed at 14 days after infection. Graph shows proportion of colonic lamina propria live CD45+ cells expressing TCRγδ (left panel) and proportion of TCRγδ+ cells co-expressing IL-23R(gfp). Bars represent mean +/-SD. Data from 1 experiment, n=8 mice in each group. Statistical significance analysed using Mann Whitney test. ** $p<0.01$.

We therefore infected TCRδ$^{-/-}$ mice, which lack all γδT-cells, with *H.hepaticus* and concurrently treated them with blocking anti-IL-10R antibody. Strikingly, we found that γδT-cell deficiency was associated with more severe intestinal disease consistent with a protective role in this model. TCRδ$^{-/-}$ animals developed more severe histological evidence of typhlocolitis (Fig 4.7A&B), with greater numbers of infiltrating cells in the lamina propria (Fig 4.7C), and significantly elevated expression of both IL-17A and IFN-γ compared to wild-type controls (Fig 4.8B). Intracellular staining of lamina propria CD4+ T-cells *ex vivo* demonstrated a significantly increased proportion of cells expressing IFN-γ in TCRδ$^{-/-}$ mice, with a reduced IL-17A^{+} population (Fig 4.8A) Analysed numerically, this was seen to reflect significantly greater numbers of IL-17A^{+}IFN-γ^{+} and IL-17A^{-}IFN-γ^{+} cells accumulating in the colons of TCRδ$^{-/-}$ mice, but similar numbers of IL-17A^{+}IFN-γ^{-} cells to those seen in WT animals (Fig 4.8A). Conversely, the absolute number of CD4^{+} T-cells expressing the Treg marker Foxp3^{+} was similar to wild-type controls, although the proportion of such cells amongst all T-cells in the colon was significantly reduced (Fig 4.8A). Colonic expression of other pro-inflammatory cytokines, including IL-17F, IL-21, IL-22, and TNF-α was similar in both groups (Fig 4.8B). In contrast to the marked differences in the intestine, comparable degrees of splenomegally and cellularity of the spleen and mesenteric lymph nodes were observed in TCRδ$^{-/-}$ and WT mice (Fig 4.7E). Importantly, *H.hepaticus* colonisation levels were slightly lower in TCRδ$^{-/-}$ mice compared to wild-type controls, confirming that the exacerbated disease was not simply the result of an increased infective burden (Fig 4.7D).

In addition to a direct role in both innate and adaptive immunity, γδT-cells have been reported to a fulfil a variety of further functions including maintenance of epithelial barrier integrity, and the elaboration of peptides and growth factors which contribute to tissue repair and wound healing.[403,404,407,807] We therefore considered that the severe colitis observed after 28 days could reflect impaired resolution of inflammation and tissue damage rather than a primary increase in inflammatory activity itself. However, we observed similar kinetics of disease development in both TCRδ$^{-/-}$ and wild type mice, with consistently higher lamina propria cell counts and greater

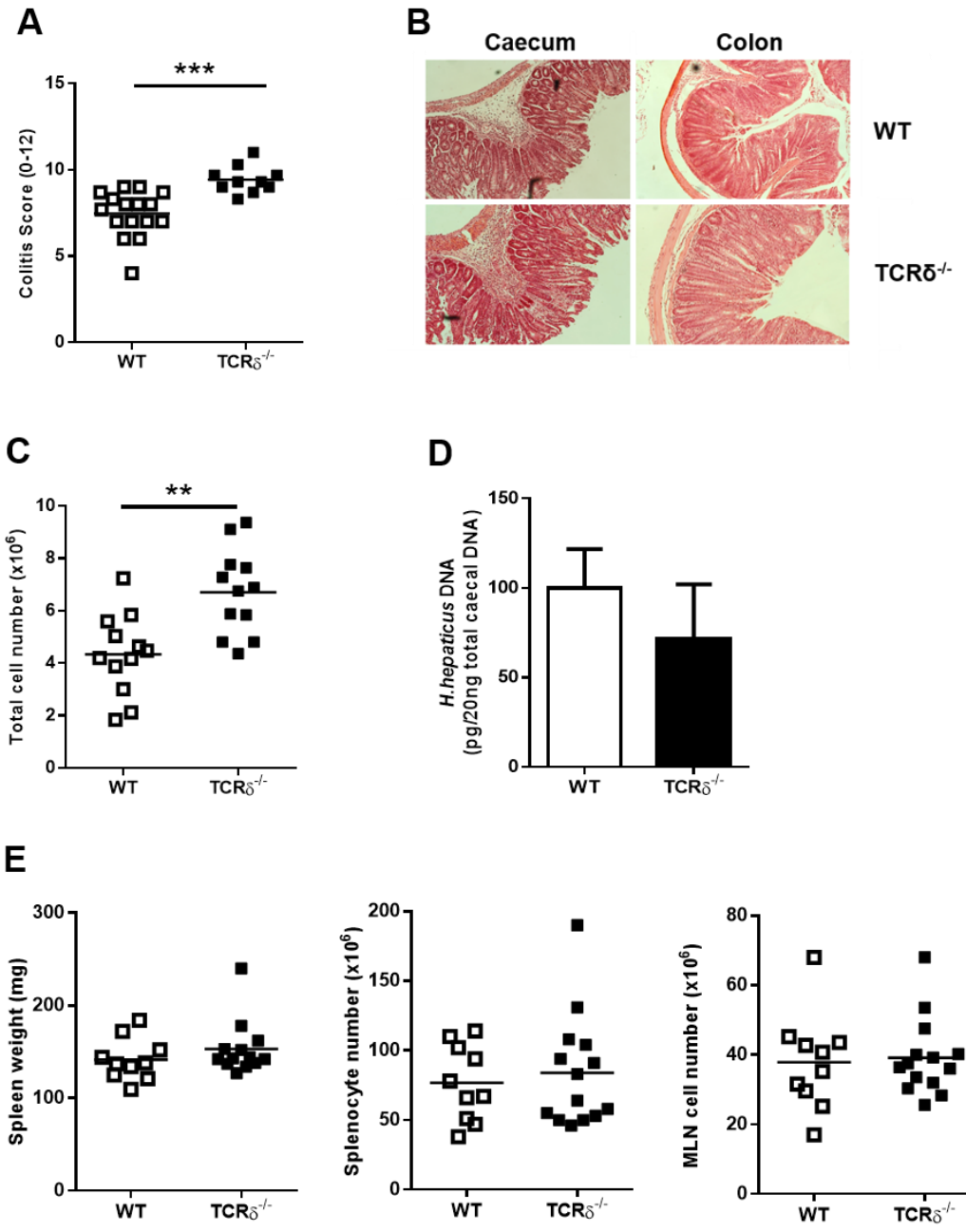

Figure 4.7 γδT-cell deficiency is associated with increased severity of *H.hepaticus* induced chronic colitis

Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and concomitantly treated with anti-IL10R (1mg/week) weekly for 4 weeks. Mice were sacrificed at 28 days after induction and tissues harvested for analysis. **(A)** Colitis scores (0-12) as assessed independently by two blinded observers **(B)** Representative colonic and caecal histology **(C)** Total colonic lamina propria cell counts **(D)** Caecal *H.hepaticus* colonisation determined by qPCR for *cdtB* gene and standardised to total caecal DNA content. Columns show mean +SD. **(E)** Spleen weight (left) and cell count (middle) and MLN cell counts (right). Data combined from 2-3 independent experiments (n=6-8 each group). Points (A,C,E) show individual mice, bars indicate mean. Statistical significance determined using Mann-Whitney Test. *$p \leq 0.05$, ***$p \leq 0.001$

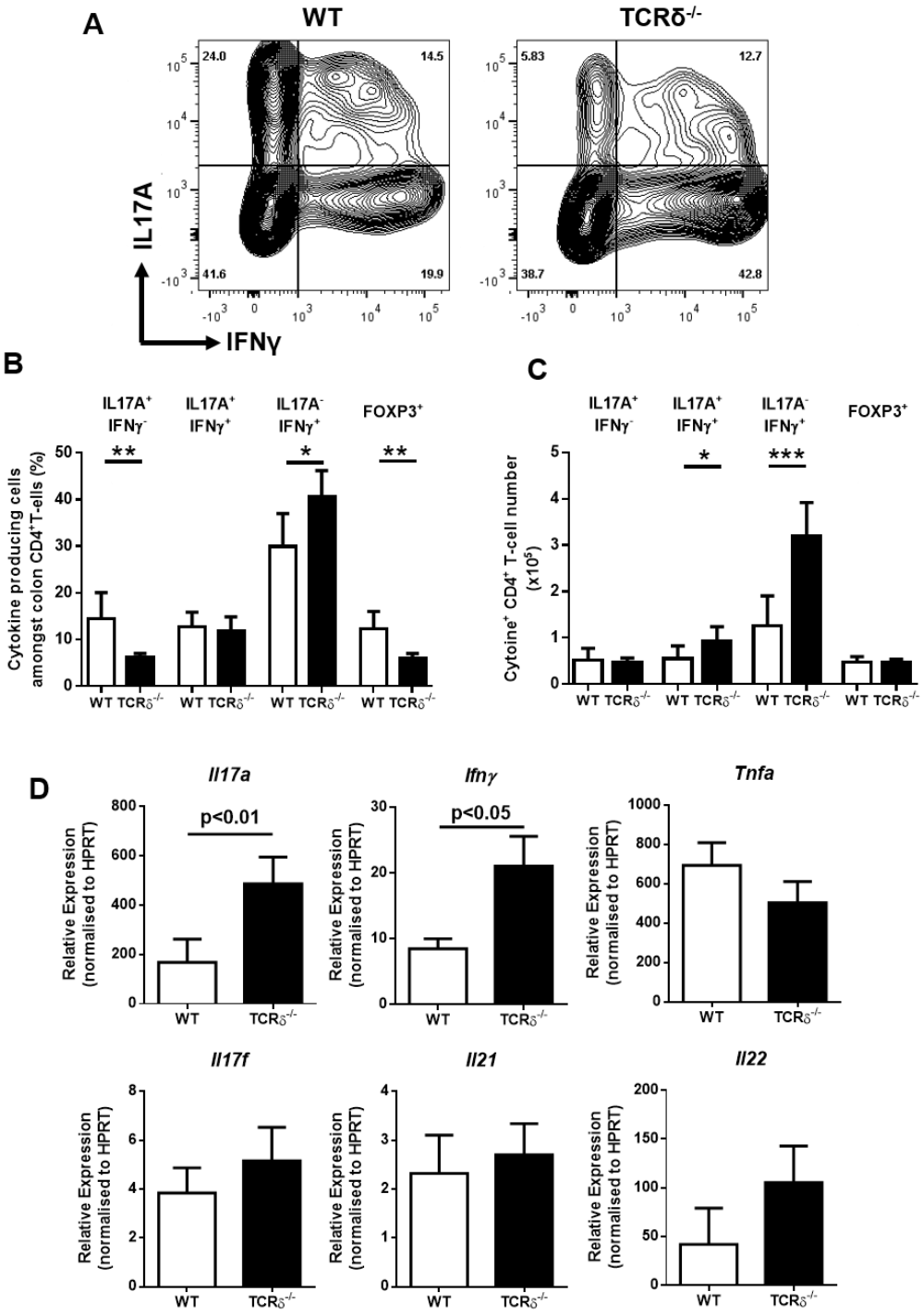

Figure 4.8 γδT-cell deficiency results in an altered CD4+ T-cell phenotype in chronic colitis
Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p.* weekly). Mice were sacrificed at 28 days after disease induction for analysis. Isolated colonic cells were restimulated in vitro using PMA/ionomycin for 4 hours in the presence of Brefeldin A, followed by surface and intracellular staining. **(A)** Representative plots, gated on live CD4+T-cells **(B)** Proportion and **(C)** number of cytokine producing cells isolated from colonic lamina propria **(D)** Colonic tissue expression of indicated cytokines, determined by qPCR and normalised to *Hprt*. Columns represent mean values +/-SD. Data representative of 3 independent experiments (n=6-8 each group). Statistical significance determined using Mann-Whitney Test. *$p \leq 0.05$, **$p < 0.01$, ***$p \leq 0.001$

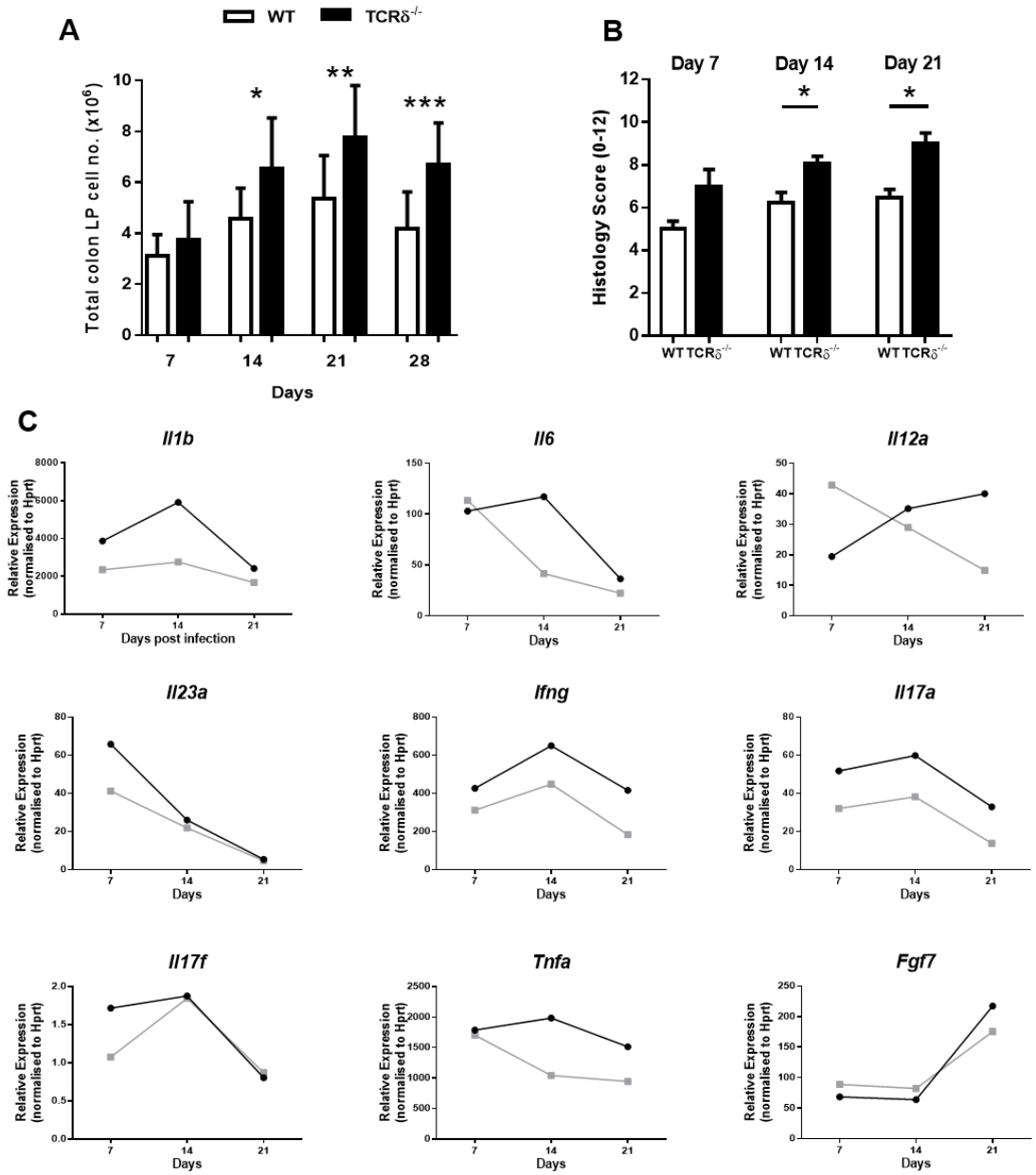

Figure 4.9 TCRδ$^{-/-}$ mice exhibit more severe colitis from early after infection with *H.hepaticus*

Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p.* weekly). Mice were sacrificed at indicated time points for analysis. **(A)** Cell numbers in colonic lamina propria **(B)** Colonic histology score (0-12) **(C)** Kinetics of expression of indicated genes within colonic tissue. mRNA levels determined by qPCR and normalised to *Hprt*. Black line shows expression in TCRδ$^{-/-}$ mice, grey line is WT expression. Columns represent mean values +/-SEM (A-B). Data pooled from 3 independent experiments (n=6-8 each group). Points (C) represent mean values from 1 experiment (n=8). Statistical significance determined using Mann-Whitney Test. *$p \leq 0.05$, **$p < 0.01$, ***$p \leq 0.001$

histological scores in TCRδ$^{-/-}$ animals from as early as day 7, with both significantly greater from day 14 onwards (Fig 4.9A&B). Expression of a panel of innate and adaptive cytokines, including IL-1, IL-6, IL-12, IL-23, IFN-γ, IL-17A, IL-17F and TNF-α demonstrated similar kinetics in both groups, but with generally greater levels observed in TCRδ$^{-/-}$ mice (Fig 4.9C). Importantly, expression of the repair factor keratinocyte growth factor (KGF; fibroblast growth factor 7), reported to be a critical mediator of γδT-cell effects on epithelial homeostasis was similar at all time points examined (Fig 4.9D).[403,824]

In total, these results suggest that γδT-cells are involved in regulating aspects of the development of disease in the *H.hepaticus*/anti-IL-10R model, and that in their absence an exaggerated host immune response occurs, resulting in severe intestinal inflammation.

4.2.5 γδT-cells play a limited role in host protection from *Citrobacter rodentium*

The finding that γδT-cell deficiency resulted in an aberrant immune response to *Helicobacter* infection led us to question whether TCRδ$^{-/-}$ mice would show a similar exacerbated phenotype following infection with other intestinal pathogens. We therefore infected TCRδ$^{-/-}$ mice with the attaching/effacing bacteria *C.rodentium*, a murine model of *E.coli* infection. In agreement with a previous report, γδT-cell deficiency had no apparent effect on weight loss or mortality rates (Fig 4.10A and data not shown), nor upon ultimate bacterial clearance (Fig 4.10D).[562] Interestingly, *C.rodentium* infected TCRδ$^{-/-}$ animals consistently developed more marked splenomegally from day 7 post infection, associated with transiently higher systemic bacterial colonisation at the earlier time point (Fig 4.10B&C). Notably, this increased colonisation did not reflect increased burdens within the intestine, with similar levels seen in TCRδ$^{-/-}$ and control mice throughout infection (Fig 4.10D). Histological assessment of TCRδ$^{-/-}$ mice revealed significantly more severe typhlitis developing at day 7 post infection compared to wild-type controls, whereas at later time points no differences were seen (Fig 4.10E). These results suggest that whilst γδT-cells play a

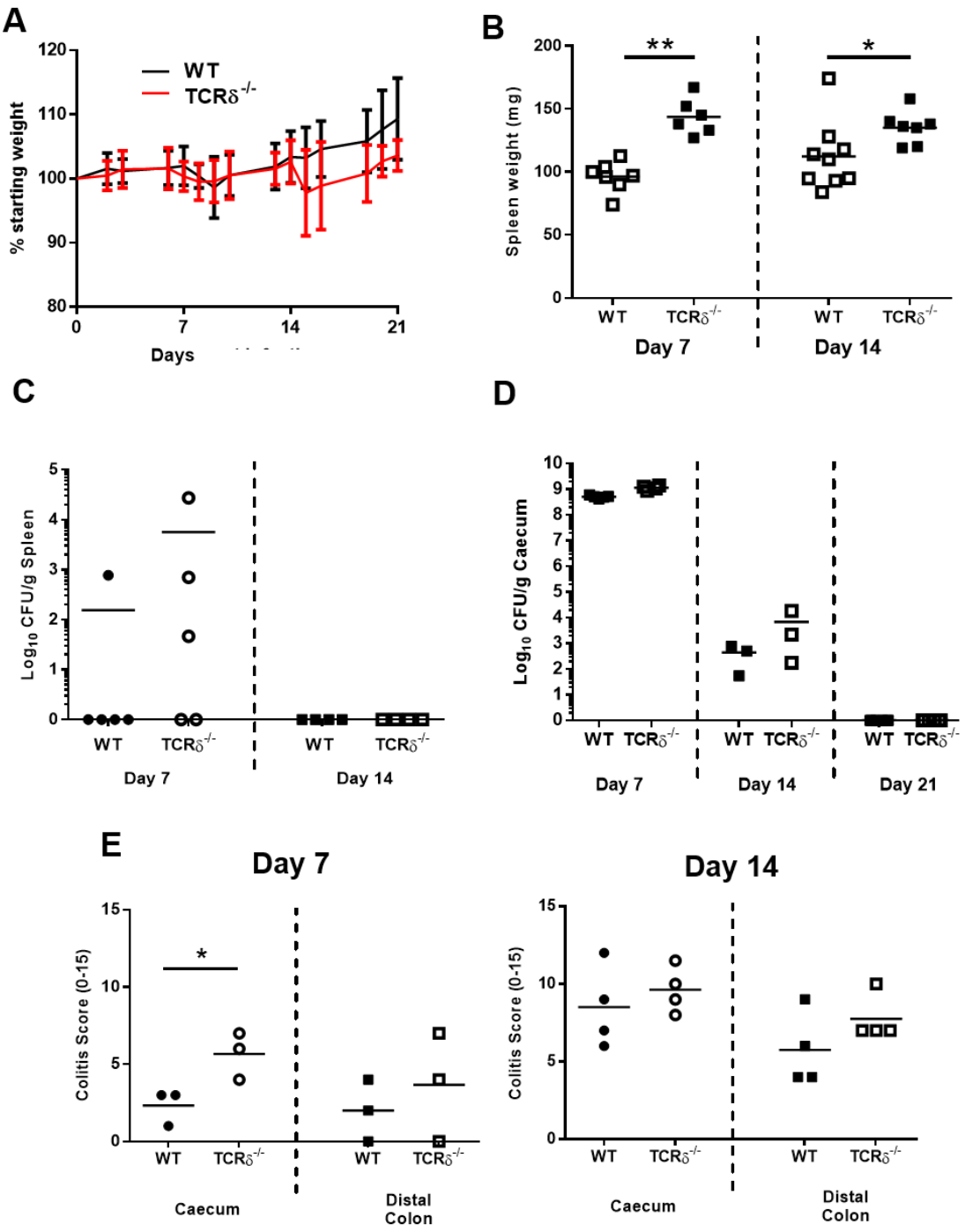

Figure 4.10 γδT-cells play a limited role in host protection from *C.rodentium* infection

Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage. Mice were monitored daily and sacrificed at the indicated time points for analysis. Tissue was harvested for histological analysis and quantification of colonisation by serial dilution plating. **(A)** Weight curves **(B)** Spleen weights at indicated time points. *C.rodentium* colonisation levels in spleen **(C)** and caecum **(D)** at indicated time points by genotype. **(E)** Histological colitis scores (0-15) in caecum and distal colon at 7 (left panel) and 14 (right panel) days after infection. Data shown from single experiment representative of results from 2 independent experiments (n=7-9 each group). Points represent mean +/-SD (A) or individual mice with bars indicating mean (B-E). Statistical significance determined using Mann-Whitney Test. *p<0.05, **p<0.01.

limited role in the overall immune response to intestinal *C.rodentium* infection, in their absence the early inflammatory response is more robust, resulting in more severe intestinal pathology. Importantly, the differing levels of disease despite similar colonisation levels in this model further support the concept that deficiency of γδT-cells may be associated with an exaggerated intestinal immune response.

4.2.6 γδT-cell deficiency does not impair the development of an adaptive Th17 response in the intestine

In models of IL-23-dependent extra-intestinal inflammation, including in the lung, joints, skin and central nervous system, γδT-cells are an important early source of IL-17A and support the development of the adaptive Th17 response, through elaboration of cytokines including IL-17 and IL-21.[387,392,614] Accordingly, TCRδ$^{-/-}$ mice are somewhat protected from disease in such models.[387,392,614] By contrast, studies of intestinal infection with *C.rodentium* and *S.typhi*, have demonstrated rapid innate-like production of IL-17A by resident CD4^{+}αβT-cells with the characteristics of effector memory T-cells, with apparently minimal contribution from γδT-cells.[548] Since our previous studies showed neither appreciable expansion of IL-17 producing γδT-cells in colitis, nor protection from disease in TCRδ$^{-/-}$ mice, we hypothesised that γδT-cells were redundant for the development of the intestinal Th17 response. We therefore isolated lamina propria T-cells from the colons of wild-type and TCRδ$^{-/-}$ mice, 7 days after infection with *H.hepaticus* and administration of anti-IL-10R antibody, the earliest point at which CD4^{+} T-cell accumulation is observed, and analysed their cytokine profiles by intracellular staining. These studies demonstrated similar proportions and numbers of T-cells demonstrating IL-17A^{+}IFN-γ^{-}, IL-17A^{+}IFN-γ^{+} and IL-17A^{-}IFN-γ^{+} phenotypes at this time point, in agreement with our earlier findings studying intestinal cytokine expression (Fig 4.11A). Furthermore, the production of additional Th17 signature cytokines, including IL-17F and GM-CSF was also similar (Fig 4.11B&C). Therefore, γδT-cells do not appear to be required for the early Th17 response in evolving intestinal inflammation.

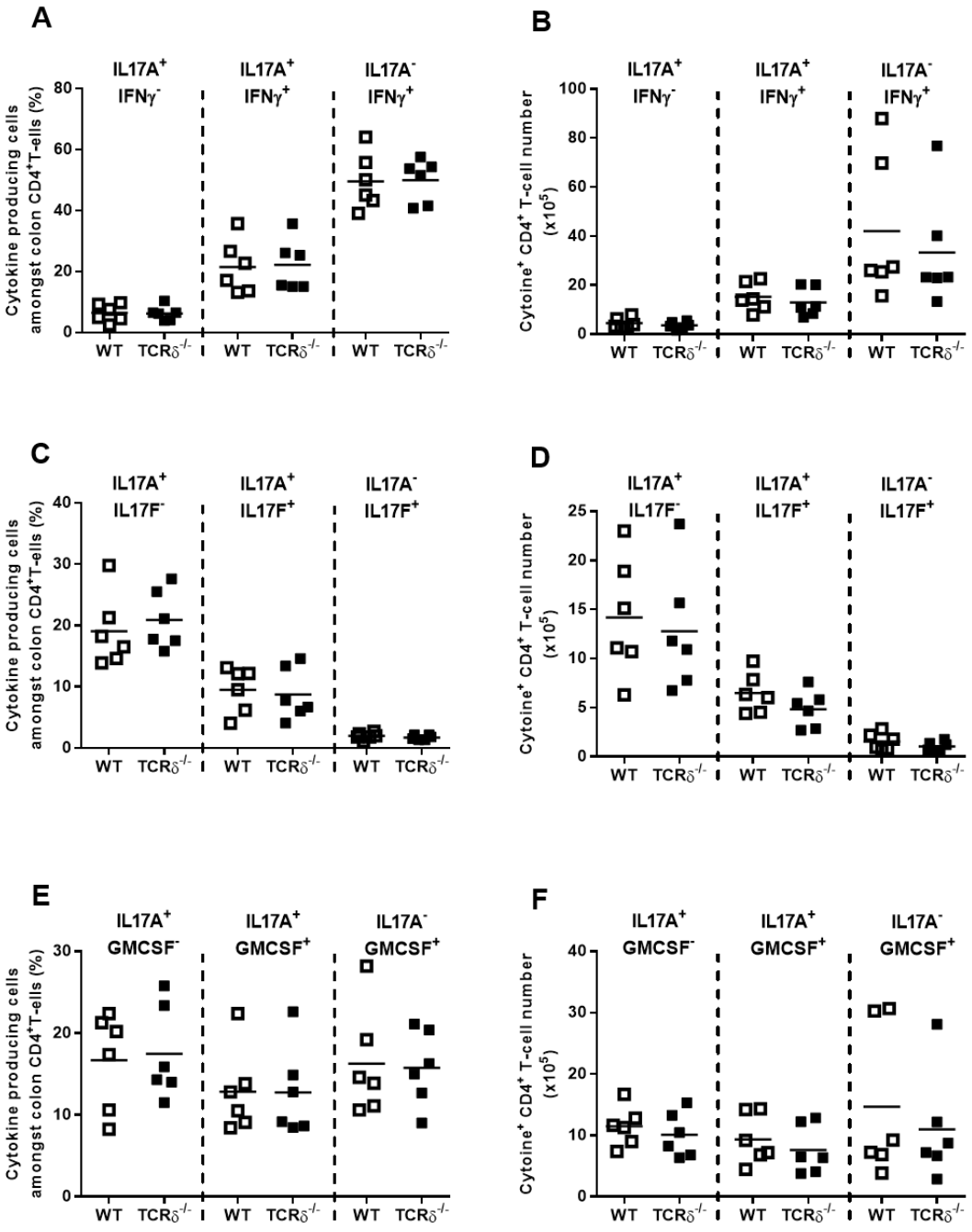

Figure 4.11 γδT-cell deficiency does not impair the early development of intestinal Th17 responses

Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p*). Mice were sacrificed 7 days after initial infection for analysis. Colonic lamina propria cells were isolated and restimulated in vitro using PMA/ionomycin for 4 hours in the presence of Brefeldin A, followed by surface and intracellular staining. **(A-F)** Proportion amongst colonic CD4+ T-cells (left panel) and total numbers (right panel) of cytokine producing cells. Points indicate individual mice, bars show mean. Data representative of 2 independent experiments (n=6 each group). Statistical significance determined using Mann-Whitney Test, with no comparison yielding $p<0.05$.

4.2.7 The inflammatory infiltrate induced by *H.hepaticus* infection differs significantly in the absence of γδT-cells

Next, we were interested to better define the basis of the more severe inflammatory response occurring in the absence of γδT-cells. A previous study addressing the function of γδT-cells in the spontaneous chronic colitis occurring in TCRα$^{-/-}$ mice concluded that disease was promoted through effects on myeloid cell accumulation and function.[810] Although we observed the opposing phenotype from γδT-cell deficiency in *H.hepaticus*/anti-IL10R induced disease, we questioned whether this might also be occurring through effects on myeloid cell populations. We therefore further analysed the inflammatory infiltrate in the colons of mice infected with *H.hepaticus* and treated with anti-IL-10R antibody, during the chronic phase of disease.

Overall, the proportion of total accumulating cells expressing the myeloid cell marker CD11b was consistently reduced in TCRδ$^{-/-}$ mice to a modest but statistically significant degree (Fig 4.12A), however due to the overall increase in cellularity, total numbers were still greater than in wild-type controls. The reduction in CD11b^{+} cells was largely balanced by an increase in the proportion of total CD3^{+} T-cells, with similar B220^{+} B-cells present amongst total colonic leukocytes in both groups (Fig 4.12A). Whereas CD11b^{+}Gr1^{hi} neutrophils were similarly represented in both TCRδ$^{-/-}$ and wild-type mice, we observed a consistently smaller proportion of cells expressing an inflammatory monocyte/dendritic cell CD11c^{+}MHCII^{+} phenotype (Fig 4.12B), or the surface marker F4/80 (Fig 4.12C). Although F4/80 has been proposed as a macrophage marker, it is also highly expressed on intestinal eosinophils.[825] Assessing MHC-II expression (Fig 4.13A), we found a smaller proportion of F4/80^{+} cells positive for this marker in TCRδ$^{-/-}$ mice, consistent with defective macrophage accumulation or activation. Since eosinophils demonstrate characteristic forward/side scatter appearance on flow cytometry, we assessed the morphology of colonic F4/80^{+} cells, and found that the proportion identified as eosinophils was significantly increased in TCRδ$^{-/-}$ animals, whereas other F4/80^{+} cells were significantly reduced in proportion (Fig 4.13B&C). Numerically, this reflected accumulation of such cells, with no apparent difference in

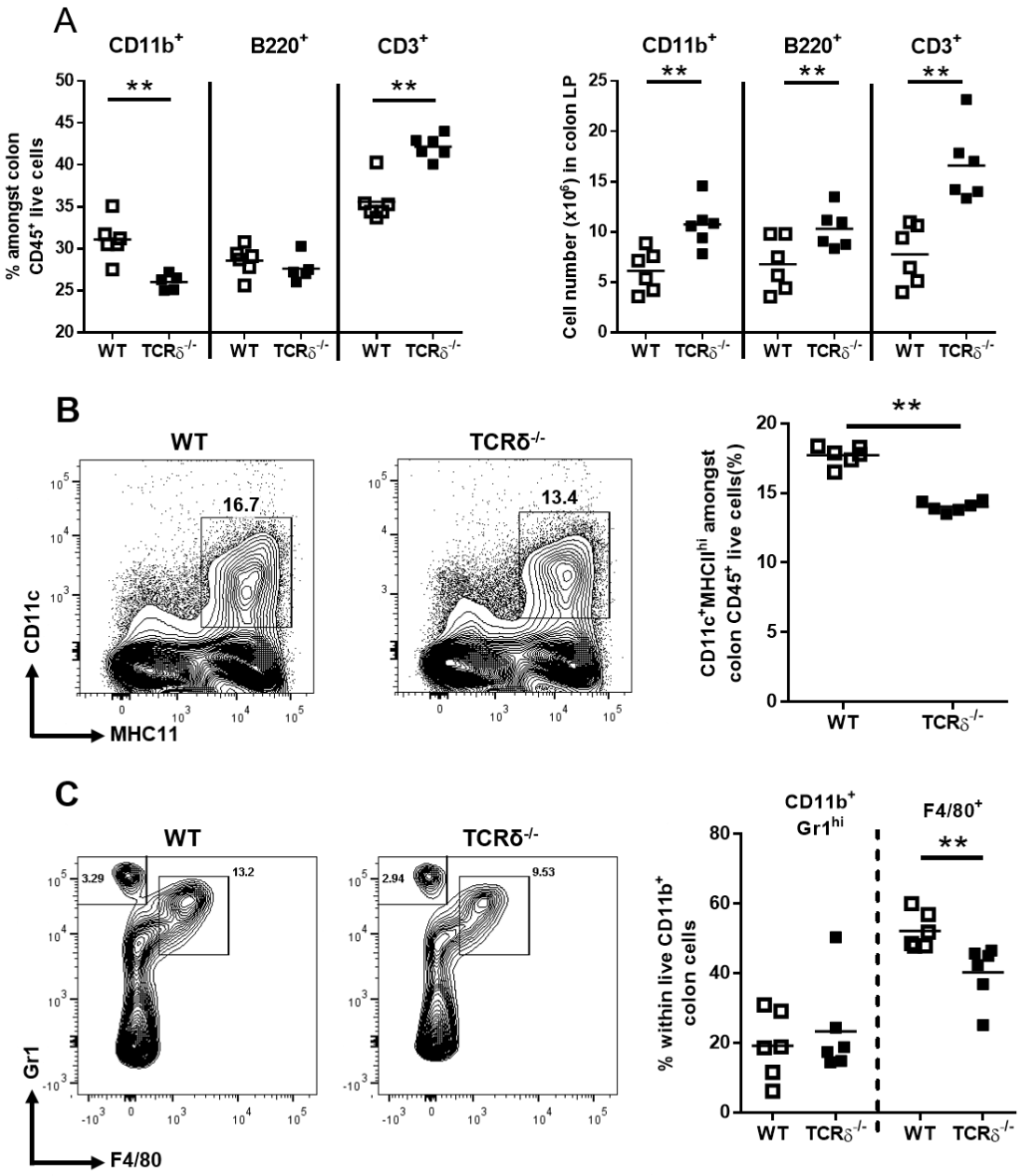

Figure 4.12 γδT-cell deficiency results in an altered colonic infiltrate in *H.hepaticus* induced colitis

Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p*). Mice were sacrificed 28 days after initial infection and colonic lamina propria cells isolated for analysis. **(A)** Proportion (left) and total numbers (right) expressing markers of major leukocyte subsets within live CD45+ cells in colonic lamina propria. **(B)** & **(C)** Representative plots and proportions of colonic cells expressing CD11chiMHCIIhi phenotype amongst live CD45+ cells(B), or F4/80+ or Gr1^{hi} phenotype amongst CD11b+ live cells (C). Points indicate individual mice, bars show mean. Data representative of 3 independent experiments (n=6 each group). Statistical significance determined using Mann-Whitney Test. ***p*<0.01.

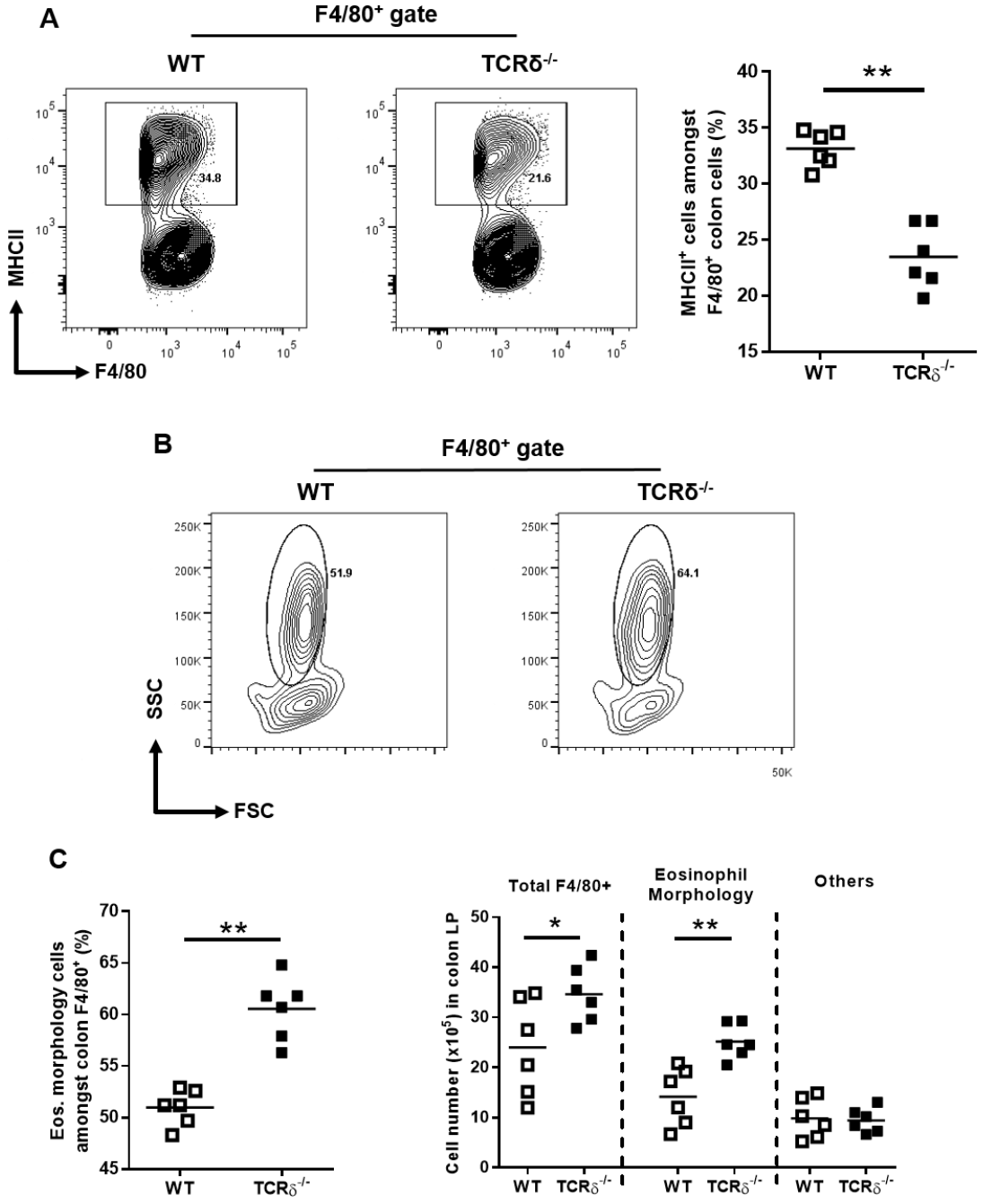

Figure 4.13 Reduced macrophage accumulation in γδT-cell deficient mice infected with *H.hepaticus*

Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p*). Mice were sacrificed 28 days after initial infection and colonic lamina propria cells isolated for analysis. **(A)** Representative plots, gated on F4/80+ cells (left) and proportions (right) of colonic F4/80+ cells co-expressing MHCII. **(B)** Representative plots showing FSC/SSC distribution of F4/80+ cells accumulating in colitis **(C)** Proportion of colonic F4/80+ cells with eosinophil morphology by genotype (left) and cell numbers (right) of F4/80+ subsets in colonic lamina propria. Points indicate individual mice, bars show mean. Data representative of 3 independent experiments (n=6 each group). Statistical significance determined using Mann-Whitney Test. *p<0.05, **p<0.01.

numbers of other F4/80^{+} cells compared to wild type controls, despite the overall increased cellularity in TCRδ$^{-/-}$ mice (Fig 4.13D). This finding suggests impaired accumulation or survival of other F4/80^{+} cells including macrophages may be occurring. Overall, in the absence of γδT-cells, the leukocyte population accumulating in chronic colitis differs markedly from that observed in wild-type animals.

4.2.8 TCRδ$^{-/-}$ mice exhibit a skewed chemokine profile in evolving inflammation

The differences observed in colonic leukocyte populations in TCRδ$^{-/-}$ mice could reflect pre-existing differences in intestinal myeloid cells due to a non-redundant role for γδT-cell in their homeostasis, or a requirement for such cells to regulate the production of cytokines and chemokines determining their accumulation in inflammation. Alternatively, the altered composition might be appropriate and secondary to a skewed immune response earlier in disease. Our previous studies had shown a more robust inflammatory response to be present from early in disease, but had not identified a molecular basis for this finding.

We therefore examined the myeloid populations present in the colon at day 7 of disease, a point at which inflammation is similar in severity to that seen in wild-type animals, and just before the more severe phenotype occurs. At this point, all major T-cell and myeloid subsets examined were similar in their proportion amongst total colonic leukocytes, with the exception of F4/80^{+} cells, which were less frequent in the colons of TCRδ$^{-/-}$ mice (data not shown). Since we had observed more profound differences subsequently developing in the inflammatory infiltrate, we analysed the expression at this point of a wide panel of chemokines implicated in the recruitment of leukocytes to homeostatic and inflammatory environments (Fig 14.4). These experiments revealed a large number of chemokine genes which were down (*Ccl11*) or up-regulated (*Ccl2*, *Ccl3*, *Ccl4*, *Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl5*, *Cxcl17*, *Cxcl10*) to similar levels in wild type and TCRδ$^{-/-}$ mice. A small number of chemokines, including *Ccl12* and *Ccl20* were upregulated only in wild

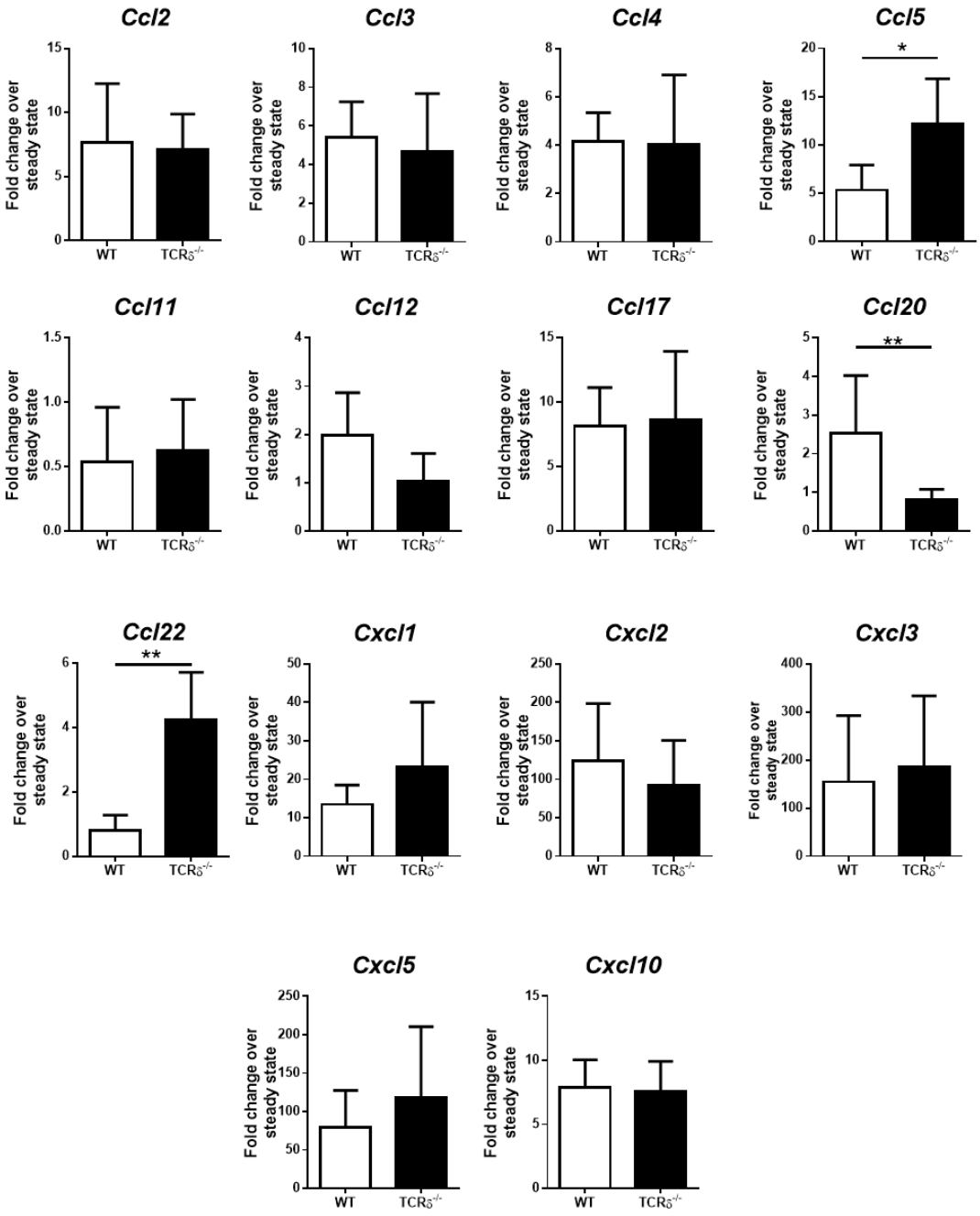

Figure 4.14 Skewed chemokine profiles develop in evolving *H.hepaticus* colitis in TCRδ$^{-/-}$ mice

Wild-type (WT) C57BL/6 or TCRδ$^{-/-}$ mice were infected with 3 doses of ~10^8 CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p.* weekly). Mice were sacrificed 7 days after initial infection and colonic tissue harvested for analysis. Expression of indicated genes within whole colonic tissue. mRNA levels determined by qPCR and normalised to *Hprt*. Columns represent mean values +/-SD. Data from single experiment (n=6 each group). Statistical significance determined using Mann-Whitney Test. *$p \leq 0.05$, **$p < 0.01$.

type mice, whereas *Ccl5* and *Ccl22* were expressed at significantly higher levels in the colons of TCRδ$^{-/-}$ animals. Although we were unable to further investigate the functional significance of these chemokine profiles, they identify a number of potential contributors to the exacerbated phenotype observed, and are potentially therefore important targets for future study in the context of intestinal inflammation.

4.3 Discussion

Despite the well documented presence of significant populations of γδT-cells within the intestine, their role in intestinal homeostasis, immunity and inflammation is incompletely defined. Whereas the biology of such cells present within the IEL compartment of the small intestine has received significant attention, less is known about γδT-cell populations at other locations. During our previously described studies of IL-23R expression, we noted the presence of a sizable population of γδT-cells within the colonic lamina propria. Concurrently, a series of important studies described significant functional roles for γδT-cells, particularly those producing IL-17A, in a number of murine models of infectious and inflammatory disease, including those linked to the IL-23/Th17 axis. We therefore undertook studies to better define the colonic γδT-cell populations and to examine their function.

Previous studies of γδT-cells in multiple anatomical compartments have shown significant variation exists between locations in terms of the cytokine production, surface phenotype, activation status, and Vγ repertoire of resident cells, with functional aspects often correlating with such markers.[332,338,798] Although previous studies have examined aspects of colonic γδT-cells, both within the lamina propria and IEL, and noted differences from small intestinal cells, these populations remain incompletely characterised.[14] Therefore, we initially examined the phenotype of colonic γδT-cell populations, and found striking differences between such cells and those found in the small intestine or lymphoid tissues, with IEL resident γδT-cells and those recovered from

the lamina propria also clearly distinct. In contrast to their abundance in the small intestine,[818,826,827] γδT-cell comprised only a minor fraction of the colonic IEL, with conventional αβT-cells the predominant population. Furthermore, γδT-cell cells within the colonic IEL were demonstrably different, lacking significant expression of the CD8αα homodimer which is such a prominent feature of small intestinal populations.[14] Although its exact function continues to be explored, CD8αα is not a true TCR-co-receptor and expressing cells are not MHC-I restricted. Instead, it exhibits high affinity binding of the MHC-like thymic leukaemia (TL) antigen, and has been proposed to mediate suppressor effects upon TCR signals, with expression enriched upon cells with potentially self-reactive TCR specificities.[828] Signals through the TCR-CD3 complex upregulate CD8αα in proportion to the signal strength, which is then maintained by cytokines including IL-7 and IL-15.[828] Therefore, the comparative paucity of CD8αα γδT-cells within the colonic IEL could therefore reflect differences in the antigenic specificity of resident cells, abundance of TCR-activating antigen, or local availability of relevant cytokines. Furthermore, although the significance is uncertain in normal mice, small intestinal IEL γδT-cells may uniquely be generated through extra-thymic pathways within the intestine itself, highlighting potentially differing origins and developmental pathways for small intestinal and colon IEL cells.[829,830] Alternatively, γδT-cells present within the colon may be occupying a different functional niche, favouring accumulation of a quite different population without significant regulatory effects or self-reactivity. The distribution of $CD4^+$ populations exhibits similar anatomical variation between intestinal immune compartments,[15,831] implying regulatory requirements and mechanisms controlling their accumulation are highly site specific. Further support for the concept that intestinal γδT-cell niches show anatomical restriction is provided by adoptive transfer experiments using γδT-cells derived from various compartments, in which reconstitution of the recipient mouse selectively occurred in the sites from which the transferred cells originated, with minimal repopulation at other locations.[832]

In contrast to their abundance in the IEL, γδT-cell were relatively uncommon within the small intestinal lamina propria, whereas there was notable enrichment of this population in the colon. Although accounting for only ~3-6% of $CD45^+$ cells at this site, this was a significantly greater proportion than seen within other sites including lymphoid organs. We considered the possibility that this could reflect contamination from IEL resident cells not adequately removed during the initial isolation process. However, the protocol employed to remove the epithelial layer and IEL has been previously shown to result in little cross-contamination. Furthermore, we consistently noted major differences in the surface phenotypes and response to ex-vivo restimulation between IEL and lamina propria cells, largely excluding this possibility. Notably, the Vγ-repertoire used by IEL and lamina propria cells demonstrated significant differences. Tissue specific patterns of Vγ-expression have been extensively noted in studies of γδT-cells within other anatomical locations, although the significance of this is debated. Importantly, diverse Vγ subsets demonstrate marked differences in their developmental kinetics, populating tissues at specific stages of development, including during embryonic and early post-natal life.[338] Since the Vγ usage of intestinal lamina propria cells has not previously been described we investigated this. However, using available reagents, we could only identify the Vγ expression on ~50% of cells. Unfortunately, few reliable antibodies directed at specific Vγ chains are currently available, and we were only able to conclude that a significant proportion of colonic lamina propria cells may be using a less well characterised or possibly novel Vγ. Importantly, when we later attempted to identify the Vγ expression of IL-$23R^+$γδT-cells in the gut, both by flow-cytometry and RT-PCR, we were only successful in a small fraction, highly suggestive of the uncharacterised population using a single, possibly colon specific receptor.

Despite clear differences in the Vγ repertoire of intestinal and systemic IL-$23R^+$γδT-cells, expression of surface markers previously positively or negatively associated with IL-23R expression or IL-17A production, including CD27, CCR6 and NK1.1 demonstrated expected patterns amongst intestinal cells.[392,410,617,833] This would be consistent with existing data

suggesting that Th1 or Th17 phenotypes amongst γδT-cells are determined during thymic development, and once acquired remain fixed. Initial studies on the differences between these phenotypes, using lymphoid tissue γδT-cells, reported antigen-experience to be a determinant of cytokine production, with TCR triggering driving development of IFN-γ^+ cells.[817] However, despite near universal expression of activation markers including CD44, CD69 and CD25 on colonic γδT-cells, >50% of such cells produced IL-17A upon re-stimulation, with a tiny fraction expressing IFN-γ. This would either suggest that this mechanism is not relevant to lamina propria cells, or that cells are undergoing TCR-independent activation within the colon. Analysis of Th17 associated molecules largely confirmed expected patterns of expression within intestinal γδT-cells sorted on the basis of IL-23R status, suggesting that intestinal IL-23R$^+$γδT-cells are similar to systemic populations. However, it was notable that the proliferative response to IL-23 was significantly attenuated amongst cells isolated from the intestine. The basis of this observation is unclear and will require better definition of the mechanisms by which IL-23 normally induces proliferation in order to identify how this is being inhibited. This would be an important area for future transcriptional and proteomic studies, since driving proliferation of CD4$^+$αβT-cells is one mechanism by which IL-23 promotes inflammatory disease, and this pathway may be amenable to therapeutic manipulation.

Studying the changes occurring to lamina propria γδT-cell populations in colitis, we found that these cells accumulated with remarkably similar kinetics to other T-lymphocytes. Importantly, no significant early increase in cell numbers was seen, even at very early time points, arguing against significant recruitment from the circulation, a mechanism by which γδT-cells have been proposed to contribute to early immune responses and bridge to the development of adaptive immunity outside of the intestine. The later rapid increase in γδT-cell numbers in the colon was characterised by the emergence of a sizable population of IFN-γ^+ cells, which were effectively absent in the steady state. The lack of plasticity of γδT-cells would suggest that this change arises either from expansion of the small population of IFN-γ^+ cells present in the lamina propria in the steady state,

or through recruitment of circulating cells. The molecular determinants of γδT-cell recruitment to sites of inflammation are incompletely defined, and include mechanisms shared with conventional T-cells.[332,338] Therefore, we questioned whether this accumulation was of functional importance, or might be occurring as a bystander phenomenon due to mechanisms primarily up-regulated to drive αβT-cell accumulation. Remarkably, when we induced chronic colitis in TCRδ$^{-/-}$ mice we observed an exacerbated phenotype, suggesting the dominant effect of intestinal γδT-cells is actually a protective or regulatory role. Such an increased severity of disease in the absence of γδT-cells has previously been noted in a number of animal models, including infections with listeria,[401] mycobacteria[834] or klebsiella,[835] and in experimental orchitis,[836] myocarditis[837,838] and lupus.[839] In the intestine, TCRδ$^{-/-}$ mice infected with the enterocyte parasite *Eimeria* are reported to develop more severe intestinal injury which can be rescued by adoptive transfer of γδT-cells from the SI-IEL.[394,813] Similarly, deficiency of γδT-cells has been associated with impaired bacterial control and epithelial barrier dysfunction in the intestine, with increased severity of disease due to infection with *Toxoplasma* or *Salmonella*, specifically related to the absence of Vγ7^{+} IELs.[406] However, unlike these organisms *H.hepaticus* is neither directly pathogenic to epithelial cells nor an invasive organism, and usually remains within the intestinal crypts even in active colitis. Importantly, we did not find differences in overall colonisation levels between wild-type and TCRδ$^{-/-}$ animals, suggesting that increased disease is not the direct result of failure to control intestinal infection.

An important role for γδT-cells within the IEL in regulating the intestinal microbiota has recently been demonstrated, linked to production of antimicrobial peptides including Reg3γ.[407] The increased immune tone in the intestines of TCRδ$^{-/-}$ mice could therefore reflect underlying differences in the composition of the gut flora, particularly if γδT-cell deficiency selects for species linked to susceptibility to intestinal inflammation. Indeed, mice deficient in Ahr, which lack IEL γδT-cells, display increased susceptibility to epithelial injury driven by IFN-γ^{+}CD4^{+}TCRβ^{+} cells, linked to an altered intestinal flora.[840] Similarly, it is possible that the spatial

distribution of bacteria is affected by the absence of γδT-cells, allowing interaction with populations of cells with which they do not normally interact. Alternatively, the increased intestinal permeability[406] observed in TCRδ$^{-/-}$ mice might facilitate interaction of bacterial products or other stimuli originating in the intestinal lumen and cells of the innate immune system, resulting in increased basal activation of such cells, and hyper-responsiveness towards *H.hepaticus*. In support of such a concept, TCRδ$^{-/-}$ mice also developed more severe initial disease when infected with *C.rodentium*, despite similar colonisation levels, suggesting the increased inflammatory response in such animals is neither pathogen specific nor simply related to anti-IL-10R treatment. Notably, within some facilities TCRδ$^{-/-}$ develop spontaneous colitis in later adult life, compatible with a breakdown of tolerance towards the intestinal flora. However, the remarkable range of extra-intestinal diseases exacerbated by the absence of γδT-cells does point to a more generalised systemic effect on immune regulation. Importantly, the failure of B6.Rag$^{-/-}$ mice to develop significant colitis upon infection with *H.hepaticus* confirms that γδT-cells are not intrinsically required to control the host response to infection in the absence of adaptive T and B-cells.

Although a protective role for γδT-cells has been reported in DSS and TNBS colitis, these models are primarily driven by epithelial injury, and would be predicted to be highly sensitive to the absence of IELs, which are a critical cellular source of growth factors to promote wound healing.[403,808,832] In other models of intestinal inflammation contrasting effects have been described, with pro-inflammatory activity in the spontaneous colitis seen in TCRα$^{-/-}$ mice,[810] and an essential role in driving intestinal disease in CD4^{cre}Pdk1$^{-/-}$ mice, which lack functional CD4+ T-cells, including Tregs.[815] Adoptive transfer of CD3ε transgenic γδT-cells results in a Crohn's disease like pathology,[841] whilst co-transfer of lymphoid tissue γδT-cells with naïve CD4^{+}T-cells results in the development of more rapid and severe disease than in mice receiving naïve CD4^{+} T-cells alone.[811,812] However, these models present significant challenges to interpret, since they occur either in the absence of conventional αβT-cells, where γδT-cells become a predominant

lymphocyte population and assume a role which may not be recapitulated in the presence of intact host immunity, or they rely upon adoptive transfer of large numbers of lymphoid derived γδT-cells. Whilst these results reveal potential biological effects of such cells, their occurrence in such non-physiological settings precludes definitive conclusions of the true effects of γδT-cells in intestinal inflammation.

An alternative explanation for the increased inflammation occurring in the absence of γδT-cell would be that it reflects hyper activation consequent to an inadequate initial immune response towards the infection. Notably, such a mechanism has been proposed to underlie intestinal inflammation occurring in chronic granulomatous disease and in Crohn's disease.[459,842] However, analysis of an extensive panel of cytokines, including innate cytokines of the IL-12 family known to critically regulate immunity towards *H.hepaticus*, demonstrated elevated levels in the intestine at time points before inflammation could be detected histologically, somewhat arguing against this possibility, and supporting the idea of increased resting immune tone. Furthermore, the accumulation of neutrophils, which would be the primary responding cell in the earliest phases of disease, was not apparently affected by γδT-cell deficiency. This is an important observation, since impaired bacterial clearance from both lung and peritoneum has been reported in TCRδ$^{-/-}$ mice, linked to reduced neutrophil accumulation and proposed to occur primarily due to loss of IL-17 producing γδT-cells.[383,843] We did however note consistent differences in the accumulation of other myeloid populations, including CD11c^{+}MHCII^{+} cells and F4/80^{+} cells. Alterations in myeloid cell accumulation have previously been reported in inflammatory disease in TCRδ$^{-/-}$ mice, linked to alterations in chemokine expression,[400,802,803,843] although whether γδT-cells are directly producing such mediators in biologically relevant quantities *in vivo* has not been shown. Indeed, an indirect effect through tissue conditioning is possible. Specific subsets of Vγ4^{+} and possibly Vγ2^{+} cells have been shown *in vitro* to directly interact with F4/80^{+} macrophages to augment activation and cytokine production in response to infection.[803] Other subsets appear to oppose this effect, and Vγ1^{+}cells may directly induce cytolysis of infected macrophages during

resolution of infectious disease.[845] We were unable to fully explore the mechanisms and functional significance of observed differences in myeloid accumulation in TCRδ$^{-/-}$ mice, but the finding of significantly skewed chemokine profiles would provide a basis for further study. Importantly, we examined these profiles at an early time point, prior to the large scale accumulation of cells in the colon. As such, this suggests that the biological effects of γδT-cell deficiency influence the subsequent inflammatory response from the very earliest stages of disease induction.

Our finding of an increased severity of disease in TCRδ$^{-/-}$ mice contrasts strikingly with the protective effect of γδT-cell deficiency in several models of IL-23 dependent disease, including experimental psoriasis and EAE.[387,392] In these models, γδT-cells are both an early source of IL-17A and related cytokines at the site of inflammation, and contribute to the development of full, pathogenic Th17 responses through a number of mechanisms including releasing Th17 cells from Treg control. Accordingly, TCRδ$^{-/-}$ mice are largely protected from developing disease. Whilst we considered that the apparently paradoxical phenotype in IL-23 dependent colitis might reflect a more profound pro-inflammatory effect from the absence of other subsets of IL-23R^{-} or IL-17A^{-} γδT-cells, our studies of IL-23R expression in colitis had raised questions as to the requirement for γδT-cells for the early 'type-17' response in the intestine. Indeed, we could not detect any defect in the accumulation of CD4^{+} Th17 cells during the early stages of colitis, nor in the levels of Th17 type cytokines. These results provide evidence supporting the concept that intestinal IL-23R^{+}γδT-cells are neither essential for disease, nor do they contribute significantly to the mucosal 'type-17' response in this model, even early in disease. These results are also in agreement with studies showing that gut resident conventional CD44^{+}CD4^{+}TCRαβ^{+} cells are a primary source of rapidly produced Th17 cytokines in intestinal infection rather than γδT-cells,[548] although the effect of γδT-cell deficiency was not explored in that work.

Ultimately, γδT-cells represent a diverse and heterogenous population of cells, even within specific anatomical compartments, with distinct subsets exhibiting pro- and anti-inflammatory or regulatory properties. The overall effect of γδT-cells deficiency will therefore reflect the relative contribution of opposing populations to the specific immune response examined. Marked phenotypic differences have been noted in mice lacking specific subsets of cells (i.e. Vγ1 or Vγ4) compared to TCRδ$^{-/-}$ animals which lack all populations.[332,338] However, the most significant difficulty in interpreting our results in TCRδ$^{-/-}$ mice arises from the absence of both IEL and lamina propria γδT-cells. To address this issue, we attempted to reconstitute the IEL of TCRδ$^{-/-}$ animals, by adoptive transfer of γδT-cells from the IEL of wild-type mice. Unfortunately, we were unable to achieve sufficient reconstitution in adequate numbers of mice to permit conclusions to be drawn from these experiments, and this remains an important outstanding issue. Indeed, the lack of available tools to manipulate γδT-cell responses is an important barrier to more fully elucidating their role in intestinal disease. Whilst mice carrying mutations in a limited numbers of specific Vγ segments have been described, none are commercially available. Furthermore, cell specific ablation using the *Cre-loxP* system, which has proven so powerful in studies of other cells types, is not currently possibly for γδT-cells. Separating their functions, particularly as producers of effector and regulatory mediators, from those of other cells is therefore challenging. Although depletion of γδT-cells and specific subtypes using antibodies has been widely employed, recent studies using TCRδ^{H2Bgfp} reporter mice which mark cells rearranging genes within the δ-locus, suggest this approach results in TCR downregulation, rendering cells 'invisible' rather than actually depleting them.[846] Since the function of the γδTCR is unclear, these cells may retain some biological activity, and indeed since the antibodies used bind to the TCR itself, they could result in cellular activation prior to receptor down-regulation.

Due to their highly tissue specific characteristics γδT-cells represent an attractive therapeutic target in the treatment of inflammatory disease, with the potential to minimise effects away from the site of disease. However, the potential translational implications of the findings presented here

are difficult to fully assess. Studies in patients with IBD have identified alterations in circulating and gut resident γδT-cells,[847,848] with accumulation occurring within tertiary lymphoid tissue forming in the inflamed colon.[849] However, significant differences exist between murine and human γδT-cells in terms of their TCR genetics and structure and indeed tissue distribution.[329] Therefore, the diversity and heterogeneity of functional γδT-cells subsets in mice may not be recapitulated in humans. Speculatively, it is likely that any role for γδT-cells in human IBD would be best demonstrated in paediatric disease, since γδT-cells appear to be more functionally relevant in a range of diseases in this age group. Although direct translation may not be possible, better understanding of human γδT-cells function and their contribution to inflammatory bowel disease would be useful, and a number of drugs and ligands have already been described which can activate or manipulate the human γδT-cell response, most notably in the setting of chronic viral infections[850-852] and cancer therapy.[853-855] However, our results caution that emergent γδT-cell based therapies which ameliorate extra-intestinal inflammatory disease may be of limited effect or even paradoxically harmful in IBD.

In conclusion, we have shown that intestinal γδT-cells represent a unique cell population, whose overall function is homeostatic rather than pro-inflammatory. We have demonstrated, using two models of bacterially driven colitis, that mice deficient in γδT-cells display an exaggerated inflammatory response, associated with alterations in the nature of CD4+ T-cell and myeloid cell accumulation. Furthermore, we found Th17 activity was not compromised in the absence of γδT-cells, highlighting the unique nature of intestinal immune responses compared to other systemic and mucosal surfaces.

Chapter 5. The Role of Interleukin-21 in Chronic Intestinal Inflammation

5.1 Introduction

Amongst factors regulating the differentiation and function of T-cells, IL-21 has received significant recent attention. Derived from activated $CD4^+$ T-cells,[649,653] IL-21 was initially characterised as a factor regulating the switch from innate to adaptive immune responses,[674] through effects upon NK-cells and cytotoxic $CD8^+$ T-cells,[649] and as a determinant of B-cell antibody isotype switching.[667,676] However, more recent studies have revealed a vast array of effects upon the development and function of multiple subsets of leukocytes, including macrophages, dendritic cells, NK/NKT cells and B and T-cells.[856,857] Important functions consistently demonstrated for IL-21 are the regulation of B-cell and T-cell proliferation and survival through activation of pro- and anti-apoptotic pathways,[680,682-684] the promotion of transition of B-cells to plasma cells,[656,673,695] promotion of NK and $CD8^+$ T-cell cytotoxicity,[649,674] and prevention of $CD8^+$ T-cell exhaustion during chronic viral infection.[651,691,692]

Studies of the effects of IL-21 on $CD4^+$ T-cell biology have demonstrated numerous and often apparently paradoxical effects on various subsets. In initial investigations it was characterised as a Th2 cytokine,[657] based upon its ability to suppress IFN-γ production through specific down-regulation of eomesodermin, although other key Th1 genes were unaffected.[685] In contrast, other studies demonstrated a conflicting role as an initiator of genes regulating Th1 differentiation, driving IFN-γ production.[664,675] *In vivo* studies of humans and mice have reported IL-21 to be predominantly derived from IFN-γ^+ Th1 cells rather than other subsets.[295,658]

The recent description of a critical role for IL-21 in the development of Th17 cell responses, whilst at the same time antagonising Treg development[176,177,611] and function has heightened interest in understanding its function in immune and inflammatory responses, and as a potential

therapeutic target. IL-21 activates STAT3 in $CD4^+$ T-cells to upregulate *Il23r* and drive transcription of further Th17 genes including RORγt, *Il17a*, *Il17f* and *Il22*.[175] A similar effect was initially described for IL-6, and it is now clear that IL-21 may act both downstream of or in place of IL-6 in Th17 differentiation.[175] Following initial IL-6 stimulation, upregulation of both *Il21* and *Il21r* establishes autocrine signalling in developing Th17 cells, and is needed for the generation of optimal Th17 responses, through effects on both differentiation and sustenance of such cells.[176,654] However, IL-6 and IL-21 fulfil non-redundant roles, as demonstrated by the finding of defective Th17 responses in IL-$6^{-/-}$ mice, possibly related to differential requirements at various stages of development.[177,857] IL-21 production is therefore now widely considered part of the 'signature' of Th17 cells, and may represent an important factor antagonising the development of alternative cell fates. IL-21 is a potent inhibitor of Foxp3 expression, and has been reported to be an important factor *in vivo* in the regulation of the reciprocal pathways of Th17 and Treg cell development.[176,611,710] However, analysis of Th17 cells in steady-state mice deficient in IL-21R demonstrated attenuated peripheral and lymphoid tissue populations, but increased Th17 populations in the intestinal lamina propria, implying a potentially compartmentalised requirement for IL-21 in steady state Th17 cell biology.[177,858]

These results suggest IL-21 may exert highly context specific effects in immune and inflammatory reactions. Accordingly, studies of the *in vivo* effect of blockade or ablation of IL-21 or IL21R report highly variable and often paradoxical effects. IL-21 is highly pathogenic in murine models of arthritis, lupus and autoimmune diabetes,[668,711,717,718] whereas in EAE IL-21 appears pathogenic, redundant or protective depending upon the specific variation of the model used and the timing of IL-21 inhibition.[677,707,708] In colitis, IL-21 deficiency may be protective in models of disease induced by the administration of chemical irritants such as DSS or TNBS, proposed mechanistically to reflect impaired Th17 differentiation.[609,612] Similarly, in T-cell transfer disease, IL-$21^{-/-}$ T-cells are less pathogenic, although the cellular and molecular mechanisms are unknown.[611,612]

Therefore, IL-21 might represent an important therapeutic target in IL-23/Th17 driven diseases such as IBD. However, greater understanding of the contribution of IL-21 to intestinal inflammation may assist in defining the optimal use of such a strategy.

5.2 Results

5.2.1 IL-21 drives intestinal inflammation induced by *Helicobacter hepaticus* infection

Infection of wild-type C57BL/6 mice with *H.hepaticus* and concomitant blockade of IL-10R signalling results in chronic intestinal inflammation, with accumulation of Th1, Th17 and a hybrid IL-17A$^+$IFN-γ^+ Th1/17 population in the inflamed colon.[183,859] In particular, the IL-17A$^+$IFN-γ^+ Th1/17 population has been implicated as a key pathogenic population in intestinal inflammation, developing in an IL-23 dependent manner.[183] Since IL-21 may play a critical role in Th17 cell function, through up-regulation of IL-23R on CD4$^+$ T-cells, we hypothesised that IL-21 might be critical to the development of disease in this model.

Initially, we examined expression of IL-21 in the inflamed colonic mucosa in established disease (day 28), and found increased expression at this point (Fig 5.1A). Kinetic analysis demonstrating the highest levels of expression occurred from around day 12-14, in keeping with production by accumulating CD4$^+$ T-cells, but was sustained above baseline throughout disease (Fig 5.1A). Next, we sought to assess the functional significance of this finding, by infecting wild-type C57BL/6 and age and sex-matched IL-21R$^{-/-}$ mice with *H.hepaticus* and administering an anti-IL-10R antibody. These experiments revealed that the absence of IL-21 signals resulted in both attenuated intestinal inflammation (Fig 5.1B) and reduced systemic markers of disease including splenomegally and liver pathology (Fig 5.1C). The total leukocyte population isolated from the colonic lamina propria of IL-21R$^{-/-}$ mice was approximately half that of WT animals (Fig 5.1C).

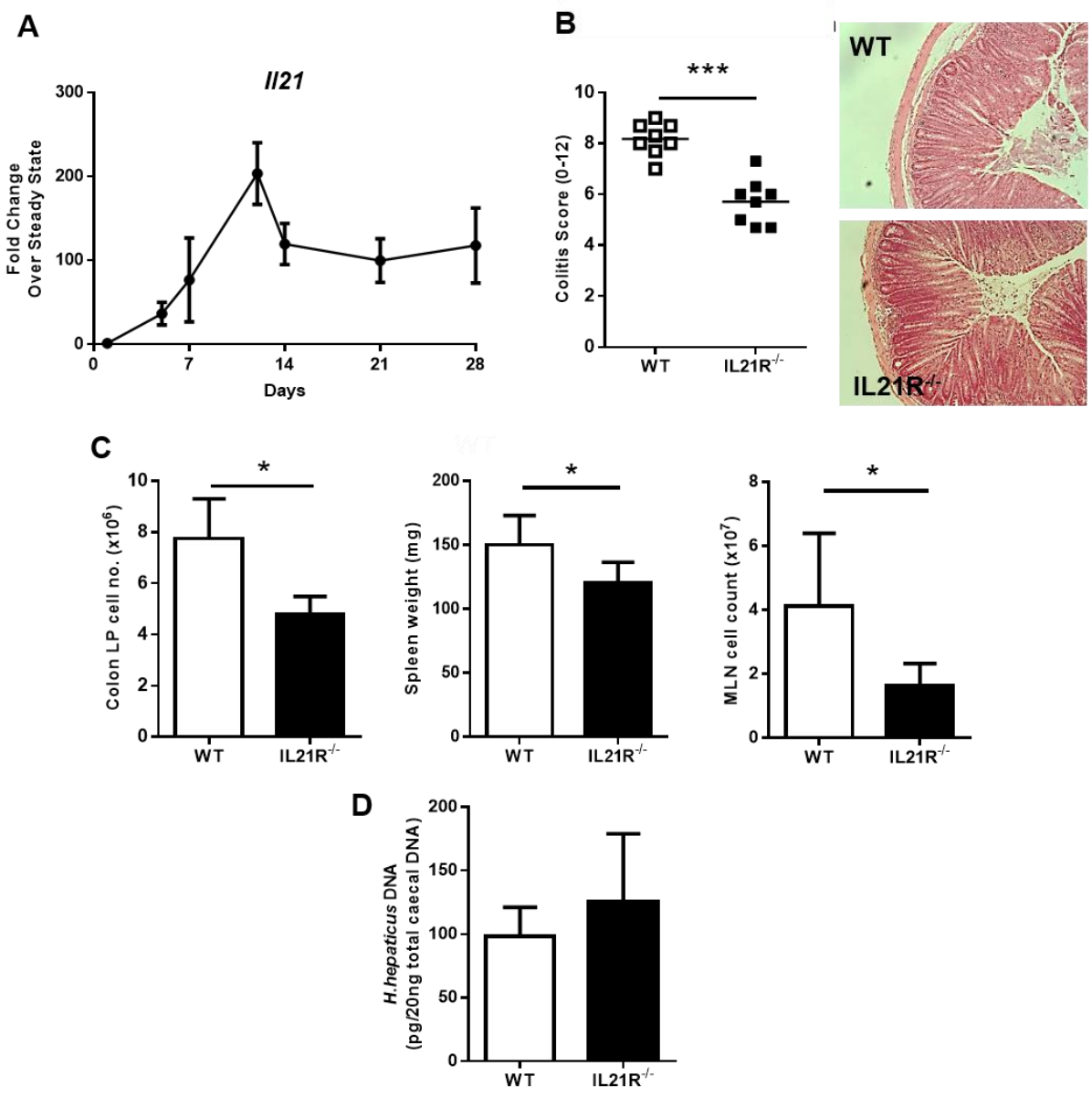

Figure 5.1 IL-21 drives intestinal inflammation induced by *H.hepaticus* infection

Wild-type (WT) C57BL/6 or IL-21R$^{-/-}$ mice were infected with ~10^{9} CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p.* weekly). Mice were sacrificed at indicated time points and tissues harvested for analysis. **(A)** Colonic *Il21* expression determined by qPCR. Results normalised to *Hprt* expression and displayed as fold-change over steady state. Points represent mean values +/-SD **(B)** Colonic histopathology scores at day 28 with representative photomicrographs. Points represent individual mice, bars indicate mean **(C)** Total colonic lamina propria leukocyte counts (left) spleen weights (middle) and MLN cell count (right) at day 28. **(D)** Caecal *H.hepaticus* colonisation determined by qPCR for *cdtB* gene and standardised to total caecal DNA content. Columns represent mean values +/-SD. Data representative of 1 (A) (n=4-6 each time point) or 2 (B-D) independent experiments (n=8 each group). Statistical significance determined using Mann-Whitney Test. *$p \leq 0.05$, ***$p \leq 0.001$

Importantly, colonisation with *H.hepaticus* was equivalent in IL-21R$^{-/-}$ and wild-type mice, demonstrating that severity of disease did not simply reflect differences in bacterial load (Fig 5.1D). However, whilst IL-21R$^{-/-}$ animals consistently developed less severe typhlocolitis, this protection was incomplete, with IL-21R$^{-/-}$ animals exhibiting histological features of mild to moderate disease, somewhat greater in severity than seen in IL-23R$^{-/-}$ mice in this model (Fig 5.1B & Fig 3.1E). This suggests that IL-21 is an important mediator of both intestinal and systemic disease in this model, but that additional pathways may partially compensate for its absence.

5.2.2 IL-21 influences the accumulating T-cell phenotype in *Helicobacter* induced typhlocolitis

IL-21 has been shown both *in vitro* and *in vivo* to be a critical regulator of T-cell polarisation, with effects upon the balance of Th1, Th2, Th17, Tfh and TReg populations reported, although such effects appear highly context dependent. We therefore examined the phenotype of colonic lamina propria T-cells in mice infected with *H. hepaticus* and treated with anti-IL-10R, by isolation and ex-vivo re-stimulation with PMA/ionomycin (Fig 5.2). Intracellular staining for the signature cytokines IL-17A (Th17), IFN-γ (Th1) and the transcription factor Foxp3 (Treg), revealed IL-21R$^{-/-}$ mice harboured significantly reduced populations of IL-17A^{+} Th17 cells and IL-17A^{+}IFN-γ^{+} Th1/17 cells, with an increased proportion of cells expressing the Treg marker Foxp3 (Fig 5.2). No significant differences were seen in the proportion of cells expressing single Th1 or Th2 signature cytokines, suggesting the dominant effect of IL-21 on CD4^{+} T-cells in this model is in regulating the balance of Th17 and Treg accumulation in the lamina propria.

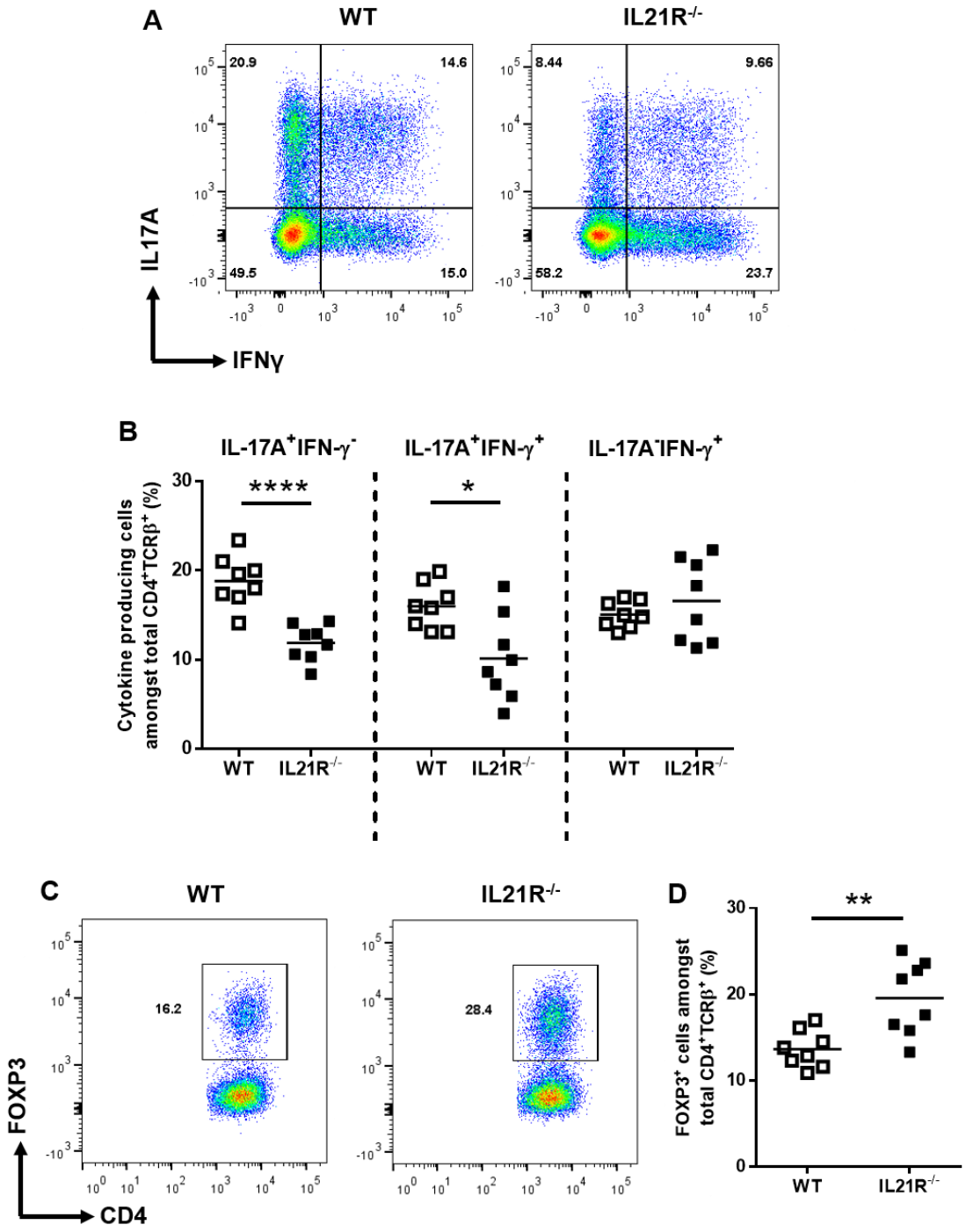

Figure 5.2 IL-21 influences the accumulating T-cell phenotype in intestinal inflammation induced by *H.hepaticus* infection

Wild-type (WT) C57BL/6 or IL-21R$^{-/-}$ mice were infected with ~10^{9} CFU *H.hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p.* weekly). Mice were sacrificed at 28 days and colonic lamina propria lymphocytes were isolated and restimulated in vitro with PMA/ionomycin with Brefeldin A. **(A)** Representative plots of intracellular cytokine staining. Gated upon live CD4+ T-cells. **(B)** Frequency of colonic cytokine producing T-cells **(C)** Representative staining of intracellular FOXP3 staining. **(D)** Frequency of FOXP3+ cells amongst colonic CD4+ T-cells. Data representative of 2 independent experiments (n=8 mice each group). (B) & (D) Points represent individual mice. Statistical significance determined using Mann-Whitney Test. *p<0.05, **p<0.01, ****p<0.0001.

5.2.3 IL-21 influences colonic chemokine expression in *Helicobacter* induced typhlocolitis

T-cell accumulation may occur through a number of mechanisms including recruitment of circulating cells from the blood and local proliferation of memory T-cells resident within the lamina propria. Differential expression of chemokine receptors by T-cell subsets may determine their relative accumulation and hence the nature of the T-cell infiltrate in inflammation. Furthermore, T-cells are themselves capable of chemokine synthesis, amplifying the accumulation of specific subsets. In Crohn's disease, IL-21 has been reported to drive epithelial cell production of the Th17 attracting chemokine CCL20.[670] Therefore, whilst IL-21R deficiency might be influencing the intestinal T-cell populations through direct effects upon the differentiation of such cells, we considered that alterations in the expression of chemokines directing the recruitment of cell populations might be an alternative mechanism contributing to our observations.

We therefore analysed the colonic expression of a panel of chemokines involved in T-cell recruitment by quantitative RT-PCR (Fig 5.3). This revealed significantly lower levels of transcripts for *Ccl5* (RANTES) and *Ccl20* in the inflamed colons of IL-21R$^{-/-}$ mice, chemokines implicated in the recruitment and activation of naïve and memory T-cell populations, particularly Th1 cells, and in the attraction of Th17 cells respectively. Importantly, no significant difference was seen in the other chemokines analysed, including *Ccl2* (MCP-1), *Ccl 19*, *Ccl22* (eotaxin-2) and *Ip10* (CXCL10) suggesting this was not simply a universal reduction in pro-inflammatory chemokines secondary to the reduced inflammation in IL-21R$^{-/-}$ colons.

In summary, these results suggest that IL-21R deficiency is characterised by an altered T-cell population in the inflamed colonic lamina propria, associated with a significantly different colonic chemokine profile to wild-type animals. Importantly, it was not possible from these experiments to determine the direction of this association, since chemokines such as CCL5 and CCL20 may

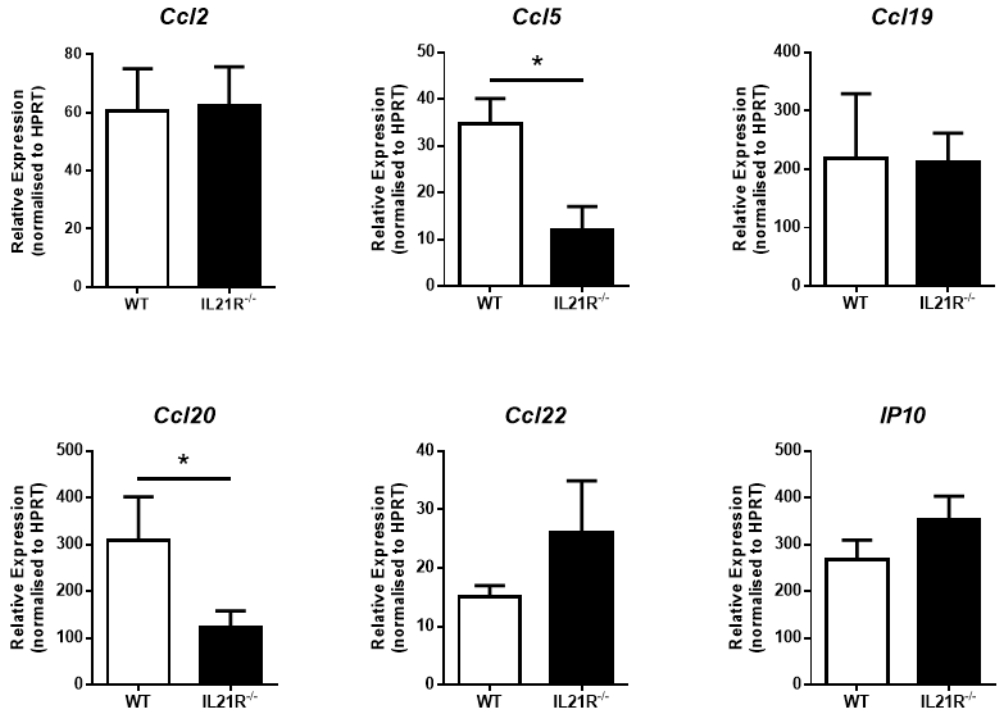

Figure 5.3 IL-21 influences colonic chemokine expression in *H. hepaticus* induced inflammation

Wild-type (WT) C57BL/6 or IL-21R-/- mice were infected with ~10^9 CFU *Helicobacter hepaticus* by oral gavage and treated with anti-IL-10R antibody (1mg *i.p.* weekly). Mice were sacrificed at 4 weeks and colonic tissues harvested for analysis. RNA was extracted from pooled proximal, mid and distal colonic segments of mice, and gene expression determined by quantitative RT-PCR, with results normalised to the housekeeping gene *Hprt1*. Results shown represent single experiment (n=8 per group). Columns show mean +/- SD. Significance determined using Mann-WhitneyTest. *$p<0.05$

be produced by accumulating T-cells cells and by multiple cell types, downstream of T-cell derived cytokines. Similarly, the analysis of samples of whole colon tissue precludes conclusions as to the critical IL-21 responsive cell type in chronic colitis.

5.2.4 IL-21R$^{-/-}$ signalling into T-cells is required for disease in T-cell transfer colitis

Whilst the *H.hepaticus*/anti-IL-10R model of disease provides a powerful tool to assess the consequences of IL-21R deficiency in an otherwise immunologically intact animal, this complexity also presents difficulties in assessing the role of IL-21 in different cell populations. Notably, IL-21R expression determined by immunohistochemistry or quantitative RT-PCR has been consistently reported on a variety of leukocytes, including T-, B-, and NK-cells, dendritic cells, and possibly upon stromal and epithelial cells, particularly during inflammation.[649,650,667,668,694] It is therefore possible that the protection noted in the *Helicobacter hepaticus*/anti-IL10R model reflects a requirement for IL-21R expression on a specific cell type other than the T-cell. To address this, we utilised the T-cell transfer model of disease, in which the transfer of CD4^{+}CD25^{-}CD45RB^{hi} naïve T-cells into a *Rag*$^{-/-}$ host results in intestinal and systemic disease characterised by the accumulation of Th1, Th17 and mixed Th1/17 cells. Previous studies in the lab have demonstrated significant elevation of IL-21 in the colonic lamina propria in this model. Transferring IL-21R$^{-/-}$ naïve T-cells into wild-type *Rag*$^{-/-}$ hosts allowed us to restrict IL-21R deficiency to the T-cell population, leaving other cell potentially responsive to IL-21.

These studies demonstrated that compared to recipients of wild-type cells, mice transferred with IL-21R$^{-/-}$ T-cells developed significantly attenuated colitis associated with reduced accumulation of both CD4^{+} T-cells and total leukocytes in the colonic lamina propria (Fig 5.4A-C). However, although weight loss was significantly more severe in animals reconstituted with wild-type T-cells (Fig 5.4D), other indices of systemic disease, including spleen weight and cell numbers in

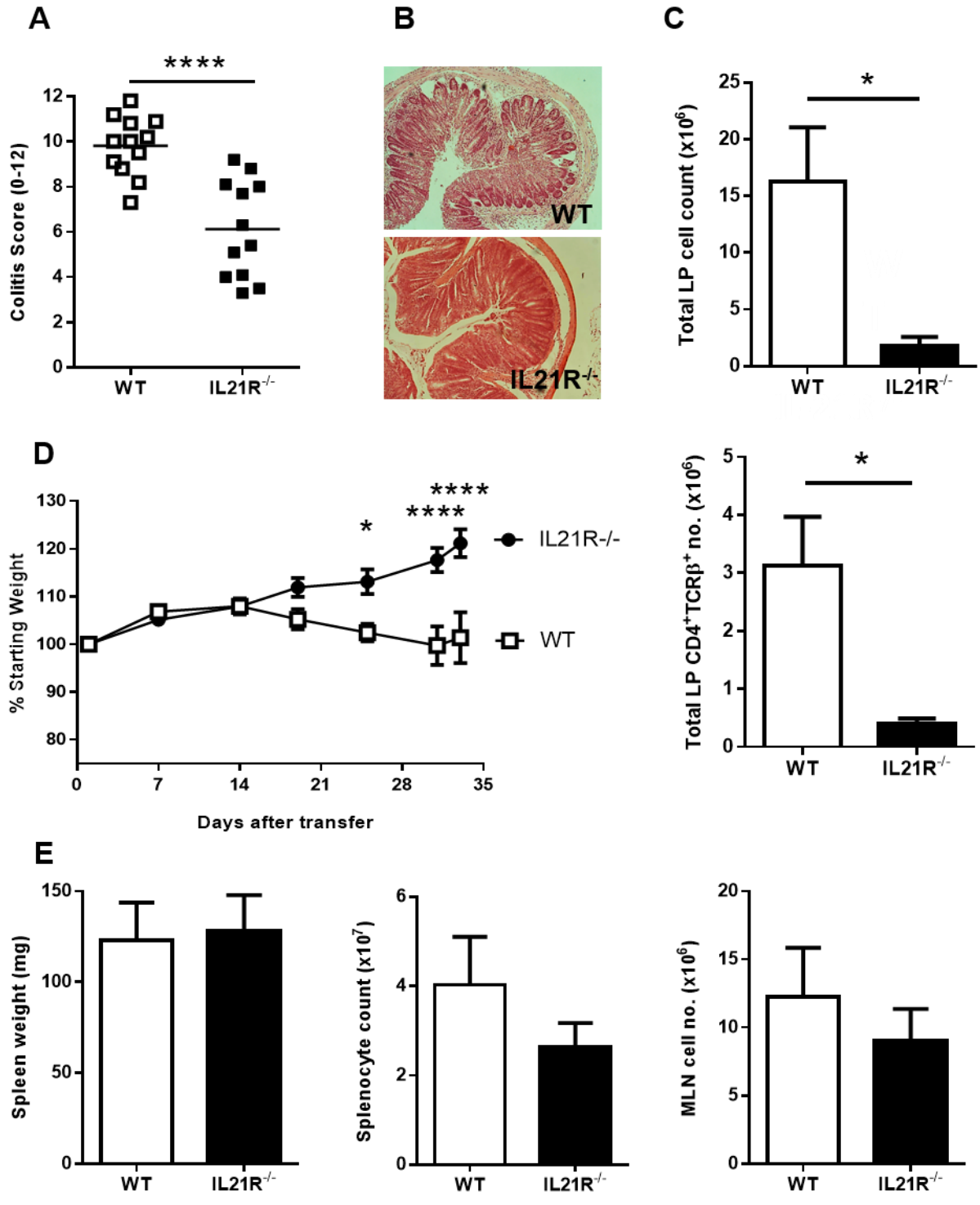

Figure 5.4 IL-21 signalling into T-cells is required for intestinal disease in T-cell transfer colitis
C57BL/6.Rag1-/- mice were transferred with 4x10^5 CD4+CD25-CD45RBhi cells from WT or IL-21R-/- donors. Mice were monitored and sacrificed upon development of disease in WT controls (6-8 weeks). **(A)** Colonic histopathology scores (0-12). **(B)** Representative photomicrographs of colonic pathology. **(C)** Total cell counts in colonic lamina propria. **(D)** Weight curves. **(E)** Spleen weight (left), splenocyte counts (middle) and MLN cell counts (right). Results shown are pooled data from 2 of 3 representative experiments (n=12-16 per group). Points indicate individual mice (A) or mean +/-SD (D) Columns show mean +/- SD. Significance determined using Mann-Whitney Test (A) or ANOVA (D). *p<0.05, ****p<0.0001.

spleen and MLN were similar in recipients of IL-21R$^{-/-}$ or wild-type T-cells (Fig 5.4E). These results show that IL-21 signals into T-cells are critically required for the development of intestinal inflammation, but may be dispensable for aspects of the extra-intestinal features of this model.

5.2.5 IL-21 regulates the balance of effector and regulatory T-cells in intestinal inflammation

In view of our findings of a significant effect upon the T-cell subsets present in the intestine in the *H. hepaticus*/anti-IL10R model of disease, we next sought to assess what effect isolated T-cell IL-21R deficiency might have upon accumulating T-cell populations in the T-cell transfer model (Fig 5.5). *Ex vivo* flow-cytometric phenotyping of T-cells from spleen, MLN and colonic lamina propria from mice transferred with either wild-type or IL-21R$^{-/-}$ T-cells revealed a significantly increased proportion of cells in all compartments expressing the regulatory T-cell transcription factor Foxp3 in recipients of IL-21R$^{-/-}$ T-cells (Fig 5.6). Conversely, after re-stimulation and intracellular cytokine staining, a significantly lower proportion of IL-21R$^{-/-}$ T-cells in all tissues analysed were found to be IFN-γ^{+}. In contrast to the findings in the *Helicobacter hepaticus*/anti-IL10R model, no significant differences were apparent in the proportion of T-cells displaying an IL-17A^{+} or IL17A^{+}IFN-γ^{+} phenotype (Fig 5.5A&B). Similarly, no change in the frequency of cells expressing the Th2 cytokine IL-5 was observed (Fig 5.5C).

These findings were supported by analysis of the expression of key cytokines in the intestine, which revealed a highly significant reduction in expression of *IFN-γ* transcripts in the colon of mice transferred with IL-21R$^{-/-}$ T-cells, whereas expression of the Th17 cytokines *Il17a, Il17f* and *Il22* were similar to wild-type (Fig 5.7). Furthermore, *Il21* transcripts were significantly reduced in mice receiving IL-21R$^{-/-}$ T-cells, compatible with ablation of the autocrine IL-21 signals reported to inducing its expression from CD4+ T-cells. Notably, despite the increased propensity of IL-21R$^{-/-}$ T-cells to differentiate into Foxp3^{+} iTregs, colonic *Il10* transcripts were similar

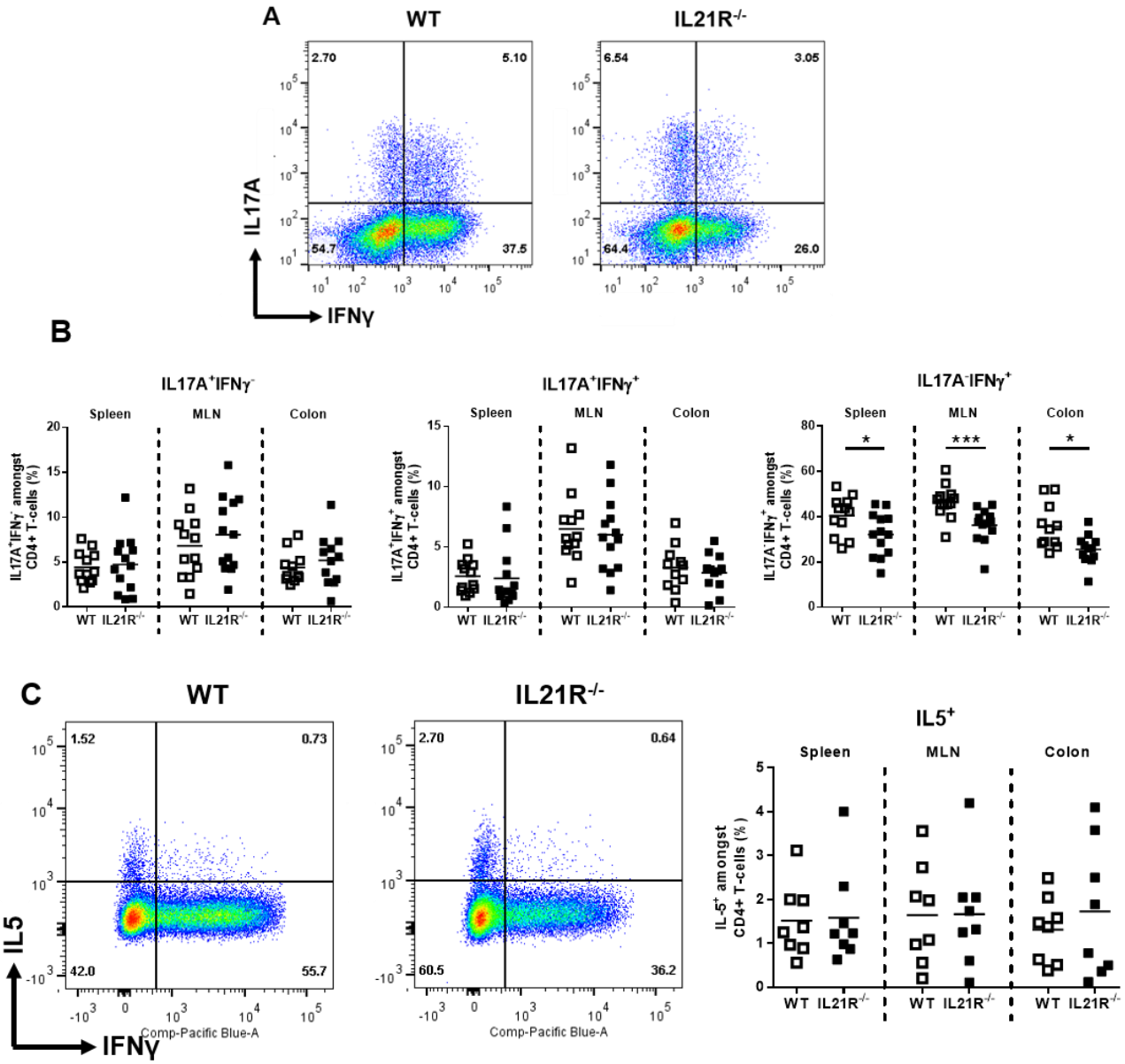

Figure 5.5 IL-21 regulates the effector T-cell phenotype in T-cell transfer colitis

C57BL/6.Rag1$^{-/-}$ mice were transferred with 4x10^5 CD4+CD25-CD45RBhi cells from WT or IL-21R$^{-/-}$ donors. Mice were monitored and sacrificed upon development of disease in WT controls (6-8 weeks). Cells were isolated from tissues and restimulated in vitro using PMA/ionomycin for 4 hours in the presence of Brefeldin A, followed by surface and intracellular staining. **(A)** Representative plots of intracellular IL-17A and IFN-γ staining in colonic CD4+ T-cells. **(B)** Frequencies of cells producing indicated cytokines by organ. **(C)** Representative plots of intracellular IL-5 and IFN-γ staining in colonic cells (left) frequency of IL-5+ T-cells by organ. Results shown are pooled data from 2 of 3 representative experiments (n=12-14 per group). Points indicate individual mice, bars show mean. Significance determined using Mann-Whitney Test. *p<0.05, ***p<0.001.

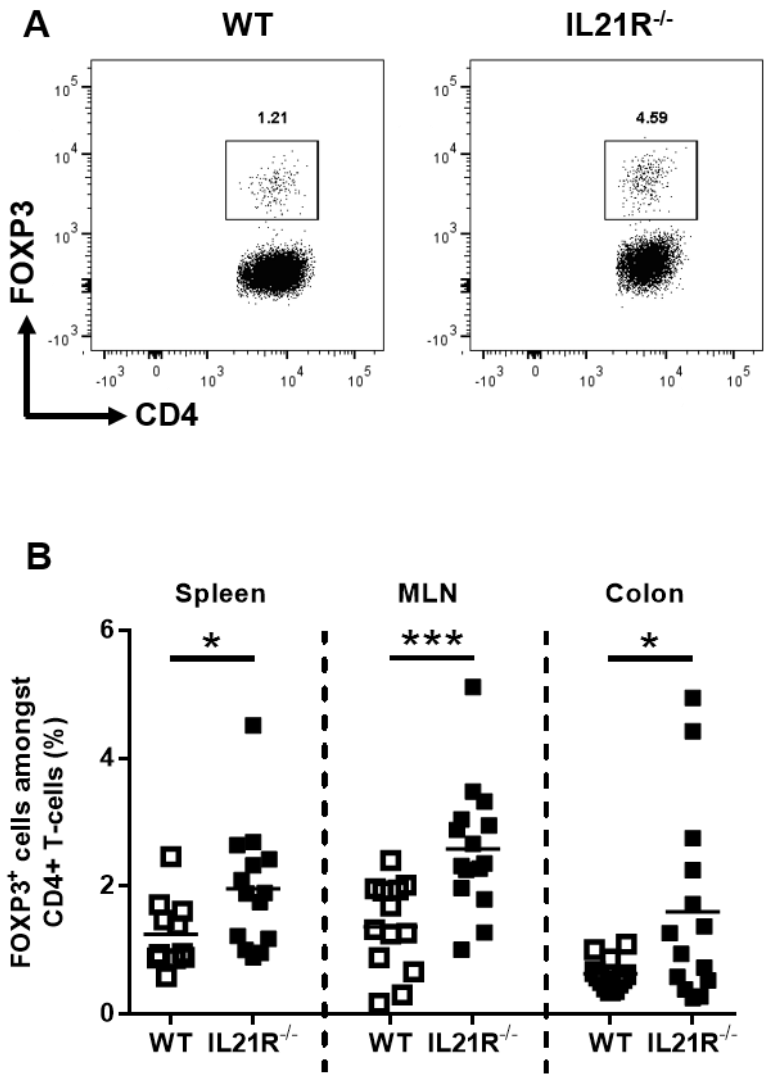

Figure 5.6 IL-21 inhibits regulatory T-cell differentiation in T-cell transfer colitis

C57BL/6.Rag1$^{-/-}$ mice were transferred with 4x10^5 CD4+CD25-CD45RBhi cells from WT or IL-21R$^{-/-}$ donors. Mice were monitored and sacrificed upon development of disease in WT controls (6-8 weeks). Cells were isolated from tissues and restimulated in vitro using PMA/ionomycin for 4 hours in the presence of Brefeldin A, followed by surface and intracellular staining. **(A)** Representative plots of Foxp3 expression in colonic CD4+ T-cells, gated on CD4+ T-cells. **(B)** Frequencies of Foxp3+ cells amongst total CD4+ T-cells by tissue. Results shown are pooled data from 2 of 3 representative experiments (n=12-14 per group). Points indicate individual mice bars show mean. Significance determined using Mann-Whitney Test. *p<0.05, ***p<0.001.

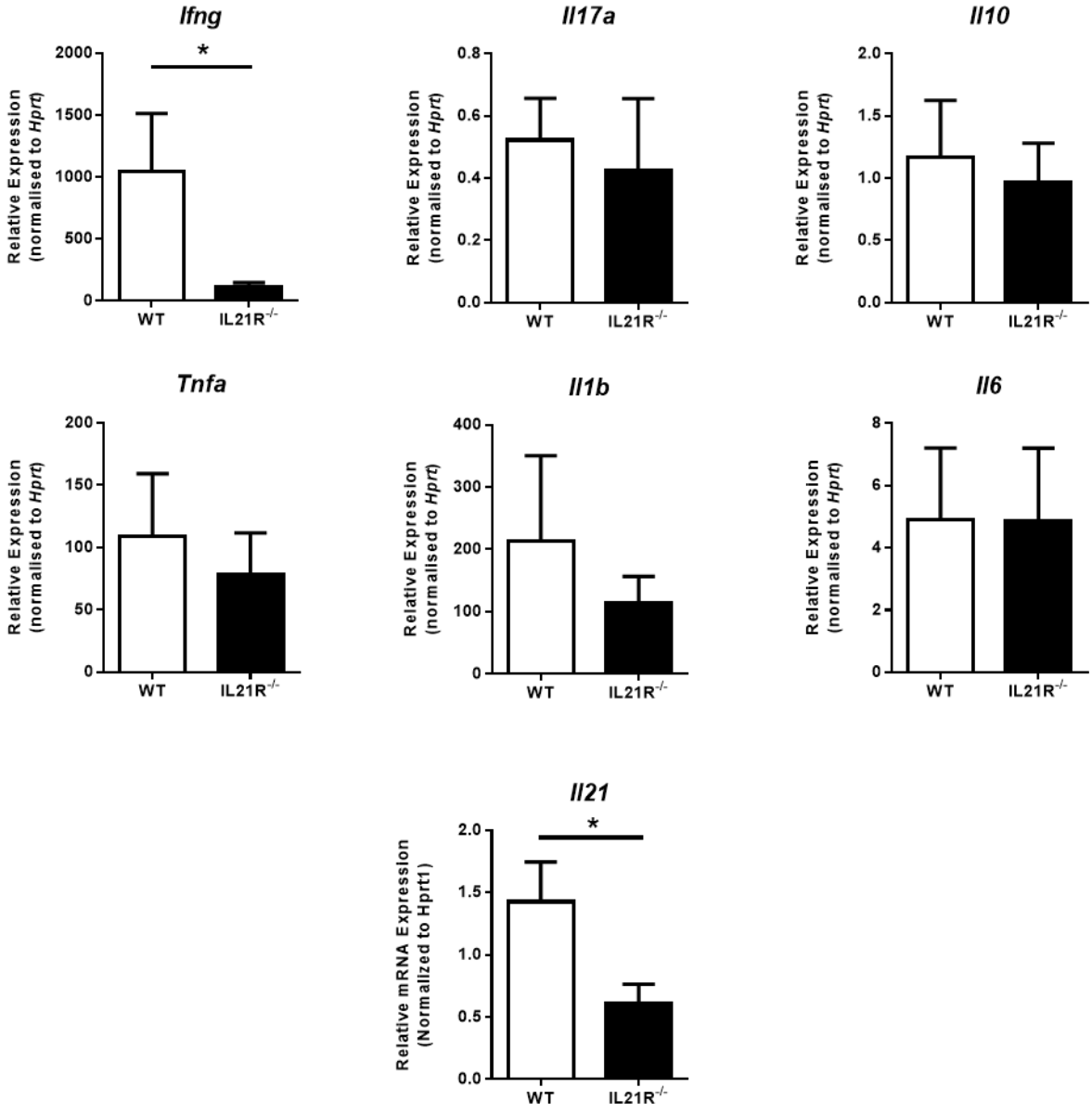

Figure 5.7 IL-21R deficiency on T-cells results in reduced colonic IFN-γ production

C57BL/6.Rag1$^{-/-}$ mice were transferred with 4x10^5 CD4+CD25-CD45RBhi cells from WT or IL-21R$^{-/-}$ donors. Mice were monitored and sacrificed upon development of disease in WT controls (6-8 weeks). Segments of tissue from proximal, middle and distal colon were isolated and total RNA extracted. Expression of indicated genes in whole colon tissue, determined by qPCR and normalised to *Hprt*. Results shown are from single experiment (n=8 per genotype). Columns indicate mean +SD. Significance determined using Mann-Whitney Test. *$p<0.05$.

between both groups, consistent with the lower total number of such cells, and the abundant non-T-cell sources of this cytokine present in the intestine. Expression of other inflammatory cytokines, including *Il1b*, *Il6* and *Tnfa* were similar between the two groups.

In total, these results show that T-cell dependent IL-21 signals regulate intestinal inflammation in the T-cell transfer model of IBD.

5.2.6 IL-21R$^{-/-}$ T-cells are not impaired in their ability to accumulate in the lamina propria

IL-21, in common with other members of the IL-2 family of cytokines which signal via receptors incorporating the common gamma chain, including IL-4, -7 and -15, has been reported to have significant effects upon maintenance of T-cell populations via effects on cellular proliferation and apoptosis.[860] It is therefore possible that the failure of IL-21R$^{-/-}$ naïve T-cells to induce significant colitis was due to defective accumulation in the intestine related to a failure to receive appropriate proliferative or survival signals. We therefore examined the comparative ability of IL-21R$^{-/-}$ and WT T-cells to accumulate in the intestinal lamina propria by transferring a 1:1 mix of congenic CD45.1^{+} naïve wild-type cells with either CD45.2^{+} naïve IL-21R$^{-/-}$ cells or CD45.2^{+} naïve wild-type cells as a control (Fig 5.8A). Analysis of the contribution of each of these congenically marked populations to the total colonic CD4^{+} T-cell population revealed that IL-21R$^{-/-}$ T-cells were similar to WT cells in their ability to accumulate in the lamina propria (Fig 5.8B), showing that IL-21R$^{-/-}$ cells are not intrinsically impaired in their ability to populate the intestinal mucosa in inflammatory settings, but do require a cell extrinsic IL-21 driven T-cell response for this.

5.2.7 IL-21 regulates the balance of intestinal Th1 and Treg cells in a cell intrinsic manner

The significant differences observed in inflammation occurring in the colons of mice transferred with WT or IL-21R$^{-/-}$ T-cells might itself influence the T-cell phenotype observed; therefore we

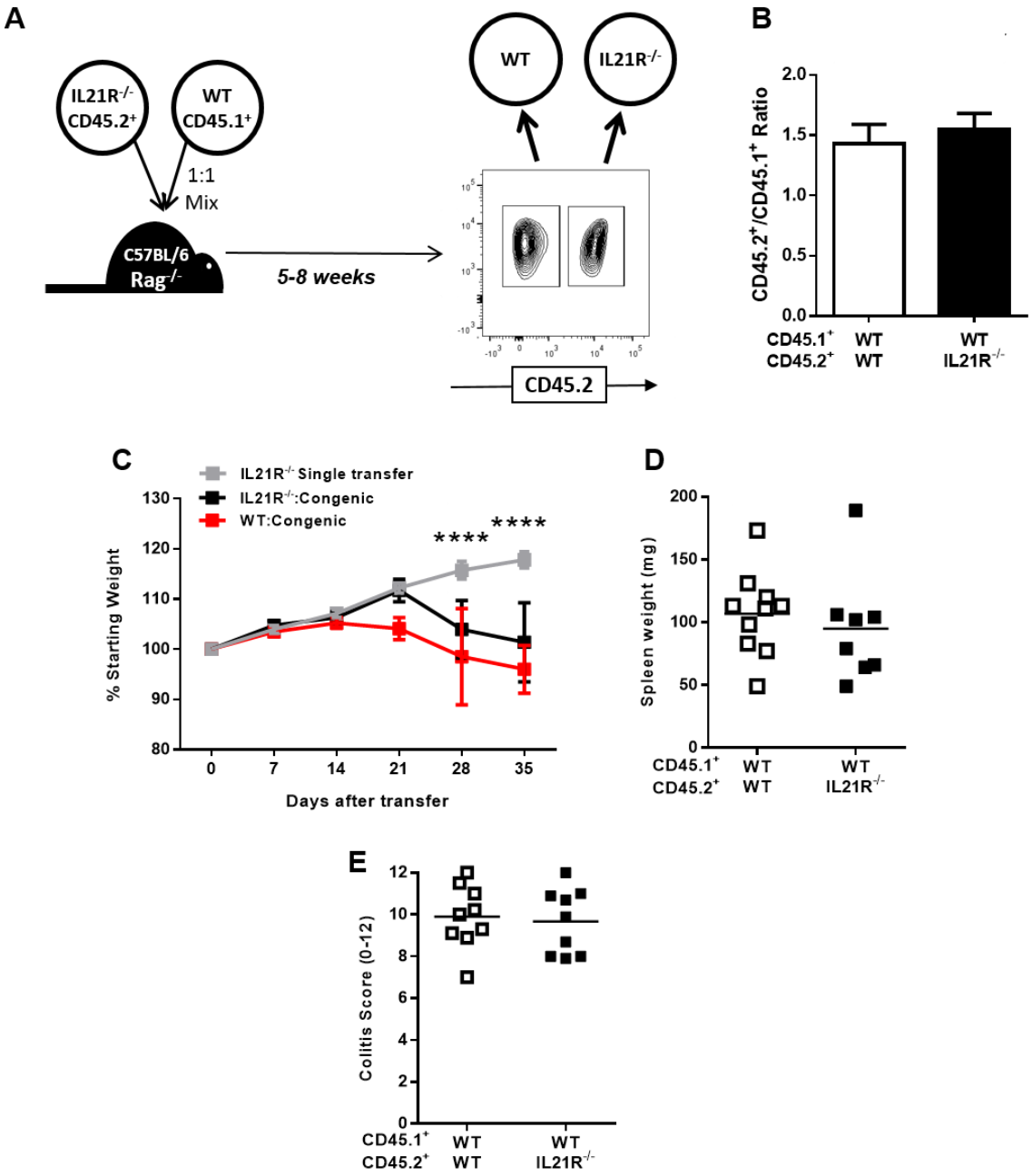

Figure 5.8 IL-21R$^{-/-}$ are not intrinsically impaired in their ability to accumulate in colitis

C57BL/6.Rag1$^{-/-}$ mice were transferred with 5x10^5 CD4+CD25-CD45RBhi cells from a 1:1 mixture of cells from CD45.1+WT (congenic) and either CD45.2+WT or CD45.2+IL-21R$^{-/-}$ donors. Control mice received 5x10^5 IL-21R$^{-/-}$ cells alone. Mice were monitored and sacrificed upon development of disease in WT controls (6-8 weeks). **(A)** Experimental schema **(B)** Ratio of CD45.2$^+$:CD45.1$^+$ cells amongst colonic T-cells **(C)** Weight curves according to donor cell genotype **(D)** Spleen weights **(E)** Colonic histopathology scores (0-12) Results shown are from one of two experiments with similar results (n=10 per co-transfer genotype, n=4 IL-21R$^{-/-}$ controls). Columns indicate mean +SD. (B) Points indicate mean +/- SD (C) or individual mice (D&E). Significance determined using ANOVA. ****p<0.0001.

sought to determine the T-cell intrinsic effect of the loss of IL-21R signalling, utilising the co-transfer system described above (Fig 5.8A). Co-transfer of a 1:1 mix of either CD45.1^{+}WT/CD45.2^{+} WT or CD45.1^{+}WT/CD45.2^{+}IL21R$^{-/-}$ cells resulted in a similar degree of both systemic disease and colonic inflammation (Fig 5.8C-E). However, the alterations previously noted in the accumulating T-cell phenotype within the colonic lamina propria amongst IL-21R$^{-/-}$ T-cells were again present, including an increased proportion of Foxp3^{+} Treg cells (Fig 5.10) and a reduced population of IFN-γ^{+} cells (Fig 5.9A&B), demonstrating that IL-21 regulates these T-cell phenotypes in a cell intrinsic manner. Importantly, despite the presence of an increased proportion of Foxp3^{+} Treg cells within the CD45.2^{+} fraction of mice transferred with CD45.2^{+}IL-21R$^{-/-}$ cells, these cells were insufficient to provide the dominant suppression seen when IL-6R deficient T-cells are used in this model (Sofia Buonocore, unpublished observations).

Interestingly, we also noted a small but significant increase in the proportion of IL-17A^{+} cells amongst IL-21R$^{-/-}$ cells in this co-transfer system (Fig 5.9B), suggesting the intriguing possibility that IL-21 may be acting in a cell intrinsic manner to suppress or extinguish IL-17A production, however the proportion of IL17A^{+}IFN-γ^{+} double-expressing cells was similar in both WT and IL-21R$^{-/-}$ cells. This double-positive population appears particularly reliant upon IL-23 signals, suggesting that IL-21 deficiency does not result in a functionally significant reduction in IL-23R expression or activity in this setting.

Recently, GM-CSF has been identified as a novel product of Th17 cells, mediating their pathogenic effects in EAE.[185,186] We therefore examined the effect of IL-21 on GM-CSF production by T-cells in our co-transfer system (Fig 5.9C). These studies showed that T-cell IL-21R deficiency did not influence GM-CSF amongst any subset. Importantly, in the context of our observations for IL-17A in this system, this result shows that IL-17A and GM-CSF are differentially regulated in intestinal Th17-cells.

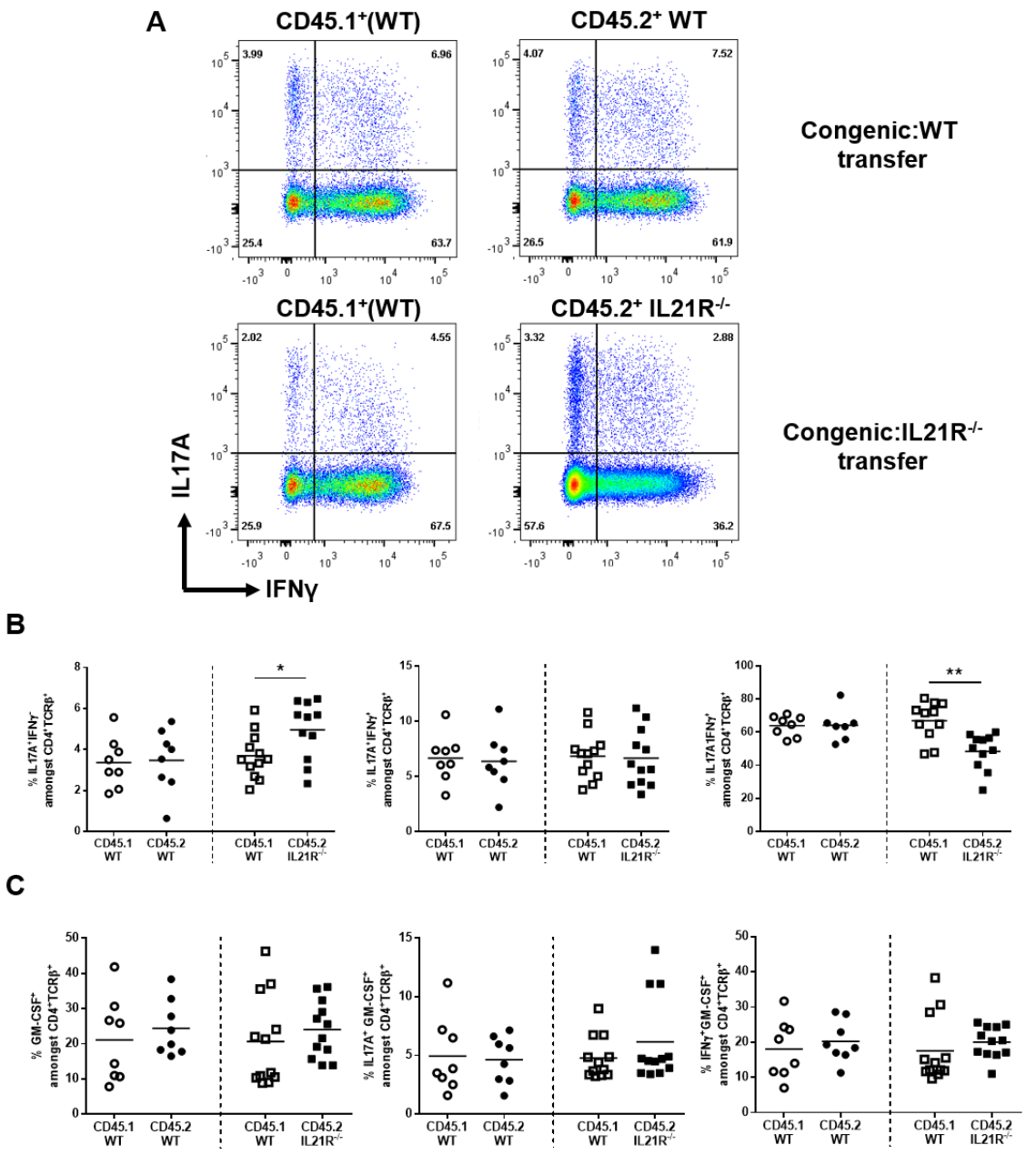

Figure 5.9 IL-21 controls colonic effector T-cell phenotype through a cell intrinsic mechanism

C57BL/6.Rag1$^{-/-}$ mice were transferred with 5x10^5 CD4+CD25-CD45RBhi cells from a 1:1 mixture of cells from CD45.1+WT (congenic) and either CD45.2+WT or CD45.2+IL-21R$^{-/-}$ donors. Mice were monitored and sacrificed upon development of disease in WT controls (6-8 weeks). Colonic cells were isolated and restimulated in vitro with PMA/ionomycin for 4 hours in the presence of Brefeldin A. **(A)** Representative intracellular cytokine staining of colonic cells gated on CD4+ live T-cells. Frequencies of **(B)** IL-17A and/or IFN-γ, and **(C)** GM-CSF and/or IL-17A or IFN-γ producing CD4+ T-cells in the colon of co-transferred mice. Results shown are pooled from two experiments with similar results (n=8 (CD45.1$^+$WT:CD45.2$^+$WT) and n=12 (CD45.1$^+$WT:CD45.2$^+$IL-21R$^{-/-}$)). Points indicate individual mice. Significance determined using Mann Whitney Test. *p<0.05, **p<0.01.

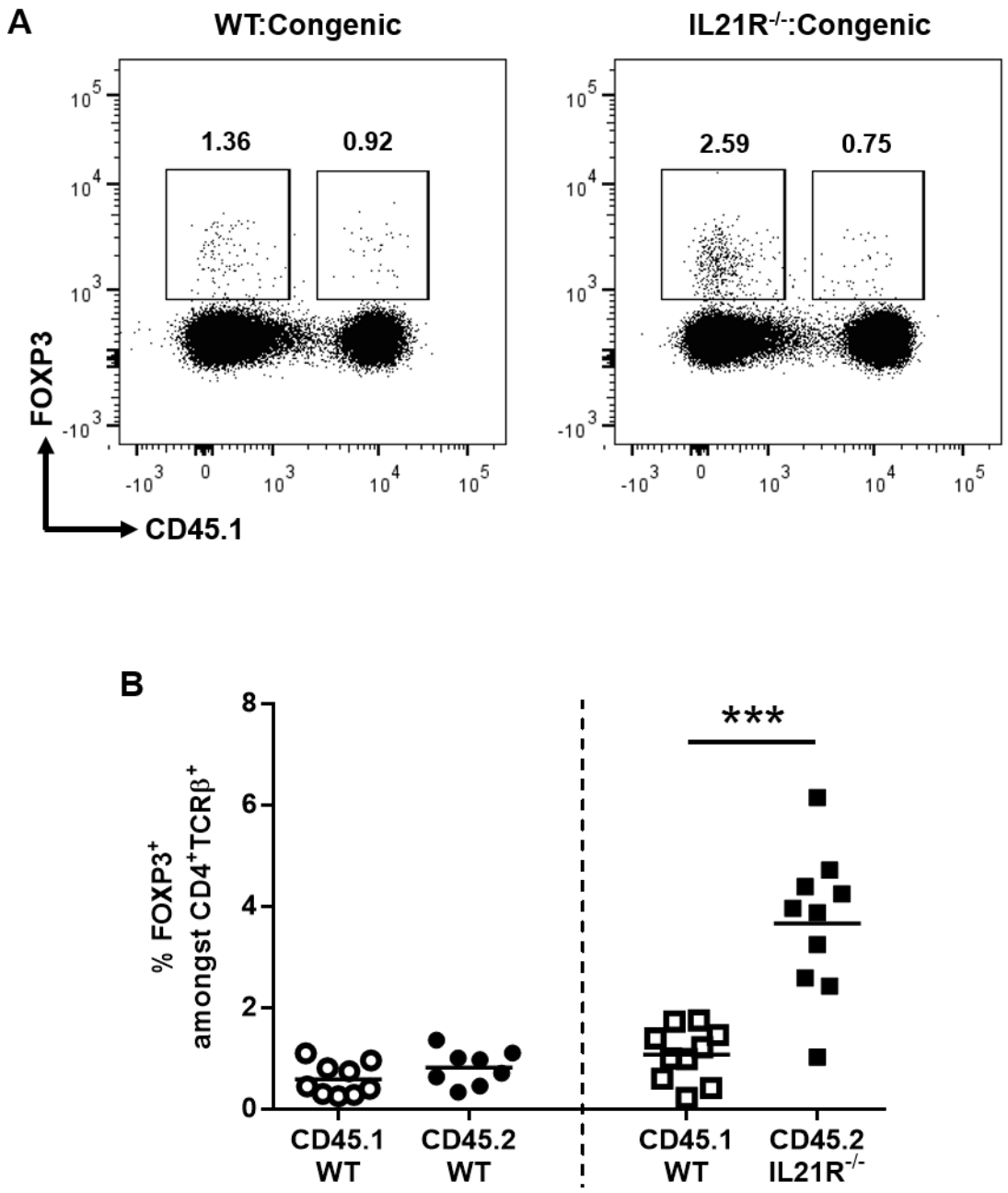

Figure 5.10 IL-21 inhibits colonic Foxp3$^+$ iTreg generation through a cell intrinsic mechanism

C57BL/6.Rag1$^{-/-}$ mice were transferred with 5x10^5 CD4+CD25-CD45RBhi cells from a 1:1 mixture of cells from CD45.1+WT (congenic) and either CD45.2+WT or CD45.2+IL-21R$^{-/-}$ donors. Mice were monitored and sacrificed upon development of disease in WT controls (6-8 weeks). Colonic cells were isolated and restimulated in vitro with PMA/ionomycin for 4 hours in the presence of Brefeldin A. **(A)** Representative intracellular Foxp3 staining of colonic cells gated on CD4+ live T-cells. **(B)** Frequencies of Foxp3+ cells amongst colonic T-cells. Results shown are pooled from two experiments with similar results (n=8 (CD45.1$^+$WT:CD45.2$^+$WT) and n=10 (CD45.1$^+$WT:CD45.2$^+$IL-21R$^{-/-}$)). Points indicate individual mice, bars show mean. Significance determined using Mann Whitney Test. ***p<0.001.

5.2.8 A molecular basis for the deviated phenotype of IL-21R$^{-/-}$ T-cells

IL-21 has been reported to influence effector and regulatory T-cell differentiation and function by promoting or inhibiting the activity of a number of key transcriptional regulators. Such findings have often indicated apparently contradictory effects on such genes, and suggest a highly context specific effect, presumably determined by additional factors including the nature of the T-cell receptor stimulus and the prevailing cytokine environment. We therefore sought to understand the molecular basis of the effects of IL-21 signals on the cell phenotypes we observed in the T-cell transfer model. Employing the congenic co-transfer system described above (Fig 5.8A), we used flow-cytometric cell sorting to isolate CD45.2^{+}IL-21R$^{-/-}$ and CD45.1^{+}WT cells from the inflamed colons of mice 5-6 weeks after initial transfer, and extracted RNA before analysing gene expression by quantitative RT-PCR.

Initially we validated this approach by examining expression of IL-21R and key genes known to be highly regulated by IL-21 in multiple experimental systems. Whereas IL-21R was highly expressed upon CD45.1^{+}WT CD4^{+} T-cells no expression was detected upon CD45.2^{+}IL-21R$^{-/-}$ cells (Fig 5.11A), confirming both that sustained expression occurs upon T-cells throughout the course of disease, and the absence of significant contamination from IL-21R sufficient cells within the sorted CD45.2^{+} fraction. Furthermore, as expected from published data,[289,673] IL-21R$^{-/-}$ T-cells demonstrated significantly reduced expression of *Prdm1* (encoding BLIMP1), and enhanced expression of the reciprocally regulated *Bcl6* gene, confirming the validity of this experimental approach (Fig 5.11B).

Next, we examined the expression of key Th1, Th2 and Th17 genes (Fig 5.11C). Confirming our inferences from the observations detailed above, IL-21R$^{-/-}$ T-cells were not impaired in their ability to express critical component of the Th17 program, including *Rorc*, *Il23r* and *Il1r*. Furthermore, expression of *Gata3, Il1rl1* (encoding the IL-33R) and *Maf*, implicated in Th2 and

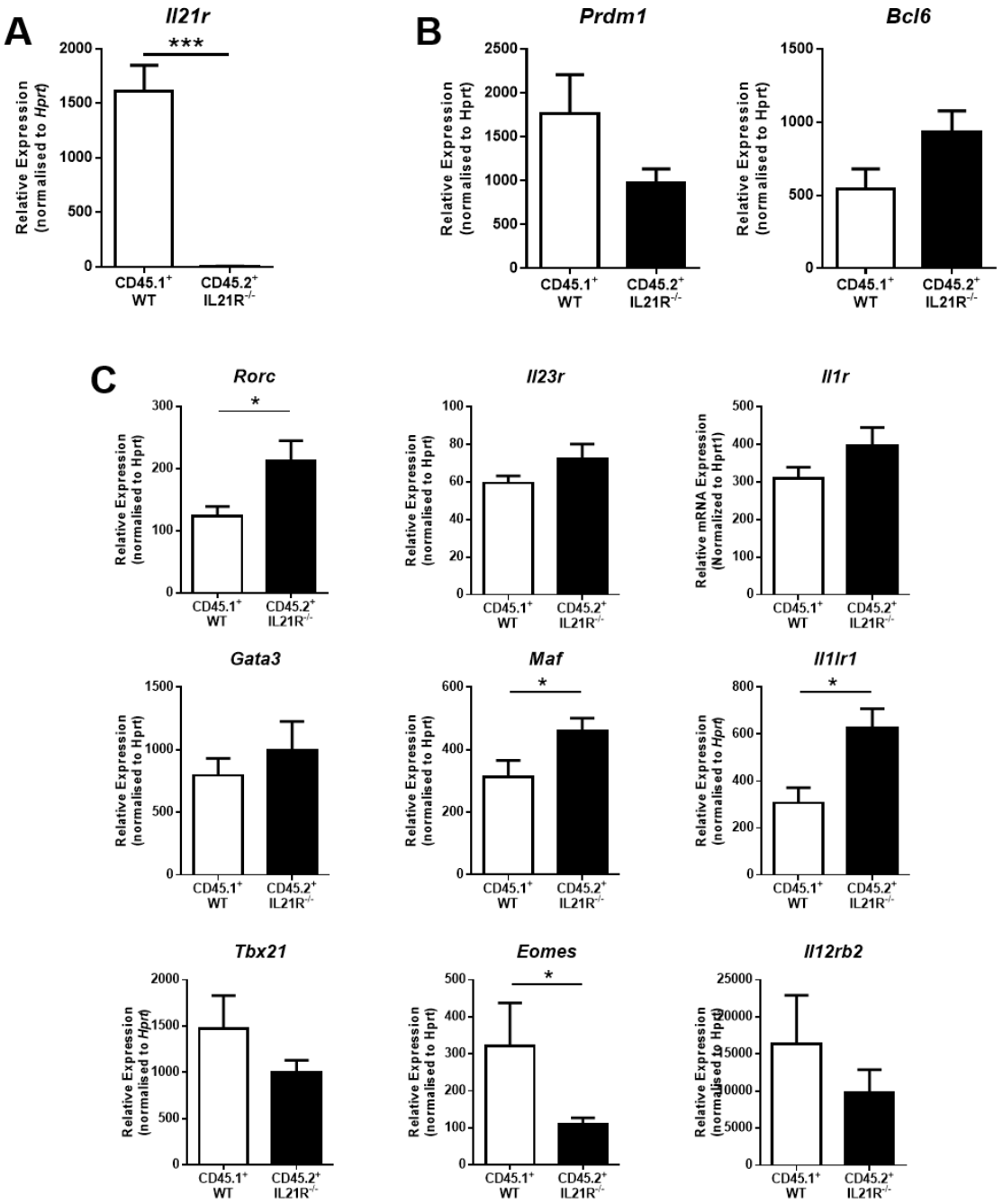

Figure 5.11 IL-21 regulates T-cell gene expression through cell intrinsic mechanisms

C57BL/6.Rag1$^{-/-}$ mice were transferred with 5x10^5 CD4+CD25-CD45RBhi cells from a 1:1 mixture of cells from CD45.1+WT (congenic) and either CD45.2+WT or CD45.2+IL-21R$^{-/-}$ donors. Mice were monitored and sacrificed upon development of disease (6-8 weeks). Colonic T-cells were isolated from recipients of WT:IL-21R$^{-/-}$ cells and sorted by flow cytometry on the basis of CD45.1 expression, and RNA extraction performed directly without further manipulation. **(A)-(C)** Expression of indicated genes determined by qPCR and normalised to *Hprt*. Results shown are from a single experiment (n=5 samples each genotype) Columns show mean +/- SD. Significance determined using Mann Whitney Test. *p<0.05, ***p<0.001.

Treg function were expressed at greater levels by IL-21R$^{-/-}$ cells. Finally, examining regulators of Th1 function and IFN-γ production, we noted reduction in both *Tbx21* (encoding T-bet) and to an even greater extent *Eomes* (coding for eomesodermin) within IL-21R$^{-/-}$ cells. There was a trend towards reduced expression of *Il12rb2*, encoding the IL-12R (Fig 5.11C).

These results conclusively demonstrate that IL-21 is not required for the activation of the Th17 programme in T-cells accumulating in intestinal inflammation, and confirm our above findings that the protective effect of IL-21R$^{-/-}$ is unrelated to the IL-23R/Th17 axis. In the context of colitis, IL-21 is required to provide direct T-cell signals to promote the Th1/IFN-γ molecular programme. Whether this is by direct activation, or through suppression of alternative cell fates requires further definition.

5.2.9 IL-21 signalling into T-cells drives intestinal chemokine expression

Since we observed significant differences in the intestinal chemokine profile in IL-21R$^{-/-}$ and wild-type mice infected with *H.hepaticus* and treated with anti-IL-10R antibody, we sought to assess whether a similar difference existed in T-cell transfer model. Analysed by qRT-PCR, we again noted reduced expression of *Ccl5* in whole colonic tissue (Fig 5.12A), however expression of other chemokines analysed was similar between the groups. Critically, innate immune, epithelial and stromal cells retained their IL-21R expression in this setting, showing that IL-21 signals into T-cells are required for optimal downstream *Ccl5* expression. The full cellular and molecular cascade mediating this will require further study to be fully defined, however it is interesting to note that using the co-transfer system we found T-cell derived *Ccl5* to be regulated by cell intrinsic IL-21 signals (Fig 5.12B).

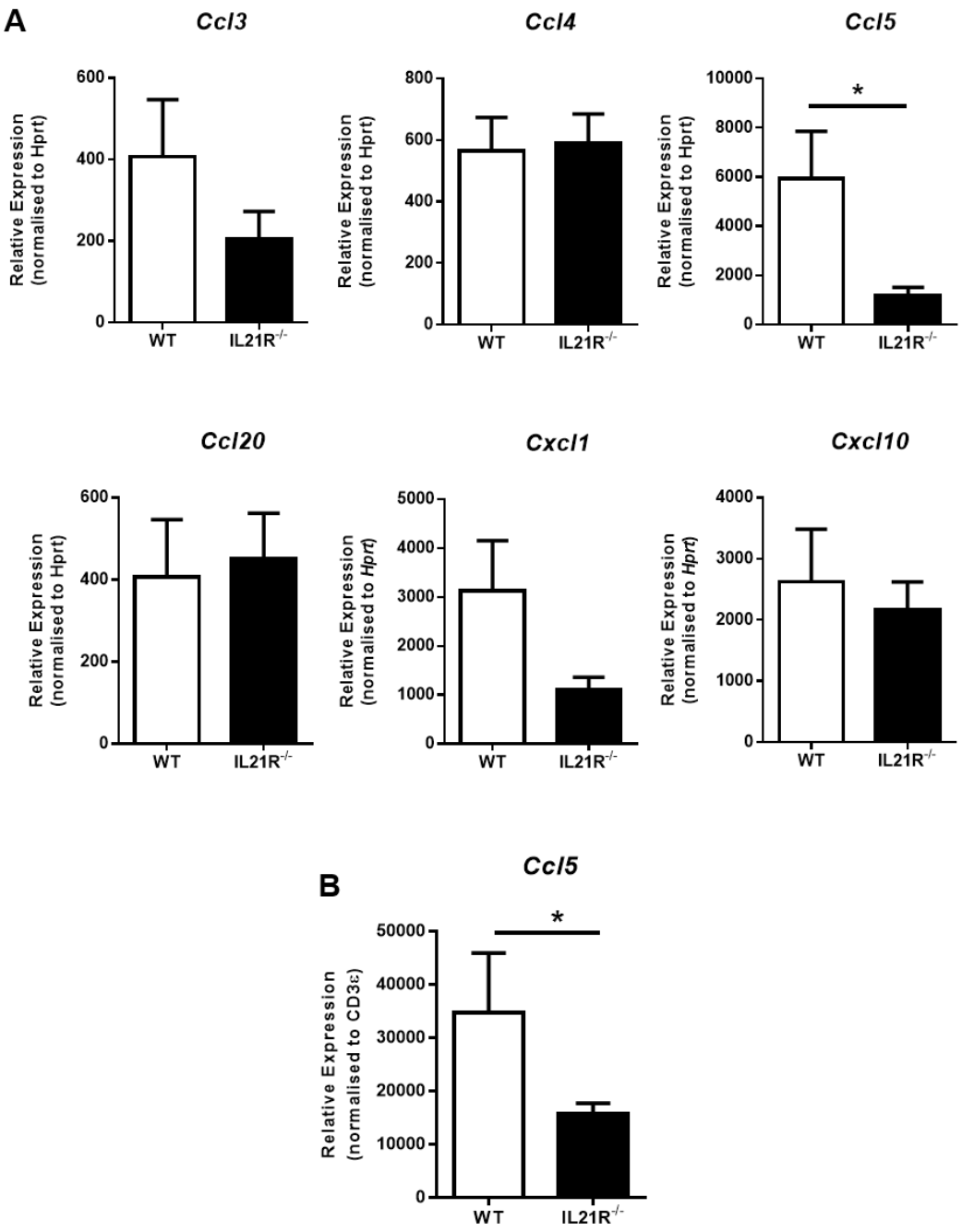

Figure 5.12 IL-21 regulates intestinal chemokine expression in T-cell transfer colitis
C57BL/6.Rag1$^{-/-}$ mice were transferred with 4x10^5 CD4+CD25-CD45RBhi cells from C57BL/6 WT or IL-21R$^{-/-}$ donors (A), or 5x10^5 cells from a 1:1 mixture of cells from CD45.1+WT (congenic) and CD45.2+IL-21R$^{-/-}$ donors (B). Mice were monitored and sacrificed upon development of disease (6-8 weeks). Section of proximal, middle, and distal colon were collected for RNA extraction (A), or colonic T-cells were isolated from recipients of co-transferred WT:IL-21R$^{-/-}$ cells and sorted by flow cytometry on the basis of CD45.1 expression, with RNA extraction performed directly without further manipulation. **(A)** Expression of indicated chemokine genes in whole colonic tissue, determined by qPCR and normalised to *Hprt*. **(B)** Expression of *Ccl5* in sorted colonic T-cells from co-transfer experiments, determined by qPCR and normalised to *Hprt*. Results shown are from a single experiment (n=6 samples each genotype in B, n=5 samples each genotype in A) Columns show mean +/- SD. Significance determined using Mann Whitney Test. *p<0.05.

5.3 Discussion

IL-21 has been proposed as a potential therapeutic target in a range of chronic inflammatory conditions, including IBD. However, limited understanding of its role in mucosal immunity exists, and is largely derived from models of acute colitis. We therefore sought to investigate the role of IL-21 signalling in chronic T-cell dependent models of disease, which recapitulate many immunological features of human IBD.

These studies revealed important information regarding the function of IL-21 in intestinal immunology, particularly as it relates to $CD4^+$ T-cell biology. We demonstrate that intestinal IL-21 expression is up-regulated in chronic inflammation, and that T-cell expression of IL-21R is sustained during active disease. Further, we show that IL-21 signalling into T-cells contributes to the development of chronic colitis, through both cell intrinsic and extrinsic mechanisms. Profound effects on the accumulating T-cell phenotype were noted in both models, however whereas expansion of $Foxp3^+$ Treg cells was a consistent effect of IL-21R deficiency, the T-cell effector subsets affected differed between models. We found that IL-21 is able to influence Th17, Th1/Th17 and Th1 subsets in particular settings. The effects of IL-21 on T-cell phenotype reflected cell intrinsic activity, however in contrast to *in vitro* studies we found IL-21 signals into T-cells were not required for expression of key Th17 molecules. Finally, we show a conserved effect between models on the production of chemokines, notably *Ccl5*, including via a T-cell intrinsic effect.

The confirmation of ongoing expression of IL-21 and T-cell expression of IL-21 receptor in chronic intestinal inflammation is not unexpected. Elevation of IL-21 expression has been noted in previous studies of the chronic DSS model, and in T-cell transfer disease.[609,612] However, sustained expression of IL-21R on T-cells has not previously been established, and is particularly interesting when contrasted with the down-regulation of IL-6R observed in established disease

(Sofia Buonocore, unpublished data). Many of the effects of IL-6 and IL-21 on lymphocyte biology seemingly overlap, and significant interest has focussed upon defining their shared and individual functions.[175,177,277,656] IL-21R is not expressed on naïve T-cells in mice, in contrast to IL-6R, and several lines of evidence support a model whereby IL-6 provides initial signals which establish IL-21 production, which thereafter mediates a number of effects previously ascribed directly to IL-6.[175,177,277,656] The relevance of such a mechanism in chronic colitis is not known, and cannot be fully discerned from our data; detailed studies of T-cell activation and function at the earliest stages of disease would be needed, combined with an approach initiating IL-6 and/or IL-21 blockade at different time points in the development of disease, or using mice with inducible ablation of IL-21 signalling pathways. However, this data does support an ongoing pathogenic role for the IL-21/IL21R axis in chronic colitis. Importantly, further support is provided by previous studies reporting efficacy of anti-IL-21 therapy in treating established T-cell transfer disease.[861]

IL-6 and IL-21 both signal via STAT3, previously shown to be essential for intestinal and systemic disease in murine colitis,[862] although the critical activation signals by anatomical location and stage of disease remain undefined. Although IL-6 signals may be provided *in trans* by the soluble IL-6R to cells expressing gp130,[863] our results suggest that IL-21 might be providing STAT3 activating signals to T-cells in established disease. Interestingly, IL-6 is redundant in the *H.hepaticus*/anti-IL10R model, whereas T-cell transfer disease is significantly reduced by IL-6 blockade (Sofia Buonocore, unpublished observation). Despite both models being T-cell dependent models of disease, we similarly noted IL-21R deficiency provided only modest protection from *H.hepaticus*/anti-IL10R disease, whereas the reduction in T-cell transfer disease was more striking. Importantly, IL-21 may exert effects through driving IL-10 production,[265,864] and the necessary blockade of IL-10 signals required for this model might mask such pathways downstream of IL-21 and attenuate the benefit of IL-21R ablation on pro-inflammatory effector function.

The T-cell transfer model allowed us to confirm that IL-21 signalling into $CD4^+$ T-cells is necessary for chronic intestinal inflammation. Despite being widely reported as a T-cell expressed receptor this was an important finding, since expression upon a range of lymphoid cells, as well as myeloid, stromal and epithelial cells has been reported to exert important functional effects in other settings. Murine epithelial cell expression of IL-21R is controversial,[865] and myeloid cell expression incompletely characterised, however the significantly attenuated disease noted with the transfer of IL-$21R^{-/-}$ cells into $Rag^{-/-}$ mice, in which IL-21R pathways in non-T-cells are functional demonstrates such expression is insufficient to drive colitis in this setting. Importantly, because IL-21 is subject to autocrine regulation,[176,654] IL-$21R^{-/-}$ T-cells produce lower levels of IL-21 itself, and it is possible the protected phenotype we observed reflects a requirement for T-cells as amplifiers for aspects of IL-21 driven immunity. The restored accumulation of IL-$21R^{-/-}$ T-cells and disease severity in the co-transfer setting would support this. More definitively, this could be addressed by crossing the IL-$21R^{-/-}$ allele onto the $Rag^{-/-}$ background to establish hosts lacking innate IL-21R expression, then transferring wild-type naïve T-cells; if IL-21 dependent non-T-cell responses downstream of T-cells are functionally important, attenuation of disease would be expected. An alternative approach would be to transfer IL-$21R^{-/-}$ T-cells into a $Rag^{-/-}$ animal and administer exogenous IL-21or induce over-expression of IL-21 by hydrodynamic injection, to test whether an IL-21 signals into non-T-cells only is sufficient to cause intestinal inflammation.

Broadly, the composition of the inflammatory infiltrate is determined by factors including cell proliferation and survival, and by signals such as chemokines and the expression of adhesion molecules and their ligands. It is therefore notable that we observed effects of IL-21R deficiency on chemokine expression within the inflamed mucosa. In the *H.hepaticus*/anti-IL-10R model, reduced expression of *Ccl20* and *Ccl5* was noted, whereas in the T-cell transfer model only the latter was significantly reduced in the absence of IL-21 signalling. IL-21 has previously been shown to influence chemokine secretion, including CCL27 in the skin,[704,705] and CCL20 within

the inflamed intestine in Crohn's disease, reportedly through an effect on epithelial cells.[670] Importantly, levels of other chemokines analysed were unaffected, suggesting this to be a specific effect of IL-21. However, we were unable to determine the cellular origins, and since both Ccl20 and Ccl5 can be produced in an autocrine manner by T-cells,[866,867] the reduced levels might be reflecting rather than driving skewed T-cell accumulation. Indeed, Ccl20, as part of the Th17 transcriptional signature driven by STAT3 and RORγt, would be expected to be downstream of IL-21 signalling into such T-cells.[866] The significant reduction in colonic *Ccl5* in IL-21R$^{-/-}$ T-cell recipients may be of functional importance, as diminished Th1 responses were seen in this model, and T-cell responses to IL-21 appear necessary for optimum Ccl5 expression. Importantly, analysis of cells from the co-transfer system experiments showed T-cell production of this chemokine to be a cell autonomous effect of IL-21. The contribution of T-cell and non-T-cell sources of Ccl5 to disease are unknown, and analysis of the transcriptional program induced by IL-21 signals into additional cells such as APCs may be enlightening. Similarly, analysis of the effect of exogenous IL-21 administration on Ccl5 levels in Rag$^{-/-}$ mice, or in mice lacking expression of IL-21R only in the T-cell compartment could provide valuable insights as to whether this mechanism is contributing to disease.

Although we did not formally examine the effects of IL-21 on aspects of cellular proliferation and death, it is notable that whilst T-cell accumulation in the intestine was attenuated in mice transferred with IL-21R$^{-/-}$ naïve T-cells, the number of such cells in the spleen and MLN was similar to wild-type levels. This demonstrates that IL-21 signals are dispensable for general reconstitution of the T-cell compartment, and that IL-21R$^{-/-}$ T-cells are not intrinsically impaired in their ability to proliferate. Furthermore, co-transfer experiments confirmed that IL-21R$^{-/-}$ T-cells display equivalent fitness to compete with wild-type cells in terms of proliferation and accumulation in the intestine, showing that IL-21 does not directly provide this signal, although it is required in a cell-extrinsic T-cell dependent manner. Examination of the effects of IL-21R deficiency on aspects of the cell cycles could be further probed using CFSE labelling of cells prior

to transfer, or immunohistochemical analysis of markers of cell turnover including Ki67, caspase activation, *Bim*/*Bid*/*Bcl-2* and TUNEL staining for apoptotic cells.

In both models of disease reported here, deficient IL-21 signals resulted in expanded Treg populations, shown using the T-cell co-transfer system to be a cell-intrinsic phenomenon. This effect of IL-21 has been previously demonstrated, although predominantly in studies performed *in vitro*, in which it acts to directly inhibit TGF-β induced Foxp3 expression.[611,710] More detailed dissection of the molecular basis of this inhibition is awaited, but pathways downstream of STAT3 (DNA methyltransferase 1) have been implicated in preventing Foxp3 up-regulation.[867,869] This is notable, since multiple STAT3 activating signals including IL-6, IL-23 and IL-27 are potentially available to the T-cells, and it is difficult to reconcile how loss of one such factor would be so critical. However, ablation of IL-23R or IL-27R results in similar expansion of $Foxp3^+$ Treg populations.[183,630] Notably, IL-27 drives IL-21 production from $CD4^+$ T-cells, suggesting that deficient downstream IL-21 signals could mediate this effect in $IL\text{-}27R^{-/-}$ animals.[265,660] Paradoxically, IL-21 can also activate STAT5, which is crucial to Treg development and maintenance.[665,856] However, only deficiency of IL-2/IL-2Rα, but not other STAT5 activating cytokines such as IL-7 and IL-15 is individually sufficient to reduce Treg numbers, demonstrating significant redundancy exists for this function.[860] In addition, IL-21 can activate STAT1 and STAT4, therefore the balance of activation of multiple STAT proteins contributed by IL-21 may be a critical underlying determinant of its effects.[675]

An alternative explanation for this finding would be that $IL\text{-}21R^{-/-}$ T-cells are more able to respond to factors driving Treg accumulation or survival than wild-type cells. IL-2 and IL-15, with which IL-21R shares the common gamma chain (γ_c) subunit, are such factors positively regulating Treg accumulation and function.[860,871] Importantly, IL-21 has been shown to augment or inhibit IL-15 driven effects, confirming interaction of these cytokines signals.[649,674,675,690] It might even be hypothesised that in the absence of IL-21R the availability of the γ_c chain and its downstream signalling molecules may be increased for other γ_c containing cytokine receptors, including the

IL-2R, IL-7R and IL-15R complexes, increasing such signals into IL-21R$^{-/-}$ cells. Any such effect is unproven, but could be explored easily using an in vitro approach.

Although highly reproducible, Treg cell expansion in T-cell transfer disease was modest and insufficient to provide dominant suppressive effects. Indeed, the presence of IL-21R$^{-/-}$ T-cells did not have a noticeable effect upon the wild-type fraction of T-cells in co-transfer experiments, arguing against a functional role for Treg cell expansion in the protection afforded by IL-21R ablation. Similarly, the marked Treg cell expansion observed in IL-6 deficiency provides dominant suppression via T-cell derived IL-10 (Sofia Buonocore, unpublished observations), and although we did not measure T-cell specific IL-10, the similar total levels of this cytokine present in the colonic lamina propria of IL-21R$^{-/-}$ and wild-type cell recipients, further suggests this is unlikely to be a mechanism underlying protection.

In contrast, the reduced population of IFN-γ^{+} single-positive cells we observed in T-cell transfer colitis was associated with a highly significant reduction in total IFN-γ, which is known to be pathogenic in this model,[872] implicating this as an important mechanism for the protective effect of IL-21R deficiency. A cell-intrinsic effect, molecular analysis of cells sorted from the intestine in the co-transfer system confirmed reduced expression of transcriptional regulators of the Th1 programme, including T-bet and Eomesodermin in IL-21R$^{-/-}$ cells. Importantly, this defect could not be overcome by the presence of other STAT1, STAT4 and T-bet activating signals in the co-transfer system, demonstrating a critical role for IL-21. This contrasts with descriptions of specific inhibition of IFN-γ production by IL-21, via suppression of Eomes.[685] Other studies have reported IL-21 to be an important initiator of Th1 differentiation and function,[664,675] and in models of graft-versus-host disease, IL-21 deficiency is associated with reduced Th1 effector functions, most pronounced within the gastrointestinal tract.[710] However, in the *H.hepaticus*/anti-IL-10R model, no effect on Th1 cells was seen, and instead reduced proportions of IL-17A^{+}IFN-γ^{-} and IL-

$17A^{+}IFN\text{-}\gamma^{+}$ cells occurred, consistent with the description of a requirement for IL-21 in Th17 development.[176,177] In the steady state, IL-21 deficiency has been associated with reduced peripheral lymphoid tissue Th17 populations but increased proportions within the intestine, suggesting the requirement for IL-21 signals is site and context specific.[177,858] We confirmed that steady state intestinal Th17 populations were unaffected by IL-21R deficiency (data not shown), and that in the T-cell transfer model, expression of Th17-associated molecules was not impaired in $IL\text{-}21R^{-/-}$ cells. Therefore the reduced Th17 response in the *H.hepaticus*/anti-IL-10R model appears to reflect a specific requirement for IL-21 signals for accumulation of Th17 cells in the inflamed intestine, although the mechanism for this requires further definition.

These seemingly paradoxical findings likely reflect context specific effects for IL-21 related to the origins and development of T-cells contributing to each model. In contrast to the *H.hepaticus*/anti-IL-10R model, which develops in the setting of an intestine already populated by a physiological T-cell pool including Th1, Th17 and Treg cells, the T-cell transfer model requires massive proliferation and differentiation of naïve T-cells in the setting of a lymphopoenic environment, and subsequent homing to the intestine. Study of the effects of IL-21 on steady state intestinal T-cells, including Th17 and Th1 cells as opposed to cells developing purely in an inflammatory setting may provide important mechanistic insights in this regard.

The developmental origins of intestinal Th1 cells are incompletely described, but an important study using a fate-mapping approach in EAE recently reported that $IL\text{-}17A^{+}IFN\text{-}\gamma^{+}$ and $IL\text{-}17A^{-}$ $IFN\text{-}\gamma^{+}$ cells derived from $IL\text{-}17A^{+}$ cells in the inflammatory setting, through an IL-23-dependent T-bet mediated mechanism.[298] The relevance of this finding to intestinal T-cells awaits demonstration, although circumstantial evidence supports such a process in humans and mice.[183,301,302] The reduction in $IFN\text{-}\gamma^{+}$ cells in the absence of IL-21 signals in the T-cell transfer model raises the possibility that it might ordinarily contribute to this progression, possibly through effects on IL-23R or T-bet expression. However, we did not detect significant deficits in the expression of *Il23r* amongst $IL\text{-}21R^{-/-}$ T-cells. We cannot exclude the possibility that Th17 cells

developing in the absence of IL-21 in vivo are subtly different from wild-type cells in further functions that we did not assess, which could determine their ability to 'progress' to a Th1 phenotype. Interestingly, although there was no excess accumulation of IL-17A single-positive cells in the IL-21R$^{-/-}$ cell recipient mice, the co-transfer system did demonstrate a small but significant increase in IL-17A^{+} cells within the IL-21R$^{-/-}$ fraction, which could support the hypothesis that IL-21 allows IL-17A^{+} cells to acquire further effector functions, and extinguish IL-17 production. Full analysis of the transcriptional profiles of Th17 cells developing in vivo in the absence of IL-21 signals would be enlightening in this regard. Alternatively, isolation of cells from the co-transfer setting, followed by purification into IL-21R$^{-/-}$ and wild-type cells and further adoptive transfer into Rag$^{-/-}$ animals could reveal functional differences in the pathogenicity of cells developing in the absence of IL-21. However, the overall findings presented here do not suggest the paucity of IFN-γ^{+} cells in the absence of IL-21R expression is likely to reflect a major failure to progress through such a phenotypic transition.

In conclusion our studies reveal that IL-21 plays an essential but highly context dependent role in mediating chronic intestinal inflammation, exerting multiple effects on T-cells. A number of potential mechanisms for its pathogenic effects were identified, and further studies are required to define the specific contribution of each to disease. Importantly, whilst the studies presented here support the IL-21/IL-21R axis as a valid therapeutic target in IBD, future translational studies should be carefully designed to target therapy at those patient groups most likely to benefit, and to define the molecular characteristics underlying any biological effects seen.

Chapter 6. The Role of IL-21 in Host Defence Against Intestinal Bacterial Infection

6.1 Introduction

Our observations detailed in Chapter 5 demonstrate that IL-21 may mediate pathogenic immune and inflammatory responses in murine models of chronic colitis. Targeting of this axis may therefore hold promise as a potential novel approach to the treatment of IBD. However, observations from humans and murine studies in which biological pathways implicated in driving chronic inflammatory diseases have been therapeutically manipulated clearly demonstrate the essential nature of such mediators for host defence in specific contexts, with ablation or blockade resulting in increased susceptibility to infectious disease or malignancy.

This dichotomy has been well demonstrated in the intestine by studies of the IL-23/Th17 axis. Whereas IL-23p19 or IL-23R deficiency protects against T-cell transfer disease and *H.hepaticus* induced colitis,[183,508,859] such mice are highly susceptible to disease induced by infection with *C.rodentium*, developing overwhelming intestinal and systemic infection whereas wild-type mice develop a mild self-limiting colitis.[541,873] This protection is mediated by IL-22 production by innate lymphoid cells and $CD4^+$ T-cells, which acts to fortify the mucosal barrier and drive production of antimicrobial peptides.[69,541,545] Notably, beyond the initial week of infection, IL-22 may be dispensable for host protection.[545] In contrast, IL-22 demonstrates pathogenic effects in both a variant of T-cell transfer disease, and in disease induced by *H.hepaticus* infection.[603] Similarly, whereas IL-17 may be pathogenic in DSS and TNBS colitis,[596] it may be protective in the T-cell transfer model,[522] and in *C.rodentium* infection.[560] Mirroring these observations, IL-17 blockade appears ineffective in Crohn's disease, and may be required for host protection, with anti-IL17A therapy associated with increased rates of mucocutaneous fungal disease,[594] as is observed in patients with auto-antibodies against IL-17A, IL-17F or IL-22.[748,749] Importantly, in

models of lung inflammation, the effects of IL-22 are highly dependent upon the presence or absence of IL-17A, demonstrating the context specific nature any individual cytokine effects.[874]

In addition to the risk of increased host susceptibility to infection or malignancy, blockade of specific cytokines and molecules which act within highly regulated networks may result in skewing of the immune response, and exacerbate disease driven by alternative immunological mechanisms. In the setting of infection, inappropriate host immune responses drive excess tissue damage in a variety of natural and experimental viral, parasitic and bacterial diseases, often without clear effect on pathogen clearance.[394,395,875] In many settings, the failure to mount the appropriate initial immune response can lead to persistence of infection and damaging inflammation through mechanisms including reactive oxygen species generation and the ongoing elaboration of inflammatory mediators and cytokines. This concept has relevance for understanding the pathophysiology of IBD, since studies in patients with Crohn's disease demonstrate deficits in systemic and mucosal innate immune function, postulated to lead to an over-reactive adaptive response towards uncleared antigen, with consequent chronic tissue damage, similar to the mechanisms underlying intestinal disease in -chronic granulomatous disease.[842]

The role of IL-21 in host defence has so far received little scrutiny, and accurate prediction of its potential effects is difficult. The numerous and highly context dependent effects of IL-21 on various cellular and molecular mediators has been well demonstrated, and although many studies show IL-21 to be pro-inflammatory, a large body of literature supports a major role in controlling or suppressing immune responses, particularly in viral infection.[651,691,692] IL-21 may influence APC function,[668,694] which is critical for establishing appropriate initial immune tone, and drives IL-10 production and regulatory Tr1 cell function to limit effector cell function.[265,864] Later effects

including production of high-affinity class switched immunoglobulins might also be expected to influence the course of some infections and consequent host response.[656,676,695,696]

We therefore sought to better define the function of IL-21 in intestinal defence against bacterial infection. We chose the *C.rodentium* model, since it is a well-defined, reproducible system, in which any disease results not from invasive bacterial infection, but from the consequences of the interaction of the bacteria with multiple aspects of host innate and adaptive immunity.[532]

6.2 Results

6.2.1 IL-21 is required for a normal host response to *Citrobacter rodentium*

Initially, we sought to understand whether IL-21 signals were involved in the host response to intestinal infection with *C.rodentium*. In this model, wild-type C57BL/6 mice develop a transient, self-limiting mild typhlitis and distal colitis, with minimal systemic dissemination of bacteria, and no significant weight loss.[532] We therefore infected IL-21R$^{-/-}$ and WT mice with 1-2x10^{9} CFU *C.rodentium* orally and assessed weights daily (Fig 6.1). These experiments revealed a striking phenotype in IL-21R$^{-/-}$ animals, with severe wasting developing during the second week of infection, with mean weight loss of up to 20%, compared to minimal loss in WT controls (Fig 6.1A). Furthermore, a mortality of ~30% occurred in IL-21R$^{-/-}$ animals, versus 100% survival in WT mice (Fig 6.1B). Analysis of mice sacrificed at intermediate time points revealed marked splenomegally developing in IL-21R-/- animals, from as early as 7 days post-infection (Fig 6.1C).

In view of the role of IL-21 in driving IL-23R expression, and published observations of high mortality rates and severe wasting in *C.rodentium* infected mice lacking IL-23p19 or IL-23R,[541,866] we next sought to understand whether the phenotype we observed might simply reflect

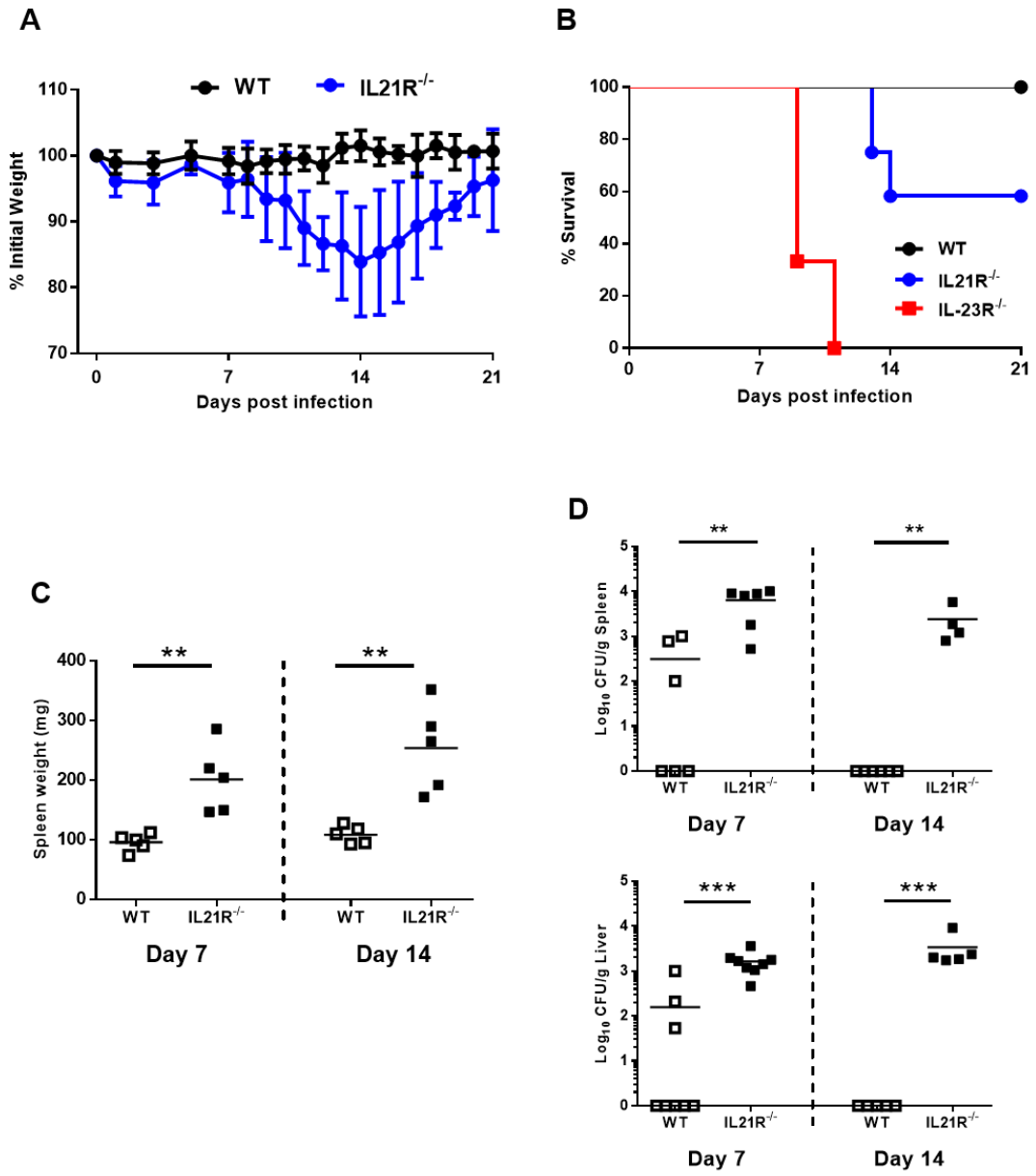

Figure 6.1 IL-21 is required for host defence in *C.rodentium* infection

C57BL/6 wild-type, IL-21R$^{-/-}$ and IL-23R$^{-/-}$ mice were infected with ~10^{9} CFU *C.rodentium* by oral gavage. Mice were monitored and sacrificed at the indicated time points for analysis. Organs were harvested and colonisation determined by serial dilution plating. **(A)** Weight curves **(B)** Survival curves. Note: mice losing >20% initial body weight were euthanised as a humane end point **(C)** Spleen weights at indicated time points **(D)** Systemic colonisation in spleen (top) and liver (bottom) at indicated time points. Data from single experiment representative of results from >5 experiments (IL-23R$^{-/-}$ data in B representative of 2 experiments). (n=10 each genotype). Points show mean +/-SD (A) or individual mice where bars indicate mean (C&D). Significance determined by Mann Whitney Test. **p<0.01, ***p<0.001.

impairment of this critical downstream axis. However, when we repeated these experiments including IL-23R$^{-/-}$ controls, we observed IL-21R$^{-/-}$ mice did not phenocopy IL-23R$^{-/-}$ animals in either the timing or degree of weight loss, nor mortality (Fig 6.1B and data not shown), suggesting that the observed effect of IL-21R deficiency does not reflect simple impairment of IL-23R expression or global failure of the IL-23/Th17-axis.

We next investigated the basis of the wasting phenotype we observed in IL-21R$^{-/-}$ mice. A number of immunodeficient mouse strains are reported to develop a similar phenotype due to uncontrolled systemic dissemination of *C.rodentium*.[556,562] We therefore sacrificed WT and IL-21R$^{-/-}$ animals at 7 or 14 days post-infection, to assess the kinetics of any such dissemination. Remarkably, whereas viable bacteria were rarely recovered from the spleen or liver of WT mice, IL-21R$^{-/-}$ animals demonstrated high systemic bacterial loads, present from 7 days post-infection, and sustained at 14 days (Fig 6.1D). These results therefore suggest that IL-21 is critically required for prevention or control of systemic dissemination of *C.rodentium* infection from the intestine.

6.2.2 IL-21R deficiency results in severe colitis in *Citrobacter rodentium* infection

Although bacterial dissemination resulting in wasting and mortality is a feature of *C.rodentium* infection in a number of genetically modified mouse strains, the development of intestinal inflammation is more variable. We therefore examined intestinal segments from IL-21R$^{-/-}$ and WT mice sacrificed at 14 days post-infection, reported to be the peak of disease in C57BL/6 mice, for histological evidence of inflammation (Fig 6.2A&B). This revealed severe inflammation of the caecum and distal colon of IL-21R$^{-/-}$ mice, in contrast to the mild disease seen in WT animals. However, the distribution of disease, which predominantly affects the caecum and distal colon with relative sparing of small intestinal and intervening colonic segments in WT animals, was similar in IL-21R$^{-/-}$ mice (data not shown).

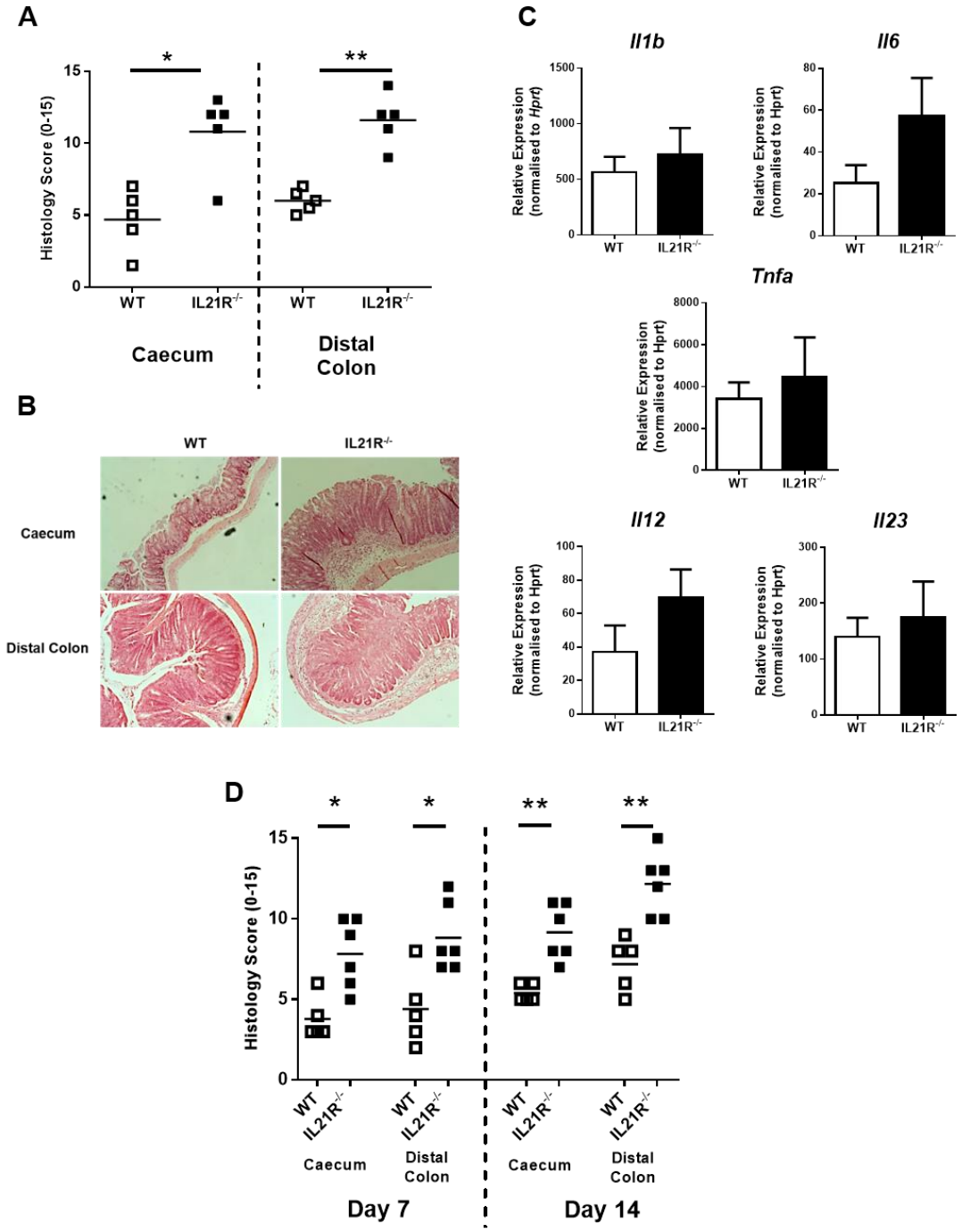

Figure 6.2 IL-21R deficiency results in severe colitis in *C.rodentium* infection

C57BL/6 wild-type (WT) or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage. Mice were monitored and sacrificed at day 14 or other indicated time points for analysis. Tissue was harvested for histological analysis and segments of caecum and distal colon removed for RNA extraction. **(A)** Colitis scores (0-15) in caecum and distal colon. **(B)** Representative photomicrographs of distal colonic and caecal histology. **(C)** Expression of indicated genes in pooled distal colonic and caecal tissue, determined by qPCR and normalised to *Hprt*. **(D)** Colitis scores (0-15) in indicated intestinal segment at indicated time point. Data from single experiments representative of results from >3 experiments. (n=5 each genotype at each time point - (A&D), n=6 each genotype - (C)). Points show individual mice where bar indicates mean (A&D). Columns indicate mean +/-SD. Significance determined by Mann Whitney Test. *p<0.05, **p<0.01.

The more severe typhlitis and distal colitis seen in IL-21R$^{-/-}$ mice was associated with increased expression of a number of pro-inflammatory cytokine genes in these segments, including IL-1β, IL-6, IL-12, IL-23, and TNF-α (Fig 6.2C).

Next, we asked whether intestinal inflammation developed with differing kinetics in IL-21R$^{-/-}$ mice compared to WT controls (Fig 6.2D). Indeed, in contrast to WT mice, in which histological markers of intestinal inflammation were minimal prior to day 14, severe inflammation was already present in both the caecum and distal colon at day 7 post-infection, although further increases in severity at both sites occurred by day 14.

6.2.3 IL-21 signals are required for early control of intestinal *C.rodentium* infection

Noting the more severe colitis which characterised infection in IL-21R$^{-/-}$ mice, we next sought to understand whether this reflected altered bacterial colonisation levels in the intestine, or whether increased inflammation was occurring despite equivalent bacterial burdens. We therefore studied the kinetics of bacterial colonisation following oral gavage with *C.rodentium*. Repeated experiments demonstrated that whilst tissue colonisation kinetics in IL-21R$^{-/-}$ progressed similarly to WT controls (initial colonisation of the caecum, then later distal parts of the colon, with levels peaking at day 8-10), intestinal segments from IL-21R$^{-/-}$ mice harboured significantly increased colonisation at all time points (Fig 6.3A&B). This increased colonisation was apparent from as early as 3 days post-infection, and persisted throughout the course of infection, with delayed clearance occurring in IL-21R$^{-/-}$ mice. Ultimately all IL-21R$^{-/-}$ mice were able to clear infection within 3 weeks, a similar time frame to that seen in WT animals (Fig 6.3C). Importantly, these differences in colonisation were present in groups of mice co-housed for up to 12 weeks prior to experimentation, reducing the likelihood that observed differences were simply reflective of variations in the intestinal flora between strains or cages.

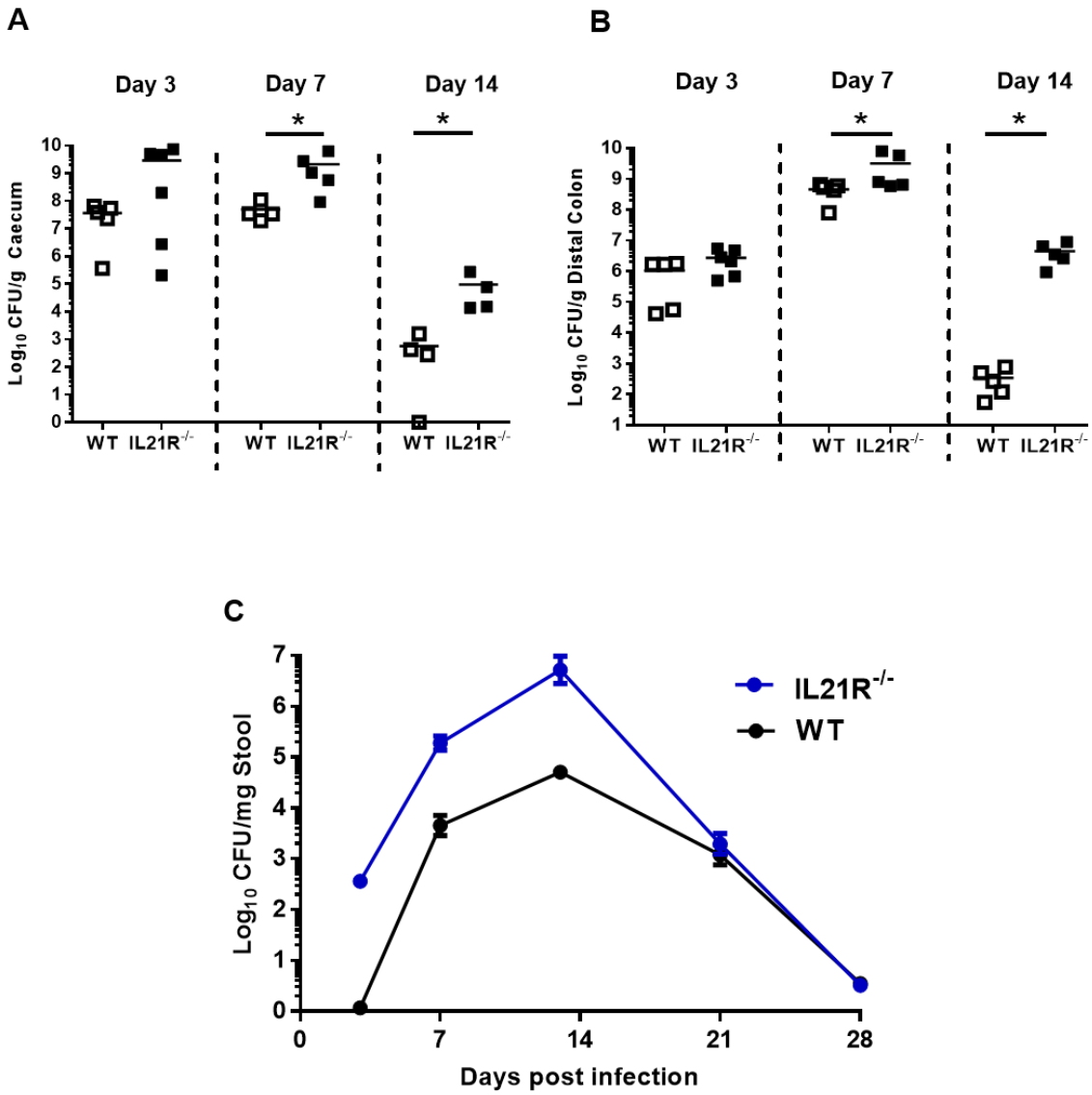

Figure 6.3 IL-21R$^{-/-}$ mice demonstrate greater intestinal bacterial burdens throughout *C.rodentium* infection

C57BL/6 wild-type (WT) or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage. Mice were monitored and stool collected for colonisation estimation as indicated, or sacrificed at the indicated time points for tissue analysis. *C.rodentium* colonisation levels were determined by serial dilution plating of tissue homogenates. **(A)** Caecal colonisation **(B)** Distal colon colonisation **(C)** Stool colonisation. Data from single experiments representative of results from 2 experiments. (n=5-6 each genotype at each time point (A-C)) Points show individual mice, bar indicates mean (A&B). Points represent mean +/-SD (C). Significance determined by Mann Whitney Test. *p<0.05.

In total, these experiments show that IL-21 driven signals are required for an appropriate early host response to intestinal *C.rodentium* infection, and that in its absence initial colonisation levels are higher, associated with the development of more rapid and severe intestinal inflammation and systemic infection.

6.2.4 Intestinal antimicrobial peptide responses are not impaired in the absence of IL-21R

The presence of significant colonisation differences so early after oral infection pointed to a requirement for IL-21 in innate immune responses in the intestine. Previous studies have shown early control of *C.rodentium* infection requires intact IL-23 driven, IL-22 dependent production of antimicrobial peptides (AMPs) including *Reg3β* and *Reg3γ*.[541] Since our previous studies suggested that IL-21 deficiency showed similarities to, but did not fully recapitulate features of IL23R deficiency, we questioned whether partial or selective impairment of the IL-23R/IL-22/AMP axis might be contributing to the phenotype we observed. However, qPCR assessment of mRNA for components of this pathway in colonic samples at the very earliest stages of disease revealed significant up-regulation of key molecules including *Il22*, *Reg3β* and *Reg3γ* in IL-21R$^{-/-}$ mice, confirming this aspect of anti-*C.rodentium* immunity was intact in the absence of a functional IL-21R (Fig 6.4).

Furthermore, although Reg3 family peptides are perhaps the best characterised AMPs for host defence in *C.rodentium* infection, additional types of AMPs have been shown to be required for bacterial control.[560] However, examination of the expression of a range of such peptides, including lipocalin, S100A8 and S100A9, and B-defensins did not reveal significant differences between wild-type and IL-21R$^{-/-}$ mice in the early stages of disease (Fig 6.4). Therefore, the phenotype in IL-21R$^{-/-}$ mice does not appear to be due to impairment of AMP expression.

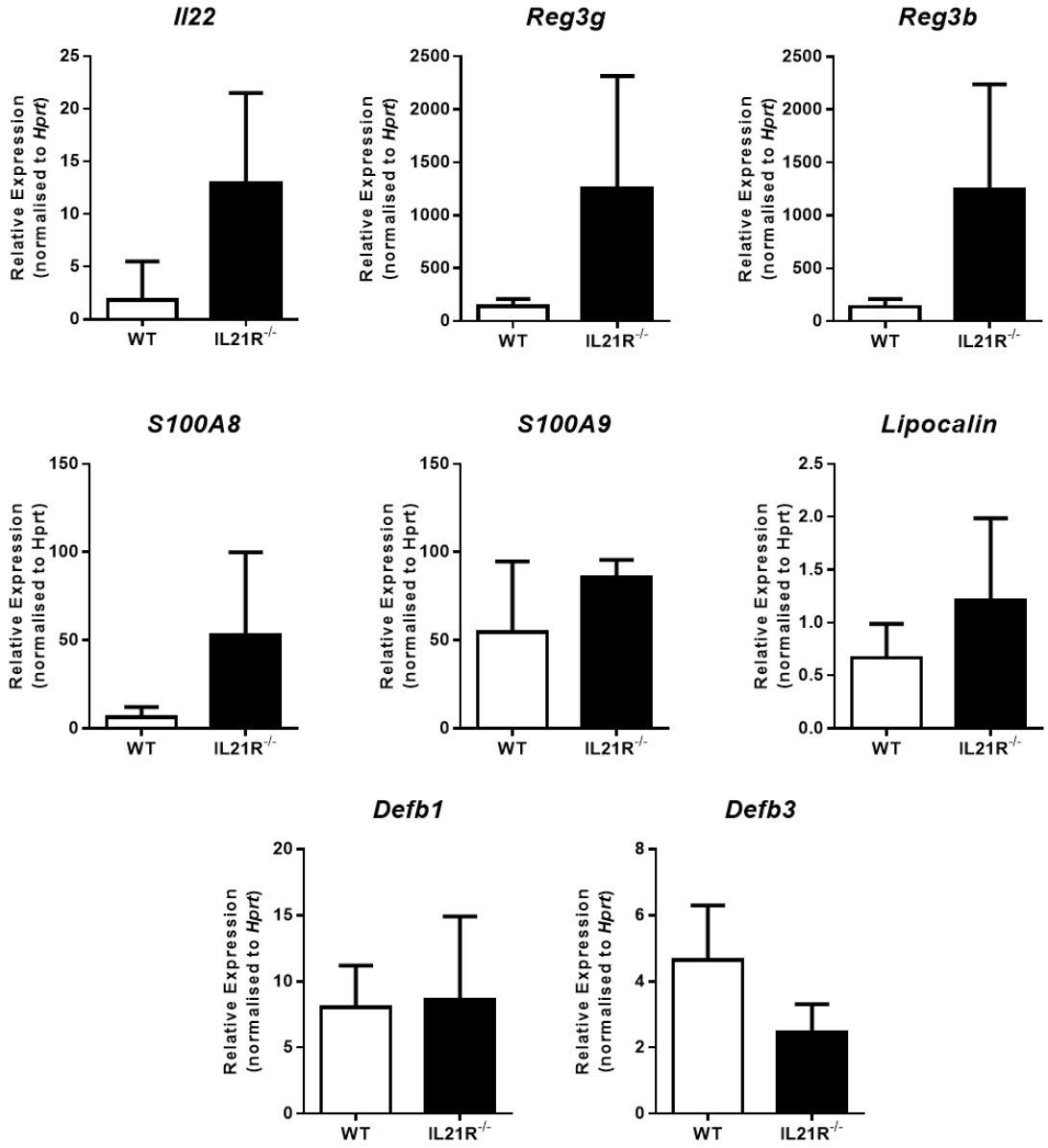

Figure 6.4 Unimpaired antimicrobial peptide responses in IL-21R$^{-/-}$ mice infected with *C.rodentium*

C57BL/6 wild-type (WT) or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage. Mice were sacrificed 3 days after infection, and caecal tissue collected for RNA extraction. Gene expression was determined by qPCR and normalised to *Hprt*. Data pooled from 2 experiments. (n=7 each genotype). Bars show mean +/-SD.

6.2.5 IL-21 is redundant for steady state intestinal IgA production

Secretory IgA provides an important defence against enteric bacteria, including *C.rodentium*, and may influence the intestinal flora, itself a determinant of the success of *C.rodentium* colonisation. Although IgA deficiency has been reported to result in paradoxically reduced early colonisation levels, no overall effect was observed from J-chain or pIgR deficiency (resulting in reduced mucosal IgM and IgA levels),[561] and supra-physiological IgA levels have been suggested to impart protection from *C.rodentium* infection.[539] Importantly, these results reflect experimental approaches utilising mice on mixed or poorly defined genetic backgrounds, and in facilities of uncertain microbiological status. Since IL-21 exerts profound effects on B-cells, including upon IgA,[876,877] we tested whether differences in intestinal IgA might underlie our observations.

However, total faecal IgA content, previously shown to closely correlate with levels in the colonic mucosa,[878] was equivalent in un-manipulated wild-type and IL-21R$^{-/-}$ mice, demonstrating no overt quantitative steady-state deficit in IgA existed (Fig 6.5A).

The generation of serum antibody against *C.rodentium* has been shown to be critical for clearance of infection, particularly IgG.[561,562] Since IL-21 has been shown to exert profound effects on B-cell function, we questioned whether any defect in immunoglobulin production might be contributing to the observed phenotype. However, with the exception of IgG1, the isotype most closely associated with IL-21 activity,[656,676] no defect in *C.rodentium* specific immunoglobulins was apparent at day 14 after infection in IL-21R$^{-/-}$ mice, indeed greater levels of IgG2b, IgG2c and IgG3 were observed (Fig 6.5B). The successful ultimate clearance of infection despite impaired IgG1 responses in IL-21R$^{-/-}$ mice also argues against an essential role for this isotype in host protection from *C.rodentium* infection.

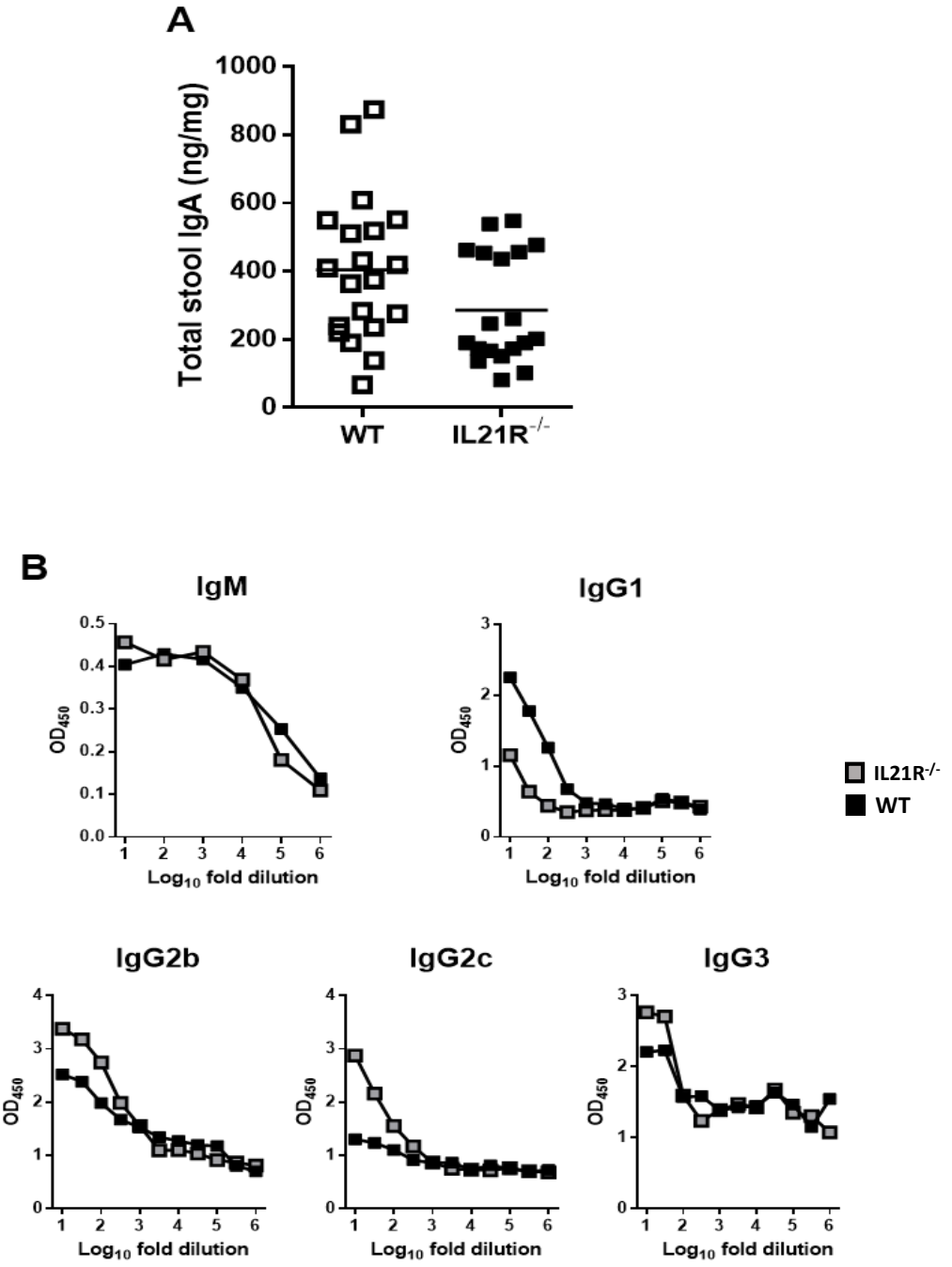

Figure 6.5 IL-21R$^{-/-}$ mice demonstrate quantitatively normal steady state IgA expression and develop *C.rodentium* specific serum antibodies

Stool was collected from co-housed age matched unmanipulated C57BL/6 and IL-21R$^{-/-}$ mice and homogenised before determination of total IgA levels by ELISA (A). C57BL/6 wild-type (WT) or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage, and sacrificed after 14 days. Serum was collected and *C.rodentium* specific immunoglobulin levels determined by ELISA (B). **(A)** Steady state total faecal IgA levels. **(B)** Serum *C.rodentium* specific immunoglobulins of indicated type. Data from single experiments. (n=20 each genotype (A), n=6 each genotype (B)). Points show individual mice, bar indicating mean (A) or points indicate mean (B).

6.2.6 IL-21 is not required for steady state IEL or lamina propria ILC or T-cell populations

Innate lymphoid cells including $CD4^+$LTi cells and $NKp46^+$ cells, and resident memory $CD4^+$ T-cells have all been reported to be required for appropriate host responses to *C.rodentium* infection.[69,72,545,548] Interestingly, IL-21 related cytokines including IL-2, IL-7 and IL-15 each exert profound effects upon the development and maintenance of these cell populations,[860] and whilst previous phenotyping studies of IL-$21R^{-/-}$ mice report intact thymic, splenic and peripheral lymphoid tissue leukocyte populations, effects on the mucosal immune system are minimally characterised.[649,676,858] We therefore speculated that the early defect in controlling *C.rodentium* might reflect absence or dysfunction of a T-cell or ILC subset in the steady state mucosa of IL-$21R^{-/-}$ mice.

The colonic IEL compartment was unaffected by IL-21R deficiency (Fig 6.6A), with similar populations of $CD8\alpha\alpha^+TCR\gamma\delta^+$, $CD8^-TCR\gamma\delta^+$, $CD8\alpha\alpha^+TCR\beta^+$ and $CD8\alpha\beta^+TCR\beta^+$ cells in both wild-type and IL-$21R^{-/-}$ animals (Fig 6.6A). Studies of colonic lamina propria cells revealed similar populations of $TCR\gamma\delta^+$, $CD4^+TCR\beta^+$ and $CD8^+TCR\beta^+$ subsets regardless of IL-21R status (Fig 6.6B). Upon ex vivo re-stimulation of steady-state colonic T-cells, similar proportions of $TCR\gamma\delta^+$ and $TCR\beta^+$ cells produced IL-17A or IFN-γ in IL-$21R^{-/-}$ and wild-type mice, demonstrating that IL-21 is dispensable for the development and function of the steady-state intestinal T-cell compartment (Fig 6.6C&D). Next, we analysed the innate lymphoid cell populations in the steady state colonic mucosa. Defining such cells as $CD45^+Lin(CD3\varepsilon CD45RCD11bCD11c)^-$ cells, we observed maintained frequencies of total ILCs, and proportions of the major subpopulations, defined by expression of CD4, NKp46, Thy1 and Sca-1 (Fig 6.7).

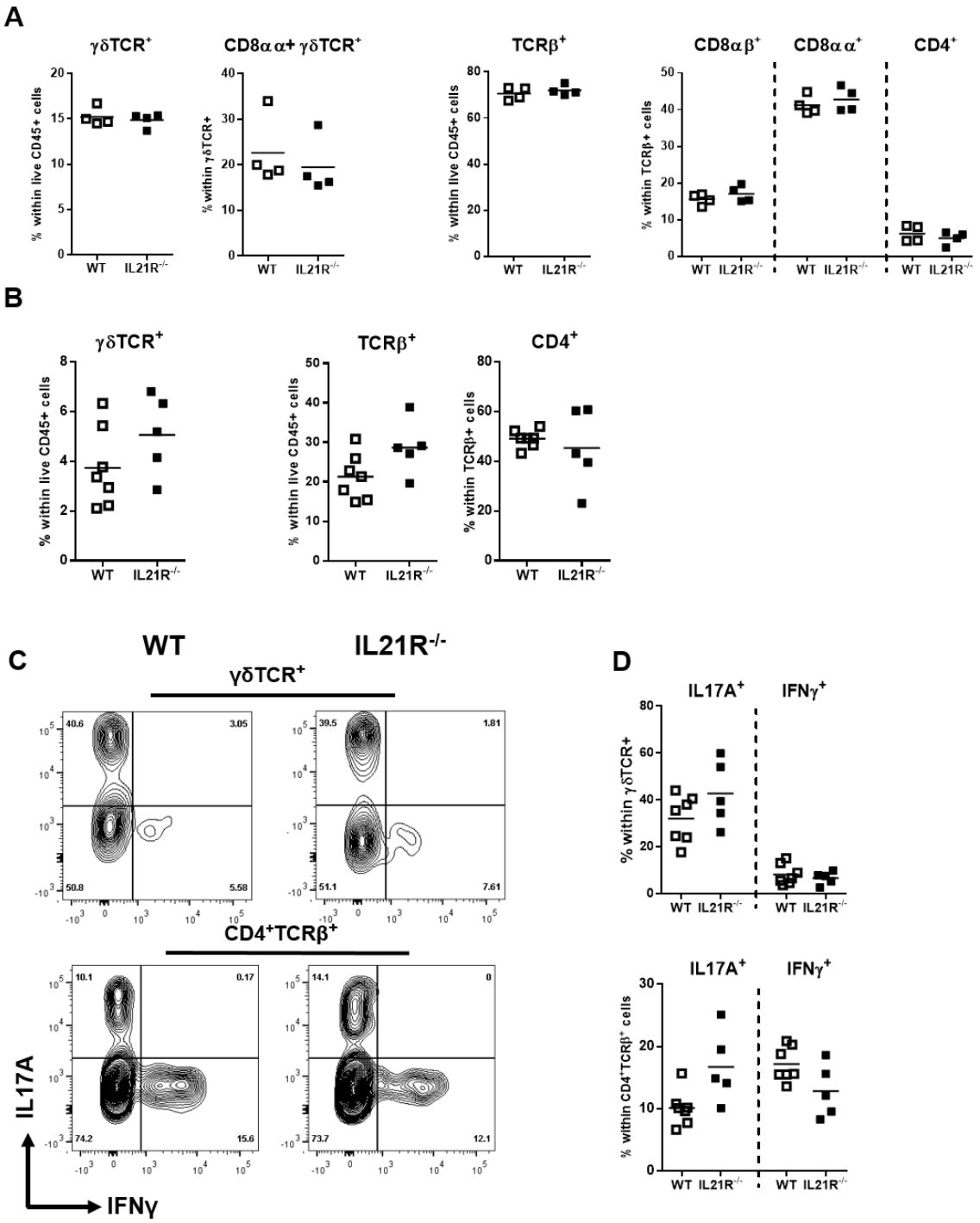

Figure 6.6 IL-21R$^{-/-}$ mice have normal colonic IEL and lamina propria T-cell populations
Co-housed unmanipulated C57BL/6 and IL-21R$^{-/-}$ mice were sacrificed at 8 weeks of age, and the colon harvested. IELs and lamina propria cells were isolated and analysed by flow cytometry (A&B), or restimulated in vitro with PMA/ionomycin for 4 hours in the presence of Brefeldin A prior to intracellular cytokine staining (C&D). Frequency of cells with indicated surface phenotype within colonic IEL **(A)**, or colonic lamina propria **(B)**. **(C)** Representative plots of intracellular staining, gated on TCRγδ+ (top) or CD4+TCRβ+ (bottom) cells **(D)** Frequency of cytokine producing cells within indicated gate in colonic lamina propria. Data from single experiment. (n=5 (IL-21R$^{-/-}$) or 7 (WT)). Points show individual mice, bar indicates mean. Statistical significance was assessed using Mann-Whitney Test, with no comparison yielding results with $p<0.05$.

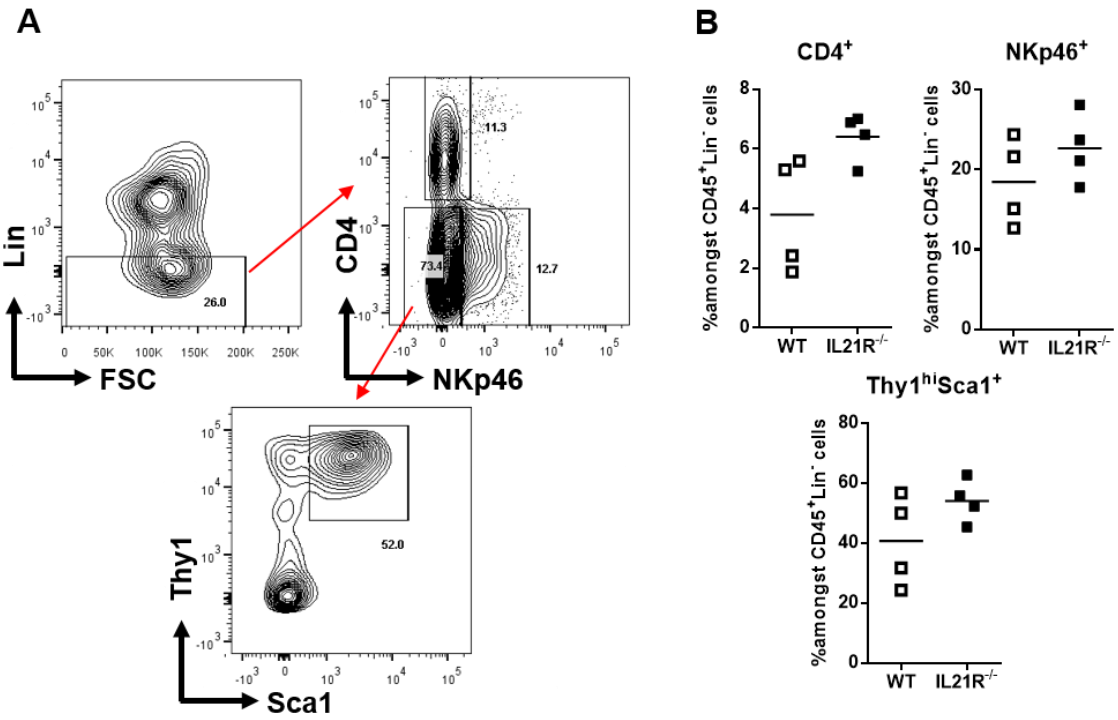

Figure 6.7 IL-21R$^{-/-}$ mice have maintained colonic lamina propria innate lymphoid cell populations

Co-housed unmanipulated C57BL/6 and IL-21R$^{-/-}$ mice were sacrificed at 8 weeks of age, and the colon harvested. Lamina propria cells were isolated and analysed by flow cytometry. **(A)** Gating strategy for identification of ILC populations **(B)** Frequency of cells with indicated surface phenotype amongst colonic Lin-CD45+ cells. Data from single experiment. (n=4 replicates of pools of 4 mice for each genotype). Points show individual pools with bar indicating mean. Statistical significance was assessed using Mann-Whitney Test, with no comparison yielding results with $p<0.05$.

In total, these studies do not suggest that the increased susceptibility of mice lacking IL-21 signals is related to a gross defect in the presence or function of IELs, mucosal ILCs or lamina propria resident T-cells prior to infection.

6.2.7 Innate cytokine production in the intestine in response to *C.rodentium* is unimpaired by IL-21R deficiency

Antigen presenting cells, fibroblasts and epithelial cells are all capable of generating cytokines and immune mediators upon interaction with bacteria, influencing the evolving immune and inflammatory response through effects on cellular recruitment, activation and polarisation. We therefore analysed the early expression of a range of cytokines and chemokines produced by innate immune cells at just 3 days post infection, the earliest point at which colonisation differences were noted, and importantly, before differences in histological markers of inflammation were apparent. At this point, tissue expression of mRNA for cytokines including IL-1β, IL-6, IL-12, IL-23p19, IL-27, and TNF-α, as well as myeloid cell chemoattractants including GM-CSF, IL-17A, CXCL1 and CXCL2 was consistently similar or greater in IL-21R$^{-/-}$ compared to wild-type animals (Fig 6.8). Similarly, iNOS expression was unimpaired in the absence of IL-21 (Fig 6.8). Therefore, the intestinal hypercolonisation and intestinal inflammation observed in the absence of IL-21 signalling does not appear to reflect a gross defect in innate immune sensing or cytokine production within the intestine.

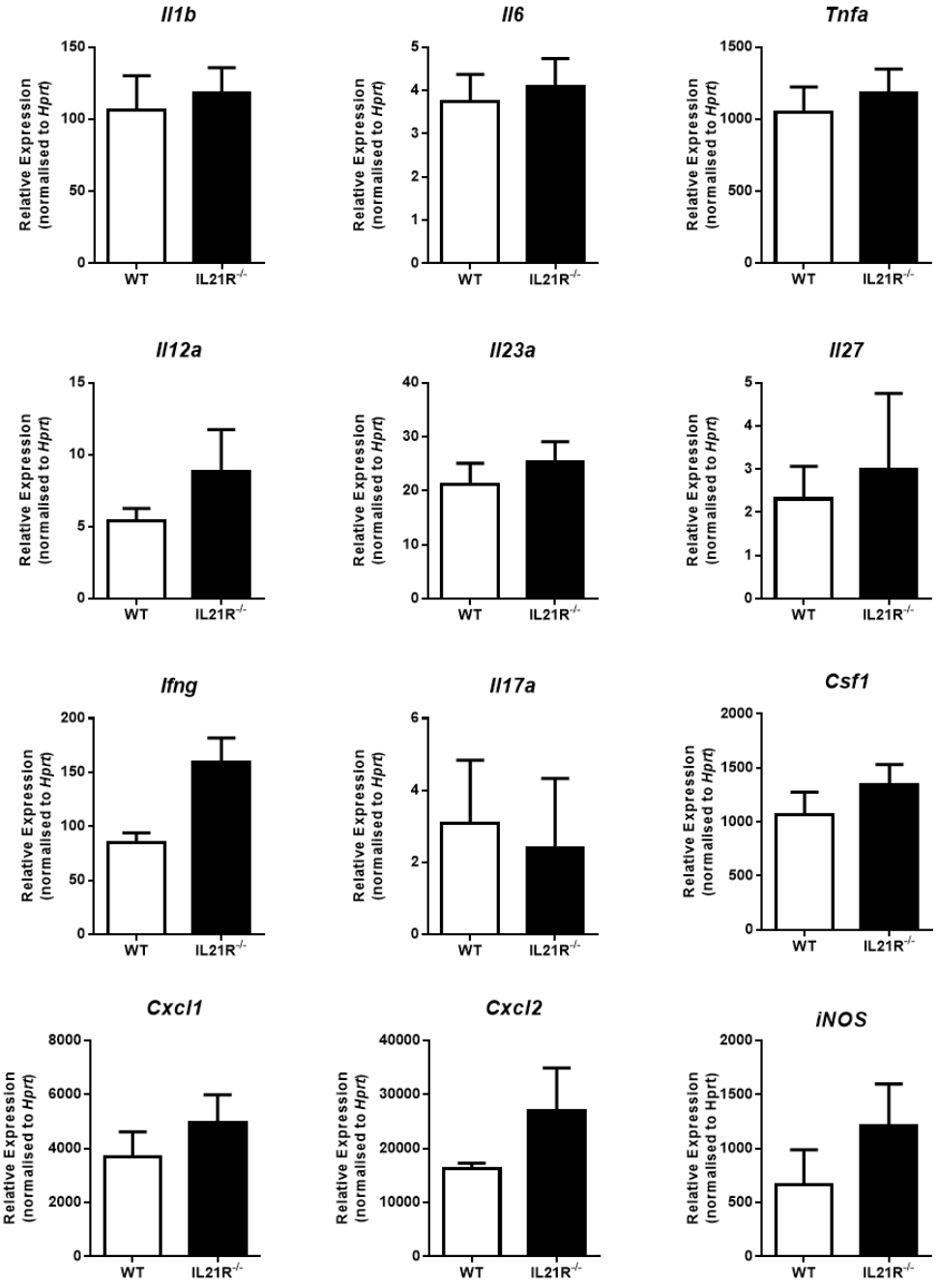

Figure 6.8 Unimpaired innate cytokine production in early *C.rodentium* infection in IL-21R$^{-/-}$ mice

C57BL/6 wild-type (WT) or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage. Mice were sacrificed 3 days after infection and caecal tissue collected for RNA extraction. Expression of indicated mRNA in caecal tissue, determined by qPCR and normalised to *Hprt*. Data from single experiment. (n=8 each genotype). Bars show mean +SD. Significance determined by Mann Whitney Test. *p<0.05.

6.2.8 Altered T-cell responses develop in the absence of IL-21 signals in *C.rodentium* infection

Adaptive CD4+ T-cell immune responses are critical for the control and eradication of C.rodentium infection, and mice deficient in all lymphocytes, αβT-cells or $CD4^+$ cells develop fatal overwhelming infection.[556,562] Mechanisms underlying this include a requirement for T-cell dependent antibody production,[556,562] and T-cell production of cytokines including IFN-γ, IL-17A, IL-17F and IL-22.[545,558,560] In chronic intestinal inflammation, we had observed that IL-21 exerted profound effects on $CD4^+$ T-cell responses, including Th1 or Th17 subsets, therefore we examined the influence of IL-21 on the T-cell infiltrate in *C.rodentium* infection.

Re-stimulation of colonic lamina propria lymphocytes from wild-type mice infected with *C.rodentium* revealed accumulating populations at the peak of disease were strongly polarised towards a Th17 phenotype, with a smaller population of IFN-γ^+ Th1 cells (Fig 6.9A&B). In marked contrast, IFN-γ^+ Th1 cells predominated in IL-$21R^{-/-}$ mice (Fig 6.9B). In addition, a greater proportion of lamina propria CD4+ T-cells from IL-$21R^{-/-}$ mice were positive for additional cytokines including GM-CSF and TNF-α (Fig 6.9D). Since the specific roles of single T-cell cytokines such as IFN-γ and IL-17A in C.rodentium infection remain unsettled, and the difference between a protective and pathogenic T-cell response may relate to the production of additional cytokines by the cell, we questioned what effect IL-21 deficiency might be having on the further mediators these accumulating cells might be making. This analysis revealed that the reduction of IL-$17A^+$ T-cells observed in IL-$21R^{-/-}$ mice was primarily reflective of a reduction in cells which co-expressed IL17F, with approximately 50% of IL-$17A^+$ cells also IL-$17F^+$ in wild-type animals, compared to less than 30% in IL-$21R^{-/-}$ cells (Fig 6.9B&C). However, the proportion of IFN-γ^+IL-$17A^+$ cells was similar (Fig 6.9B). These results suggest that the colonic T-cell population induced by *C.rodentium* infection differs significantly in the absence of IL-21 signals, in terms of both the predominant major subset, and the expression of additional cytokines.

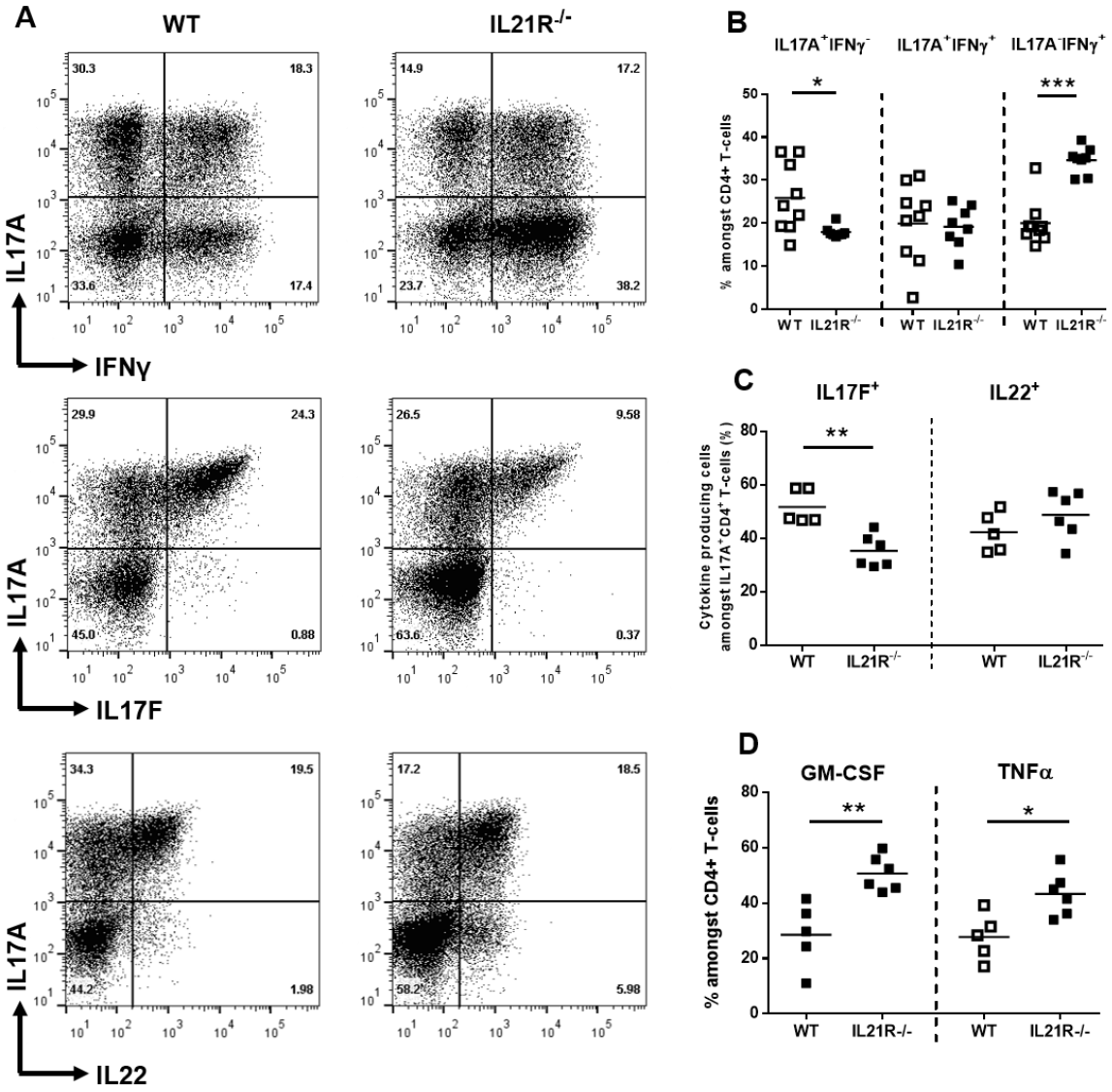

Figure 6.9 IL-21R deficiency results in altered colonic effector T-cell populations in *C.rodentium* infection

C57BL/6 wild-type or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage, and sacrificed after 14 days. Colonic lamina propria cells were isolated and restimulated in vitro with PMA/ionomycin for 4 hours in the presence of Brefeldin A prior to surface and intracellular cytokine staining. **(A)** Representative intracellular staining, gated on colonic live CD4+ T-cells. **(B)** Frequency of indicated cytokine producing phenotype within colonic CD4+ T-cells. **(C)** Frequency of IL-17F or IL-22 producing cells amongst IL-17A+ colonic CD4+ T-cells. **(D)** Frequency of GM-CSF or TNFα producing cells amongst colonic CD4+ T-cells. Data from one experiment representative of results of independent 3 experiments. (n=5-9 each genotype). Points show individual mice, bar indicating mean (B-D). Statistical significance determined by Mann Whitney Test *p<0.05, **p<0.01, ***p<0.001

6.2.9 Skewed T-cell subsets in IL-21R$^{-/-}$ mice are not secondary to bacterial load nor T-cell intrinsic IL-21 signals

In chronic models of colitis, we observed a T-cell intrinsic effect of IL-21 on the accumulating intestinal T-cell phenotype. However, in the *C.rodentium* model, we considered that the differing T-cell phenotype might be arising from the marked colonisation differences between wild-type and IL-21R$^{-/-}$ animals. To explore this possibility, we constructed chimeras by reconstituting lethally irradiated *Rag*$^{-/-}$ mice with a 1:1 mix of bone marrow from CD45.2^{+}IL-21R$^{-/-}$ or CD45.2^{+}IL21R$^{+/+}$ wild-type mice, and from CD45.1^{+}IL-21R$^{+/+}$ congenic wild-type animals (Fig 6.10A). Animals were left to reconstitute for 12 weeks, followed by oral infection with *C.rodentium*. Analysis of the origins of the reconstituted haematopoetic compartment based upon expression of CD45.1 and CD45.2 at this point revealed no impairment of the fitness of IL-21R$^{-/-}$ cells to compete with wild-type bone marrow (Fig 6.10B).

Next, we analysed the behaviour of IL-21R deficient and sufficient cells exposed to the same level of bacterial colonisation and prevailing cytokine environment (Fig 6.10C). These studies showed that the proportion of colonic lamina propria T-cells demonstrating IFN-γ^{+}, IL-17A^{+} or IFNγ^{+}IL17A^{+} phenotypes was similar within both CD45.1^{+}IL-21R sufficient cells and CD45.2^{+}IL-21R$^{-/-}$ cells isolated from the same mouse, demonstrating that the differences in these markers previously noted in IL-21R$^{-/-}$ mice in this model are not due to a T-cell intrinsic effect of IL-21 signalling (Fig 6.10C).

Surprisingly, weight loss at day 14 after infection was significantly greater in mice transferred with the mixture of CD45.2^{+}IL-21R$^{-/-}$ and congenic cells than in those receiving CD45.2^{+} wild-type and congenic cells (Fig 6.11A). Although spleen weights were similar between both groups, systemic dissemination of viable bacteria was more commonly observed amongst recipients of IL-21R$^{-/-}$ containing bone marrow, although this showed marked variation between animals (Fig

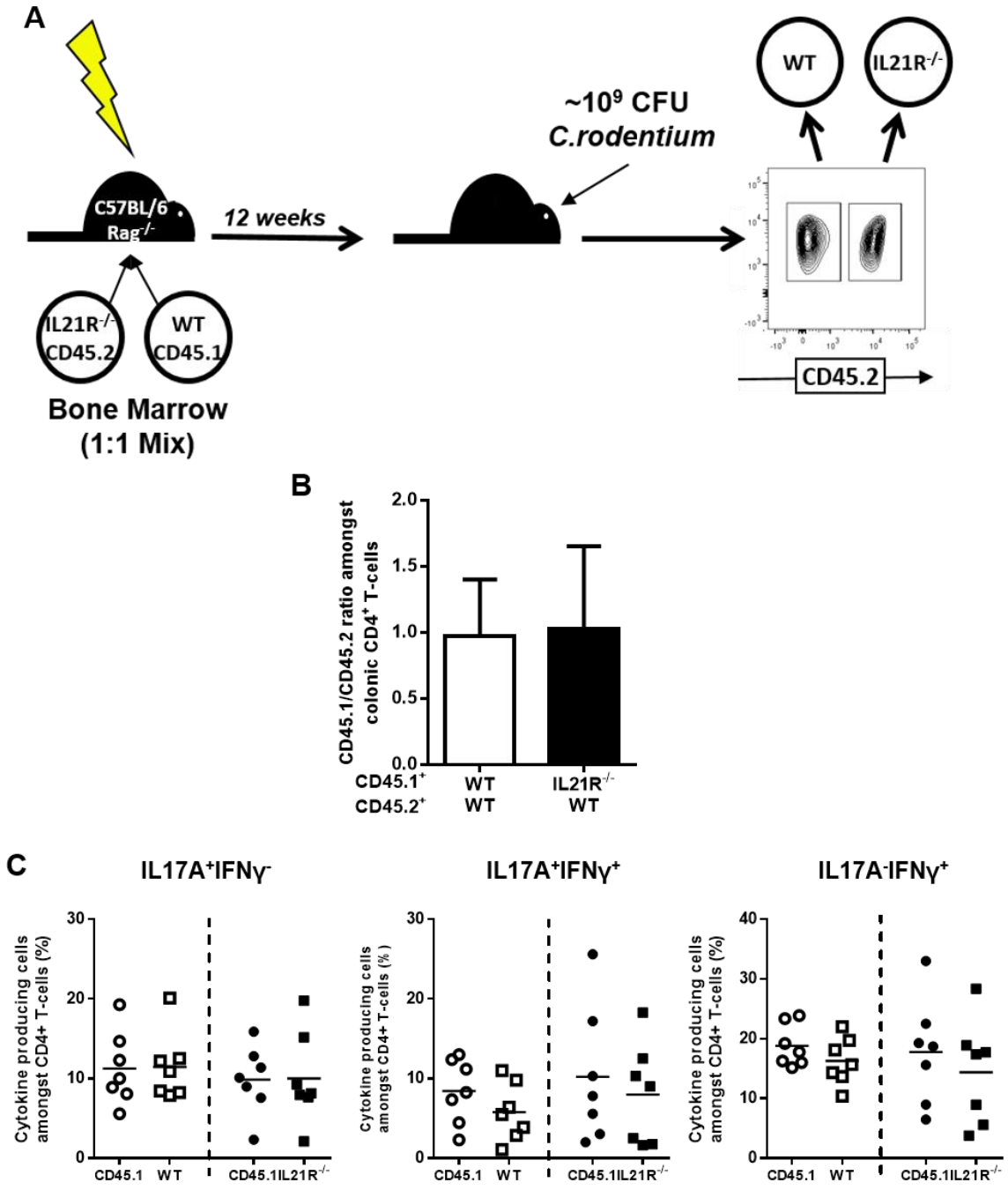

Figure 6.10 Altered effector T-cell phenotype in *C.rodentium* infection in IL-21R deficiency is not a cell intrinsic effect

Mixed bone marrow chimeras were constructed by lethal irradiation of C57BL/6.Rag$^{-/-}$ mice followed by iv injection of a 2x10^{6} cells from a 1:1 mixture of CD45.1+WT and either CD45.2+WT or CD45.2+IL-21R$^{-/-}$ bone marrow. After reconstitution, mice were infected with ~10^{9} CFU *C.rodentium* by oral gavage, and sacrificed 14 days after infection. Colonic lamina propria cells were isolated and restimulated in vitro with PMA/ionomycin for 4 hours in the presence of Brefeldin A prior to surface and intracellular cytokine staining. **(A)** Experimental schema **(B)** Ratio of CD45.1:CD45.2 cells amongst colonic CD4+ T-cells. **(C)** Frequency of cells exhibiting indicated cytokine profile amongst colonic CD4+ T-cells, according to bone marrow genotype. Data from single experiment, (n=7 recipients of each genotype). Bars show mean +SD (B). Points show individual mice with bar indicating mean (C). Statistical significance determined by Mann Whitney Test, with no comparison yielding $p<0.05$.

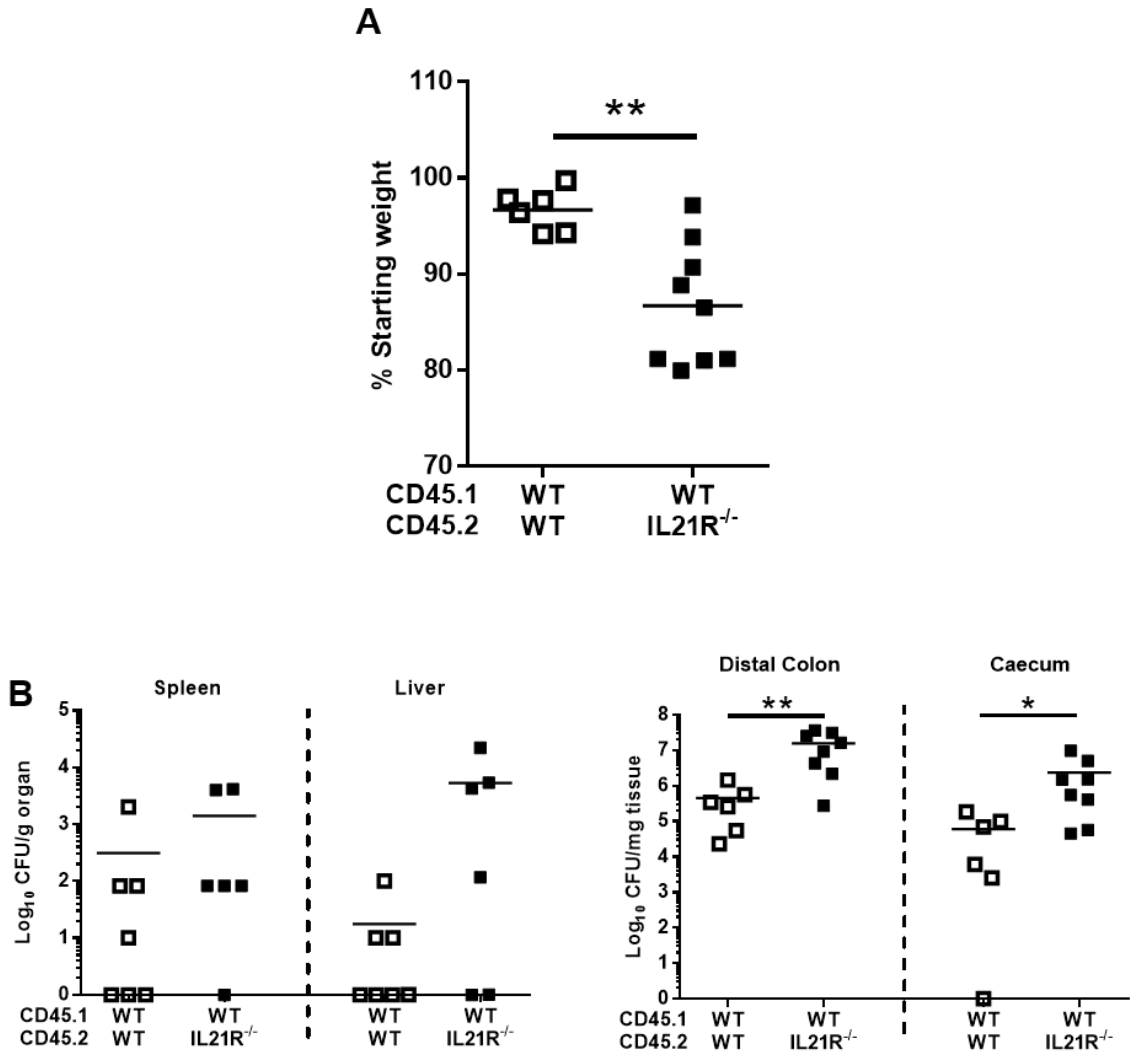

Figure 6.11 Increased bacterial burdens are evident in chimeras reconstituted with IL-21R$^{-/-}$ containing bone marrow

Mixed bone marrow chimeras were constructed by lethal irradiation of C57BL/6.Rag$^{-/-}$ mice followed by iv injection of a 2x10^{6} cells from a 1:1 mixture of CD45.1+WT and either CD45.2+WT or CD45.2+IL-21R$^{-/-}$ bone marrow. After reconstitution, mice were infected with ~10^{9} CFU *C.rodentium* by oral gavage, and sacrificed 14 days after infection. Tissue samples were harvested for estimation of *C.rodentium* burden by serial dilution plating of homogenates. **(A)** Body weight at 14 days relative to pre-infection (day 0) weight. **(B)** *C.rodentium* burden in indicated tissues by genotype of bone marrow received. Data from single experiment, (n=6-8 recipients of each genotype). Points show individual mice with bar indicating mean. Statistical significance determined by Mann Whitney Test. *p<0.05, **p<0.01

6.11B). However, intestinal colonisation was significantly greater in both caecum and distal colon in mice reconstituted with IL-21R$^{-/-}$:congenic bone marrow compared to mice receiving entirely wild-type cells (Fig 6.11B). Importantly, despite the noted differences in intestinal colonisation, T-cell responses were similar between mice receiving CD45.2^{+} wild-type containing bone marrow and CD45.2^{+}IL-21R$^{-/-}$ recipients (Fig 6.10C).

Although preliminary and in need of confirmation, these results suggest that whereas the presence of IL-21R$^{-/-}$ cells of haematopoetic origin exerts a dominant effect which facilitates increased levels of intestinal colonisation by *C.rodentium*, in the presence of IL-21 responsive haematopoetic cells the T-cell phenotype is not determined by the colonisation level.

6.2.10 IFN-γ drives exacerbated intestinal and systemic disease in IL-21R$^{-/-}$ mice infected with *C.rodentium*

The role of IFN-γ in *C.rodentium* infection is uncertain, with conflicting reports of protective or pathogenic roles.[543,556-559] We were therefore interested to understand the significance of the increased Th1 response we had observed in IL-21R$^{-/-}$ mice. Previous studies outside of the intestine have shown that IFN-γ induces epithelial hyperplasia,[705] which if occurring in the gut might facilitate increased colonisation by C.rodentium. However the role of IFN-γ beyond an effect on initial colonisation is unclear. We hypothesised that the severe colitis occurring in IL-21R$^{-/-}$ mice might reflect damaging immunopathology due to an over-exuberant IFN-γ driven Th1 response, developing due to the increased bacterial load in these animals.

We therefore infected IL-21R$^{-/-}$ and WT mice with *C.rodentium* and monitored their intestinal bacterial burden by daily stool collection. At day 5, as expected, all IL-21R$^{-/-}$ mice showed around 2 log higher stool burdens of *C.rodentium* (Fig 6.13A). From this point, mice were treated with either blocking anti-IFN-γ antibody or an irrelevant isotype control, with body weight and stool CFU monitored daily. Strikingly, IL-21R$^{-/-}$ mice treated with anti-IFN-γ had an attenuated wasting

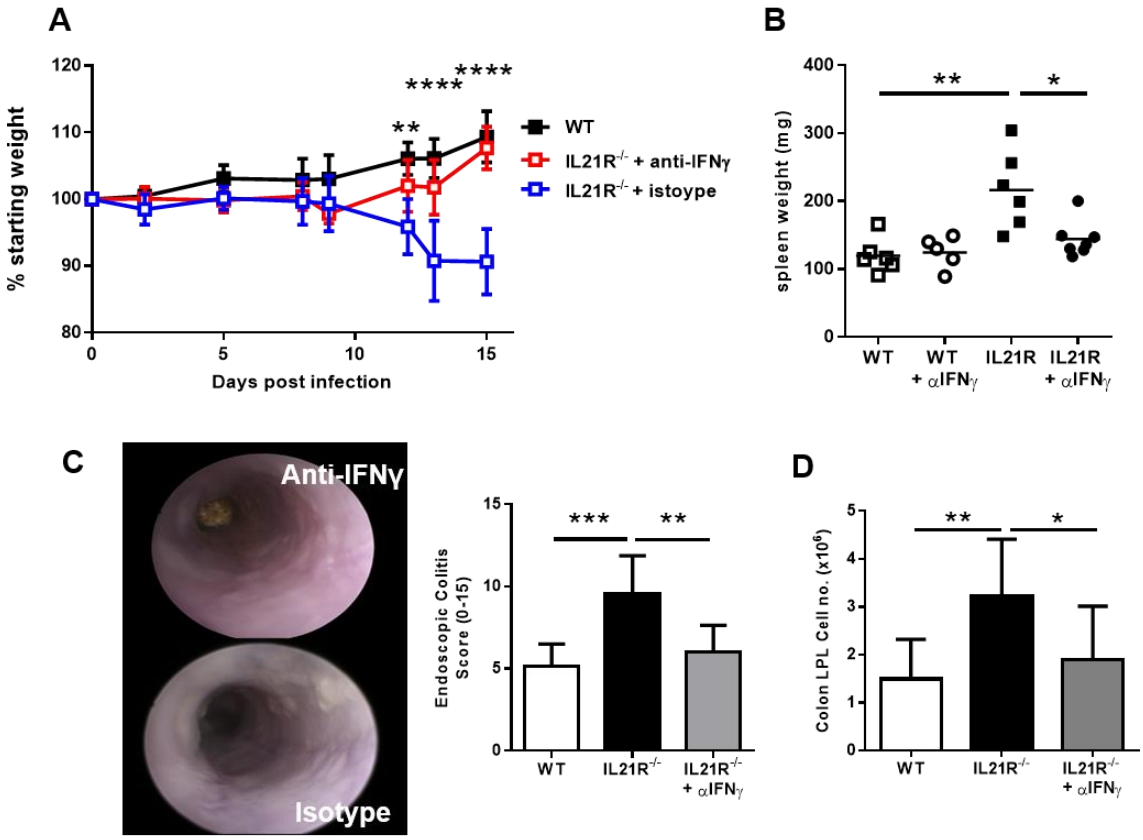

Figure 6.12 IFN-γ drives exacerbated intestinal and systemic disease in IL-21R$^{-/-}$ mice infected with *C.rodentium*

C57BL/6 wild-type or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage. Groups of mice of each genotype were treated with anti-IFN-γ antibody or isotype control *i.p.* 3x/week. Mice were monitored and sacrificed at day 14, and colonic disease assessed by endoscopic examination, prior to harvesting of organs and isolation of cells. **(A)** Weight curves. **(B)** Spleen weights.**(C)** Representative endoscopic images and endoscopic colitis severity scores (0-15) **(D)** Colonic lamina propria total cell counts. Data from single experiment representative of results from 2 experiments. (n=5-7 each genotype/treatment group). Points show mean +/-SD (A) or individual mice (B). Bars indicate mean +SD (C&D). Significance determined by ANOVA (A) or Mann Whitney Test (B-D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

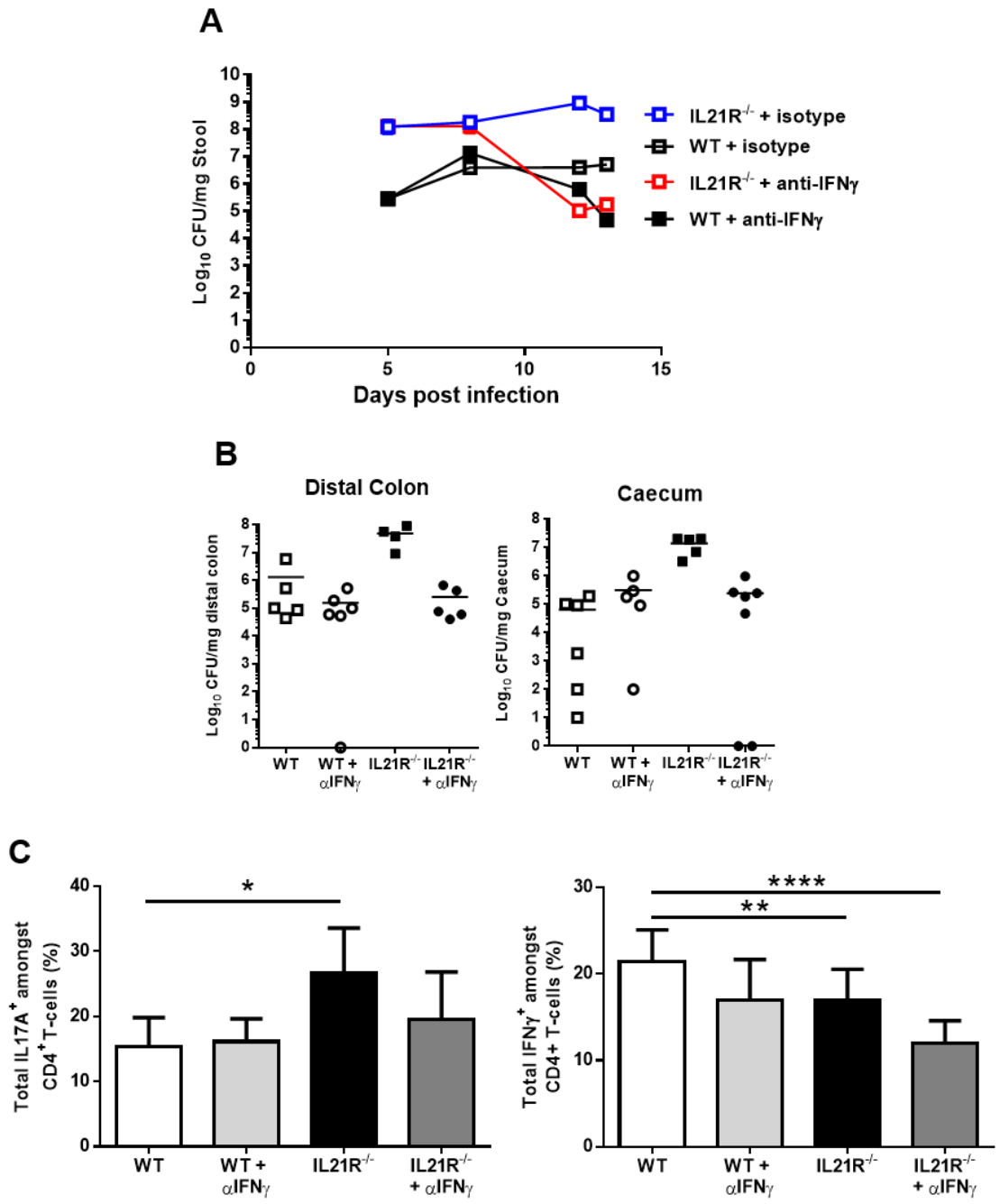

Figure 6.13 Anti-IFN-γ treatment reverses the hypercolonisation occurring in *C.rodentium* infected IL-21R$^{-/-}$ mice

C57BL/6 wild-type or IL-21R$^{-/-}$ mice were infected with ~10^9 CFU *C.rodentium* by oral gavage. Groups of mice of each genotype were treated with anti- IFN-γ antibody or isotype control *i.p.* 3x/week, and were monitored with stool collected at indicated time points. Mice were sacrificed at day 14 and organs harvested. Colonisation was assessed by serial dilution plating of tissue homogenates. Lamina propria cells were isolated and restimulated in vitro with PMA/ionomycin for 4 hours in the presence of Brefeldin A, before intracellular staining. **(A)** Stool colonisation kinetics **(B)** Colonisation of distal colon and caecum **(C)** Frequency of cells producing IFN-γ (top) and IL-17A (bottom) amongst CD4+ T-cells in the colonic lamina propria. Data from single experiment representative of results from 2 experiments. (n=5-7 each genotype/treatment group). Points show mean (A) or individual mice (B). Bars indicate mean +SD (C). Significance determined by Mann Whitney Test (B&C). *p<0.05, **p<0.01, ****p<0.0001.

phenotype compared to animals treated with isotype, and 100% survival, compared to 90% amongst isotype treated mice (Fig 6.12A and data not shown). Splenomegally was significantly reduced amongst IL-21R$^{-/-}$ mice treated with anti-IFN-γ compared to isotype treated animals (Fig 6.12B). Colonoscopic assessment revealed equivalent pathology in wild-type mice and IL-21R$^{-/-}$ mice receiving anti-IFN-γ, in contrast to the more severe disease occurring in the isotype treated group (Fig 6.12C). Lamina propria cellularity was significantly reduced by anti-IFN-γ treatment in IL-21R$^{-/-}$ mice, and was comparable to that observed in wild-type animals (Fig 6.12D). Importantly, no significant effect on body weight, colitis or spleen size was seen in wild-type mice treated with anti-IFN-γ (Fig 6.1 and data not shown). However, daily assessment of *C.rodentium* load by measurement of stool CFUs revealed anti-IFN-γ treatment reduced bacterial burdens and actually promoted bacterial clearance, both in IL-21R$^{-/-}$ and WT controls (Fig 6.13A). Analysis of the colonic T-cell infiltrate confirmed the reduced proportion of IL-17A^{+} cells expected in IL-21R$^{-/-}$ animals, but interestingly, this was further reduced by IFN-γ treatment (Fig 6.13C). A non-significant reduction in IL-17A^{+} cells in wild-type mice receiving anti-IFN-γ treatment was also noted. The proportion of IFN-γ^{+} Th1 cells in IL-21R$^{-/-}$ mice was normalised by anti-IFN-γ treatment.

These experiments demonstrate that IFN-γ contributes directly to both the severity of intestinal inflammation in IL-21R$^{-/-}$ infected with *C.rodentium*, and to clearance of infection.

6.3 Discussion

Although widely described to exert pathogenic effects in a number of models of immune and inflammatory disease, including chronic intestinal inflammation, the results presented here demonstrate IL-21 plays a critical host-protective role in acute infective colitis. We demonstrate that the IL-21/IL-21R axis is required for both the initial control of the attaching/effacing bacteria *C.rodentium* within the intestine, and for the prevention of disseminated infection, but not for bacterial clearance. We show that in the absence of IL-21 signals mice develop severe intestinal inflammation, associated with a predominant Th1-response, and that this severe pathology is dependent on IFN-γ. Preliminary experiments suggest IL-21 acts in a T-cell extrinsic manner, via a cell of haematopoetic origin, to regulate the accumulating cellular infiltrate.

Unravelling the function of IL-21 in host defence in this model was challenging due to the wide expression of IL-21R, its protean described effects and limited understanding of aspects of the immunology of the model. Importantly, we observed two significant aspects to the phenotype of IL-21R$^{-/-}$ mice; the initial increased intestinal colonisation, and the subsequent exaggerated inflammatory response.

Initially we speculated that the increased mortality rates and severe wasting we observed in mice lacking IL-21R might simply reflect impairment of the IL-23/IL-22 axis, which has previously been demonstrated to be essential for control of initial *C.rodentium* infection.[541] However, the differing phenotypes of IL-21R$^{-/-}$ and IL-23R$^{-/-}$ mice, and the preserved intestinal expression of both IL-22 and AMPs excluded this possibility. In the light of findings presented in previous chapters this was not unexpected, since we did not find an essential role for IL-21 in regulating IL-23R expression or Th17 function amongst intestinal CD4^{+} T-cells. However, Th17 cells may not be the critical IL-23R expressing cells in *C.rodentium* infection, with a number of RORγt^{+} innate lymphoid cells including CD4^{+} LTi cells and NKp46^{+} cells reported instead to be

essential.[69,72] Whilst not directly tested here, our results suggest that IL-21 may be dispensable for IL-23R expression by such innate cells, although very recent studies have shown $CD4^+$ T-cells to be an important source of IL-22 in a low-dose variant of the model used here.[545] The effect of IL-21 on IL-23R expression by non-Th17 cells awaits investigation.

Despite the functional IL-23/IL-22/AMP pathways we observed, IL-$21R^{-/-}$ mice had a clear and reproducible difference in the initial level of infection established within the intestine. This abnormality was apparent from as early as 3 days, the initial point at which infection could reliably be detected. We therefore questioned the role played by the steady state intestinal immune tone in wild-type and IL-$21R^{-/-}$ mice.

In common with other bacterial enteric infections, the influence of the host microbial flora on this model is profound, and likely underlies the sometimes strikingly different results reported with identical murine strains in different animal facilities.[537-540] Multiple mechanisms may underlie this effect, including differences in the development of structural or cellular components of the intestinal immune system in the presence or absence of specific microbial components, or simply the availability of a metabolic or nutritional niche allowing colonisation to be established. A clear example of this is the impaired immune response to *C.rodentium* seen in mice lacking SFB, and therefore Th17 cells.[421] Our breeding strategy, maintaining the IL-$21R^{-/-}$ line as a fixed homozygous line, did not permit the ideal scenario of using littermate IL-$21R^{+/+}$ and IL-$21R^{-/-}$ mice. In order to minimise the potential that the flora might be contributing to the differing phenotypes we observed, mice were mixed at weaning, and co-housed for at least 6 weeks prior to experimental use. This interval has been shown to be adequate for the acquisition of similar microbial communities, and for the normalisation of some underlying immune abnormalities.[419] However, it is important to note that without detailed analysis of the microbiota, such as by deep sequencing, it is impossible to fully exclude an effect of absent IL-21 signals on the composition

of the pre-morbid flora as a contributory factor to our findings. Understanding of the role of host genetics in influencing the acquisition and composition of the flora is currently limited, but is rightly an area of intense study, particularly as it pertains to IBD.

We were interested to note the apparent similarities in our phenotype to that reported for B-cell deficient mice,[562] particularly since IL-21 has such profound effects on multiple aspects of B-cell biology. Experiments in our facility confirmed such mice harboured increased intestinal and systemic bacterial loads similar to those we observed in IL-21R deficiency, but thereafter mortality rates of 90-100% occurred at 12-14 days after infection (data not shown). Although serum immunoglobulin responses have been shown to be essential for clearance of infection, the mechanism underlying these previously noted increased levels of early colonisation in B-cell deficient mice is unknown, and has not been fully investigated.[562,879] Studies of mice deficient in immunoglobulins which might realistically contribute to host defence at such an early stage, chiefly IgA and IgM, do not demonstrate increased colonisation levels; indeed IgA deficiency may be associated with reduced early colonisation.[561] Despite reported effects of IL-21 on IgA production,[877] we did not observe quantitative or qualitative effects on steady state intestinal IgA in IL-21R$^{-/-}$ mice. Indeed, when we examined the development of *C.rodentium* specific antibodies later in disease, excepting reduced levels of IgG1, which were of no apparent consequence for the clearance of infection, IL-21R$^{-/-}$ were unimpaired in their production of multiple serum isotypes. Overall, these results did not support a critical role for IL-21 in driving antibody responses at any stage of *C.rodentium* infection, and furthermore argue against significant impairment of Tfh function in this model. However, beyond immunoglobulin production, B-cells fulfil further immunological functions, including innate type functions including antigen-presentation. Although the importance of this in the already APC rich intestine is not clear, the deficiency of MAIT cells noted in B-cell deficient mice does support a non-redundant role in the processing or presentation of particular antigens to specific cells.[321] Regulatory B-cells producing IL-10 have been shown to fulfil an essential role in some models of infection, as have B-cells with

characteristics of innate cells, termed B1 cells, however any role for these mechanisms in the intestine remain to be established.[880]

Understanding of the contribution of IL-21 to epithelial and stromal cell biology is very limited, however since epithelial cells are the initial site of interaction of *C.rodentium* with the host, altered biology of such cells resulting from IL-21R deficiency could potentially contribute to our findings. However, whether IL-21R is even expressed by epithelial or stromal cells in either the steady state or inflamed murine intestine is unresolved. In studies of human intestinal tissue, IL-21 dependent effects on these cells has been described.[670] However, reagents for the detection of murine IL-21R by immunohistochemical approaches were not commercially available to allow us to explore this further. We speculated that since IL-21 is a potent activator of STAT3 in leukocytes, it might fulfil a similar function in epithelial cells, particularly since other STAT3 activating cytokines including IL-6 and IL-22 are required for intestinal epithelial homeostasis.[599] However, in contrast to the effects of IL-6 and particularly IL-22, we were unable to detect IL-21 dependent STAT3 phophorylation in epithelial cells isolated from either steady state or inflamed colons (data not shown), and did not pursue this further. Whilst primarily undertaken to examine the regulation of the intestinal T-cells, our later experiments using bone-marrow chimeras also argue against an underlying epithelial defect driving the IL-21R$^{-/-}$ phenotype. In these experiments, the presence of IL-21R$^{-/-}$ cells of haematopoetic origin was associated with greater colonisation levels, despite intact epithelial IL-21R expression. The mechanism of this latter observation is uncertain, but it suggests that in the absence of IL-21 signals some aspect of the host response strongly favours colonisation, in a manner which is not overcome by the presence of IL-21R sufficient cells, and is presumably through elaboration of a cytokine or factor promoting bacterial growth.

Although we could not determine the underlying basis of the increased intestinal colonisation in IL-21R$^{-/-}$ mice, these animals developed a rapid onset severe colitis characterised by a Th1 dominated lymphocyte infiltrate. Furthermore, Th17 cells accumulating in the inflamed intestine of IL-21R$^{-/-}$ animals demonstrated other functional differences to those observed in wild-type mice. We therefore sought to understand the basis of these differences, and in particular whether they were a primary effect, or a consequence of the increased early colonisation.

Using a bone-marrow chimera approach, we found that the effects of IL-21 on T-cell phenotype were not mediated through cell intrinsic mechanisms, since IL-21R deficient and sufficient cells displayed similar cytokine profiles when taken from within a common inflammatory environment. Intriguingly, although intestinal colonisation in mice receiving bone-marrow containing IL-21R$^{-/-}$ cells was equivalent to levels seen in entirely IL-21R$^{-/-}$ animals at the peak of disease, neither wild-type nor IL-21R$^{-/-}$ T-cells in this environment developed the Th1 dominated profile previously noted in such mice. Therefore, the altered T-cell response in IL-21R$^{-/-}$ mice is not simply the result of the greater bacterial burdens in these animals, but reflects a cell extrinsic requirement for IL-21 to regulate the accumulating T-cell population. The cellular and molecular determinants of this effect are unknown, but speculatively APCs may be likely mediators. Interest in the effects of IL-21 on APCs largely stems from studies in the NOD model of diabetes, in which IL-21 signals into APCs, including DCs, are required for effective antigen-presentation and T-cell activation including Th17 cell development.[668,669,694] Notably, this latter aspect appears to reflect an interaction of IL-21 with retinoic acid (RA) signalling pathways, with decreased transcription of RA regulated Th17 promoting molecules including *Irf4* noted using IL-21R$^{-/-}$ APCs *in vitro*.[669] Importantly, RA is critical in the regulation of the intestinal immune tone and maintenance of homeostasis, including promotion of tolerogenic responses.[881] Furthermore, IL-21 drive STAT3 signals may oppose the STAT5 mediated effects of GM-CSF signals to DCs, impacting upon their function and survival.[882] *In vitro*, IL-21R$^{-/-}$ APCs are not only less able to support Th17 development, but actively promote IFN-γ^{+} T-cell responses, further

suppressing Th17 development.[669] These observations are remarkably similar to those made in our *in vivo* studies of *C.rodentium* infection, and highlight the effects of IL-21 on the numerous and diverse intestinal APC populations as an important area for further study.

Finally, we hypothesised that the severe colitis developing in IL-21R$^{-/-}$ mice was likely to represent immunopathology rather than direct bacterial damage, and might be driven by the exaggerated Th1 response we had observed. Using antibody blockade of IFN-γ, which allowed us to largely separate any influence on initial colonisation and innate responses from its later effects, we confirmed this. Initiating treatment once colonisation was established showed that IFN-γ not only contributes to host tissue damage and systemic features of disease in IL-21R$^{-/-}$ mice, but supported more prolonged and higher levels of colonisation. Whether the reduced colonisation in mice receiving anti-IFN-γ was a direct effect of blocking this cytokine, or was secondary to a rapid reduction in tissue damage, removing the niche for bacterial colonisation could not be determined from these studies. Importantly, IFN-γ may drive epithelial hyperplasia, and although the significance of this effect in the gut is debated, if occurring it could support increased bacterial colonisation. Indeed, it is possible that the increased levels of IFN-γ occurring throughout disease in IL-21R$^{-/-}$ mice were actually promoting the colonisation abnormalities observed. Equally, it cannot be excluded that the resolution of inflammation in mice treated with anti-IFN-γ was mediated by the reduced bacterial load rather than a direct effect on host immunity. IFN-γ exerts multiple effects upon host immune responses, including augmentation of T-cell and NK-cell cytotoxicity, increased activation and antigen presentation by APCs, activation of iNOS, driving class-switching in B-cells to IgG2a and IgG3 isotypes, and activity on haemoatopeotic stem cells.[883] In addition, it reinforces Th1 responses, whilst preventing alternative T-cell fates, including Th17 functions.[139] Interestingly, anti-IFN-γ treatment did not correct the reduced populations of IL-17A^{+} cells observed in IL-21R$^{-/-}$ mice, indeed if anything this population was decreased further, which supports the conclusions of previous studies that this aspect of the Th17 response does not determine disease expression or bacterial clearance,[541]

however accumulation of IFN-γ^+Th1 T-cells in the mucosa was also significantly reduced by treatment. Overall, we observed anti-IFN-γ treatment normalised most immune parameters assessed, suggesting no single dominant mechanism was mediating its beneficial effect.

In conclusion, the studies presented here show that in contrast to a clear pathogenic role in chronic colitis, IL-21 is required for aspects of host protection in intestinal infection, and the development of severe colitis in IL-21R deficient mice illustrates the potential paradoxical consequences of failure of an apparently pro-inflammatory pathway. Therefore, whilst IL-21 may be a valid therapeutic target in inflammatory conditions including IBD, caution must be exercised in the use of such a strategy, and clinical trials need to be mindful for safety signals related to intestinal infection.

Chapter 7. General Discussion

7.1 Summary

In this thesis I have presented results from studies undertaken to explore the role of cellular and molecular mediators of intestinal immunity and inflammation, developed from attempts to better define the mechanisms by which IL-23 dependent pathways may contribute to the development of disease in murine models of inflammatory bowel disease.

Using IL-23R^{gfp} reporter mice, initial studies addressed the cellular mediators of IL-23 signals in the healthy and inflamed colon, and demonstrated both the striking enrichment for IL-23R expressing cells within the intestine even in the absence of disease and the remarkable diversity of such cells. Studies of the changes occurring in colitis demonstrated that inflammation was associated with the rapid accumulation of CD4$^+$ Th17 cells, which became the numerically dominant IL-23R$^+$ cell type from early after initiation of disease. Importantly, results from several experimental approaches suggested differences exist in the regulation of IL-23R expression on CD4$^+$ T-cells and other populations. Experiments using FTY720 to prevent lymphocyte recirculation did not show therapeutic benefit in an IL-23 dependent model of intestinal disease despite inducing peripheral lymphopenia, suggesting that colitogenic IL-23R$^+$ T-cell responses may be generated locally. Since γδT-cells are the predominant steady state IL23R$^+$ cell type, we next studied the effect of their deficiency, using TCRδ$^{-/-}$ mice. Remarkably, in the absence of γδT-cells, mice developed a more severe chronic colitis in an IL-23 dependent model of disease, and although the specific mechanism for this was not established no defect in the generation of colonic Th17 responses was apparent. Indeed, further studies highlighted an exaggerated intestinal immune response as a potential underlying basis of the phenotype in TCRδ$^{-/-}$ mice. Overall, γδT-cells do not appear to play an essential role in mediating intestinal inflammation, but may instead exert a critical regulatory or host protective effect. Finally, we studied the role of IL-21, a cytokine often described to regulate and mediate aspects of the IL-23/Th17 axis, in intestinal models of chronic inflammatory disease and host protection. We demonstrated highly context specific effects on the intestinal immune response, with IL-21R deficiency resulting in paradoxical

outcomes dependent upon the nature of the model studied, including an apparently critical role in host protection against a model of infective colitis. We showed that IL-21 exerts important effects on T-cells which go beyond the IL-23/Th17 axis, and demonstrate redundancy for aspects of Th17 cell function *in vivo.* Our studies demonstrate IL-21 to be an important regulator of intestinal Th1 and Treg function in chronic disease and validate it as an important potential therapeutic target to further explore in IBD. In total, these studies demonstrate a number of important concepts which I will discuss in more detail here.

7.2 Context specific effects of inflammatory mediators in the intestine

The most striking findings from our studies of IL-21 in various models of disease was the variation in the apparent effects depending upon the context examined. In two models of T-cell dependent disease chronic colitis, we observed differing effects of IL-21R$^{-/-}$ on the T-cell phenotype; protective effects were primarily via Treg/Th17 balance in *H.hepaticus* induced disease, but through Treg/Th1 populations in T-cell transfer. By contrast, in the *C.rodentium* model of infectious colitis, IL-21R$^{-/-}$ mice demonstrated impaired host defence against infection and developed an immunopathology driven by an exaggerated IFN-γ response.

These context specific effects of IL-21 were not unexpected as numerous apparently conflicting effects on the function of T- and B-cells have been reported, indeed IL-21 has been described to both drive[664,675] and to inhibit[657,685] Th1 and IFN-γ responses in T-cells. However, more recently its effects on Th17 function have received most attention, being described as an important co-factor in Th17 development, through STAT3 activation and IL-23R upregulation.[175-177,211,654] However, even this finding is clearly context related, since intestinal Th17 populations were reported to be unaffected or even expanded in IL-21 or IL-21R deficient animals.[177,421] This data therefore adds to the increasingly complex picture regarding the *in vivo* requirements for differentiation and population of the intestinal or systemic compartments by T-cell subsets, with further complexity arising when comparing steady state or inflammatory populations. Although

in vitro requirements for murine Th17 differentiation have been defined to include a combination of a TCR stimulus and signals from TGF-β1, IL-1β and a STAT3 signal (IL-6 or IL-21),[169,171,173-175,873] *in vivo* studies suggest highly variable effects from deficiency of each of these components. Mice lacking active TGF-β1 were initially reported to be devoid of Th17 cells[172,752], whereas more recent studies contradict this claim, suggesting Th17 cells may develop in a TGF-β1 independent manner.[202] IL-1β was initially reported not to influence Th17 development *in vivo*,[752] whereas more recent data suggests it to be necessary and sufficient for steady state intestinal Th17 development, with variable effects on systemic cell populations.[770] Studies in colitis indicate that T-cells deficient in IL-1R are unimpaired in their differentiation to Th17 cells, but are defective in their accumulation in the colon.[608] Although IL-6 had previously been reported to be essential for intestinal but not systemic steady state Th17 cell development,[175,181] other studies have refuted an essential *in vivo* role.[770] Similarly, IL-23 is reported to be an essential requirement for Th17 cell development in inflammatory settings outside of the gut,[184,306] but may not be needed for the development of IL-17A$^+$ T-cells in the intestine even in inflammation.[183] Similar redundancy has been reported for intestinal steady state Th17 cells,[421] likely related to the ability of additional poorly characterised mediators, including those derived from the microbiota, to overcome single cytokine deficiency.

However, despite confirming normal overall intestinal T-cell populations in IL-21R$^{-/-}$ mice, our initial studies in *H.hepaticus* induced colitis did reveal impaired Th17 accumulation and reduced populations of Th17/Th1 cells. A finding, combined with increased Treg populations in the absence of IL-21, similar to that reported for TNBS and DSS colitis, although somewhat less marked.[609] However, in our studies using the T-cell co-transfer model, which allowed us to dissect specific cell intrinsic effects of IL-21, we did not observe impaired expression of Th17 molecules such as *Il23r* or *Rorc*. This would suggest that whilst in specific contexts IL-21 may contribute to Th17 accumulation and function, in inflammation it is not essential to the activation of the Th17 molecular programme in intestinal Th17 cells. Functionally, IL-21 is thought to be an important

STAT3 activating signal, yet numerous further cytokines which are elevated in the inflammatory environment also signal via this pathway, including IL-6, IL-22, IL-23, IL-27, and IL-10.[177,212,264,623,862,884-887] Therefore an important fundamental question which requires addressing is the relative contribution and redundancy of such diverse signals between anatomical compartments. Importantly, the ability of each cytokine to signal may be determined by variation in T-cell expression of the necessary receptors, such as the downregulation of surface IL-6R occurring on chronically stimulated T-cells.[863,888] Furthermore, most cytokines are signalling via a panel of STAT and other proteins, therefore the net effect reflects the integration of multiple signals.[889] Systematic study of the contribution of these various cytokines to STAT signalling in T-cells at various stages of disease could reveal important insights to temporal requirements. Initial studies in our lab confirm IL-21 to be a potent STAT3 activating signal in steady state lamina propria T-cells, whereas much more attenuated effects were observed with other cytokines (data not shown).

In contrast to the *H.hepaticus* model, a much stronger protected phenotype and marked effects on Th1 differentiation occurred in T-cell transfer disease. This was a T-cell intrinsic effect, and observed in all tissues examined. Interestingly, we also observed reduced IL-21, consistent with a loss of autocrine signalling, an effect shown to be important for Th17 function.[176] Recently, Tfh cells have been implicated as a sizable and important IL-21 producing cell population within lymphoid tissues,[270,276,659] however their presence in the intestine is less well defined, and although this merits formal study, in pilot experiments defining such cells as $CD4^+ICOS^+PD1^+CXCR5^+$ [270] we could not reliably detect Tfh cells in the inflamed intestine (data not shown). However, the recent description of an IL-21-reporter mouse could permit increasingly informative studies in this regard.[659] We did not determine the cellular origins of IL-21 in our disease models, but these findings suggest that Th1 cells may be an important source in T-cell transfer disease, which would be compatible with recent reports of IFN-γ^+/IL-21^+ cells in the mucosa of patients with IBD.[658] In that study, no significant population of IL-21-producing Th17 cells was observed. This highlights

the important question as to the developmental relationship of such cells to other IFN-γ^+ or IL-17A$^+$ cells.[294,659] Ultimately, the developmental origins of all T-cell populations occurring in experimental models of colitis remain to be more fully determined. An approach using a system such as the recently described IL-17A fate mapping mouse, already studied in the context of CNS inflammation, would be informative, and should be a priority for future study.[298] Alternatively, 'ex-Th17 cells' in the CNS were reported to retain expression of specific Th17-associated molecules including IL-1R and Ahr, which if replicated in the intestine may permit their easy identification and isolation.[298]

Although therapeutic targeting of IL-21 would appear attractive on the basis of these results, predicting its likely clinical efficacy is difficult, and may itself be context related. Both models of chronic disease we used share immunological and clinical features with IBD. However, the T-cell transfer model relies upon massive proliferation of transferred naïve T-cells to induce disease,[890] whereas the *H.hepaticus*/anti-IL-10R model occurs in an otherwise intact animal, which already contains memory T-cells and other lymphocytes for which regulatory mechanisms are active. Consequently, mediators with multiple effects may reveal phenotypes skewed towards specific effects on processes such as proliferation or trafficking in the T-cell transfer model, masking potential additional roles. Importantly, IL-21 shares biological characteristics with IL-2, IL-7 and IL-15 which are known to be critical regulators of the size of the T-cell pool, and of homeostatic T-cell expansion in conditions of lymphopenia,[860,891-893] although our co-transfer experiments did not suggest any intrinsic defect in expansion resulting from IL-21R deficiency.

Notably, the major protective effect in T-cell transfer disease appears to be from reduced Th1/IFN-γ responses, and anti-IFN-γ therapy has itself proven disappointing in trials in patients with IBD.[588] Indeed, inhibition of IFN-γ is of limited effect in murine models beyond the initiation of disease.[519,579,894] Future studies of IL-21 should therefore address its ability to cure or ameliorate established colitis. Data recently presented in abstract form does suggest efficacy for anti-IL-21 therapy in treating established T-cell transfer disease.[861] Should this be confirmed, it will be important to further analyse the T-cells developing in the absence of IL-21 to ascertain what other

effector pathways it is regulating within Th1 cells. Importantly, although *Tbet* expression was reduced in IL-21R$^{-/-}$ cells in the co-transfer experiments, the *Tbet* paralogue *Eomes* was more significantly reduced. This is of interest, since *Eomes* has been described as an important specific regulator of IFN-γ,[895] and remarkably IL-21 has been reported to exert opposing effects on IFN-γ through regulation of *Eomes*.[685]

The potential benefits of inhibiting IL-21 signalling should be weighed against the finding of more severe disease in our studies using *C.rodentium* infection. However, it is important to consider that this is primarily a model of host defence against infection, and therefore unlikely to predict its effects in chronic IBD.[532] Rather our findings demonstrate the potential risk of exacerbated intestinal infection in a recipient of anti-IL-21 therapy. Somewhat frustratingly, we could not determine the underlying mechanism for the hypercolonisation occurring in IL-21R$^{-/-}$ animals. However, we were able to demonstrate that the resultant severe inflammatory response was driven by elevated production of IFN-γ, in direct contrast to chronic colitis. A similar propensity for an exaggerated Th1/IFN-γ response in the absence of IL-21/IL-21R signals has been reported in a number of other experimental systems, possibly via a mechanism involving APCs.[657,668,694,707,896] Importantly, IL-21R sufficient APCs were present in the T-cell transfer model, the results therefore simply reflect the direct T-cell dependent effects of IL-21. The apparently paradoxical effects in other models may therefore reflect the T-cell consequences of the loss of additional IL-21 dependent signals usually provided via other cell types. The results from our studies using bone marrow chimeras are preliminary and somewhat challenging to fully interpret, but do implicate a non T-cell haematopoetic cell to be critical to this response. Repeating T-cell transfer experiments using Rag$^{-/-}$IL21R$^{-/-}$ hosts is a clear next step to investigate this issue. Existing flow cytometry reagents proved unreliable in our pilot studies to identify IL-21R expression (data not shown), but assessment of *Il21r* expression amongst sorted myeloid cell populations could be a simple but informative undertaking. Our results demonstrate that in the appropriate context, IL-21R$^{-/-}$ T-cells are not impaired in their ability to differentiate to a Th1/IFN-γ^{+} phenotype. Therefore, questions again arise as to the developmental origins of these Th1 cells,

and it is important to note that the *C.rodentium* model uses an otherwise intact B6.IL-21R$^{-/-}$ mouse. Furthermore, whereas WT mice displayed more polyfunctional Th17 cells such as IL-17A^{+}IL-17F^{+} cells, IL-21R$^{-/-}$ mice appeared to deviate towards a pro-inflammatory IFN-γ^{+}GM-CSF^{+}TNFα^{+} phenotype, although the signals driving this require definition.

A further remarkable observation in our studies blocking IFN-γ in *C.rodentium* infection was the apparent benefit of this approach for bacterial clearance, which initially appears counterintuitive. However, IFN-γ is known to drive epithelial hyperplasia which might be predicted to increase colonisation, providing a possible explanation.[559,705,897] Alternatively, the generalised reduction in inflammation may have allowed unassessed homeostatic pathways to regain dominance and initiate repair and recovery, reducing the niche for colonisation. This highlights the often overlooked potential effects of immunological mediators on non-immune cells or processes in such models.

The demonstration that the severe disease in IL-21R$^{-/-}$ mice infected with the *E.coli* like organism *C.rodentium* reflected an immunopathology occurring secondary to an impaired innate immune response is of particular interest, since a chronic manifestation of this mechanism has been proposed to underlie Crohn's disease.[842] Studies of neutrophil and macrophage function in patients with Crohn's disease have reported defective accumulation in the setting of acute inflammation, with impaired clearance of experimentally inoculated *E.coli*, leading to the proposal that the disease results from immunodeficiency, and requires augmentation rather than inhibition of immune responses.[842,898] Although trials of G-CSF have proven disappointing, the recruitment of patients with long disease durations prior to study entry would appear to have reduced the likelihood of a positive result.[899] The reported association of autoantibodies against GM-CSF and paediatric IBD would support this intriguing hypothesis.[900,901] However, the finding of significant benefit from inhibition of GM-CSF in murine colitis provides a stark reminder of the contextual relevance of such mediators.[607]

A highly context specific effect for IL-21 has been reported in studies examining its potential role in colitis associated cancer (CAC). Anti-IL-21 treated WT or IL-21$^{-/-}$ mice develop fewer tumours

in the DSS/AOM model of inflammation driven carcinogenesis,[610,612] associated with less STAT3 activation in tumour infiltrating lymphocytes,[610] leading to the proposal that IL-21 may be a therapeutic target in CAC, although the finding that IL-21 ablation was associated with lower levels of inflammation provides a simple alternative interpretation of the mechanism of benefit. In other studies, overexpression of IL-21 in tumour cells reduced growth and enhanced anti-tumour immunity leading to the opposite conclusion that IL-21 administration itself could be beneficial.[720,721,902] A further study suggested that despite usually being protected from chronic DSS colitis, IL-21$^{-/-}$ mice developed similar colitis when intestinal tumourogenesis was induced using AOM, yet had reduced tumour formation, paradoxically linked to increased CD8^{+} T-cell driven inflammation and IFN-γ levels in the absence of IL-21.[612] In this scenario, the context specific effects of IL-21 therefore appear to primarily reflect the predominant responding cell type.

An important final consideration in assessing the implications of these results is the similar paradoxical observations made in studies of the roles of IL-23,[183,515,520,873] IL-17A[521,522,560,591,592,596] and IL-22[541,603,903] in intestinal inflammation. Although such findings may primarily relate to the differing importance of the numerous mechanisms of disease pathogenesis and repair active in each model, studies in experimental lung inflammation have highlighted the risks of artificially modifying the effect of other cytokines active within a network when targeting a single effector molecule.[874]

Ultimately, the overall effect of manipulating IL-21 signalling in IBD will be determined in clinical studies. Our data, along with previously published studies provide adequate indication of potential benefit in IBD, yet also provides important data which may assist in designing and analysing the outcomes of such studies, which should be undertaken within an experimental medicine setting to determine the relevance of such mechanisms to activity in IBD.

7.3 The intestine as a unique inflammatory site

Although the cellular and molecular mediators investigated in the studies described here are conserved between anatomical locations, a number of our results highlight the concept of the intestine as a site where underlying inflammatory responses develop through site specific mechanisms. Whilst the presence of large populations of leukocytes in the uninflamed gut has long been recognised, along with the existence of mechanisms controlling their activation, our understanding of their specific contributions to chronic intestinal inflammation or homeostatic functions remains limited. Importantly, our early finding of significant enrichment of the healthy colonic mucosa for IL-23R expressing cells contrasted with the much lower proportions and numbers of IL-23R$^+$ cells reported to be present in the steady state at other sites where IL-23 has been implicated in the pathogenesis of disease, such as the skin and CNS.[387,392,617] Indeed, the healthy CNS is virtually devoid of lymphocytes, mandating recruitment of all cells in the setting of developing inflammation.[904] Expression of IL-23 itself is enriched in the healthy gut,[638] suggesting a potential homeostatic role, which is well demonstrated by studies of intestinal infection with *C.rodentium*.[541,873] Further, these findings would suggest the existence of mechanisms preventing inappropriate activation of gut resident IL-23R expressing cells in the steady state. Our studies of IL-23R$^+$ cell kinetics in evolving disease were enlightening, since the steady state IL-23R$^+$ populations showed minimal expansion compared to IL-23R$^+$CD4$^+$ T-cells, suggesting that the switch between protective and pathogenic effects of IL-23 may relate to changes in the primary responding cell type. Only CD4$^+$ T-cells are reported to show appreciable plasticity in terms of effector function,[297] with IL-23 signalling driving the acquisition of a pathogenic phenotype by Th17 cells,[183,299,753] a finding confirmed here, whereas the dominant intestinal IL-23R$^+$ cell type, γδT-cells, did not appear to acquire activity beyond IL-17A production, even in disease, confirming published reports.[365,410,614,747] The nature of this 'switch' was not fully addressed, but our studies showing the cell specific suppressive effect of IL-10 on CD4$^+$ T-cell expression of IL-23R highlight a plausible candidate, in agreement with data showing a cell-intrinsic effect for IL-10 signalling in regulating Th17 cell function.[622] The lack of effect

from blocking IL-10 on IL-23R expression by other cell types was notable, raising the question as to what regulatory signals control such cells in the intestine. Interestingly, Foxp3$^+$ Tregs appear able to exert regulatory effects over some γδT-cell populations in the intestine, in part through IL-10 dependent mechanisms.[815] Alternatively, it is possible that IL-23R expression is constitutive upon the cells other than CD4$^+$Th17 cells, and not subject to active regulation. Indeed, γδT-cell expression of IL-23R has been reported to be a developmentally fixed characteristic.[410,747,905] IL-23 is a potent proliferative signal for Th17 cells, and the lack of significant proliferation by any other colonic IL-23R$^+$ cell type, particularly in developing disease, raises the question as to the differences in downstream signalling occurring in Th17 cells compared to the more innate steady state populations. This presents an important aspect for further study, since it may reveal natural pathways regulating pathogenic IL-23 driven molecular effects, and potential molecular targets to specifically manipulate Th17 cell function.

In contrast to findings in extra-intestinal models of inflammation, our results also suggest that colonic γδT-cells play little if any role in mediating the effects of IL-23 in the setting of evolving chronic colitis.[387,389,392] Whereas in CNS inflammation IL-23R$^+$ γδT-cells promote early inflammation through elaboration of cytokines including IL-17 and inhibition of the suppressive effects of Treg cells, with TCRδ$^{-/-}$ mice largely protected from disease,[392] their absence had no effect on the aspects of the intestinal Th17 response we analysed. This points to differences in the mechanisms governing CD4$^+$ Th17 cell accumulation in the intestine compared to other inflammatory sites (Figure 7.1). Indeed, inflammation in CNS mandates accumulation of cells with characteristic kinetics,[905] whereas all relevant populations are already potentially resident in the intestine. Preliminary studies using pharmacological inhibition of lymphocyte recirculation provided additional support for this assertion, since despite inducing peripheral lymphopenia, this did not affect the severity of *H.hepaticus* induced colitis, nor the number or phenotype of accumulating CD4$^+$ T-cells. This would imply that the Th17 population in the uninflamed colon of SPF mice already contains cells with colitogenic potential. If confirmed, this identifies an opportunity to better understand the concept of homeostatic and pathogenic Th17 signatures.

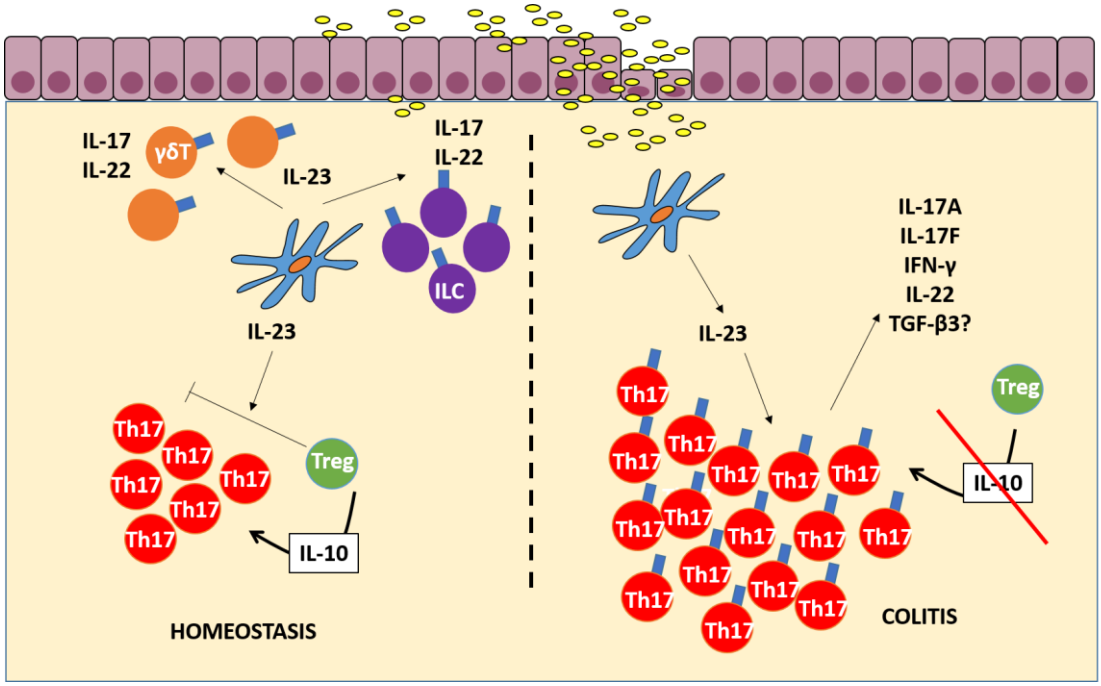

Figure 7.1 Proposed model of how colitogenic effects of IL-23 may relate to ability to signal into resident Th17 cells

In homeostasis, a small population of Th17 cells, defined by production of IL-17A or expression of IL-23R is present in the colonic lamina propria. Intestinal APCs constitutively express IL-23 under the influence of signals from the microbiota. The expression of IL-23R on Th17 cells, and hence the ability to respond to IL-23 signals is controlled by IL-10, postulated to be from Treg cells. In contrast, the homeostatic IL-23R expressing populations, which are more innate in nature and include γδT-cells and ILCs may not be subject to similar control by IL-10, possibly because of their more limited pathogenic potential. Therefore, IL-10 signals limit the potential of resident steady state Th17 cells to induce inflammation. In the setting of inadequate or perturbed IL-10 signalling, Th17 cells upregulate their expression of the IL-23R, allowing proliferation and attainment of a pathogenic effector profile, driving colitis.

Although IL-23 driven Th17 pathogenicity has been linked to acquisition of additional effector characteristics such as IFN-γ[183,298,299] or GM-CSF expression,[185,186] or autocrine production of TGF-β3,[753] the developmental relationship between steady state intestinal populations and Th17 cells accumulating in disease is incompletely defined. Analysis of the transcriptional or signalling profiles of unstimulated and IL-23 stimulated Th17 cells from steady state, and from the diseased mucosa would be informative. In addition, it would be of significant interest to examine the molecular and epigenetic characteristics of $CD4^+$ Th17 cells present in the intestine *after* the resolution of colitis, an important feature of the *H.hepaticus*/anti-IL-10R model used in these studies, to determine whether the biology of resident populations are altered by inflammatory experience.

These findings also raise the important question of the anatomical basis of the inflammatory response in the models of colitis studied. Adoptive transfer experiments have shown colitogenic memory cells to be present in bone marrow and lymphoid tissue reservoirs,[603,775,780-782,906,907] with our results further suggesting the uninflamed colon itself already contains such cells. Importantly, the preceding studies involved transfer of T-cells into 'empty' immunodeficient hosts, whereas the findings here represent what may be considered a more physiological scenario. Studies of the dynamics and recirculation characteristics of intestinal lymphocytes represents an important area for future study, and will be facilitated by the recent description of an actin-kaede murine reporter system which irreversibly marks cells upon exposure to 405nm wavelength light, which can be achieved using a rectally introduced endoscope or fibre-optic, allowing real time detection of cells originating in the intestine.[908-910] Notably, the efficacy of a number of current potent therapies used for the maintenance of remission in IBD has been linked to their ability to induce long term depletion of intestinal effector memory T-cells, rather than through effects on peripheral lymphocyte numbers.[886,911-914] This issue also has potential relevance in predicting the optimal use and likely benefit of therapies targeting lymphocyte circulation and recruitment. Inhibitors of α_4-integrin were shown to be of some clinical benefit in Crohn's disease,[915,916] although their use is limited by severe adverse effects,[917,918] and trials of agents targeting $\alpha_4\beta_7$-integrin are ongoing,

with mixed early results.[919,920] Similarly, based upon their efficacy in treating multiple sclerosis,[795,796] trials of S1P-inhibitors including fingolimod (FTY720) are in progress in IBD,[921] and the results are awaited with interest.[922] Whilst inhibition of lymphocyte migration to the intestine is clearly a promising and active area of drug development, better definition of the basic biology and role of T-cells resident in the uninflamed intestine in IBD is an important area for further study.

7.4 Interpreting the translational implications of animal models of IBD

Whilst the studies reported here provide insights into aspects of the basic biology of intestinal inflammation, the translational implications of such studies, undertaken in murine models systems, remain to be determined in human studies. The potential difficulty in predicting effects from murine studies is well demonstrated by the wide range of effects of IL-21R deficiency we observed between models, and the marked differences in the contribution of γδT-cells to intestinal inflammation we noted compared to that reported in other models. Indeed, despite the widespread use of animal models to explore mechanistic aspects of the pathogenesis of intestinal inflammation, successful clinical translation of findings from such studies has been limited. A significant number of therapies previously predicted by murine models to be effective for the treatment of IBD have proven somewhat disappointing in clinical trials, including targeting of IFN-γ,[588] IL-12/23p40,[586] and IL-17A.[594] Furthermore, paradoxical effects occur, at odds with clinical observation. As described above, the benefit from GM-CSF blockade in murine models of colitis[607] contrasts with the effects of G-CSF treatment in subsets of IBD patients.[923] Although aspects of trial design, patient selection and pharmacokinetics might each contribute to such negative results, it is important to ask why findings in animal models of IBD have often proven not to translate well in human disease.

Despite the description of a large number of models of intestinal inflammation, including in excess of 100 murine systems of varying complexity, in many cases it remains uncertain as to

how each relates to human disease.[496,499,500,924,925] The majority of models require the genetic ablation or blockade of specific mediators, treatment with irritant chemicals or infection with pathogens or pathobionts.[496,499,500,924,925] Whilst the pathogenesis of IBD remains uncertain, debate exists as to the validity of each approach, with the histological similarity of each model to some aspect of human disease often cited as simple validation. However, whereas some models are highly dependent upon environmental factors such as the intestinal flora, others occur in germ free conditions. Similarly, some models only develop in the presence of intact populations of specific cells, most commonly T-cells, whereas others occur in $Rag^{-/-}$ mice.[496,499,500,924,925] In general findings from chemical injury models, including DSS and TNBS, which are T-cell independent, contrast with results using more complex systems, which may be more informative regarding human disease. Exemplifying this, whereas IL-17A may be pathogenic in DSS and TNBS systems,[596] it has either a protective or redundant effect in T-cell dependent models,[520-522] an apparently similar role to that observed in subsequent human studies.[594]

Therefore, despite any recapitulation of histological features of IBD, it seems unsurprising that models which can arise in the absence of the intestinal flora or in animals lacking adaptive immunity may not accurately reflect the processes underlying IBD. However, even in these systems there is clear conservation of pathogenic mechanisms, as demonstrated by the finding of $ROR\gamma t^{+}$ ILC populations in Crohn's disease,[87] first described in innate models of disease occuring in $Rag^{-/-}$ mice.[67] Similarly, the description of a role for IFN-γ^{+} ILCs in human IBD[70] parallels findings in Tbet deficient $Rag^{-/-}$ (TRUC) mice.[71]

A key message from the multiple highly informative studies recently undertaken to decipher the genetic basis of IBD is that environmental factors remain a significant, but less well understood contributing factor to the development of disease.[441,442,462] Prime amongst implicated factors is the composition of the intestinal flora, with clear differences apparent between normal controls and each subtype of IBD.[413] Representation of specific species of clostridia and bacteroidets appears significantly different in IBD patients compared to healthy subjects.[430,483,484,926] Importantly, interaction of such species with the host may induce specific immune modifying effects. Studies

in mice clearly demonstrate essential host species specific characteristics of the flora are required for normal maturation and intestinal immune function. Importantly, components of the flora may also directly influence the significance of host genetic factors for disease risk, as elegantly demonstrated in studies of Atg16L1 hypomorphic mice. In this strain, which carries a mutation implicated in susceptibility to Crohn's disease, the severity of DSS induced ileocolitis was dependent on colonisation with murine norovirus, through an effect on Paneth cell function, experimentally demonstrating the concept of host genetic interaction with microbial and further environmental factors in colitis susceptibility.[573,574] It is therefore unsurprising that conflicting results occur between mouse colonies despite apparently using the same strains and protocols.

Importantly, whereas murine colitis is induced in mice kept in SPF conditions, which by definition have not previously been exposed to intestinal pathogens, human disease develops in patients who will have experienced and resolved previous infective episodes. The immunological consequences of such exposure for the risk of IBD are unknown, although epidemiological studies suggest an increased risk of developing IBD may exist in patients following enteric infection,[920,921] and that relapse of quiescent disease may be triggered by infection. Similarly, the development of post-infectious irritable bowel syndrome (IBS), which is linked to subtle but well replicated immunological alterations within the colon following infection with *Campylobacter, Salmonella* or *Shigella* demonstrates the potential of such infections to induce long-term alterations in the host immune response.[929,930] Such findings raise important questions as to whether specific pathways and effectors remain of consistent relevance regardless of previous immunological experience and activity. For instance, whereas steady state colonic Th17 cells do not feature significant numbers of the presumed pathogenic IL-17A^{+}IFN-γ^{+} cells, it is unclear whether with resolution of disease such populations would contract to leave T-cells which predominantly demonstrate single IL-17A production and again require specific signals to adopt a pathogenic phenotype, or whether resultant cells could rapidly expand as IL-17A^{+}IFN-γ^{+} cells without the developmental signals originally required.

The development of experimental colitis as a single event without prior episodes of intestinal inflammation is a notable variation from the situation in humans, and may therefore contribute to translational difficulties. Considering the results of the studies reported in this thesis, I would hypothesise that the mechanisms driving inflammation in the previously inflamed intestine would differ from those active in initial inflammatory events in naïve animals. The significance of this could be tested experimentally by the induction of single or repeated episodes of colitis, followed by full macroscopic recovery prior to induction of further colitis (Figure 7.2). The susceptibility to and severity of developing disease would be the initial parameters assessed. If previous inflammatory activity influences the risk of disease, it might do so in one of two ways; it could increase the severity and speed with which disease develops due to non-antigen specific activation of existing cell populations, or alternatively, previous inflammation may increase the regulatory tone or favour homeostatic and repair promoting pathways, resulting in attenuated disease. Importantly, in such an approach the combination of initial and subsequent insults would need to be carefully selected. Were differences in disease severity to be noted, mechanistic studies should address the cellular populations present following resolution of initial disease, in particular the APC and T-cell phenotypes, as well as the effect of previous inflammation on tertiary lymphoid tissue formation, and the cells populating such structures. Epigenetic analysis of cells resident in the colon following resolved inflammation may be informative.

If differences in the immunology of initial and recurrent disease were confirmed, such an experimental system might also provide a platform to test the relative contribution of effector molecules to different stages of disease, potentially improving translational relevance. Notably, many effectors which contribute to initial disease development may be redundant for ongoing inflammation. This is paralleled by observations in human IBD, where the efficacy of anti-TNF-α therapy may decline with disease duration.[931] Similarly, T-cell clones derived from paediatric patients with new onset IBD or infective colitis are phenotypically similar, whereas in established IBD, cytokine receptor expression and effector responses differ significantly.[932]

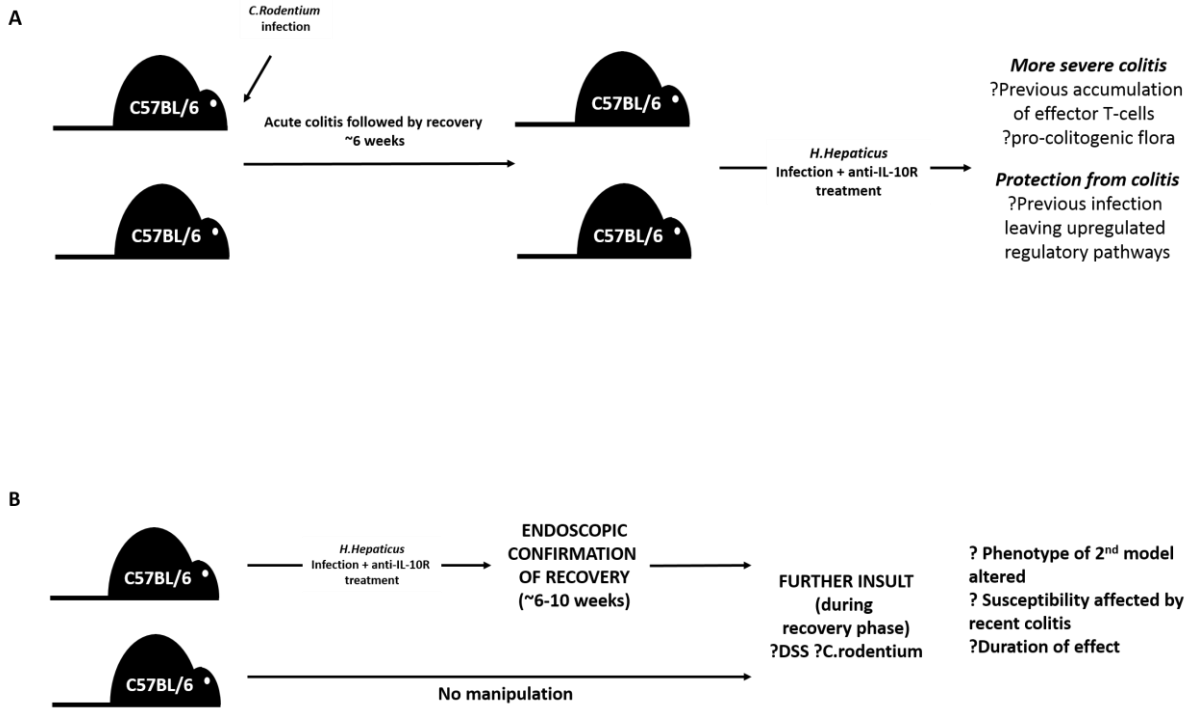

Figure 7.2 Proposed Experimental Approach to Analysis of Effect of Prior Inflammatory Episodes on Development of Colitis

Mice would be treated or exposed to an initial insult, followed by a period of recovery before a further, different model was utilised. **(A)** A proposed approach would be to use *C.rodentium* infection which results in mild self-limiting colitis followed by a period of recovery (~6 weeks), then induction of a model of chronic disease such as *H.hepaticus*/anti-IL-10R. A strength of this approach is that this concept tests the concept from epidemiological studies that recent enteric infection, which *C.rodentium* models, may increase the risk of developing chronic disease. The susceptibility, severity and kinetics of disease development and recovery in the 2nd model would be initial parameters analysed. **(B)** Alternatively, a chronic model of disease could be the initial approach, with endoscopic confirmation of mucosal healing prior to induction of acute disease. Following this with an acute model of disease as the second insult may reveal a more obvious phenotype than approach (A).

Such studies may also better clarify similarities and differences in the mechanistic basis of IBD relapse rather than initial disease development. This remains a further important limitation of murine studies in which manipulations aim to prevent initial disease development, in contrast to the clinical scenario in which resulting therapies are largely used, in treating refractory disease and preventing relapse.

7.5 Conclusions

In conclusion, I have presented work in this thesis which serves to highlight the unique and complex nature of intestinal immunology, with implications for the treatment of human intestinal inflammation, including IBD. These findings demonstrate the cellular basis by which IL-23 contributes to both homeostatic processes and inflammation in the colon, and identify mechanisms of disease development which may be amenable to therapeutic intervention. These results, along with studies undertaken to define the role of γδT-cells, also provide some insight into why findings in intestinal disease may differ from those observed in other inflammatory sites, which again has potential implications for selecting pathways for the treatment of IBD. Furthermore, these results have generated testable hypotheses regarding novel approaches to studying the immunology of IBD. Finally, studies identify the IL-21/IL-21R pathway as a potentially important target in IBD, through a number of highly context specific effects. In the light of the earlier findings, this pleurality of effects may be advantageous to the biological activity of IL-21 targeting therapies in a range of clinical scenarios in IBD treatment, and now requires study in humans.

Chapter 8. References

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