Molecular networks regulating T cell responses at barrier sites

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Abstract

Inflammatory bowel disease is a complex syndrome characterized by chronic inflammation of the intestine with an unclear aetiology. GWAS and preclinical studies revealed how IL-23 is one of the main drivers of the deranged Th17 inflammatory response that characterizes this disease, although the molecular and cellular pathways involved are still unclear. With a combination of *in vitro* and *in vivo* systems, in this study we identified *Timp1* and *Ltb4r1* as IL-23-induced genes in Th17 cells and tested their pathogenic potential in IL-23/Th17 driven intestinal inflammation. *Timp1*$^{-/-}$ and *Ltb4r1*$^{-/-}$ mice were tested in models of T-cell dependent colitis showing minimal impact on overall inflammation and Th17 cytokines production. With this study we also showed how another gene of interest, *Dgat1*, was enriched in CD4^{+} T cells and Foxp3^{+} Tregs at barrier sites. While *Dgat1* deficiency in CD4^{+} cells did not have an impact on T cell induced colitis, mice carrying the specific ablation of *Dgat1* in Foxp3 expressing cells developed a systemic type 2 inflammatory response characterized by accumulation of GATA3^{+} Foxp3^{+} Tregs at barrier sites and high levels of serum IgE. Thus, with our study we identified a novel role for barrier site Tregs in inducing immune tolerance through DGAT1. Finally, we investigated the effect of IL-23 signalling on intestinal Th2 cells. Our data suggest that IL-23, while sustaining Th17 inflammation, restrains the activity of intestinal Th2 cells. Using an adoptive T cell transfer model of colitis, we observed a cell intrinsic inhibitory effect of IL-23 on Th2 cells, suggesting that in the intestine, the Th17/Th2 balance is controlled by IL-23. With this study we revealed new insights in the complex regulation of inflammatory responses at barrier sites, where the function of T cells and Tregs is tightly regulated to maintain homeostasis.

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Chapter 1: General Introduction

1.1 The intestinal immune system

The intestine is part of the gastrointestinal tract, a system of organs that provide the human body with two fundamental functions: digestion and uptake of nutrients, and protection from environmental factors. Along its length, the intestine comprises different regions and exhibits structural differences that reflect the multiple functions of this organ. It is composed of two main organs, the small intestine, where digestion of food and uptake of nutrients occur, and the large intestine, whose function is primarily water reabsorption and processing waste products into faeces.

In both the small and the large intestine, a single layer of columnar epithelial cells separates the intestinal lumen from the deeper layers of the organ. The surface of the small intestine is characterized by finger-like structures known as villi, that increase the surface area of the epithelium facilitating the absorption of nutrients. In all the length of the intestine, the epithelium is continuously renewed by stem cells residing at the bottom of invaginations known as crypts. Underneath this thin layer of cells is the lamina propria (LP), primarily composed of loose connective tissue, and characterized by the presence of blood vessels, lymphatic vessels and nerves. The LP is also home to most of the immune cells of the intestine [1]. All together, the epithelium, the LP and a thin layer of muscle cells, known as the muscularis mucosae, constitute the intestinal mucosa, a unique microenvironment where the immune system is in constant contact with the outside world. In fact, the epithelium is the only barrier that separates the immune system from the immense and varied community of microbes that colonize the intestine, that are collectively called microbiota. The total number of microbes and the diversity of species of the gut microbiota increase down

the length of the intestine, with the colon having the most abundant and diverse community. It has been estimated that the intestine harbours between 10^{13} and 10^{14} microbes [2]. Therefore, the intestinal immune system has the challenging task of protecting from harmful pathogens while maintaining tolerance to the innocuous resident bacteria. This balance is known as immune homeostasis, and its breakdown is considered to be the culprit of many intestinal inflammatory disorders [3]. The lamina propria and the epithelium are the main effector sites of the intestinal immune system, while the initiation and priming of immune responses take place in associated structures like the mesenteric lymph nodes (MLN) and the gut-associated lymphoid tissue (GALT), comprehending Peyer's Patches, isolated lymphoid follicles and cryptopatches. Here, the presentation of antigens by antigen presenting cells (APC) to adaptive immune cells induces the activation of gut-homing T cells and B cell humoral responses.

In the intestine, myeloid APCs are a heterogeneous population composed of dendritic cells (DC) and macrophages. Both populations can uptake, process and present antigens on major histocompatibility complex class I or II (MHC-I and MHC-II) molecules, yet they are phenotypically and functionally different cells that can be distinguished by the expression of specific surface markers function and migratory capacity. Under homeostatic conditions, intestinal macrophages arise from the infiltration of lymphocyte antigen 6C-high ($LY6C^{hi}$) blood-derived monocytes, that upon entering the lamina propria upregulate the expression of major histocompatibility complex II (MHCII), lose LY6C expression and upregulate the macrophage markers F4/80, CD64 and the CX3C-chemokine receptor 1 (CX3CR1) [4, 5].

Another important population of mononuclear phagocytes is represented by the $CD11c^{+}CD103^{+}CX3CR1^{-}$ DCs, cells that upon uptake of an antigen migrate to the mesenteric lymph nodes (mLN) and the Peyer's patches, where through antigen presentation they induce the differentiation of effector $CD4^{+}$ T cells and the production of immunoglobulin A (IgA) by B cells [6, 7]. These DCs can also induce expression of homing receptors on the surface of activated T cells, such as CCR9 and α4β7, allowing their migration to the intestinal LP (Mora et al., 2003, Johansson-Lindbom et al., 2005). In addition, $CD103^{+}$ DCs are the main source of IL-23, a master proinflammatory cytokine that drives intestinal $CD4^{+}$ T cell responses [8, 9].

As millions of bacteria colonize the lumen of the intestine, maintaining intestinal homeostasis is a crucial function of the immune system, and tolerogenic immune responses have to be dominant in the colonic LP. $CX3CR1^{+}$ macrophages contribute to the tolerogenic environment of the intestinal mucosa by producing high levels of interleukin 10 (IL-10), which suppresses effector responses and promotes Treg survival and function [10, 11]. In contrast to the migratory nature of $CD103^{+}$ DCs, monocyte-derived $CXCR3^{+}$ macrophages reside in the colonic LP close to IECs [7, 12] where they sample antigens via transepithelial dendrites that can reach through the lumen and scan for pathogens [12, 13].

The intestine also harbors a large proportion of the total innate lymphoid cell (ILC) population, a subset of innate cells that share characteristics with adaptive immune cells. These cells have a lymphoid appearance and express CD45, but do not depend on an antigen specific receptor for their function. Similarly to $CD4^{+}$ T helper cells, they can be classified into three main categories based on their expression profiles: group 1, 2 and 3 ILCs (ILC1, ILC2, ILC3) that express the transcription factors T-bet,

GATA3 and RORγt respectively. In addition, ILC1 produce IL-12 and IL-18, tipically associated to a Th1 phenotype, ILC2 express the Th2 cytokines IL-5 and IL-13, and IL-C3 express the Th17 associated cytokines IL-17 and IL-22[14]. These cells can amplify effector responses in the intestine and have been shown to be involved in the development of colitis. In fact, IFN-γ producing ILC1 and IL-17 producing ILC3 have been shown to accumulate in the intestine of Crohn's disease patients [15, 16]. However, ILCs can also contribute to intestinal barrier integrity, as ILC3 express the barrier promoting cytokine IL-22 and are involved in protection from *Citrobacter rodentium* infections [17, 18], while IL-33-activated ILC2 ameliorated intestinal disease by producing amphiregulin (AREG) in a mouse model of intestinal damage and inflammation [19].

Adaptive cells are also crucial players in the landscape of intestinal immune responses. The importance of B cells relies primarily on their ability to produce secretory IgA antibodies, which are highly concentrated in the intestinal mucus layer and can bind non-specific as well as microbe-specific glycans to facilitate the recognition of pathogens and induce immune responses [20, 21]. Bacteria that are highly coated with IgA are suggested to represent the species with the highest pathogenic potential, indicating that the B cell-mediated humoral response direct the identification and clearance of colitogenic bacteria [22].

1.2 $CD4^+$ T cells

The intestine harbours a large amount of lymphocytes, including multiple specialised T cell populations. T cells reside in both the epithelial layer, where $\gamma\delta^+$ T cell receptor (TCR) T cells are abundant, and in the lamina propria, where most T cells are $CD4^+$

$TCR\alpha\beta^+$ although a smaller $CD8^+$ $TCR\alpha\beta^+$ population is also present. These cells all contribute to maintaining intestinal homeostasis and play a role in inflammatory responses. Effector $CD4^+$ T cell responses begin when naïve $CD4^+$ T cells exit the thymus and migrate to the periphery where they recirculate through the lymphatic system to the lymph nodes. Here, T cells scan for peptide:MHCII complexes on the surfaces of APCs like DCs, which migrate from the periphery to the lymph nodes where they present the captured antigen to T cells [23]. Upon recognition of their cognate antigen via their TCR, APCs provide a co-stimulatory signal via the surface molecules CD80/CD86. This interacts with CD28 on the surface of T cells to induce an activation process and clonal expansion that results in a T effector clone [23]. Several other costimulatory molecules have activating or inhibiting properties and are involved in the activation process of T cells. These include CTLA-4, CD40-L, OX-40, GITR and RANKL expressed by T cells and interact with CD40, OX-40L, GITRL, RANK on APCs [24]. TCR engagement in the absence of a co-stimulatory signal induces an anergic state that renders T cells inactive [25]. T cell activation and generation of an effective immune response require not only TCR activation and costimulation, as the cytokine milieu further dictates the subsets of T helper (Th) cells that will emerge from these interactions (Fig.1.1).

$CD4^+$ T helper cells are fundamental components of the effector immune response, and a balance within different Th subsets is crucial for maintaining immune homeostasis. When proinflammatory effector pathways supersede, or when regulatory mechanisms are impaired, the risk of developing deranged and pathological inflammatory responses increase. This is especially true in the intestine, where a tight

regulation of $CD4^+$ effector responses is needed to avoid the development of chronic inflammatory diseases like inflammatory bowel disease (IBD) [26].

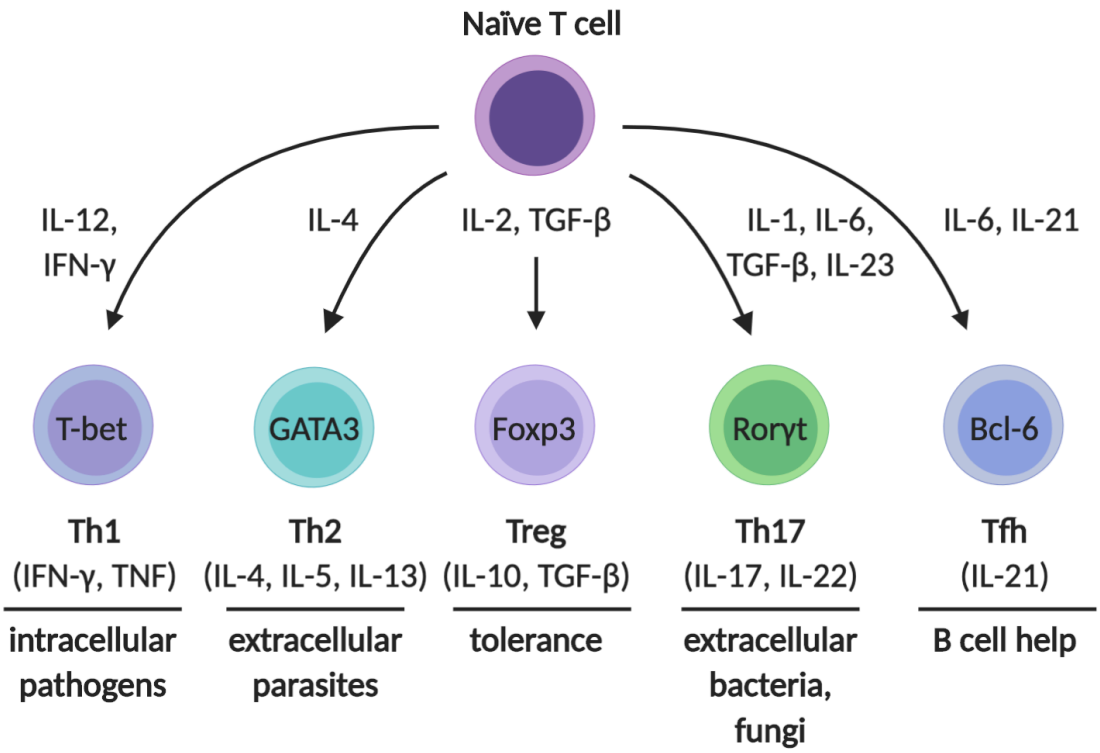


Figure 1.1: $CD4^+$ T helper cell subsets

Upon TCR engagement and in the presence of appropriate polarizing cytokines, naïve $CD4^+$ T cells express specific transcription factors that dictate their differentiation in distinct T helper cell subsets. Each subset is characterized by the expression of signature cytokines that mediate their effector function.

1.2.1 Th1

$CD4^+$ $TCR\alpha\beta^+$ naïve T cells, in the presence of TCR stimulation and IL-12, differentiate into Th1 cells, a subset of T cells whose main function is to mediate immune responses against intracellular bacteria and viruses. Th1 cells are

characterized by expression of the T-box transcription factor T-bet and production of high amounts of IFN-γ [27]. Their differentiation and transcriptional profile is driven by IL-12 and IFN-γ that signal via the STAT4 and STAT1 [28, 29] pathway respectively and induce the expression of T-bet. T-bet regulates transcription of *Ifng* [30, 31] and its expression is fundamental for Th1 differentiation [32], as T-bet deficient mice have defects in Th1 differentiation and function [33]. T-bet has also been shown to stabilize the Th1 phenotype, as it inhibits GATA3 expression, which is the master transcription factor of Th2 cells [30].

Deranged Th1 responses have been implicated in the development of inflammatory diseases like IBD. Studies on mouse models of colitis showed how $CD4^+$CD45RBhigh naïve T cells adoptively transferred into SCID mice produce high levels of IFN-γ contributing to disease [34], and this is consistent with clinical observation, as IFN-γ producing Th1 cells accumulate in the intestine of CD patients [35]. IFN-γ has a functional role in driving IBD-like diseases, as blocking of IFN-γ in *H. hepaticus* induced colitis reduces the severity of disease [36]. In contrast, the adoptive transfer of IFN-γ deficient T cells into immunodeficient hosts does not affect the development of colitis, suggesting a non-essential role of T cell-derived IFN-γ in driving intestinal inflammation [37]. In addition, both the blockade of IL-12 signaling and the STAT4 cascade ameliorate disease, confirming the IL-12/STAT4 pathway as crucial for Th1 driven intestinal inflammation [37, 38]. The functional role of T-bet in inducing intestinal inflammation is controversial: while one study showed that T-bet deficient $CD4^+$ T cells fail to induce colitis in the adoptive T cell transfer model [39], Krausgruber *et al.* demonstrated that T-bet modulates IL-23 driven responses rather

than driving disease, as in the same model T-bet deficient T cells are able to induce pathology at a comparable level to WT counterparts [40].

1.2.2 Th2

TCR activation in the presence of IL-4 induces a phenotype in $CD4^+$ T cells known as Th2. Th2 cells are involved in host defense against extracellular parasites and in orchestrating allergic reactions. They coordinate humoral immune responses by inducing immunoglobulin class-switching to immunoglobulin E (IgE) in B-cells, and can attract innate immune cells such as eosinophils and mast cells to the site of inflammation [41]. Induction of Th2 differentiation is through activation of a receptor complex consisting of the IL-4Rα chain and the common gamma chain (γc). IL-4R engagement activates the STAT6 signaling pathway, which mediates expression of Th2 master transcription factor GATA binding protein 3 (GATA3) [42]. Th2 cells are characterized by the expression of cytokines like IL-4, IL-5 and IL-13 [43, 44], all of which are regulated by GATA3 [45]. While induction of the Th2 signature is IL-4/STAT6 dependent, cytokines such as IL-6 and IL-21, that signal through STAT3, can synergise with IL-4 and promote Th2 differentiation [46] while epithelium-derived alarmins such as TSLP, IL-33 and IL-25 can potentiate Th2 immune responses by enhancing the production of Th2 cytokines [41, 47].

In the intestine, Th2 cells are not abundant without a relevant driver such as parasites or immunogenic food antigens [48]. In the intestine of patients with UC, IL-5 and IL-13 have been reported to be upregulated [35], however the main source of these cytokines are iNKT cells, that drive an atypical Th2 response in UC patients [49].

The induction of Th2 responses may be beneficial in Th1/Th17 inflammation, as GATA3 prevents the production of IFN-γ [50]. Indeed, IL-4 gene therapy inducing expression of this cytokine by intraperitoneal injection of an *Il4* coding plasmid reduced the severity of TNBS-colitis, which is a Th1-mediated model of intestinal inflammation [51].

Th2 cells also play a functional role in driving the pathogenesis and exacerbation of skin disease like as atopic dermatitis (AD). By inducing IgE production in B cells, Th2 cells can contribute to the IgE-mediated sensitization that characterizes AD [52].

In inducing allergic reactions, both Th2 cytokines and cell-cell interactions have a role. In fact, Th2 cytokines and cell-contact dependent CD40/CD40L mediate activation of B-cells leading to IgE class-switch, crucial for activation of mast cells and eosinophils [53].

1.2.3 Th17

The identification of IL-17 producing T cells (Th17) as a separate lineage of T cells challenged the original Th1/Th2 paradigm proposed by Mosmann & Coffman in 1989 [54]. Th17 cells were first discovered by studies that questioned the role of Th1 cells in the development of autoimmune diseases like experimental autoimmune encephalomyelitis (EAE). Initial studies showed how development of EAE was dependent on IL-23 rather then IL-12 [55], and that IL-23 deficiency correlated with a decrease in IL-17 production, suggesting that a T cell population other than Th1 was involved in the disease [56]. In 2005, Th17 were first identified as a distinct lineage of $CD4^+$ T cells able to produce IL-17 and induce EAE [57].

Since then, Th17 have been implicated in several inflammatory and autoimmune diseases, but they also have a functional role in host defence against extracellular

bacteria and fungi. $CD4^+$ naïve T cells differentiate into Th17 cells in presence of TCR activation and TGF-β and IL-6 signaling [58], which induce expression of the master Th17 transcription factor RAR-related orphan receptor gamma RORγt [59]. IL-6 stimulation results in the activation of the STAT3 signaling cascade [60], while TGFβ leads to activation of the Smad pathway [61]. Further exposure to cytokines like IL-21 [62] and IL-23 [63, 64] enhance the effector function of Th17 cells. Upon activation, Th17 cells produce cytokines like IL-17A, IL-17F, IL-21 and IL-22 [65]. As IL-21 enhances Th17 differentiation and has been shown to be produced by Th17 cells themselves, it was proposed that it might act as a proliferative stimulus in a positive feedback loop [66].

IL-17A and IL-17F signal through the complex IL-17RA/RC, a heterodimeric receptor that is ubiquitously expressed; however, non-hematopoietic cells like epithelial cells and fibroblasts are generally the main responders to IL-17 [67, 68].
The pro-inflammatory capacity of IL-17 was first identified in fibroblasts, where IL-17 stimulation induces NF-kB-dependent cytokine production [69]. Both IL-17A and IL-17F are involved in the development of inflammatory responses and host defence by inducing the expression of pro inflammatory cytokines like TNF-α, IL-1, IL-6, G-CSF, and GM-CSF, and chemokines such as CXCL1, CXCL5, IL-8, CCL2, and CCL7 [70]. IL-17A can attract neutrophils to the site of inflammation by inducing a specific pattern of activation in endothelial cells that leads to the expression of CXCR2-activating chemokines [71]. The role of IL-17 in protecting against pathogens is also exerted by inducing the expression of antimicrobial peptides in epithelial cells. IL-17 induced molecules like defensins, S100 proteins, occludin, regenerating islet-derived protein 3g (Reg3g), and mucin 1 all contribute to pathogen

clearance and maintenance of epithelial barrier integrity in the intestine [72]. In addition, IL-17 can induce expression of matrix metalloproteinases in different tissues including smooth muscle [73] and fibroblasts [74].

Although they share many of their functions, IL-17A and IL-17F seem to have distinct biological effects, as studies comparing *Il17a*$^{-/-}$ with *Il17f*$^{-/-}$ mice showed that IL-17A has a more relevant role in autoimmunity than IL-17F, possibly due to its more potent strength of signalling (10–30-fold weaker in terms of downstream gene activation) [67, 75]. By contrast, it was reported that blocking of IL-17F, but not of IL-17A, induced protection in both chemically induced colitis and adoptive T cell transfer colitis models [76]. However, a complete understanding of their distinct role is yet to be determined.

IL-22 is a Th17 derived cytokine whose production is regulated by RORγt and induced by IL-23 [77]. IL-22 is expressed in a variety of tissues, including the GI tract, skin and lung, and its receptor, a heterodimeric complex composed of IL-22R1 and IL-10Rβ, is expressed on the stromal and epithelial cells of those tissues. The function of IL-22 partially overlaps with that of IL-17, as it can induce the expression of proteins involved in antimicrobial defence, cellular differentiation, and expression of mucins [78]. IL-22 plays a fundamental role in regulating intestinal inflammation and homeostasis through the production of antimicrobial peptides, enhancement of epithelial regeneration, and regulation of wound healing [79].

Based on studies on experimental models of colitis, recent findings have hypothesized a possible protective role of IL-22 in IBD. In the DSS-induced colitis model, IL-22 deficiency leads to enhanced colitis with extensive epithelial destruction [80]. In addition, adoptive transfer of naïve T cells from *Il22*$^{-/-}$ mice causes a more aggressive

form of colitis than transfer of WT naïve T cells [80]. IL-22 protects from infection with *Citrobacter rodentium*, a model of attaching-effacing bacteria, by inducing the production of antibacterial Reg family proteins in epithelial cells [81].

Following the discovery of IL-23 as one of the main driver of pathogenic Th17 responses, Th17 cells have been recognized as important players in intestinal inflammation. The importance of the IL-23/Th17 immune axis in the intestine is discussed in detail in a separate section of this thesis.

1.2.4 T regulatory cells

$CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) are a specialized subset of T helper cells whose main function is to regulate immune responses and induce peripheral tolerance. By modulating both adaptive and innate immunity, Tregs restrain the development of unnecessary inflammatory responses to self- or non-harmful antigens. Therefore, they play a crucial role in protecting against autoimmune responses and maintaining homeostasis at barrier sites.

The first evidence of a subset of immune cells with regulatory capacity was observed in studies by Sakaguchi *et al*., who showed how self-reactive immune responses in mice could be prevented by adoptive transfer of T cells isolated from the thymus or spleen [82, 83]. Further studies confirmed the existence of a subset of T cells with the ability to suppress the effector response of $CD4^+$ naïve T cells adoptively transferred into immunodeficient mice [84-86]. These $CD4^+$ T cells had an antigen experienced phenotype, expressed high levels of the IL-2Rα (CD25) and were able to suppress immune responses to self-antigens and to food and microbial antigens [87, 88].

The transcription factor forkhead box P3 (Foxp3) was identified as the master regulator of T regulatory cells, involved in their development, maintenance and suppressive functions [89, 90]. This discovery was confirmed by observations made in a Foxp3 deficient mouse strain and in mice with a specific T cell restricted Foxp3 deletion, which develop lethal autoimmunity as a consequence of a failure in peripheral tolerance [91, 92]. Clinical evidence of a crucial role of Foxp3 and Tregs in maintaining immune homeostasis came from patients affected by IPEX syndrome (immune dysregulation, entheropathy, X-linked), who have mutations in Foxp3 and therefore are affected by autoimmune manifestations [93, 94]. In both mice and humans, deficiencies in Foxp3 often lead to autoimmune responses at barrier sites like the skin or the intestine, further suggesting a fundamental role of Tregs in maintaining immune tolerance at barrier sites [85, 94].

As with other $\alpha\beta^+$ T cells, Treg cell development takes place in the thymus, where two distinct pathways of differentiation begin [95]. A majority of $Foxp3^+$ Tregs develop in the thymus, where TCR affinity to self-antigens determines the selection of T cell clones that leave the thymus as naïve $Foxp3^+$ thymus-derived tTregs [96]. Peripherally induced Tregs (pTregs) follow a different path, as they exit the thymus as $CD25^-Foxp3^-$ Naïve T cells and are induced into $CD25^+Foxp3^+$ pTregs in the periphery following recognition of their cognate antigen and stimulation by IL-2 and TGF-β [97, 98]. For both tTreg and pTreg, TCR stimulation happens in secondary lymphoid organs (SLOs) such as mesenteric lymph nodes and spleen. Upon TCR stimulation, Treg cells acquire an activated phenotype characterized by the expression of activation markers like CD44, ICOS, GITR and IL-10 [99]. Activated Treg cells also upregulate the expression of tissue homing receptors, such as CCR9 and $\alpha4\beta7$ for

trafficking to the intestine, and migrate to tissues to exert their regulatory function [11, 100, 101]. Phenotypically, tTregs and pTregs are not easy to distinguish, and to date only the transcription factor Helios and the surface marker Neuropilin 1 (Nrp-1) have been suggested to be preferentially expressed by tTregs and allow distinction between the two subsets [102-104]. However, the reliability of these markers has been questioned by the observation of peripherally induced Tregs that can express Helios and Nrp-1 [105, 106]. tTregs and pTregs have non-redundant roles in the regulation of immune responses [107]. There is evidence that pTregs bear TCRs with specificity to non-self antigens such as microbe-derived peptides [108]. Several studies have shown how bacteria from the genus *Helicobacter* can induce pTreg regulatory responses in an antigen specific manner [109, 110]. On the contrary, tTregs are believed to have self-specific TCRs, although displaying reactivity to gut microbes [111]. This can be possibly explained by the fact that self-specific TCRs can cross-react to food and microbial antigens [99]. In support of this hypothesis is the observation that Tregs with TCR reactivity to one bacterially derived antigen can cross-react to peptides from other bacteria or food antigens [112, 113]. All together these observations highlight the critical function of Tregs in limiting reactivity to self-antigens and restrain excessive immune response to innocuous non-self antigens.

T regulatory cell mediated suppression consists of several mechanism of action that span both cellular-dependent and cellular-independent pathways. Tregs can modulate the response of both adaptive and innate immune cells, and while their activation requires TCR stimulation, their effector function does not require further exposure to their cognate antigen, enabling a wide array of regulatory effects.

The cell-contact-dependent immunosuppressive function of Tregs is mediated by the upregulation of cell-surface molecules that have an inhibitory effect on target cells. Upon engagement, these molecules can directly restrain the immune response of target cells or induce a transcriptional program that leads to an immunosuppressive state in effector cells or APCs [114]. Among these molecules are CTLA-4 and LAG-3 [114]. CTLA-4, which is constitutively expressed on the surface of Tregs and binds CD80 and CD86 on APCs, inducing downregulation of those same molecules and thus decreasing the costimulatory potential of APCs [115, 116]. The *in vivo* relevance of this molecule was shown in mice with a deletion of CTLA-4 specifically in $Foxp3^+$ Tregs, which spontaneously develop a lethal systemic autoimmune response [117]. Treg mediated immune suppression in a T cell transfer model of colitis was abrogated by treatment with an anti-CTLA-4 antibody, leading to enhanced intestinal inflammation [118]. Furthermore, Tregs were shown to restrain Th2 responses in the intestine via CTLA-4, as Treg specific deletion of CTLA-4 in mice resulted in a higher frequency of $GATA3^+$ T cells and higher serum levels of IgE [119]. Another cell surface molecule that plays a role in suppressing the function of APCs is LAG-3, a CD4 homologue that can bind MHC-II on the surface of immature DCs and induces an inhibitory signal that suppresses DCs maturation and their antigen-presentation capacity [120].

In addition to cell-cell interactions, Treg cells also produce immunosuppressive cytokines such as IL-10 and TGF-β. Production of IL-10 is of fundamental importance to maintain immune homeostasis at environmental interfaces. It was shown that IL-10 deficiency in $Foxp3^+$ Tregs does not induce systemic autoimmunity but leads to deranged immune responses at barrier sites like the intestine, lungs and

skin [121]. In addition, observation in adoptive T cell transfer colitis showed a central role of locally produced T-cell derived IL-10 in restraining inflammation of the intestinal lamina propria [122]. IL-10 also acts in an autocrine way directly on Treg cells: under inflammatory conditions, IL-10 signaling induces Foxp3 transcription in Tregs via STAT3, stabilizing the regulatory phenotype of Tregs and inducing further production of IL-10 [10, 123]. Treg cells are a rich source of TGF-β, another immunosuppressive cytokine whose expression is enriched in the intestine. Initial observations showed how TGF-β deficient mice develop a lymphoproliferative autoimmune disorder that leads to early death [124, 125]. Further studies revealed how Treg derived TGF-β restrained the effector response of T cells and contributed to Treg-dependent maintenance of intestinal immune homeostasis [126]. In addition, TGF-β has been shown to act as a promoting factor for expression of Foxp3 [127] and can induce the differentiation of peripheral naïve $CD4^+$ T cells into Tregs [97, 128], suggesting that it plays a role in both the differentiation and effector function of Treg cells. TGF-β is initially secreted in its latent form, consisting of TGF-β bound to the latency-associated peptide (LAP). Upon TCR activation, $Foxp3^+$ Tregs express the glycoprotein A repetitions predominant (GARP) which binds latent TGF-β. Association of TGF-β with GARP induces activation of the latent complex via integrin $\alpha_v\beta_6$ or integrin $\alpha_v\beta_8$, allowing the release of the biologically active form of TGF-β [129]. $GARP^{-/-}$ or $\alpha_v\beta_8$ Tregs still have the capability of secreting latent TGF-β, however it does not have biologic activity, highlighting the importance of the GARP complex in empowering the functionality of Treg cells [130, 131].

Metabolic disruption has also been revealed to be a mechanism through which Tregs regulate immune responses. Tregs express high amounts of CD25, the IL-2Rα

chain [88], and thus compete with effector T cells for availability of IL-2, a survival factor for T cells [132]. Other cell-surface molecules involved in the Treg mediated metabolic disruption of immune cells are CD39 and CD73. These molecules are ectoenzymes highly expressed on Treg cells that have been shown to take part in Treg mediated immune suppression by catalyzing the sequential degradation of ATP to AMP, mediated by CD39 [133], and AMP to adenosine, mediated by CD73 [134]. As a consequence, adenosine signals through the A2A receptor and directly inhibits effector responses in T cells. Through this mechanism, Tregs shift ATP-driven pro-inflammatory immune cell activity toward an anti-inflammatory state mediated by adenosine [135].

1.2.5 $CD4^+$ T cells adaptation to tissue sites

$CD4^+$ T helper cells are highly plastic cells and can adapt their phenotype to the tissue of residence or to environmental cues. A good example of this phenomenon is the ability of Tregs to upregulate the expression of transcription factors usually associated with other cell types and acquire tissue-specific characteristics. By comparing the transcriptional profile of lymphoid organ associated Tregs to non-lymphoid tissue-resident Tregs, several studies have revealed how Tregs display a phenotypical trajectory in their development from the thymus to tissues, where they progressively become highly specialized and develop tissue-specific function (Fig. 1.2). In a recent publication, Li *et al.* [136] showed how a subpopulation of spleen Tregs express low level of the adipocyte associated transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), which is then upregulated when Tregs reach the visceral adipose tissue (VAT). The same group also showed that VAT-associated Treg cells acquire a PPARγ dependent transcriptional program that leads to the acquisition of

specific tissue-restricted properties and unique metabolic functions [137]. Miragaia *et al.* described a similar scenario for skin and colon Tregs, as they identified non-lymphoid-tissue (NLT) -like Tregs in the draining lymph nodes that showed distinct degrees of commitment to the tissue phenotypes, such as the upregulation of skin-specific or colon-specific homing receptors. These studies revealed a two-step scenario for tissue Tregs diversification, where Tregs express partial tissue-specific signatures in secondary lymphoid organs and later give rise to fully specialized Tregs upon migration to the tissues.

Treg heterogeneity is also highlighted by the fact that they can acquire the expression of transcription factors usually restricted to other T helper cell subsets. Treg cells expressing GATA3, the master transcription factor of Th2 cells, are found at different tissue sites but represent a relevant population in both the intestine and skin. The expression of GATA3 in Treg cells is associated with a tissue-healing program and has been proposed to promote tissue repair [138]. In the skin, $GATA3^+$ Tregs represent the vast majority of Tregs, up to 80% of the total dermal population [139], and are $Helios^+$ $Nrp1^+$, thus *bona fide* tTreg [140, 141]. Dermal $GATA3^+$ tTreg have been shown to restrain local aberrant inflammatory responses, as mice with a specific GATA3 deficiency in $FOXP3^+$ Tregs develop a type 2 inflammatory response driven by commensal specific $CD4^+$ T cells [142]. $Helios^+$ $GATA3^+$ tTreg are an appreciable population of intestinal Tregs too. In the intestine, they display a signature that is associated with a tissue-repair program, as they express the IL-33 receptor ST2 and amphiregulin (AREG) [139, 143, 144]. These cells are also involved in regulating intestinal inflammatory responses, as GATA3 stabilizes $Foxp3^+$ expression and is important for accumulation in the intestinal mucosa [139, 145].

Tregs that express the Th17 associated transcription factor RORγt represent the most abundant Treg population of the intestine [146]. Foxp3$^+$ RORγt$^+$ Tregs are highly immunosuppressive, and Rorγt expression was shown to be necessary for their regulatory function in adoptive transfer model of colitis [146]. Intestinal RORγt$^+$ Tregs have been shown to be necessary to inhibit Th1/Th17 intestinal inflammation in trinitrobenzenesulfonic acid (TNBS)–induced colitis [143]. These cells are Helios$^-$ peripherally induced Tregs and their induction and maintenance is dependent on the microbiota [119, 143]. Interestingly, the small intestine harbours a population of RORγt$^-$ pTreg whose maintenance is, on the contrary, dependent on food antigens [147]. In addition, Treg cells can express the Th1 associated transcription factor T-bet, and Tbet$^+$ Tregs are crucial in restraining Th1 inflammatory responses [148, 149]. All together these data indicate that Tregs are a very heterogeneous population, and that, by upregulating non-canonical transcription factors, Treg cells mirror the phenotype of those immune cells they regulate.

As previously mentioned, the microbiota can shape the immune system and affect the development of a T cell subset over another. Bacteria and their products are in fact one of the main factors determining the tissue adaptation of T cells. Studies on germ-free (GF) mice revealed how the absence of a commensal flora leads to an underdeveloped immune system, with reduced numbers of CD4$^+$ T cells. This condition can be reversed by colonization with a specific pathogen free (SPF) flora that induces the accumulation of intestinal CD4$^+$ T cells [150, 151]. T regulatory cells and Th17 cells are thought to be the most susceptible CD4$^+$ T cells to changes in the microbiota composition, and several species of bacteria have been shown to promote

or suppress them [108, 152]. The Treg cell pool in the colon of germ-free or antibiotic treated mice is dominated by Helios$^+$ tTreg cells [143, 153], while the maintenance of both RORγt$^+$ pTreg cells and RORγt$^-$ pTreg cells is dependent on the microbiota. RORγt$^+$ Treg cells especially seem to be sensitive to microbial shifts, and colonization of GF mice with an altered Schaedler flora (ASF) or a selected mixture of 17 human *Clostridia* strains have been shown to induce their appearance in the colon [154, 155]. GF mice have also been given *Bifidobacterium*, *Bacteroides*, *Lactobacillus* and *Helicobacter* which are all genera known for promoting intestinal pTreg [109, 143, 154]. *Helicobacter hepaticus* has been shown to induce intestinal pTreg cells in an antigen-specific manner, as the adoptive transfer of CD4$^+$ naïve T cells carrying a *H. hepaticus*-reactive TCR differentiate in RORγt$^+$ pTreg cells in the colon of *H. hepaticus* colonized host mice [110]. Whether other bacteria induce Tregs in an antigen-specific manner is yet to be determined. A characteristic of *H. hepaticus* is to be associated with the intestinal epithelium, thus providing constant source of antigen for APCs.

Bacteria can induce intestinal Tregs in an antigen independent way too. In fact, components of bacterial origin have been shown to promote intestinal Tregs in an indirect way. *H. hepaticus* produces a large polysaccharide that induces innate cells to produce IL-10, which could promote the maintenance of Treg cells [156]. In addition, *Bacteroides fragilis* polysaccharide A and cell-surface β-glucan and galactan polysaccharides from *Bifidobacterium bifidum* have a similar effect, as they induce IL-10 production in innate immune cells via TLR2 signalling thus promoting the accumulation of Treg cells [113, 157]. Colonization with *Bacteroides fragilis* protects mice from the development of intestinal inflammation in a model of *H. hepaticus*

induced colitis, while mutant strains that do not express PSA on the contrary promote Th17 responses [158].

There is large evidence that metabolites of microbial origin can also induce intestinal Tregs. Short chain fatty acids (SCFAs) originate from the fermentation of fibres carried out by certain bacteria [159]. Butyrate, a SCFA produced by commensal bacteria has been shown to induce intestinal Treg cells [160, 161]. Interestingly, administration of SCFAs to SPF mice induces an emergence of Treg cells and promotes their immunosuppressive function, further supporting the idea that bacterial metabolites play a role in maintaining the intestinal Treg population [162]. The presence of a commensal flora is also essential for Th17 cells, as demonstrated by several studies on GF mice. Mice lacking a bacterial community of the intestine, display almost undetectable levels of IL-17 expressing $CD4^+$ Th17 cells [163, 164]. Interestingly, identical mouse strains in different animal facilities can show variable frequencies of intestinal Th17, suggesting that different microbiome compositions can have different effects on the generation of Th17 cells [165]. Mono-colonisation experiments showed that the commensal segmented filamentous bacteria (SFB) alone can induce the emergence of IL-17 and IL-22 producing $CD4^+$ T cells in the intestinal lamina propria [166]. SFB is a member of the *Clostridium* species that preferentially colonizes the terminal part of the ileum and is in close association with the epithelium. SFB has been shown to induce expression of serum amyloid A (SAA), a member of acute-phase response proteins induced during infection, which acts on DCs to induce Th17 differentiation [166]. Adhesion to the epithelium seems to be a common trait of Th17-promoting bacteria, as other adhesive species like *Citrobacter rodentium* and *Escherichia coli* O157 trigger Th17 responses. Mutant strains that lose

the ability of adhering to epithelial cells, also fail to induce Th17 responses [167]. Remarkably, a selection of 20 bacteria isolated from the faeces of UC patients based on their Th17-inducing potential, also exhibited epithelial cell adhesive characteristic [167]. Depending on the context, microbiota-induced Th17 responses have been shown to be both protective and detrimental. Mice colonized with SFB-containing microbiota increased their resistance to infection with *C. rodentium* by inducing Th17 differentiation [166], while colonization of the genetically predisposed strain K/BxN with SFB induced a severe autoimmune arthritis via Th17 [168]. Commensal-reactive Th17 cells have also been implicated in the development of inflammatory diseases affecting immune privileged sites, such as uveitis and autoimmune encephalomyelitis [169, 170]. By contrast, SFB colonization in the diabetes predisposed NOD mice correlated with protection [171].

All together these data show how host-microbiota interactions are highly context-dependent, and by influencing the intestinal immune system they can have systemic effects and contribute to the development of inflammatory disease.

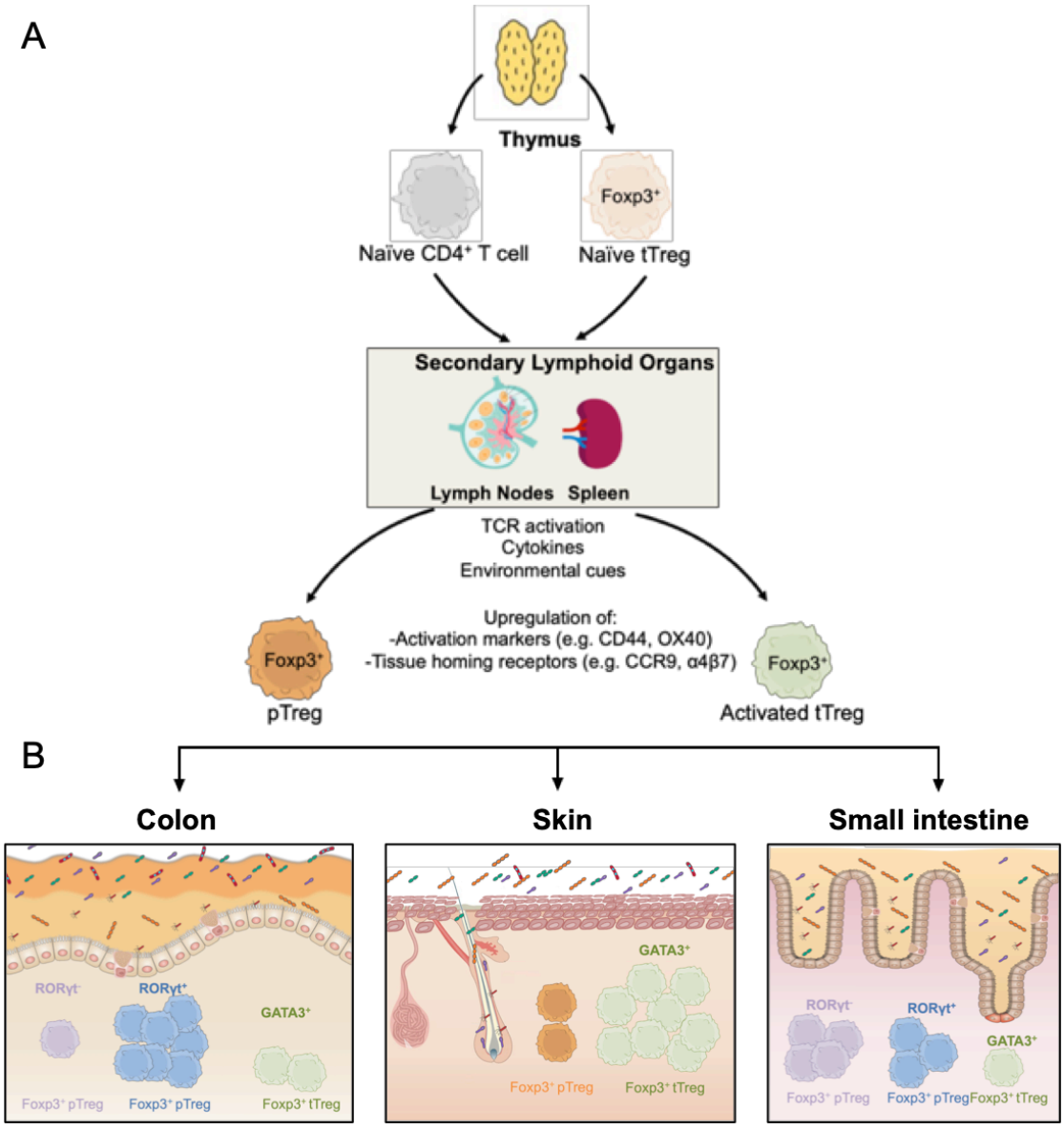


Figure 1.2: Treg cells development and adaptation to tissue sites

(**A**) The development of Foxp3$^+$ Treg cells start in the thymus, where two trajectories are recognized. tTreg cells differentiate into Foxp3$^+$ Tregs in the thymus, while pTregs migrate from thymus as naive CD4$^+$ T cells to the SLOs where they differentiate to Foxp3$^+$ Treg cells. Upon activation in the SLOs, both tTregs and pTregs upregulate activation markers and tissue-homing receptors that drive their migration to tissue sites. (**B**) Different tissue sites harbour different specialized subsets of Treg cells. In the small intestine, most Treg cells are RORγt$^+$ pTregs and

RORγt$^-$ Tregs, while GATA3$^+$ tTregs represent a minor fraction of the total population. In the colon most Treg cells are RORγt$^+$ pTregs, while in the skin this population is not detectable, and the vast majority of Tregs are GATA$^+$ tTregs.

1.3 IgE responses and their regulation

IgE is one of the five classes of human antibodies, and it is the least abundant immunoglobulin isotype. IgE levels are tightly regulated and concentrations of serum IgE are ~50–200ng per ml of blood in healthy humans and mice, compared with ~1–10 mg per ml of blood for other isotypes [172]. Generally, IgE responses are transient and IgE have a half life of ~5–12 hours in mice and ~2 days in humans, compared to ~10 days in mice and ~20 days for IgG in humans [172]. The low concentration level of serum IgE and the transient nature of IgE responses are thought to help minimize IgE-cross reactivity that could lead to unwanted anaphylactic reactions. IgE is produced in two possible forms: as membrane B cell receptor, expressed on the surface of B cells that have undergone class switching to IgE or as secreted immunoglobulins produced by plasma cells.

IgE responses arise initially from low-affinity, short-lived IgE plasma cells that generate from naïve B cells in secondary lymphoid organs' extra-follicular foci, following antigen recognition and secondary stimulation. Late IgE responses arise from the germinal centre, where activated B cells that received help from T follicular helper cells undergo class-switch recombination to IgE. Through the process of somatic hyper mutation, IgE B cells in the germinal centre undergo affinity maturation and differentiate into short-lived IgE plasma cells that produce high-affinity IgE. Some IgE germinal centre B cells differentiate into long-lived IgE

plasma cells that migrate to the bone marrow, while others differentiate into IgE memory B cells in order to retain immunological memory [173].

The main role of IgE in host defence is mediating reactions that result in protection against helminth infections and the expulsion of environmental toxic substances such as toxins, venoms, irritants and xenobiotics. An uncontrolled IgE response to innocuous allergens can lead to allergy, which represent the pathogenic detrimental response of IgE, and in severe cases can result in systemic anaphylaxis and death [174, 175].

IgE bind to immune cells via two receptors, a high-affinity Fc receptor for IgE (FcεRI) and the low-affinity Fc receptor for IgE (FcεRII; also known as CD23)[176, 177]. Activation of the FcεRI on mast cells and basophils mediates cellular degranulation, eicosanoid production and cytokine production. On dendritic cells and macrophages, FcεRI engagement induces the internalization and processing of IgE-bound antigens for presentation on the cell surface, as well as production of cytokines that mediate type-2 immune responses [177].

Basophils are mainly circulating cells but home to areas of inflammation during various types of IgE-mediated allergic inflammatory responses, including atopic dermatitis, allergic rhinitis, and asthma [178]. Activated basophils produce several different cytokines such as IL-4, IL-6, IL-13 and TNF-α, and act as mediators of inflammation by recruiting other inflammatory cells like eosinophils and neutrophils [178]. Another feature of basophils is the ability to degranulate and release immune mediators such as histamine, which induces smooth muscle contraction and mucous secretion [179].

In contrast to basophils, mast cells are tissue-resident inflammatory cells. They are characterized by expression of the transmembrane receptrr KIT (CD117) and are implicated in host responses to pathogens, autoimmune diseases, fibrosis, and wound healing [179]. Their activation depends on FcεRI engagement and IL-4 signalling, but they are also activated by TLRs ligands. The major cytokine stored and secreted by mast cells is TNF-α, which induces upregulation of endothelial and epithelial adhesion molecules, increases bronchial responsiveness and enhances T cell activation [180]. Other cytokines produced by mast cells are IL-3, GM-CSF and IL-5, which are critical for eosinophil development and survival, and IL-6, IL-10 and IL-13 [180]. Degranulation is also a characteristic of activated mast cells, which release mediators such as histamine and heparin. Excessive activation of mast cells is central to the pathogenesis of IgE-mediated diseases like hypersensitivity reactions, including rhinitis, urticarial and asthma [181].

Tregs cells are able to regulate allergic reactions through different mechanisms. They can suppress the effector function and allergen-induced degranulation of activated immune cells such as mast cells [182] and inhibit the influx of eosinophils and effector T cells into inflamed tissues [183]. Treg cells can also promote the generation of tolerogenic DC phenotypes via CTLA-4 [117], thus blocking the development of allergic reaction. In addition, by inhibiting allergen-specific Th2 cells [119] and by directly acting on B cells, Tregs can block excessive production of IgE [184].

1.4 IL-23 and its receptor IL-23R

IL-23 is an APC derived pro-inflammatory mediator member of the IL-12 family of cytokines. Both IL-12 and IL-23 are heterodimeric molecules, sharing the IL-12p40

subunit, that in IL-12 is covalently bound to IL-12-p35 [185], while in IL-23 is linked to the IL-23p19 subunit [186] (Fig. 1.3). Despite their structural similarity, IL-23 and IL-12 induce different inflammatory responses. All three subunits are expressed by DCs and macrophages, and a tight regulation of p35 and p19 dictates whether IL-12 or IL-23 is expressed [187]. Sensing of microbial molecules by toll-like receptors (TLRs) induce DCs and macrophages to produce IL-12 and IL-23 [188], although certain stimuli induce preferential expression of one over the other. As an example, engagement of TLR4 in human DCs induces a preferential expression of IL-12, while TLR2 agonists induce IL-23 [189]. Preferential production of IL-23 was also shown to be induced by nucleotide oligomerization domain 2 (NOD2) agonists [190] and by activation of the fungal beta-glucan receptor Dectin-1, both pathways that depend on CARD-9 signaling [191]. In inflammatory conditions, endogenous pathways also contribute and reinforce production of IL-23 in APCs. Activation of the CD40-CD40L pathway by T cells has been shown to sustain IL-23 expression in a positive feedback loop [192], as do the damage associated patterns like ATP and prostaglandin E2, which act directly on DCs to induce IL-23 production [193-195].

The receptor for IL-23 is composed of the common subunit IL-12Rβ1, which is shared with the receptor for IL-12 and binds p40, and an exclusive IL-23Rα chain, that specifically binds the p19 subunit [196]. Engagement of the IL-23 receptor induces a signalling pathway that is dependent on the activation of Tyrosine kinase (Tyk) 2 and Janus kinase (Jak) 2, which phosphorylate STAT3, and to a lesser degree STAT1, STAT4 and STAT5 [196]. The IL-23R is highly expressed in memory T cells although its expression has been reported on other immune cells like NKT cells, DCs, macrophages and innate lymphoid cells [196, 197]. In T cells, expression of the IL-

23R is induced by IL-6 and IL-21 and it is dependent on RORγt [198]. TGF-β has been shown to have opposing effects on IL-23R expression: low levels of TGF-β promote the upregulation of IL-23R by synergizing with IL-6 and IL-21 and promoting Th17 polarization, while at high concentrations, TGF-β inhibits its expression of IL-23R and induces Treg differentiation [199]. Interaction of IL-23 with its receptor activates STAT3, which in turn promotes the transcription of IL-23R and RORγt, generating a positive feedback loop that stabilizes gene expression required for Th17 cell activation and effector functions [200].

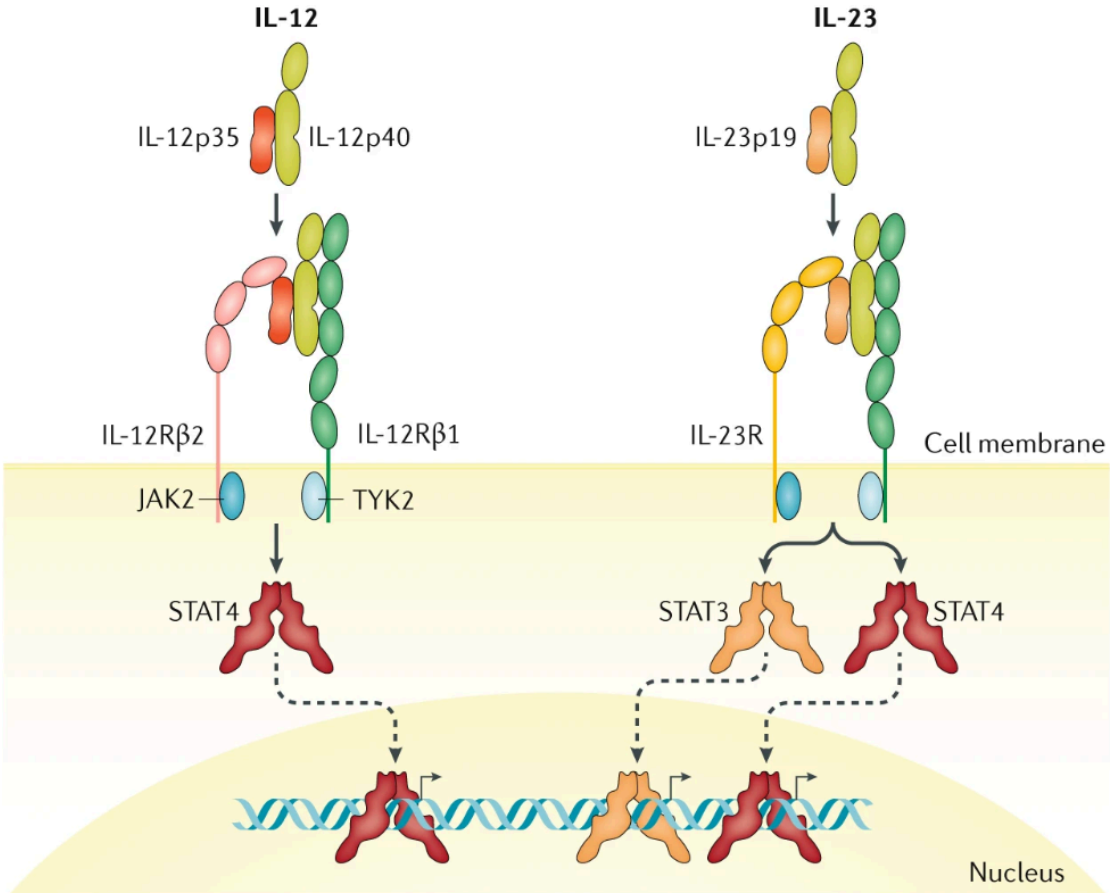


Figure 1.3: Structure and signalling of IL-12 and IL-23 receptors

IL-12 is composed of the two subunits IL-12p40 and IL-12p35, and binds to the IL-12R, which is heterodimer of IL-12Rβ1 and IL-12Rβ2. IL-23 consists of the IL-12p40 and IL-

23p19 subunits and binds to the IL-23R heterodimer composed of IL-23R and IL-12Rβ1. IL-12 signals through activation of STAT4 while IL-23 signals predominantly through STAT3, although IL-23 induced STAT4 signalling has been reported. Figure adapted from *Moschen et al*., 2019 [201].

1.5 The IL-23-Th17 immune axis

Before the discovery of IL-23, many autoimmune and chronic inflammatory disorders like EAE and colitis were thought to be driven by IL-12. Most of these studies were however based on mouse models deficient in IL-12p40 [202], the shared subunit of IL-12 and IL-23, generating confounding results. Early studies on the function of IL-23 showed how it induced IFN-γ in activated T cells, suggesting an overlapping role with IL-12 [186]. However, it was subsequently revealed that IL-23 has a specific effect on T cells in promoting Th17 responses [68]. The first study to describe the specific role of IL-23, rather than IL-12, in driving chronic inflammation was that of Cua *et al.*, who demonstrated how IL-23p19 deficient mice are resistant to EAE [55]. Later studies identified IL-17 producing Th17 cells as the main target of IL-23 in driving organ specific autoimmune inflammation in the central nervous system [56]. In fact, IL-23 induces the expression of proteins that define the Th17 lineage, like IL-17A, IL-17F, GM-CSF, TNF-α and the IL-23R [56]. It was also shown that IL-23, rather then IL-12, is responsible for the development of Th17-driven intestinal inflammation [192].

In vivo studies showed that IL-23 is dispensable for Th17 induction [203] but in the absence of IL-23, there is a reduced activation of Th17 cells in the periphery [63], suggesting a role in expanding and sustaining the activation of Th17 responses. The

role of IL-23 in Th17 responses was also confirmed by the generation of *in vitro* Th17 cells with different combination of cytokines. Culturing of naïve $CD4^+$ T cells with IL-6 and TGFβ gives rise to Th17 cells that, when adoptively transfered, do not induce EAE, while cultures containing IL-6, IL-1β and IL-23 give rise to pathogenic Th17 cells that, upon adoptive transfer, can drive EAE [204]. With a similar *in vivo* approach, another study further supported the hypothesis of a population of IL-23-dependent pathogenic Th17 cells, which are functionally and molecularly different from TGFβ induced non-pathogenic Th17 cells [205]. Therefore, several studies aimed to identify the transcriptional and molecular signature of pathogenic Th17 cells. Single-cell genomics allowed the description of the transcriptional profile of *in vivo* and *in vitro* generated pathogenic Th17 cells, and allowed the identification of molecules, such as the glycoprotein CD5L, that are involved in IL-23 driven Th17 responses [206, 207]. *In vivo* models of disease revealed how the IL-23-Th17 pathway is implicated in many inflammatory disorders, including multiple sclerosis [55, 208] colitis and IBD [64, 209], and arthritis [210].

At steady state, the intestine harbours almost all the Th17 cells of the body [211]. Here, a complex microenvironment where immune cells and microbes coexist can lead DCs to produce IL-23 and trigger Th17 responses. It is now widely accepted that IL-23 is one of the main drivers of T cell dependent colitis. In fact, IL-10 deficient mice, as well as mice treated with an IL-10R blocking antibody and infected with the pathobiont *Helicobacter hepaticus,* develop an IL-23 dependent colitis characterized by activation of IL-17 and IFN-γ producing $CD4^+$ T cells [64, 212]. The adoptive transfer of naïve $CD4^+$ T cells in immunodeficient *Rag1*$^{-/-}$ mice also leads to IL-23 dependent accumulation of Th17 cells and severe inflammation of the intestine [209].

In vivo studies showed how several pro-inflammatory cytokines traditionally associated with Th1 and Th17 responses such as IFN-γ, IL-17A, IL-1β and TNF-α are decreased in the intestine when IL-23 is blocked [64]. Similar results were obtained when *Rag1*$^{-/-}$ mice were adoptively transferred with CD4^{+} naïve T cells from I0-deficient mice and concomitantly administered recombinant IL-23: expression of IL-17A, IFN-γ, IL-1β and TNF-α in the colon were increased and colitis exacerbated [64]. IL-23 has been shown to induce colitis but it is also required for the maintenance of intestinal inflammation, as neutralization of IL-23 with monoclonal antibodies reversed active colitis in a model of adoptive T cell transfer [213].

All together, these studies showed how IL-23 driven T cell dependent colitis is characterized by the accumulation of Th1/Th17 cells. However, which T cell derived effector cytokine mediates the inflammatory response in colitis has not been fully established yet. In fact, blocking of inflammatory mediators like IFN-γ gives opposing results depending on the disease model and experimental conditions: in *H. hepaticus* induced colitis, IFN-γ neutralization protects from colitis [36], while the adoptive transfer of IFN-γ deficient naïve T cells does not prevent the development of intestinal inflammation [37]. Similarly, IL-17A expression has been associated with development of DSS induced colitis [214] and the blocking of IL-6 and IL-17A was shown to be protective in a model of T cell transfer colitis [64]. In contrast, it has been reported that loss or inhibition of IL-17A or IL-17RA signalling in different models of T cell dependent experimental colitis results in worsening of disease [215, 216], indicating a possible protective role of IL-17A in those settings. Others reported that blocking of IL-17A and IL-17F results in amelioration of colitis, while blocking of IL-17A or IL-17F alone was inefficient in protecting from intestinal inflammation [217, 218].

These studies suggest that not one but rather a combination of cytokines contributes to the IL-23-Th17 driven response. We cannot however exclude that yet unknown factors could be fundamental drivers of IL-23-Th17 induced colitis.

Despite its association with autoimmune and chronic inflammatory responses, the IL-23-Th17 immune pathway has been shown to be critically involved in host defence against pathogens and fungal infections. In mice, IL-23 activated Th17 cells have a protective role against *Citrobacter rodentium* infection, a bacteria that resembles the symptoms of entheropathogenic *Escherichia coli* infection. Bacterial clearance in *C. rodentium* infected mice is dependent on Th17 derived IL-22 that induces production of the antimicrobial proteins RegIIIβ and RegIIIγ in epithelial cells [81]. A functional IL-23-Th17 response has also been shown to be critical for protection against *Klebsiella pneumoniae* infection, as *p19*$^{-/-}$ infected mice showed an impaired production of IL-17A and IL-17F in the lung and substantial mortality to a sublethal dose of *K. pneumoniae* [219]. Similar results have been observed in mice infected with *Mycoplasma pneumoniae*: Il-23p19 blocking reduces the ability of Th17 to produce IL-17 and leads to impaired clearance of the bacteria [220]. IL-23p19 deficient mice are susceptible to oropharingeal candidiasis, a disease caused by the opportunistic fungus *Candida albicans*, as they fail to induce a protective Th17 response [221]. The critical role of IL-23 in protecting from *C. albicans* has been shown in other settings such as the skin, where optimal resolution of *C. albicans* infection is dependent on IL-23p19 and $CD3^+$ T cell derived IL-17A [222]. As previously mentioned, IL-17A deficiency in $CD4^+$ T cells exacerbates T cell transfer colitis [215], and this has also been shown for T cell derived IL-22 [80].

Together these data suggest a complex IL-23-dependent network in intestinal immunity, that mediates both protective and pathologic responses. Under homeostatic

conditions, low levels of IL-23 contribute to intestinal host defense and barrier function through T-cell activation and production of IL-17 and IL-22. However, when homeostasis is lost, inflammatory stimuli induce increased production of IL-23 and other inflammatory mediators that lead to the development of chronic inflammation involving the activation of Th1 and pathogenic Th17 responses.

Therefore, IL-23-elicited Th17 cells have a dichotomous nature and it remains unclear what determines their fate as pathogenic mediators or barrier protective cells [211].

1.6 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group of chronic and relapsing inflammatory disorders that affect the gastrointestinal tract [223]. It encompasses three main phenotypes, Crohn's disease (CD), ulcerative colitis (UC), and IBD unclassified (IBDU) [224]. IBD is a major global healthcare burden with increasing incidence and prevalence worldwide [225]. Although the aetiology of the disease is yet to be fully understood, many factors have been identified as contributing to disease pathogenesis, including complex interactions between genetic [226] and environmental factors [227]. The chronic and relapsing intestinal inflammation that characterizes IBD causes extensive tissue damage, including fistulizing and stricturing in CD, and sees life-threatening episodes of acute UC [224]. While CD can affect any region of the intestine, UC is restricted to the rectum and colon. In CD, inflammation is discontinuous and transmural, while UC involves continuous inflammation that is typically confined to the mucosa and submucosa [3]. Immunologically, CD is characterized by high expression of IFN-γ [35], IL-12 [228, 229] and T-bet [39] in the intestine of patients. In contrast, UC patients have increased levels of IL-5 and IL-13 in the intestine [35, 49].

Genome-wide association studies (GWAS) have identified more than 230 susceptibility loci in human IBD, the majority being in non-coding intronic and intergenic regions [230-232]. Candidate genes within these loci suggest a role for epithelial barrier integrity, bacterial handling, and innate and adaptive immune dysregulation. Fine mapping of IBD associated loci allowed the identification of eighteen single variants, including protein-coding variants in *NOD2*, *IL23R*, *CARD9*, *SMAD3*, and *IFIH1*[233]. The evidence of *IL23R* as a risk locus for both CD and UC comes from the combination of GWAS [234] and functional studies. SNPs in the IL-23R gene conferring increased susceptibility to disease are thought to act as gain-of-function mutations; as an example, a variant in the 3'-untranslated region of *IL23R* has been described to induce loss of miRNA regulation and enhanced protein production [78]. Interestingly, a mutation of the IL-23R gene (variant R381Q) has been associated with protection from CD [234]: stimulation of T cells with IL-23 from patients carrying the protective allele showed a decreased Th17 activation, suggesting that the R381Q variant is a loss-of-function mutation [235, 236].

IL-23 signalling is a relevant immune pathway in the intestine and has been associated with the development of pathogenic responses in several experimental models of colitis. In fact, *Rag1*$^{-/-}$ mice also deficient in IL-23p19 do not develop colitis upon adoptive transfer with naïve T cells [209]. Similarly, transfer of naïve T cells lacking the IL-23 receptor did not induce colitis, while transfer of *in vitro* polarized antigen-specific Th17 cells into SCID mice caused severe intestinal inflammation that could be prevented by an anti-IL23p19 antibody treatment [218].

IL-23 drives the activation of pathogenic Th17 cells that induce intestinal inflammation through production of pro inflammatory cytokines such as GM-CSF and IFN-γ, that in turn drive myeloid cell activation [237], and can also indirectly promote the development of intestinal inflammation by inhibiting the regulatory activity of Treg cells [238].

Clinical observations further supported an involvement in the IL-23-Th17 pathway in IBD. CD is associated with increased expression of IL-23[228] and accumulation of IL-23 producing $CD14^{+}$ intestinal macrophages has been identified in CD patients [239]. In addition, IBD patients show increased production of IL-17A and IL-22 by T cells [240, 241].

IBD is also associated with dysbiosis, a reduced diversity or altered composition of gastrointestinal bacterial communities. This change in the microbiota can disrupt host-microbe homeostasis and lead to the development of chronic inflammation. CD and UC patients have a reduction in *Bacteroidetes* and *Lachnospiraceae* [242], a lower proportion of *Firmicutes* and increased *Gammaproteobacteria* and *Enterobacteriaceae* [243-245]. Despite these associations, no single microbe has been proven to cause IBD and whether dysbiosis contributes to the development of disease, or is the result of a deranged immune response towards the commensal flora, is yet to be determined [246].

With the introduction of biological agents, therapeutic options for IBD patients shifted from non-specific immunomodulators such as glucocorticoids, thiopurines and methrotrexate toward pathway-specific anti-inflammatory approaches. The introduction of anti-TNF-α therapies have opened a new era in the treatment of IBD, with the chimeric antibody infliximab being the first shown to be beneficial in the

treatment of patients [247, 248]. In order to reduce immunogenic responses to the chimeric antibodies, fully humanized antibodies were developed. Adalimumab, a humanized anti–TNF-α monoclonal antibody showed efficacy to both CD and UC patients [249, 250], as did certolizumab pegol, a humanized anti-TNF antibody Fab′ fragment conjugated with a polyethylene glycol molecule [251-253]. Golimumab, another humanized anti–TNF-α monoclonal antibody, was shown to be effective in the treatment of UC patients. However, between 10 and 30% of patients with IBD do not have a clinically relevant response to currently available TNF inhibitors, and up to 50% of responders will lose response over time [254, 255]. Therefore, alternative targeted therapies are needed for the treatment of IBD. The role of IL-12 and IL-23 in intestinal inflammation provided a solid rationale for the development of blocking agents for IBD. Ustekinumab is a fully human anti-p40 IgG1antibody that targets IL-12p40, thus blocking both IL-12 and IL-23, which was approved for CD treatment in 2016 [256]. Several trials tested the efficacy of IL-23-specific blocking agents: risankizumab, a humanised monoclonal antibody that targets IL-23p19 [257], and brazikumab (or MEDI2070), an anti-IL-23p19 fully human IgG2 monoclonal antibody , reached phase III and phase IIa clinical trials respectively and showed clinical efficacy on CD patients, while mirikizumab is being studied in patients with moderate-to-severe UC (NCT02589665). As IL-17A and IL-17F have been implicated in the induction of colitis, they have also been considered promising therapeutic targets [258]. However, the therapeutic blocking of IL-17A with the monoclonal antibody Secukinumab showed no beneficial effect in CD and induced severe side effects in some treated patients [259]. Similar results were obtained by a trial that tested the therapeuthic blockade of IL-17RA with Brodalumab, the common receptor for both IL-17A and F, as patients in the treatment group developed

worsening of disease [260]. The lack of efficacy of these therapies is possibly due to the blockade of the barrier promoting effect of IL-17 in the intestine. In fact, IL-17A and IL-17F function is highly context dependent, as both pathogenic and protective effects have been reported.

1.7 Experimental models of Inflammatory Bowel disease

Animal models are fundamental for understanding the complexity of the systemic and mucosal immune response of IBD, and are useful for testing therapeutic concepts. Due to the genetic and physiological similarities between mice and humans and the availability of genetic tools, mice are the current standard for the development of models of colitis. Hundreds of mouse models of intestinal inflammation have been developed, including infection models, chemically induced models and immune-activation models, and each recapitulates certain aspects of human IBD. Although none of the available models fully represents the complexity of human IBD, they are powerful tools for understanding the cellular and molecular networks of the intestinal immune system.

1.7.1 Adoptive T-cell transfer

Functional studies aimed at addressing the role of different subsets of T cells in the development of inflammatory responses identified populations of T cells with the ability to both induce and prevent the intestinal inflammation when adoptively transferred into immune deficient recipient hosts. Transfer of naïve $CD4^+$ T cells ($CD4^+CD45RB^{high}$ T cells) from donor mice into syngeneic immunodeficient SCID or *Rag1*$^{-/-}$ recipient mice causes wasting disease and colonic inflammation that develops

5 to 10 weeks after the transfer. In contrast, transfer of mature $CD4^{+}CD45RB^{low}$ T cells or co-transfer of both naïve and mature T cells into the recipient mice did not cause colitis [85, 261]. This was shown to be due to the fact that the mature T-cell population contains Treg cells that expand in the intestine and suppress inflammation, while the naïve T-cell population does not include such cells, thus driving intestinal pathology [116]. Treg cells exert their suppressive function and antagonize effector T cells in the intestine through multiple mechanisms, including production of IL-10, TGF-β, and IL-35 [262]. In fact, in the adoptive transfer model, treatment with anti-IL-10 receptor or anti-TGF-β antibodies [263, 264], or adoptive transfer of $CD4^{+}$ T cells deficient in IL-10 or TGF-β into *Rag1*$^{-/-}$ recipient mice [264, 265], results in development of colitis.

In T cell transfer colitis, the inflammatory response is characterized by dense cellular infiltrates in the lamina propria, epithelial cell hyperplasia and depletion of goblet cells. Pathology affects the whole length of the colon, and it is considered a Th1/Th17 type of response, as it is associated with high levels of cytokines such as IFN-γ, TNF-α and IL-17A [209]. IFN-γ and TNF-α play a crucial role in the development of disease, as blocking of these cytokines with antibody treatment prevented induction of colitis [34]. Although the majority of T cells in the lamina propria of transfer colitis mice are IFN-γ^{+} Th1 [266], the T cell responses in adoptive T cell transfer is very plastic and appearance of a Th1/Th17 mixed population producing both IFN-γ and IL-17A has been demonstrated [267, 268]. This pathogenic T cell response is also known to be induced by IL-23, as IL-23p19 deficient *Rag1*$^{-/-}$ mice do not develop colitis upon transfer of naïve T cells [209], and adoptive transfer of naïve T cells lacking the IL-23 receptor did not lead to disease [268]. Further evidence of the Th17 nature of the

response was provided by studies that showed how the transfer of IL-17A-producing antigen-specific Th17 cells into SCID mice cause severe colitis that can be prevented by an anti-IL23p19 antibody [213], while naïve T cells deficient in RORγt fail to induce colitis in *Rag1*$^{-/-}$ hosts [218]. In addition to driving pathogenic Th17 responses, IL-23 has been shown to induce inflammation in T cell transfer colitis also by inhibiting regulatory Treg responses. *Rag1*$^{-/-}$ mice reconstituted with IL-23R deficient naïve T cells display increased number of Foxp3^{+} Tregs in the colon and have milder colitis [268].

In T cell transfer colitis, the development of disease is microbe dependent, as adoptive transfer in germ-free hosts does not lead to inflammation [269], suggesting that an epithelial barrier malfunction leads to the entry of components of bacterial origin in the lamina propria that can drive inflammation.

Although a powerful model to recapitulate many of the features of IBD, adoptive T cell transfer is not a flawless system. Immune responses in adoptively transferred *Rag1*$^{-/-}$ mice lack the contribution of a variety of cell types such as B cells, CD8^{+} T cells and NKT cells, immune components that play important roles in intestinal immunity. In addition, this model depends on cell expansion in a lymphopenic host, leading to an abnormally high level of proliferation that is far from a physiological T cell response.

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

Helicobacter hepaticus is a microaerophilic gram-negative spiral bacterium that colonizes the crypts of the caecum and colon of mice [270]. It was first isolated from the livers of A/JCr mice suffering from chronic hepatitis [271]. *H. hepaticus* is an opportunistic microbe, as it can chronically colonize the intestine of WT mice, but

induces intestinal inflammation only in immunocompromised strains [272-274]. The disease is characterized by epithelial cell hyperplasia, goblet cell depletion and leukocyte infiltration. Infection with *H. hepaticus* leads to inflammation of the colon and cecum (typhlocolitis) in IL-10-deficient mice and this can be reproduced in WT infected mice by treatment with an anti-IL-10R antibody that blocks IL-10R signaling [212]. *H. hepaticus* infection drives the emergence of antigen-specific IL-10 producing $CD4^+$ T cells with regulatory capacity, suggesting that deficiency in the IL-10 signalling pathway impairs a regulatory component of the intestinal immune system that would normally prevent inflammation upon *H. hepaticus* infection [275]. In recent work, our lab showed how a large polysaccharide produced by *H. hepaticus* engages TLR2 signalling in macrophages and induces a MSK/CREB-dependent anti-inflammatory signature, leading to IL-10 production [156].

H. hepaticus driven intestinal inflammation in IL-10 deficient mice or in the presence of IL-10R blockade induces a mixed Th1/Th17 response, with high levels of both IFN-γ and IL-17A [212]. IFN-γ has been demonstrated to be functional in driving inflammation, as treatment with IFN-γ blocking antibodies protects from disease [36], while IL-17A is suggested to have a protective role, as blocking of IL-17A exacerbates disease [276]. Interestingly, *H. hepaticus* driven colitis correlates with the emergence of double expressing IFN-γ^+ IL-$17A^+$ $CD4^+$ T cells in the intestine, although IL-17A fate-mapping mice showed how IL-17 expression in T cells is lost with the progression of disease [277].

The inflammatory response is IL-23 dependent, as *Il23r*$^{-/-}$mice are protected from disease [40], and $CD11c^+$ macrophages are the main source of IL-23 [9].

This model allows the study of both the adaptive and innate arm of the intestinal immune system in immune-competent mice, and its features closely resemble human IBD. However, blocking of the IL-10R signalling, one of the main pathways in the induction of intestinal tolerogenic responses, represents a caveat to this system.

1.7.3 DSS induced colitis

The intestinal epithelial barrier has the fundamental role of keeping the commensal microbes separated from the underlying lamina propria and an intact intestinal epithelium has been demonstrated to be important for the prevention of mucosal inflammation [278]. Breaches in the epithelium can lead to the entry of commensal microorganisms into the lamina propria and can induce an inflammatory response.

This mechanism is what drives inflammation in dextran sulphate sodium (DSS) colitis, where a toxic sulphated polysaccharide causes epithelial damage and results in alteration of immune homeostasis. DSS is usually administered to mice in the drinking water and results in acute inflammation of the colon, loss of crypts, ulcers and infiltration of granulocytes. The disease develops even in the absence of T cells, as administration of DSS to *Rag1*$^{-/-}$ mice leads to activation of an innate response that is sufficient for disease development [279]. In this model, macrophages are a source of pro-inflammatory cytokines such as IL-1β, TNF-α, GM-CSF and IL-6, and the like that orchestrate the inflammatory response [280]. In addition, it was shown that macrophage and DC derived CXCL1 and CXCL2 attract and activate neutrophils via CXCR2, as CXCR2-deficient mice or treatment with CXCR2 blocking molecules, results in a lower susceptibility to DSS induced colitis [281-283].

Several studies revealed how disruption of the TLR2 and TLR4 signalling pathways exacerbates DSS colitis, as macrophages depend on these pathways to promote

epithelial proliferation and restoration, limit the translocation of bacteria [284, 285] and recruit neutrophils to the site of inflammation via MIP-2 production [286].

In DSS colitis, TLR activation also induces inflammasome responses, resulting in the secretion of IL-1β and IL-18, cytokines that are involved in maintaining intestinal homeostasis. Mice lacking components of the inflammasome machinery have a decreased expression of IL-1β and IL-18 and are more susceptible to DSS colitis, displaying systemic dispersion of commensal bacteria, enhanced leukocyte infiltration and increased production of chemokines in the colon [287, 288]. Further suggesting a role for bacterial sensing in the development of DSS colitis is the observation that abnormalities in the NOD2 signalling pathway lead to increased susceptibility to DSS induced colitis with associated changes in the intestinal microbiome [289, 290].

Although the disease is driven by disruption of the epithelium and an innate response, T cell responses can exacerbate the inflammatory response with production of both Th1 and Th2 cytokines such as IFN-γ, IL-4 and IL-5 [291].

DSS has provided a useful system to study how the immune system maintains epithelial barrier integrity and intestinal homeostasis. However, it is a model with limitations as the sole epithelial breach and translocation of microbes is unlikely to be the main cause of human IBD [292]. In addition, this model is characterized by a marginal T cell response, while in human IBD T cell responses play a pivotal role in driving disease.

1.7.4 Citrobacter rodentium induced colitis

The mouse-restricted pathogen *Citrobacter rodentium* colonizes the intestinal mucosa by forming attaching and effacing (A/E) lesions [293]. It was identified as the

pathogenic agent of transmissible murine colonic hyperplasia and is the only known naturally occurring A/E enteropathogen of mice [294]. Infection with *C. rodentium* is used as a model of human enteropathogenic A/E infections such as *Escherichia coli* infections [294]. *C. rodentium* preferentially colonizes the distal large intestine and lies in close proximity to the apical surface of superficial enterocytes, without extending to the intestinal crypts [295]. Infection in immunodeficient mouse strain such as *Rag1*$^{-/-}$ is lethal, and these mice display increased bacterial colonization of crypts and translocation when compared to wild-type mice [295].

Upon infection with *C. rodentium*, epithelial cells are activated and release anti-microbial proteins, serum amyloid A, and reactive oxygen species as a first line of defense [296]. Subsequently, innate immune cells like macrophages and DCs use toll-like receptors to recognize and respond to *C. rodentium* infection. While phagocytes including CX3CR1^{+} macrophages, CCR2^{+} macrophages and CD11b^{+}F4/80^{+}Gr1^{+} monocytes release proinflammatory cytokines, phagocytose and lyse *C. rodentium*, DCs migrate to the draining lymph nodes to induce the response of lymphocytes [297-299]. In particular, Notch2-dependent CD11b^{+}CD103^{+} DCs are an important source of IL-23, a cytokine that has been shown to be protective in this infection model [299]. In fact, IL-23 induces the production of IL-22 from ILC3 and Th17 cells, and IL-22 has emerged as one of the main protective cytokine in this model [81, 300]. IL-22 induces epithelial cells to produce o antibacterial proteins such as S100A8, S100A9, RegIIIβ, and RegIIIγ, and promotes epithelial barrier integrity via the upregulation of the tight junction claudin-1 [301]. S100A8 and S100A9 heterodimerize to form calprotectin, which sequestrates manganese and zinc, vital supplements for some bacteria including *C. rodentium* [302]. RegIIIβ binds to lipid A

of LPS and has bactericidal properties against Gram-negative bacteria but not Gram-positive bacteria [303], while RegIIIγ binds to peptidoglycan and is bactericidal against Gram-positive bacteria but not Gram-negative bacteria [304].

C. rodentium is widely studied model of A/E enteropathogenic infections and represents an important inflammation model for understanding the dynamics of type 3 immunity in host defence.

1.8 Aims of the thesis

The immune balance between pro-inflammatory and regulatory immune responses is fundamental to maintain homeostasis at barrier sites. This is especially true in the intestine, where a strict regulation is needed to maintain the balance between different T helper subsets and avoid the development of chronic inflammation. Loss of homeostasis can in fact give rise to deranged inflammatory responses that often lead to pathology. Evidence from pre-clinical research and genetic studies in humans suggest that IL-23 is one of the main players involved in controlling T cell inflammation at barrier sites. However, the molecular and cellular mechanisms that regulate IL-23 driven T cell responses in the intestine are still unclear. Therefore, the aims of this thesis are:

- To identify IL-23-controlled molecules in Th17 cells and test their colitogenic potential
- To gain further insight into the mechanisms used by Tregs to maintain homeostasis at barrier sites
- To evaluate the functional effects of IL-23 on intestinal Th2 cells

2 Chapter 2: Materials and methods

2.1 Mice

Wild type C57BL/6 (WT), B6.*Rag1*$^{-/-}$, B6.SJL-CD45.1, B6.*IL-23R*$^{gfp/gfp}$ (*Il23r*$^{-/-}$), B6.*IL-23R*$^{gfp/+}$, B6.129S4-Timp1tm1Pds/J (*Timp1*$^{-/-}$, Jackson laboratories), B6.129S4-Ltb4r1tm1Adl/J (*Ltb4r1*$^{-/-}$, Jackson laboratories), *CD4*^{Cre}*xDgat1*$^{fl/fl}$ and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice were bred and maintained under SPF conditions in accredited animal facilities at the Kennedy Institute of Rheumatology, University of Oxford, UK.

B6.129S4(Cg)-Dgat1tm2Far/J (Jackson laboratories) mice were crossed to *Foxp3*^{Cre} and *CD4*^{Cre} mice to obtain the *CD4*^{Cre}*xDgat1*$^{fl/fl}$ and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ strains.

Both females and male mice were used in the experiments. Mice were age- and sex-matched, and were at least 6 weeks old when used. Mice were negative for *Helicobacter* spp. and other known intestinal pathogens. All procedures were conducted in accordance with the UK Scientific Procedures Act of 1986 by a Personal Licence holder under a Project License authorized by the UK Home office.

2.2 *In vivo* models

2.2.1 *Helicobacter hepaticus* infection / + anti-IL-10R induced colitis

Helicobacter hepaticus NCI-Frederick isolate 1A (strain 51449) was grown on blood-agar made with laked horse blood supplemented with trimethoprim (5μg/ml), vancomycin (10μg/ml) and polymyxin B (25U/ml) (TVP, Oxoid) under microaerophilic conditions (10% CO2, 10% H2, balance N2) in a CampyPak jar

(Oxoid) at 37°C. After 24 hours, bacteria were transferred from the agar plates into a liquid culture of Tryptic Soy Broth (TSB, Sigma-Aldrich) enriched with 10% Fetal Calf serum (FCS, Sigma- Aldrich) and TVP antibiotics in vented Erlenmeyer flasks (Corning) and grown under microaerophilic conditions at 37°C in a shaking incubator (200rpm). After 24 hours optical density (OD) of the culture was measured at 600nm using a BioPhotometer (Eppendorf). Bacteria were then centrifuged at 3000rpm for 20 minutes and resuspended in PBS for infection.

Mice were infected with $1x10^8$ colony-forming units (CFU) (equal to 1 O.D.) *H. hepaticus* by oral gavage with a 22G curved needle on day 0 and day 1 of the experiment. To induce colitis, mice also received 1mg of anti-IL-10R blocking antibody (clone 1B1.2, produced in-house) via an intraperitoneal injection weekly from day 0. Mice were sacrificed on day 14, unless stated otherwise.

2.2.2 *Citrobacter rodentium* infection

A single colony of B109 *C. rodentium* (nalidixic acid resistant) (ICC169) was cultured in nalidixic acid-supplemented Luria-Bertani broth and grown overnight to saturation. The following day, the culture was diluted to an O.D. of 0.05 and grown to log phase to an O.D. of 1. *C. rodentium* was harvested by centrifugation and resuspension in PBS. Mice were orally gavaged with 10^9 CFU of *C. rodentium* in 200 ml of PBS. Following infection, mice were weighed every day and culled if weight loss exceeded 20% of starting weight.

2.2.3 DSS induced colitis

Mice were administered 2.5% DSS (wt/vol) (Sigma) through drinking water for five days. DSS was then removed to avoid excessive ulceration of the lamina propria.

Mice were sacrificed 9 days after the start of the experiment. Loss of body weight was assessed every day and mice were culled if weight loss exceeded 20% of starting weight.

2.2.4 Adoptive T cell transfer

$4x10^5$ $CD4^+CD25^-CD45RB^{hi}$ FACS sorted T cells were injected via the intra-peritoneal injection into *Rag1*$^{-/-}$ recipient mice. In co-transfer experiments $2x10^5$ $CD4^+CD25^-CD45RB^{hi}$ T cells from each source were mixed and injected i.p. into *Rag1*$^{-/-}$ hosts. Weight loss was monitored once a week for the duration of the experiment. Mice were sacrificed upon development of clinical signs of disease, namely weight loss (20% of body weight at initiation of experiment) or diarrhea. Following sacrifice the whole colon was removed and pieces of colon approximately 1 cm in length were taken from the proximal, mid and distal portions. Samples were transferred immediately into 3.6% (w/v) formaldehyde solution.

2.2.5 Calcipotriol induced dermatitis

The Calcipotriol model of atopic dermatitis was adapted after the method of Li *et al.* [305]. Calcipotriol was dissolved in ethanol at a concentration of 4nmol and 10µl were topically applied to the dorsal and ventral sides of both ears. Treatment was repeated with 20 ul solution per ear every 24 hours for 7 consecutive days. 24 hours after last application mice were sacrificed. Ear thickness was measured prior to first application and daily before treatment with a dial thickness gauge (Mitutoyo UK). The mean of two measurements was determined. Mice were weighed daily.

2.2.6 Histological assessment

To determine the extent of the intestinal pathology, colons were washed in PBS and 1cm pieces were cut from the proximal, middle and distal colon. The samples were fixed in 10% formalin solution, which was prepared by diluting 37% formaldehyde (Merck) in PBS, for more than 24 hours. Caeca were cut open longitudinally, washed, placed on blotting paper (Invitrogen) and fixed as above. Paraffin-embedded sections were cut into 4μm sections and stained with haematoxylin and eosin by the Kennedy Institute's histology facility. Pathology was scored in a blinded fashion by two researchers. Each sample was scored from 0 to 3 for the four following criteria: degree of epithelial hyperplasia and goblet cell depletion; infiltration of leukocytes in the lamina propria; area of tissue affected; and presence of markers of severe inflammation such as crypt abscesses, submucosal inflammation, and ulcers. Scores for each criterion were added to give the overall inflammation score for each mouse sample, ranging from 0 to 12. The total colonic score was calculated as the average of the individual scores from the sections of proximal colon, mid-colon, and distal colon. Pictures of the sections were acquired on an Olympus BX51 microscope.

2.2.7 Endoscopic assessment

To track disease progression, high-resolution mouse endoscopy was performed as described in Koelink et al. [306]. Mice were anesthetized with 4% isofluorane gas mixture. The endoscope (HOPKINS Optik 64019BA, Karl Storz Aida Vet) was inserted via the rectum and the colon was insufflated with air to facilitate entry of the endoscope. The endoscope was inserted 4 cm in length and a high-resolution video was recorded during the procedure. Following endoscopic assessment mice were

allowed to recover from anesthesia in a recovery cage. The pathology score was measured as described in the publication by Koelink et al. [306].

2.3 Cell isolation

2.3.1 Isolation of leukocytes from spleen and lymph nodes

Spleens and peripheral lymph nodes (including superficial cervical, axillary, brachial, inguinal lymph nodes and mesenteric lymph nodes) were collected from the sacrificed mice. Organs were mashed through a 70µm strainer and washed with PBS/0.1% BSA/5mM EDTA and centrifuged at 1500rpm for 7 minutes. Cells from the spleens were additionally incubated with 1ml ACK lysis buffer for 3 minutes at room temperature to lyse the red blood cells, followed by a wash with PBS/0.1% BSA/5mM EDTA. Single cell suspension was finally resuspended in the appropriate volume of PBS/0.1% BSA/5mM EDTA for subsequent analysis.

2.3.2 Isolation of leukocytes from the colon

For isolation of lamina propria leukocytes (LPL), colon was collected from the sacrificed mice, opened longitudinally and washed in PBS/0.1% BSA. Colon was cut into 3 pieces and stored on ice in 50ml conical tubes containing PBS/0.1% BSA/5mM EDTA. To remove epithelial cells, colons were washed in 20ml pre-warmed RPMI/10%FCS/5mM EDTA for 30 minutes at 37°C in a shaking incubator (200RPM). An additional 15 minutes wash was performed on the cells at the same conditions. To remove residual EDTA, cells were washed in RPMI/10%FCS/15MmHepes for 10 minutes and then transferred to a fresh 50 ml tube. 10ml Digestion media (RPMI/10%FCS/15mM Hepes + 100U/ml collagenase

VIII (Sigma-Aldrich) + 40ug/ml DNase (Roche)) was added and tubes incubated horizontally at 37°C in a shaking incubator at 200rpm for 60 minutes. Cell suspension was filtered through a 70um cell strainer and digestion stopped by addition of 20 ml of ice cold RPMI/10%FCS/5mM EDTA. Cells were centrifuged at1500 for 7 minutes at 4°C and then separated using a two-layers Percoll (GE Healthcare) (40%/80%) density-gradient. After centrifugation at 900G for 20 minutes without brake lymphocytes were collected at the 40%-80% interface. The cell suspension was washed thoroughly in ice-cold PBS/0.1%BSA/5mM EDTA and cells re-suspended in the appropriate buffer for further analysis.

2.3.3 Isolation of leukocytes from the small intestine

For isolation of lamina propria leukocytes (LPL), small intestine was collected from the sacrificed mice, opened longitudinally and washed in PBS/0.1% BSA. Mucus was removed by rubbing the tissue on adsorbent paper. Small intestine was cut into 1 cm pieces and stored on ice in 50ml conical tubes containing PBS/0.1% BSA/5mM EDTA. To remove epithelial cells, small intestines were washed in 20ml pre-warmed RPMI/10%FCS/5mM EDTA for 30 minutes at 37°C in a shaking incubator (200RPM). An additional 15 minutes wash was performed on the cells at the same conditions. To remove residual EDTA, cells were washed in RPMI/10%FCS/15MmHepes for 10 minutes and then transferred to a fresh 50 ml tube. 20ml Digestion media (RPMI/10%FCS/ + 48U/ml collagenase VIII (Sigma-Aldrich) + 16ug/ml DNase (Roche)) was added and tubes incubated horizontally at 37°C in a shaking incubator at 200rpm for 30 minutes. Tubes were then shaken to facilitate disruption of the tissue and the cell suspension was filtered through a 70um cell strainer and digestion stopped by addition of 20 ml of ice cold

RPMI/10%FCS/5mM EDTA. Cells were centrifuged at1500 for 7 minutes at 4°C and then separated using a two-layers Percoll (GE Healthcare) (40%/80%) density-gradient. After centrifugation at 900G for 20 minutes without brake lymphocytes were collected at the 40%-80% interface. The cell suspension was washed thoroughly in ice-cold PBS/0.1%BSA/5mM EDTA and cells re-suspended in the appropriate buffer for further analysis.

2.3.4 Isolation of leukocytes from the lung

Lungs were collected and cut into small pieces. The processed tissue was digested in RPMI/10% FCS/15mM Hepes with 100U/ml collagenase VIII (Sigma-Aldrich) at 37 °C in a shaking incubator at 200rpm. Cells were then filtered through a 70μm cell strainer, and the digestion was stopped by addition of ice-cold RPMI/10% FCS/5mM EDTA. The cells were centrifuged at 1500rpm for 7 minutes and the single cell suspension was resuspended in an the appropriate volume of PBS/0.1% BSA/5mM EDTA for subsequent analysis.

2.3.5 Isolation of leukocytes from the skin

This protocol requires the cells to be constantly on ice when not digesting in order to increase the final yield. Both ears were harvested, split in half and cut into very small pieces with surgical scissors. Ears were digested in a 50ml conical tube containing 4ml of RPMI/10% FCS/15mM Hepes and 3mg/ml collagenase D (Roche), 450ug/ml Liberase TL (Roche) at 37°C in a shaking incubator for 80 minutes. Cell suspension was aspirated and pushed through an 18 G needle 3-5 times before filtering the cell suspension through a 70um cell strainer. Digestion was stopped by adding 4°C cold PBS/0.1% BSA/5mM EDTA and cell suspension centrifuged at 1500rpm for 7

minutes. Leukocytes were then separated on a two-layers Percoll-density gradient as described in section 2.2.2. Cells from the 40/80% Percoll interface layer were collected, washed, and resuspended in the required volume of PBS/0.1% BSA/5mM EDTA for subsequent analysis.

2.4 Flow cytometry and cell sorting

2.4.1 Restimulation of cells for intracellular cytokine staining

Single cell suspensions were plated at $1x10^6$ cells/well in a 96-well round bottom plate (Corning) in complete RPMI-1640 medium supplemented with 10% FCS, 2mM L-glutamine (Life Technologies), 0.05mM 2-mercaptoethanol (Life Technologies), 100U/ml each of penicillin and streptomycin (Sigma-Aldrich). 0.1μM phorbol 12-myristate 13-acetate (PMA) and 1μm ionomycin together with 10μg/ml brefeldin A (all from Sigma-Aldrich) were added to the culture and cells were incubated for 3.5 hours at 37°C before further processing for flow cytometric analysis.

2.4.2 Surface staining

Staining and washings were performed in PBS/0.1% BSA/5mM EDTA. All centrifugations were performed at 1500rpm for 7 minutes. For myeloid cell stainings, a 10-minute incubation with anti-CD16/CD32 (eBioscience) was performed to minimize non-specific antibody bindings. Appropriate antibody mixes were added together with the Fixable Viability Dye eFluor 780 (eBioscience) and incubated for 20minutes at 4°C in the dark followed by two washes. For surface-only stainings, cells were directly analyzed on a flow cytometer.

2.4.3 Intracellular staining

Prior to the intracellular staining, the surface staining protocol was performed as described in the previous section. For transcription factors stainings, cells were fixed and permeabilised using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer's instructions. Firstly, cells were resuspended in 50µl fixation/permeabilisation buffer for 30 minutes in the dark at room temperature, followed by a wash in 1X permeabilisation buffer. For cytokine stainings, cells were fixed and permeabilised using the Cytofix/cytoperm Kit (BD Bioscience): cells were resuspended in 100µl fixation/permeabilization buffer for 20 minutes at 4°C, followed by a wash in 1X permeabilisation buffer. Antibody cocktail was prepared in the appropriate permeabilisation buffer and cells were incubated for 20 minutes at 4°C followed by a wash with the PBS/0.1% BSA/5mM EDTA to remove any residual antibody. Cells were analyzed on a flow cytometer immediately for the stainings including transcription factors, or on the following day when cytokine staining was performed.

2.4.4 Flow cytometric analysis

Stained cells were acquired on LSRII or Fortessa cytometers (BD Biosciences) run by the FACSDiva software (BD Biosciences). Before sample acquisition, spectral overlap compensation using single-coloured control CompBeads (BD Biosciences) was performed. The results were analyzed using FlowJo (Tree Star).

2.4.5 Cell sorting

Single cells suspensions were obtained as described in section 2.3. Where needed, $CD4^+$ T cell enrichment using the EasySep Mouse CD4+ T Cell Enrichment Kit

(STEMCELL Technologies) was performed as per the manufacturer's instructions. Cells were then stained with appropriate antibody mixes for 20 minutes at 4°C in the dark, washed with PBS and resuspended in PBS/0.1% BSA/5mM EDTA and filtered. 4',6-diamidino-2-phenylindole (DAPI) was added for viability assessment. Cells were sorted into PBS/10% FCS or directly into RLT for mRNA analysis. Cells were sorted using FACS AriaIII (BD Biosciences).

Specificity	**Fluorochrome**	**Clone**
CD11b	BV510	M1/70
CD25	AF647, BV421	7D4
CD4	BV785, BV605	RM4-5
CD44	BV510	IM7
CD45	AF600, BV650	30-F11
CD45.1	BV510	A20
CD45.2	PE-Cy7	104
CD45RB	AF647	C363-16A
CD49b	PE	R1-2
CD62L	FITC	MEL-14
CD8	PE	53-6.7
cKit	PerCP/Cy5.5	ACK2
FOXP3	FITC, PE-Cy7	FJK-16s
GATA3	ef660	TWAJ
GM-CSF	PE, PerCP/Cy5.5	MP1-22E9
IFN-γ	ef450	XMG1.2
IgE	BV605	R35-72
IL-10	PB	JES5-16E3
IL-13	FITC	eBio13A
IL-17A	BV605	TC11-18H10.1
IL-17F	PE, AF647	9D3.1C8
IL-22	PE	1H8PWSR
IL-4	BV421	11B11
IL-5	APC	TRFK5
RORγt	PE	Q31-378
SiglecF	BV421	E50-2440
ST2	biotinilated	101001B
TCR-β	PE-Cy7, PE-Dazzle, BV510	H57-597
TCR-γδ	PE-Cy7	GL3

TNF-α	AF700	MP6-XT22
Viability Dye (fixable)	eF780	-

Table 2.1 List of antibodies used for flow cytometric analysis and cell sorting

2.5 Analysis of gene and protein expression

2.5.1 RNA extraction

Tissue samples were homogenized in soft tissue homogenizing CK14 columns (Stretton Scientific) in 700µl RLT lysis buffer (Qiagen) for 30s using a Precellys24 homogenizer (Precellys). RNA was extracted with the RNeasy Mini RNA extraction kit (Qiagen) according to the manufacturer's instructions. RNA was finally quantified using a NanoDrop spectrophotometer (Thermo Scientific) and immediately used for cDNA synthesis or stored at -80 °C.

2.5.2 Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

cDNA was synthetized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

cDNA synthesis was performed in a C1000 Touch™ Thermal Cycler (BIO-RAD) and cDNA was stored at -20 °C.

RT-qPCR reactions were performed using mouse TaqMan gene expression assay probes (Life Technologies) with 2µl of cDNA and 2X Precision Fast qPCR mastermix with ROX low (PrimerDesign) in a final volume of 6µl. All reactions were performed in duplicates in a 384-well plate on the ViiATM 7 Real-Time PCR System (Applied Biosystems). For expression analysis, the expression levels of the gene of

interest were normalized to that of the housekeeping gene *Hprt* . Gene expression was calculated using the 2-ΔCt method.

Gene	Taqman Probe
Csf2	Mm01290062_m1
Dgat1	Mm00515658_g1
Dgat2	Mm00499536_m1
Gata3	Mm00484683_m1
Ifng	Mm01184322_m1
Il10	Mm00439614_m1
Il13	Mm00434204_m1
Il17a	Mm00439618_m1
Il17f	Mm00521423_m1
Il1b	Mm00434228_m1
Il1rl1	Mm00516117_m1
Il22	Mm01226722_m1
Il23r	Mm00519943_m1
Il33	Mm00505403_m1
Il4	Mm00445259_m1
Il5	Mm00439646_m1
Il6	Mm00446190_m1
Ltb4r1	Mm00521839_m1
Maf	Mm02581355_s1
RegIIIb	Mm00440616_g1
RegIIIg	Mm00441127_m1
Rorc	Mm01261022_m1
S100a9	Mm00656925_m1
Smox	Mm01275475_m1
Timp1	Mm01341361_m1
Tnf	Mm00443258_m1
Tslp	Mm00498739_m1

Table 2.2 List of Taqman primers used for RT-qPCR assays

2.5.3 Protein quantification by Enzyme-linked immunosorbent assays (ELISAs)

Serum IgE concentration was quantified by ELISA. Blood was collected in BD Microtainer® blood collection tubes (BD) and serum isolated by centrifugation.

Samples were diluted at the appropriate dilution and IgE concentration measured using the Mouse IgE ELISA MAX™ Deluxe Set (Biolegend), following the manufacturer's instructions. Absorbance was read at 450 nm by a spectrophotometric plate reader SPECTROstar Nano and analysis was performed using SPECTROstar Nano Data Analysis software.

2.6 *In vitro* cell cultures

For *in vitro* cultures, $CD4^{+}CD44^{lo}CD62L^{hi}$ T cells were sorted from the spleen and peripheral lymph nodes as described above. Cells were plated at a density of $2x10^{5}$ per well in flat-bottom plates (Corning) in complete RPMI-1640 media supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml of penicillin and streptomycin, with plate bound anti-CD3 (2.5 μg/ml, eBioscience) and soluble anti-CD28 (3 μg/ml, eBioscience).

To induce differentiation into specific T helper subsets, naïve T cells were cultured in the presence of lineage-specifying cytokines and blocking antibodies: Th0 (IL-2, anti-IFNγ, anti-IL-4), Th1 (IL-2, IL-12, anti-IL-4), Th2 (IL-2, anti-IFNγ, IL-4, -/+IL23, -/+IL-33), Th17/23 (anti-IFNγ, anti-IL-4, TGFβ, IL-1β, IL-6, IL-21, -/+ IL-23), Treg cells (IL-2, anti-IFNγ, anti-IL-4, TGFβ). Three days after plating, cells were lifted from anti-CD3 and anti-CD28 and seeded in a new plate with fresh media supplemented with the appropriate cytokines. Cells were harvested for analysis at day 5.

Cytokines and blocking antibodies were added to the media at the following concentrations: anti-IL-4 (10 ng/ml, Biolegend); anti-IFNγ (10ng/ml, Biolegend); TGFβ (200 pg/ml for Th17, 2ng/ml for Treg, RnD Systems); IL-23 (20 ng/ml, RnD

Systems); IL-2 (100 U/ml, Peprotech); IL-1β (20 ng/ml, Peprotech); IL-6 (20 ng/ml Peprotech); IL-21 (20 ng/ml, Peprotech); IL-33 (10ng/ml, Biolegend).

2.7 Statistical analysis

Statistical analyses of experiments were performed in Prism (GraphPad Software, SD, USA). Mann-Whitney U test, student's T test or one-way ANOVA in combination with adequate post tests were performed. Differences were considered statistically significant when $p<0.05$. Significance is indicated as * $p<0.05$; ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3 Chapter 3: Molecular analysis of the IL-23R signaling pathway in intestinal T-helper cells

3.1 Introduction

IL-23 is a cytokine known to have a central role in driving autoimmune and chronic inflammatory diseases. Its inflammatory potential has been described in murine models of autoimmune encephalomyelitis, arthritis and intestinal inflammation. Several studies on murine models of colitis have revealed how IL-23 is required for $CD4^+$ T cell mediated and bacterially triggered inflammation in the intestine. High levels of IL-23 expression are also associated with patients with active IBD [228, 239], and mutations in the *IL23R* gene have been linked to increased susceptibility to the development of chronic intestinal inflammation. Altogether, these observations suggest that IL-23 is an essential player in driving inflammatory responses and that if not tightly regulated, it can lead to colitis in both mice and humans [307].

In 2005, Langrish *et al.* identified IL-17 producing Th17 cells as the main cellular population to respond to IL-23 in the context of central nervous system autoimmune inflammation [56]. Later studies on mouse models of colitis revealed the critical role of the IL-23/Th17 pathway in the development of intestinal inflammation [64, 209]. In the intestine, Th17 cells express high levels of the IL-23R, and although IL-23 is not required for Th17 differentiation, exposure to IL-23 sustains their activation and survival. Interestingly, the function of $CD4^+ROR\gamma t^+$ Th17 cells is highly context dependent, as they are involved in both homeostasis and host defence against extracellular bacteria and fungi, but can also drive pathogenic inflammatory responses. Despite numerous studies both *in vitro* and *in vivo*, the differentiation cues

that distinguish homeostasis-promoting Th17 cells from those with pathogenic potential remains an enigma. Along these lines, IL-23 is known to be critically involved in reinforcing Th17–driven responses in the gut, but the molecular mechanism that determines IL-23-induced Th17 intestinal inflammation is still unknown. Therefore, identifying the IL-23 dependent molecular network of activated Th17 cells could reveal therapeutic targets for chronic inflammatory disease.

A number of studies have previously defined the molecular signature of pathogenic Th17 cells, yet only at the mRNA level and mainly using models of EAE rather than colitis [205, 206]. Previous work in our lab aimed to identify IL-23 regulated gene and protein networks in $CD4^+$ T cells with a transcriptomics and proteomics approach. $CD4^+$ naïve mouse T cells isolated from spleens and lymph nodes were cultured in Th17 differentiation medium in the presence or absence of IL-23 and under anti-CD3/anti-CD28 stimulation for five days (Fig. 3.1a). Total proteins extracted from the cell lysates were subjected to trypsin digestion, followed by ultra performance liquid chromatography - mass spectrometry (UPLC-MS) (Fig 3.1b). Peptides representing 3329 proteins were identified in these conditions, 70 of which were found to be upregulated in IL-23 treated vs. untreated cells. Those included IL-17F, a well-known IL-23 target, TIMP1, LTB4R1 and SMOX. An additional experiment was done on Th17 cells already differentiated in the presence of IL-23 for five days, then cytokine-depleted for four hours and re-incubated in the presence or absence of IL-23 for a further 24 hours (Fig 3.1c). In addition to total protein for UPLC-MS, in this experiment mRNA was also isolated for microarray analysis (Fig. 3.1d). Only about a hundred genes were differentially expressed in IL-23 treated cells, with *Il17a* and *Il17f* amongst the top up-regulated genes. Other interesting genes that were

upregulated in IL-23 pulsed cells are *Timp1* and *Smox*, which also came up as enriched targets in the previous experiment, and *Dgat1*. The mining of IL-23 controlled targets in Th17 cells with proteome and transcriptome analysis allowed us to identify molecules that could potentially be involved in the activation and pathogenic potential of Th17 cells. As the IL-23/Th17 axis is a central immune pathway in driving inflammation of the colon, this project aimed to test the pathogenic potential of selected genes and proteins in the intestine.

Therefore, the aims of this chapter are:

- Identify molecules whose expression is controlled by IL-23 in Th17 cells
- Test their pathogenic potential in models of intestinal inflammation

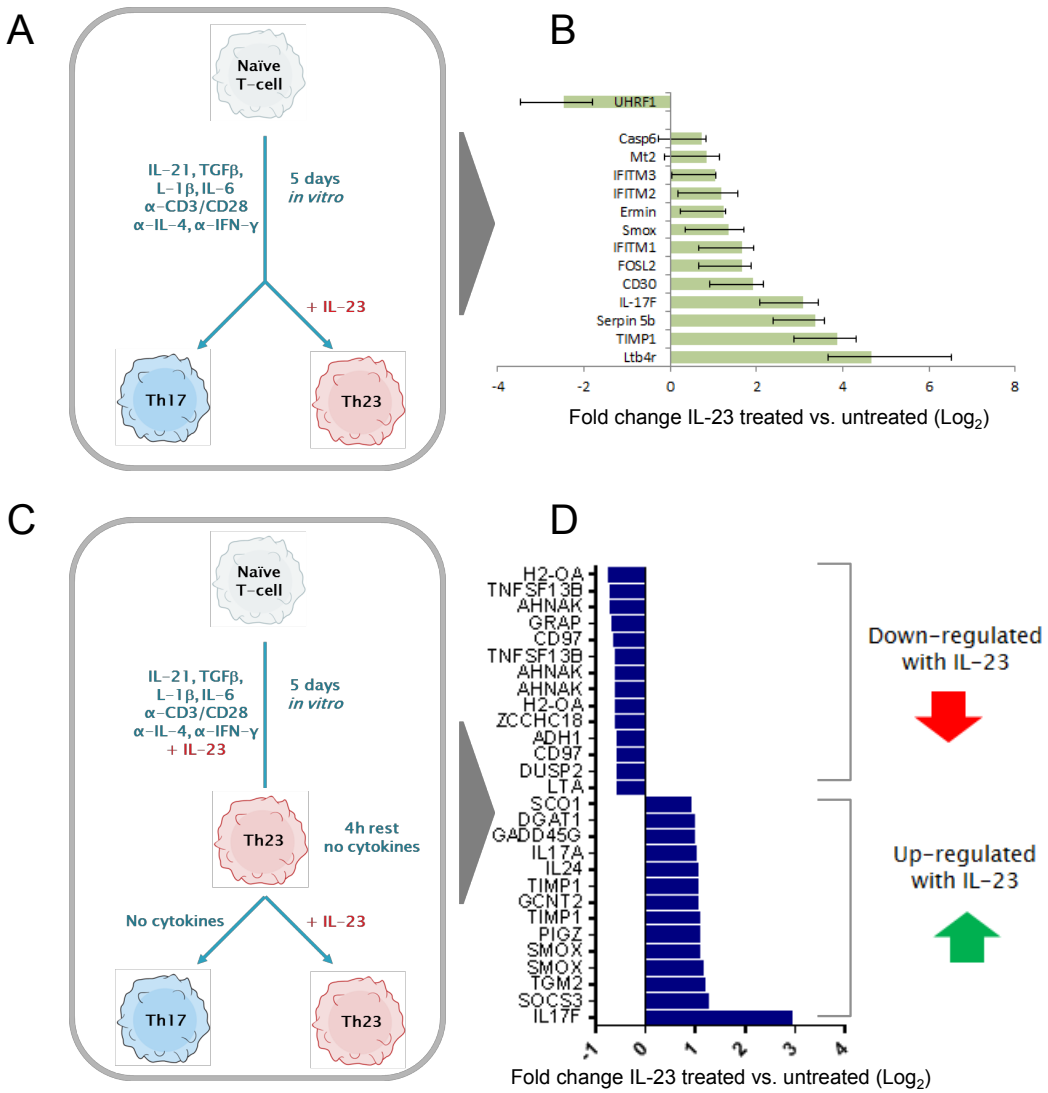


Figure 3.1: Multi-omics approach identifies novel IL-23 targets in Th17 cells

(A) Experimental setup: naïve $CD4^+$ T cells were isolated from C57BL/6 mouse spleens and lymph nodes using a naïve CD4 T Cell isolation kit and plated in Th17 conditioning media, with or without IL-23, for 5 days. **(B)** Total proteins extracted from the cell lysates were subjected to trypsin digestion, followed by UPLC-MS. Data shown as log2 transformed ratios of normalised protein abundances in IL-23 treated vs. untreated samples (average of two biological replicates). **(C)** Experimental setup: naïve $CD4^+$ T cells were plated in Th17 conditioning media for 5 days, cultured for 4 hours in cytokines free media and then incubated with or without IL-23 for additional

24 hours. **(D)** Microarray summary: data shown as log2 fold change gene expression of IL-23 pulsed vs. control. *(Data provided by Dr Grigory Ryzhakov)*

3.2 Results

3.2.1 *In vitro* and *in vivo* validation of IL-23 controlled genes

With our previous transcriptomics and proteomics analysis we identified a set of genes that are expressed by Th17 cells and are controlled by IL-23. To validate whether these genes are indeed differentially expressed in an IL-23-dependent manner we performed a qPCR analysis on fresh Th17 cells treated in the same way as our proteomics and transcriptomic experiments (*data kindly provided by Dr Grigory Ryzhakov)*. WT and *Il23r*$^{-/-}$ naïve CD4^{+} T cells isolated from spleens and lymph nodes were cultured as in Fig. 3.1a for 5 days, and mRNA isolated for qPCR analysis. Expression of *Il17a* and *Il17f* was induced by IL-23 in WT but not in *Il23r*$^{-/-}$ cells. Th17 differentiation in the presence of IL-23 also had an enhancing effect on the expression of *Timp1* and *Ltb4r1*, with *Dgat1* and *Smox* being only marginally induced (Fig. 3.2a). We also tested these genes in the IL-23 pulsed set up as in Fig. 3.1c. The effect of IL-23 on the cultured cells was evident, as genes like *Il17f*, *Il17a* and the *IL-23r* were highly induced. Other genes were confirmed as induced by IL-23 in this setting too: above others *Timp1*, while *Ltb4r1* expression did not change with IL-23 treatment (Fig. 3.2b). In order to translate these findings in an *in vivo* setting, we performed further validation in a mouse model of IL-23 driven inflammation. Infection with *Citrobacter rodentium* is an established model of attaching and effacing (A/E) enteropathogens that resembles *Escherichia coli* infections in humans [294]. C57BL/6 mice infected with *C. rodentium* develop a transient form of colitis [308] characterized by a strong IL-23 driven Th17 response that results in clearance

of the bacteria [309]. In this model, IL-23 induces the activation and production of type-3 cytokines in Th17 cells, and it has been shown how T cell derived IL-22 [81], IL-17A and IL-17F are required for protective immunity [75]. In order to isolate IL-23 responsive cells and identify IL-23 controlled genes, we used an IL-23R reporter strain, the *Il23r*$^{gfp/+}$ mouse [197], where IL-23R expression is coupled with concomitant expression of GFP and therefore traceable. We then infected *Il23r*$^{gfp/+}$ mice with *C. rodentium* and sacrificed them at the onset of inflammation (2 weeks). Leukocytes from the colon lamina propria were isolated and TCRβ^{+}CD4^{+}IL-23R^{+} and TCRβ^{+}CD4^{+}IL-23R^{-} cells were FACS sorted for mRNA extraction (Fig 3.3a). qPCR analysis confirmed that the IL-23R is a good marker to enrich for *bona fide* Th17 cells, as in TCRβ^{+}CD4^{+}IL-23R^{+} cells the expression of Th17 associated genes like the transcription factor *Rorc* and the cytokines *Il17a*, *IL17f* and *Il22* was higher than in the TCRβ^{+}CD4^{+}IL-23R^{-} population (Fig 3.2b, top). Upon infection, the expression of these Th17 genes was enhanced in IL-23R^{+} T cells, indicating an activated status induced by *C. rodentium* (Fig 3.2b, top). We then tested how IL-23R expression and *C. rodentium* infection affected the expression of a set of genes selected from our target mining. *Timp1* expression was not detected in T cells from uninfected mice, but was upregulated upon infection in both IL-23R^{+} and IL-23R^{-} T cells (Fig 3.2b, bottom). *Ltb4r1* was enriched in IL-23R expressing cells and upregulated upon infection, indicating a selective expression in IL-23 responsive cells. (Fig 3.2b, bottom). *Smox* expression was also detected as higher in IL-23R expressing cells, but was not further enhanced by *C. rodentium* infection (Fig 3.2b, bottom). Other genes were not affected by infection or did not seem to be enriched in IL-23R^{+} T cells. Among others, *Dgat1* was expressed at comparable level in T cells isolated from all the groups tested (Fig 3.2b, bottom). These validations allowed us to confirm the

results of our IL-23 target mining, identifying *Timp1*, *Ltb4r1* and *Smox* as possible genes that are involved in the activation and function of IL-23 responsive Th17 cells.

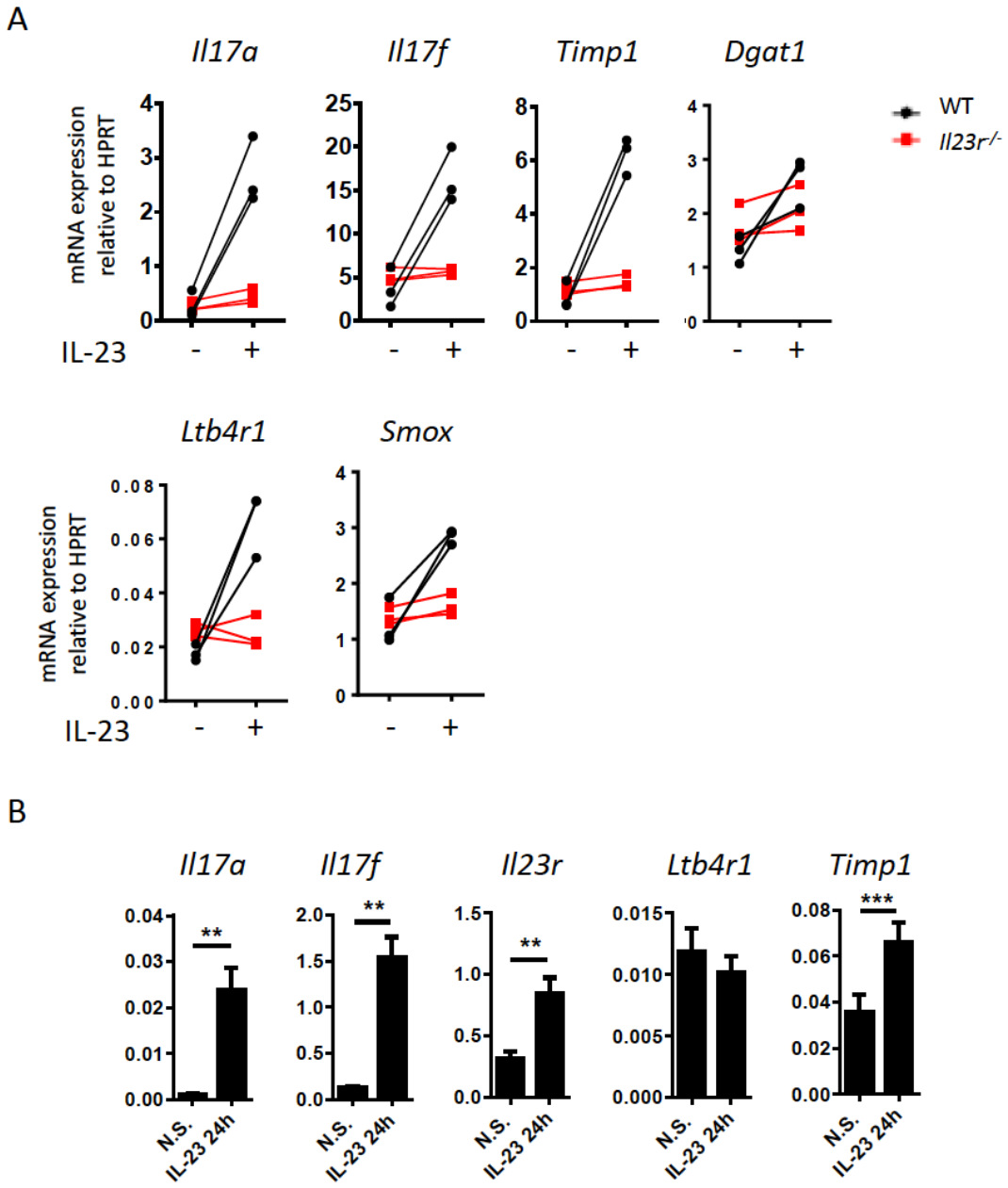


Figure 3.2: *In vitro* validation of IL-23 controlled genes

(A) Naïve $CD4^+$ T cells isolated from spleens and lymph nodes of WT and *Il23r*$^{-/-}$ mice were cultured in Th17 conditioning media with or without IL-23 for 5 days. mRNA expression of selected genes was assessed by qPCR analysis. Each dot represents a biological replicate and lines connect cells isolated from the same mouse. **(B)** Naïve $CD4^+$ T cells isolated from spleens and lymph nodes of WT mice were

cultured in Th17 conditioning media with IL-23 for 5 days. Cells were then rested for 4 hours without cytokines and further incubated in cell culture media with or without IL-23 for 24 hours. mRNA expression of selected genes was assessed by qPCR analysis. Bars represent the mean ±SEM. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. $^{**}p<0.01$, $^{***}p<0.001$. One experiment, n=3. *(Data provided by Dr. Grigory Ryzhakov)*

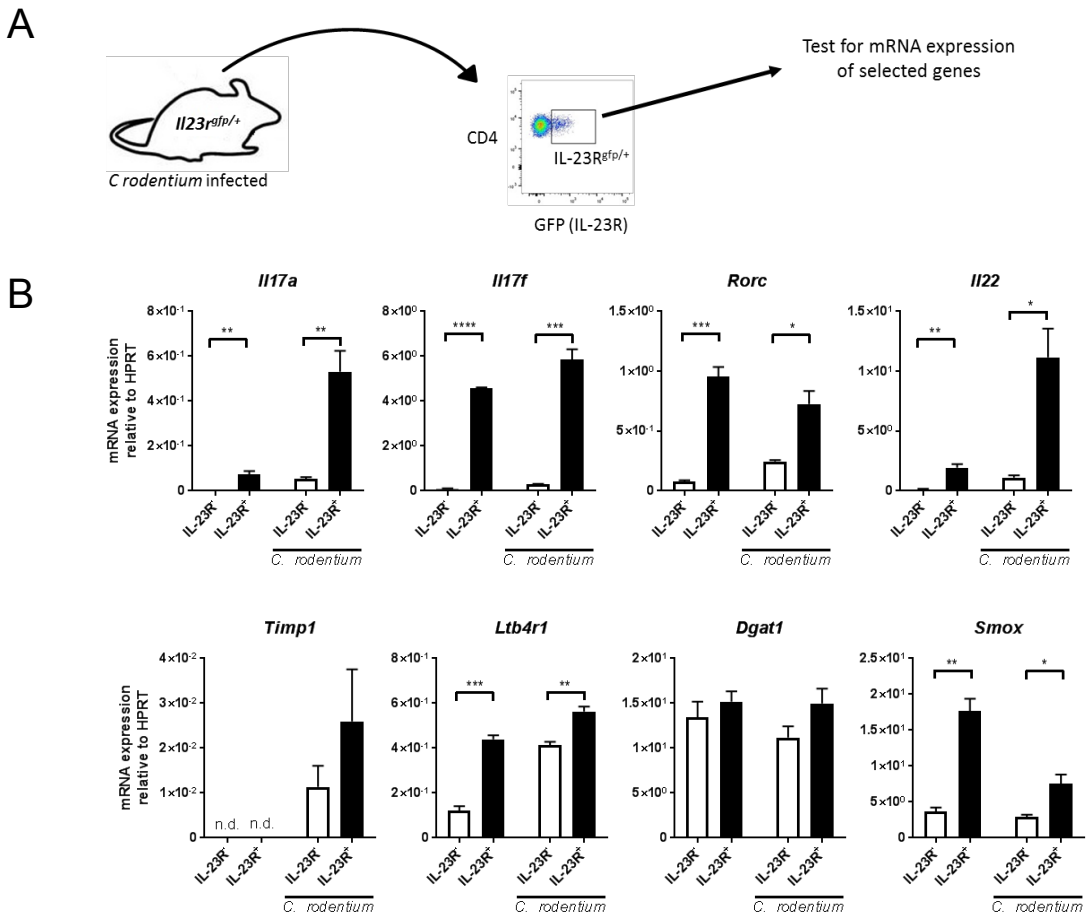


Figure 3.3: Shortlisted IL-23 targets are selectively expressed in IL-23R$^+$ T cells *in vivo*

(A) Experimental layout: *Il23r*$^{gfp/+}$ mice were infected with 5x10^9 CFU/mouse and sacrificed 14 days post infection. LPLs were isolated from the colon, CD4$^+$TCR-β$^+$IL-23$^+$ and CD4$^+$ TCR-β$^+$IL-23$^-$ cells were FACS sorted. **(B)** mRNA expression of

selected genes was assessed by qPCR analysis. Bars represent the mean ±SEM. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05, **p<0.01, ***p<0.001. One experiment, n=3 (3 mice per replicate were pooled)

3.2.2 TIMP1 as a possible mediator of IL-23 driven Th17 responses

One of the genes that our targetome analysis and subsequent validations revealed as controlled by IL-23 is *Timp1*, which encodes the protein tissue inhibitor of metalloproteinase 1 (TIMP1). TIMP1 was first described as a collagenase inhibitor [310], and it is a natural inhibitor of matrix metalloproteinanses (MMPs). It is a two-domain secreted protein [311], known to be involved in the regulation of extracellular matrix turnover [312] and ubiquitously expressed. In addition to its role in inhibiting most of the known MMPs, TIMP1 acts as a signalling molecule and it is able to promote cell proliferation in different cell types via its interaction with surface molecules like CD63 [313], CD82 [314] and LRP1 [315]. Expression of high levels of TIMP1 in the tissue and blood are associated with poor prognosis in every human cancer [316], and several studies indicated an anti-apoptotic function of this protein [317, 318]. A few studies have reported an involvement of TIMP1 in T cell responses. The study by Adamson *et al.* showed how TIMP1 is preferentially expressed in Th1 and Th17 cells and its expression enhances the pathogenic potential of auto reactive T cells in a model of experimental autoimmune encephalomyelitis, a pathology that is IL-23 dependent [197]. *Timp1* expression is also upregulated in patients with IBD [319, 320] and it has been shown to be induced in mouse models of colitis like DSS, TNBS and adoptive T cell transfer [321]. Therefore, we hypothesized that TIMP1 could be a mediator of IL-23 dependent intestinal inflammation. As shown in Fig. 3.2b, *Timp1* expression was induced in $CD4^+$ T cells upon infection with *C.*

rodentium, a pathogen that induces an IL-23 dependent protective Th17 response. We therefore infected C57BL/6 WT mice and *Timp1*$^{-/-}$ mice with 5x10^{9} CFU/mouse of *C. rodentium*. In order to track the progress of inflammation, body weight was measured throughout the course of the experiment, but no significant difference in the change of body weight was observed between WT and *Timp1*$^{-/-}$ mice (Fig 3.4a). When mice showed signs of disease (2 weeks post infection), they were sacrificed for analysis. By assessing the mRNA expression of *Timp1* in the colon of WT mice we confirmed that this gene is upregulated upon infection (Fig. 3.4b), suggesting a possible role in mounting a protective immune response to *C. rodentium*. Both WT and *Timp1*$^{-/-}$ mice showed signs of inflammation in the intestine, as assessed by pathology scoring of H&E stained colon sections, but no difference in the level of pathology was observed between the two groups (Fig. 3.4c). In line with this finding we observed a comparable number of CFUs from the stool, colon and spleen of infected WT and *Timp1*$^{-/-}$ mice (Fig. 3.4d). To assess the effect of TIMP1 deficiency on the activation of CD4^{+} T cells, we performed flow cytometric analyses on colonic lamina propria leukocytes (LPLs). When compared to the WT group, *Timp1*$^{-/-}$ infected mice showed a similar frequency and number of CD4^{+}TCR-β^{+} IL-17A expressing cells (Fig. 3.5). Similarly, frequency and numbers of IL-22 and IFN-γ expressing T cells were comparable between WT and *Timp1*$^{-/-}$ inflamed mice (Fig. 3.5). In order to evaluate the overall immune response in the intestine, we performed qPCR analysis of mRNA isolated from tissue samples of the colon. In both WT and *Timp1*$^{-/-}$ mice, *C. rodentium* infection induced the expression of genes encoding for pro inflammatory cytokines like *Csf2*, *Ifng*, *Il17a*, *Il17f* and *Il22*, although when comparing the expression between the two inflamed groups we did not detect any significant difference (Fig 3.6).

Taken together, these data suggest that TIMP1 expression in $CD4^+$ T is dispensable for mounting a protective immune response against *C. rodentium*.

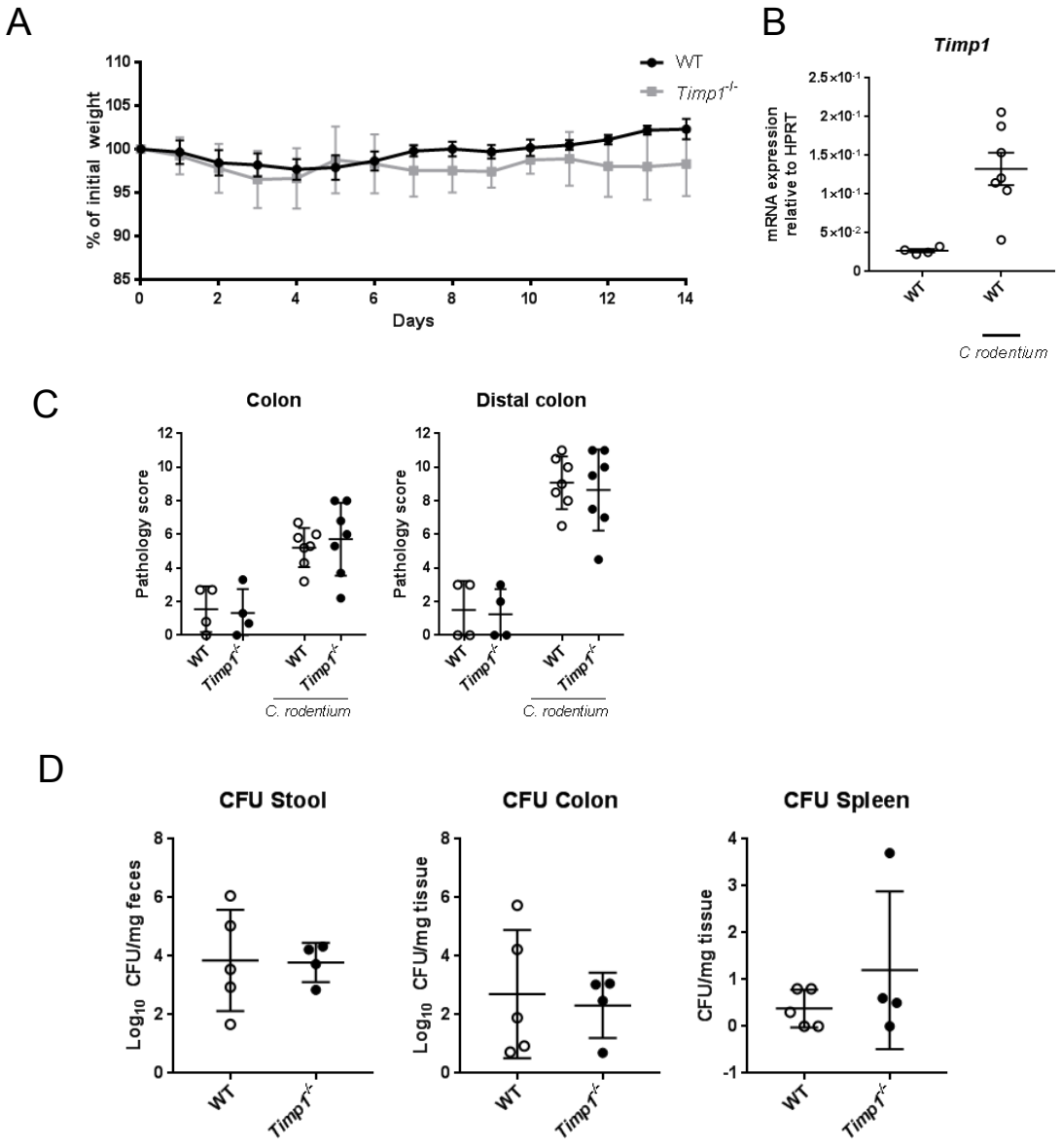


Figure 3.4: TIMP1 is dispensable in the *C. rodentium* model of colitis

WT and *Timp1*$^{-/-}$ mice were infected with 5x10^9 CFU/mouse of *C. rodentium* and sacrificed 14 days post infection. **(A)** Percentage of initial body weight (g), shown as mean of the group with SD. **(B)** mRNA expression of *Timp1* in the colon of WT mice as assessed by qPCR analysis. **(C)** Pathology score as assessed by histology scoring

of the colon. **(D)** Number of CFU/mg of tissue. Bars represent the mean ±SD and each point represents an individual mouse. Data were analysed using GraphPad Prism. Data shown are representative of two independent experiments, n=7 per group.

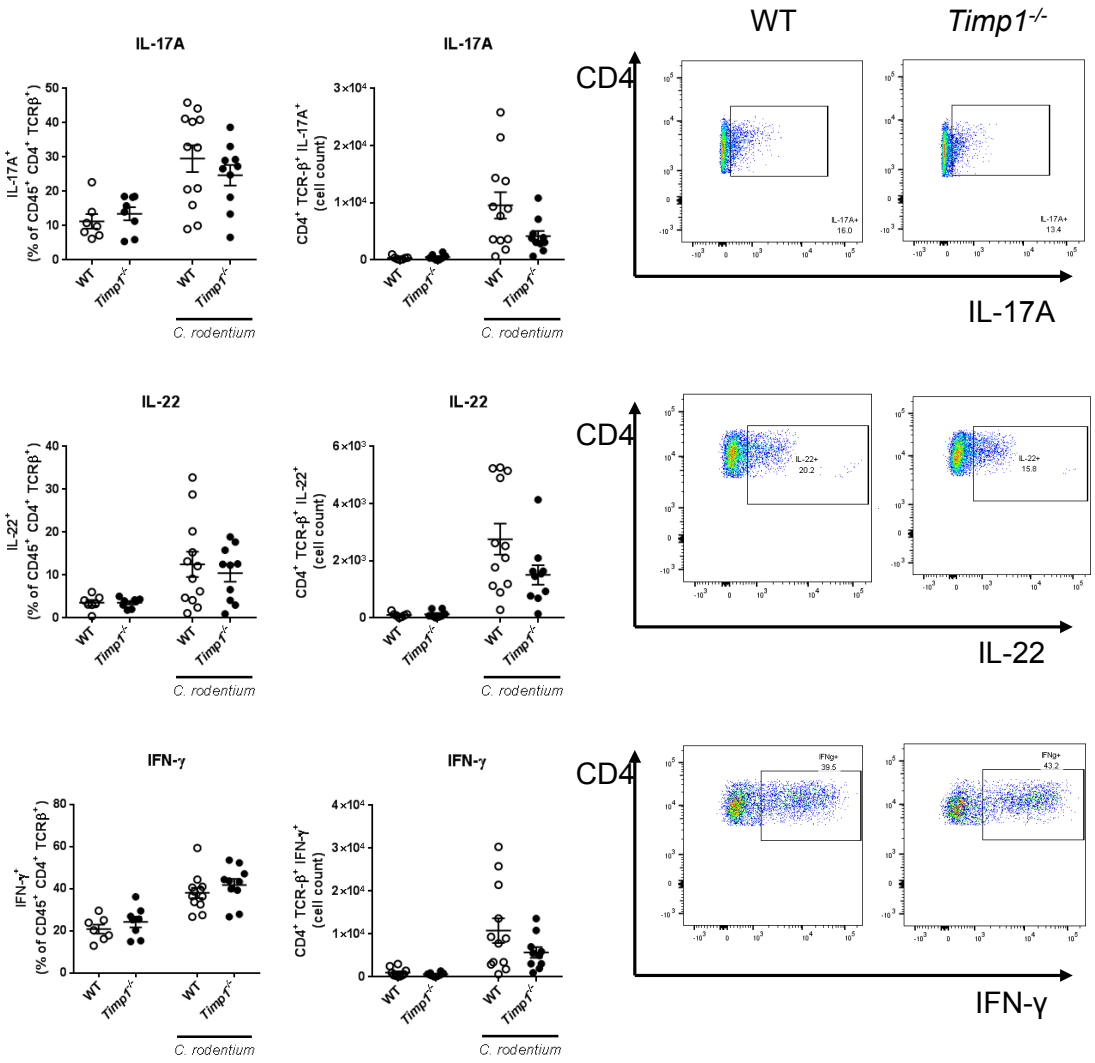


Figure 3.5: *C. rodentium* infected WT and *Timp1*$^{-/-}$ mice display a comparable Th17 response

WT and *Timp1*$^{-/-}$ mice were infected with *C. rodentium* and sacrificed 14 days post infection. LPLs were isolated from the colon for flow cytometric analysis. Frequency and numbers of IL-17A (top), IL-22 (middle) and IFN-γ (bottom) expressing T cells

are represented. Representative flow cytometry plots are also shown. Bars represent the mean ±SEM and each point represents an individual mouse. Data were analysed using GraphPad Prism. Two experiments, n=7 per group.

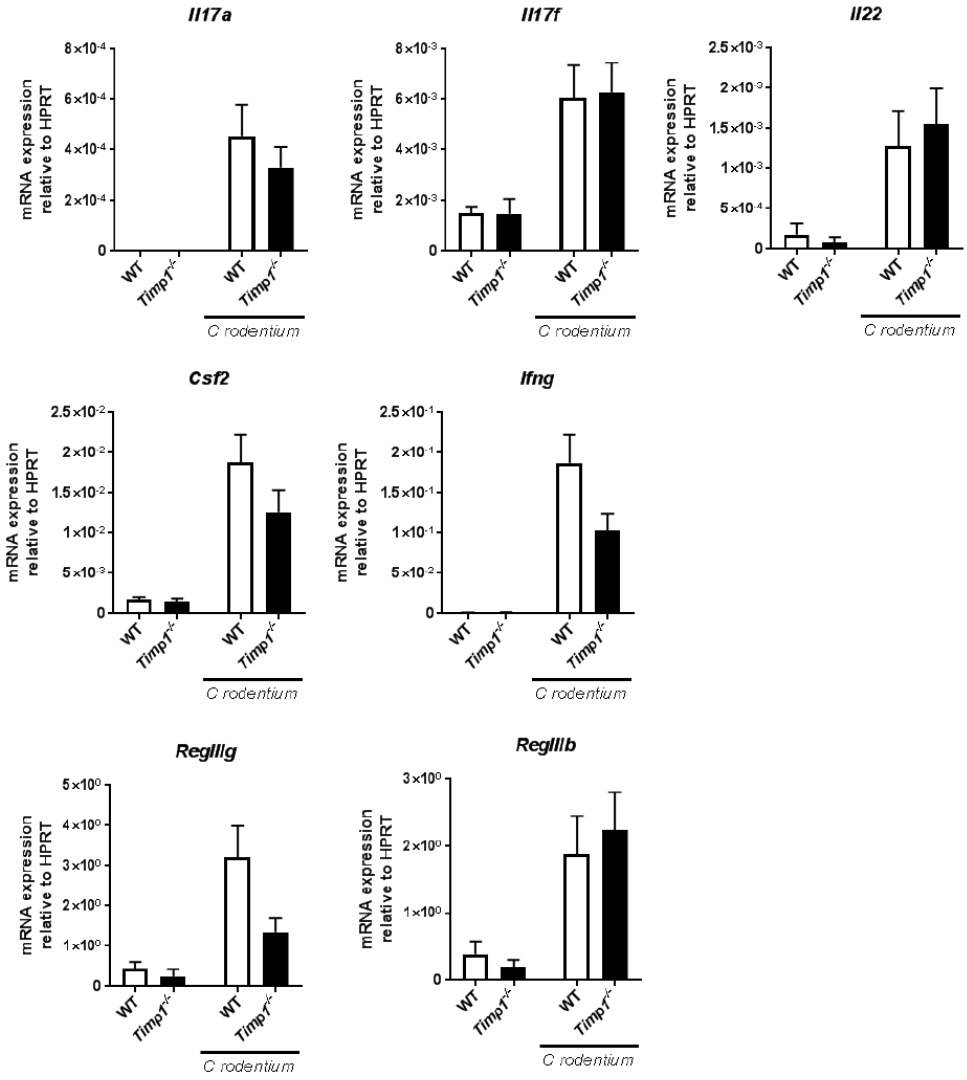


Figure 3.6: TIMP1 deficiency does not affect the overall immune response in *C. rodentium* infected mice

mRNA was purified from the colon of WT and *Timp1*$^{-/-}$ *C. rodentium* infected mice. Gene expression of selected cytokines was assessed by qPCR analysis. Bars represent the mean ±SEM. Data shown are representative of two experiments, n=7 per group.

3.2.3 LTB4R1 as a mediator of IL-23 driven Th17 responses

The target mining described in Fig. 3.1 revealed a set of genes that are directly controlled by IL-23 in Th17 cells. One of these targets is *Ltb4r1*, which encodes for the leukotriene B4 receptor 1, a G protein-coupled transmembrane receptor that has high affinity for the lipid inflammatory mediator Leukotrien B4 (LTB4) [322]. This receptor is expressed by both mouse and human leukocytes, including T cells [323], and it is involved in cell migration and recruitment of leukocytes [324]. In fact, LTB4 is a potent chemoattractant, and LTB4R1 has been shown to mediate migration of *in vitro* differentiated Th17 cells [325] and to exacerbate disease in a model of EAE by regulating the migration of immune cells [326]. In addition, elevated levels of LTB4, the ligand of LTB4R1, have been detected in the cerebrospinal fluid of patients with multiple sclerosis [327], in the synovial fluid of patients with rheumatoid arthritis [328], in the colonic mucosa of IBD patients [329], and in the skin of patients with active psoriasis [330], further suggesting a role of LTB4R1 in the development of IL-23 driven Th17 pathologies.

Our *in vivo* validation in a model of *C. rodentium* infection revealed how *Ltb4r1* expression is enriched in IL-23R expressing T cells in both steady state and infected mice (Fig. 3.2b), suggesting a role for LTB4R1 in IL-23 induced T cell responses. To test this, we infected WT and *Ltb4r1*$^{-/-}$ mice with 5x10^{9} CFU/mouse of *C. rodentium* and sacrificed them at the onset of inflammation (2 weeks). WT and *Ltb4r1*$^{-/-}$ mice showed similar levels of disease, as shown by comparable pathology score and no difference in the variation of body weight (Fig. 3.7a and 3.7b). The bacterial burden and dissemination were also similar between the two genotypes, as shown by the number of CFUs from stool, colon and spleen (Fig. 3.7c). To assess the role of LTB4R1 in T cell activation, we performed flow cytometric analysis and tested for

expression of Th17 associated cytokines. WT and *Ltb4r1*$^{-/-}$ infected mice showed comparable frequency and number of IL-17A, IFN-γ, and IL-22, cytokines that are known to be induced by IL-23 in the context of *C. rodentium* infection (Fig. 3.8). As other immune cells in addition to CD4^{+} T cells can contribute to bacterial clearance, we tested the overall immune response in the intestine by qPCR analysis. Gene expression of type 3 cytokines like *Il17a*, *Il17f*, *Il22*, as well as *Ifng* and antimicrobial proteins such as *S100a9* and *Reg3b* was not significantly different between WT and *Ltb4r1*$^{-/-}$ mice (Fig. 3.9). All together, these findings suggest that, like Timp1, LTB4R1 has a redundant role in the immune response against *C. rodentium*.

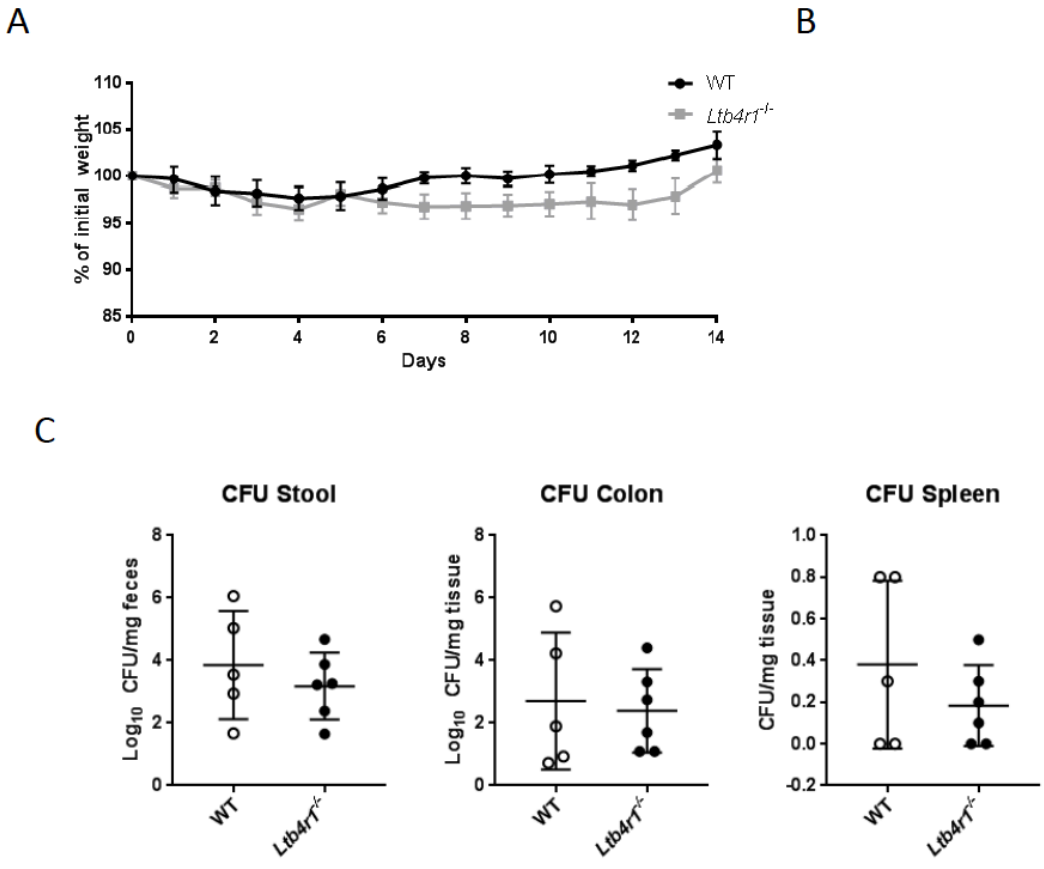


Figure 3.7: LTB4R1 in the *C. rodentium* model of colitis

WT and *Ltb4r1*$^{-/-}$ mice were infected with 5x10^{9} CFU/mouse of *C. rodentium* and sacrificed 14 days post infection. **(A)** Percentage of initial body weight (g), shown as mean of the group with SD. **(B)** Pathology score as assessed by histology scoring of

the colon. **(C)** Number of CFU/mg of tissue. Bars represent the mean ±SD and each point represents an individual mouse. Data were analysed using GraphPad Prism. Data shown are representative of two independent experiments, n=4-6 per group.

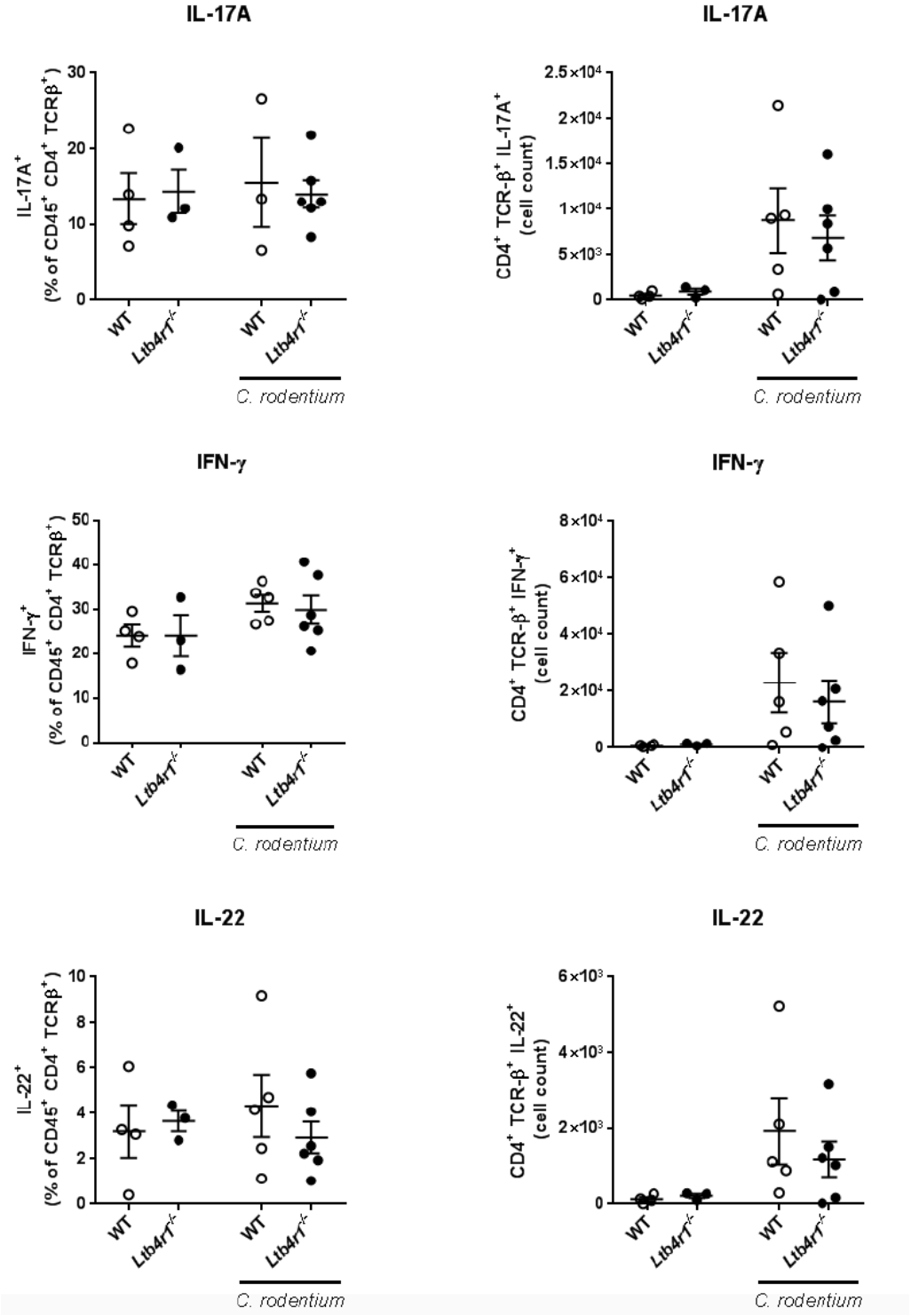


Figure 3.8: *C. rodentium* infected WT and *Ltb4r1*$^{-/-}$ mice mount a comparable Th17 response

WT and *Ltb4r1*$^{-/-}$ mice were infected with *C. rodentium* and sacrificed 14 days post infection. LPLs were isolated from the colon for flow cytometric analysis. Frequency and numbers of the indicated cytokine expressing T cell populations are represented. Bars represent the mean ±SEM and each point represents an individual mouse. Data were analysed using GraphPad Prism. Data shown are representative of two independent experiments, n=4-6 per group.

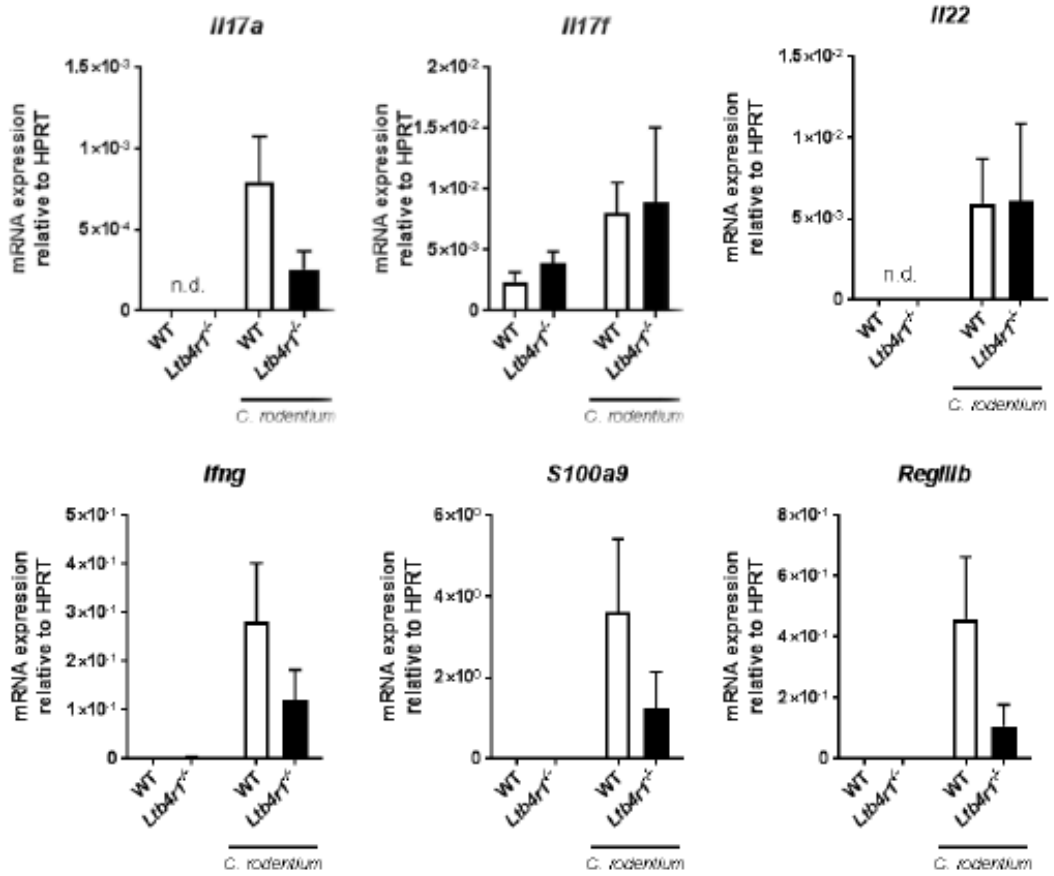


Figure 3.9: LTB4R1 deficiency does not affect the overall immune response to *C. rodentium*

mRNA was purified from the colon of WT and *Ltb4r1*$^{-/-}$ *C. rodentium* infected mice. Gene expression of selected cytokines was assessed by qPCR analysis. Bars represent the mean ±SEM. Data shown are representative of two independent experiments, n=4-6 per group.

3.2.4 TIMP1 and LTB4R1 in a model of chronic colitis

Infection with *C. rodentium* is a commonly used model of acute and self-resolving inflammation of the intestine and its dependence on IL-23 makes it a widespread tool to study Th17 responses. However, IBD is a non-resolving pathology characterized by chronic and relapsing inflammation of the intestine. Therefore, we tested the pathogenic potential of TIMP1 and LTB4R1 in the *H. hepaticus*+anti-IL-10R colitis model, a murine model of disease that resembles most of the main features of human IBD [331]. WT, *Timp1*$^{-/-}$ and *Ltb4r1*$^{-/-}$ mice were infected with *H. hepaticus* and concomitantly treated with anti-IL-10R antibody. The mice were sacrificed on day 14, during inflammation (Fig 3.10a). Mice from all groups developed colitis, as shown by pathology scoring, with WT and *Ltb4r1*$^{-/-}$ mice displaying similar levels of inflammation, while *Timp1*$^{-/-}$ showed significantly lower pathology when compared to WT (Fig. 3.10b). In order to assess in detail the Th17 response, we isolated LPLs from the colon for flow cytometric analysis. Infection with *H. hepaticus* induced expression of pro inflammatory cytokines, but all three groups of inflamed mice showed similar frequency of TNF-α IL-17 and IFN-γ expressing CD4^{+} T-cells as well as IL-17^{+} IFN-γ^{+} double expressing T cells (Fig. 3.10c), a subset of cells that is known to arise in *H. hepaticus* induced colitis [276]. *Timp1*$^{-/-}$ and WT mice displayed a comparable frequency of IL-22 expressing T-cells, while the frequency of IL-22^{+} T cells in *Ltb4r1*$^{-/-}$ mice was lower than in WT mice. Similar results were obtained

when qPCR analysis was performed on the mRNA extracted from colon samples to assess the overall immune response. WT, *Timp1*$^{-/-}$ and *Ltb4r1*$^{-/-}$ expressed comparable levels of the pro-inflammatory cytokines *Ifng*, *Il6*, *Csf2*, *Il1b*, *Tnfa*, *Il17a* at tissue level (Fig. 3.10d).

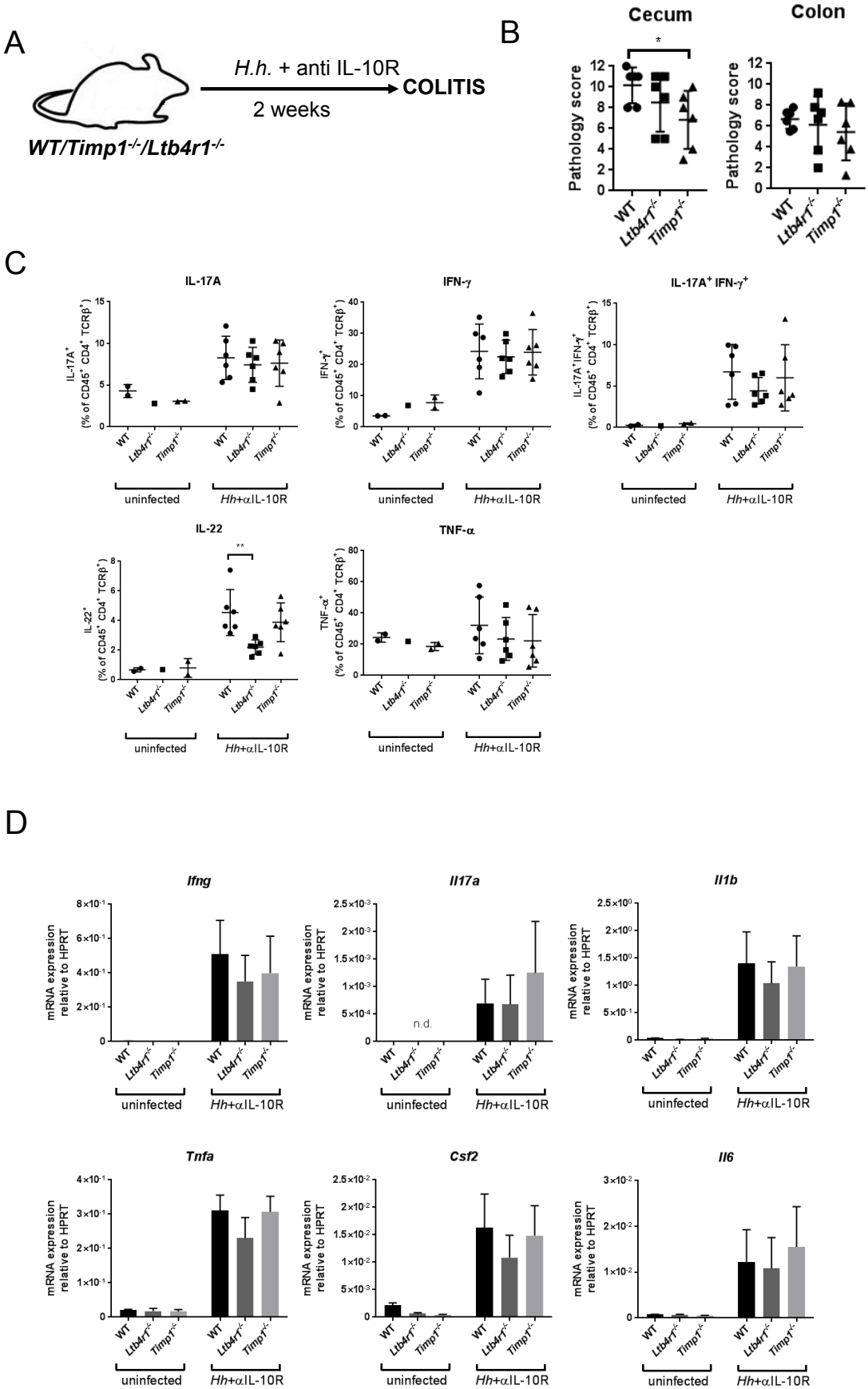

A
H.h. + anti IL-10R
2 weeks
COLITIS
WT/Timp1-/-/Ltb4r1-/-
B
Cecum
Colon
Pathology score
WT
Ltb4r1-/-
Timp1-/-
C
IL-17A
IFN-γ
IL-17A+ IFN-γ+
IL-22
TNF-α
uninfected
Hh+αIL-10R
D
Ifng
Il17a
Il1b
Tnfa
Csf2
Il6
mRNA expression relative to HPRT
n.d.

Figure 3.10: Assessing the T cell response to *H. hepaticus* in TIMP1 and LTB4R1 deficient mice

(A) Experimental layout: WT, *Timp1*$^{-/-}$ and *Ltb4r1*$^{-/-}$ mice were infected with 1x10^{8} CFU of *H. hepaticus* by oral gavage for two consecutive days. In addition, 1 mg of anti-IL-10R blocking antibody was administered by i.p. injection once weekly starting on the day of infection. **(B)** Pathology score was assessed by histology scoring of the colon and cecum. **(C)** LPLs from the colon were isolated for flow cytometric analysis. Frequency of the indicated cytokine expressing cells is shown. **(D)** mRNA was purified from the colon and gene expression of selected cytokines was assessed by qPCR analysis. Bars represent the mean ±SEM and each point represents an individual mouse. Data were analysed using unpaired Mann-Whitney test, using GraphPad Prism. ***p*<0.01. One experiment, n=3-6 per group.

3.2.5 TIMP1 and LTB4R1 deficiency does not impact development of pathology in TCT colitis

In the previous paragraph we described the impact of TIMP1 and LTB4R1 deficiency in the Th1/Th17 response that contributes to pathology in the *H. hepaticus* model of colitis. *Timp1*$^{-/-}$ and *Ltb4r1*$^{-/-}$ mice displayed expression of Th17 cytokines at a similar level as WT mice, suggesting that neither proteins are directly affecting the T cell response to *H. hepaticus*. However, in this system we used full knock-out mice, and since TIMP1 and LTB4R1 are molecules expressed by several immune cell types, this was a limitation that added a confounding effect to our analysis and could have masked a T cell specific phenotype. Therefore, in order to restrict the genetic deficiency to CD4^{+} T cells, we performed an adoptive T cell transfer model of colitis by transferring naïve CD4^{+} T cells isolated from the spleen and lymph nodes of WT,

Timp1$^{-/-}$ and *Ltb4r1*$^{-/-}$ mice into *Rag1*$^{-/-}$ recipients (Fig. 3.11a). The mice were sacrificed at the onset of inflammation (8 weeks) for analysis. Mice transferred with WT, *Ltb4r1*$^{-/-}$ and *Timp1*$^{-/-}$ naïve T cells all developed colitis, as shown by pathology scoring and H&E stained sections of the colon, but no difference in the severity of pathology was observed between the three groups (Fig. 3.11b). Flow cytometric analysis on LPLs isolated from the colon of inflamed mice revealed no difference in the frequency and numbers of IL-17A, IL-17F, IFN-γ and TNF-α, as well as in the frequency of IL-17A^{+} IFN-γ^{+} double expressing T cells (Fig. 3.11c). These findings suggested that, although not ruling out a possible functional role in T cell biology, the expression of TIMP1 and LTB4R1 is not required for T cell mediated pathology in the intestine.

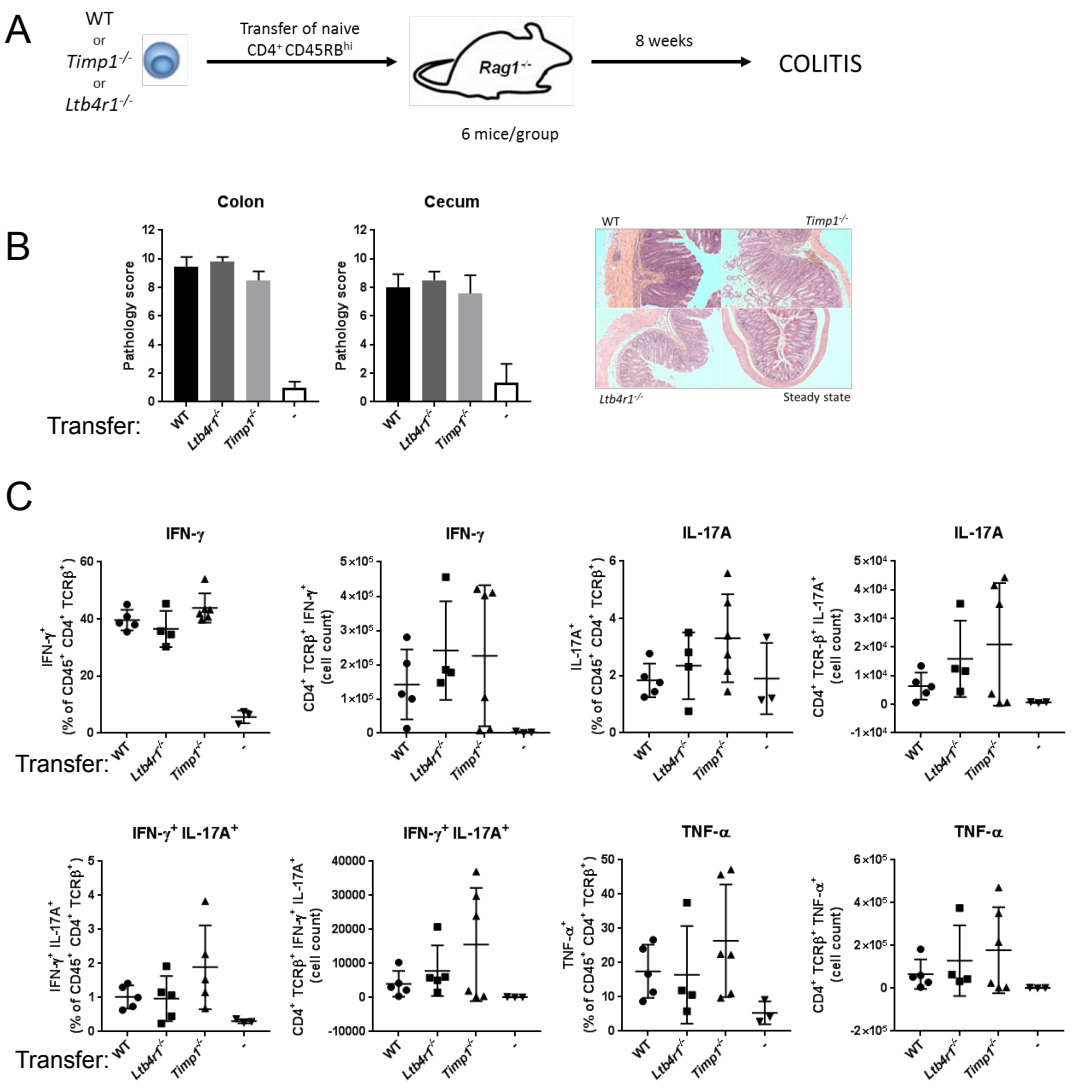


Figure 3.11: TIMP1 and LTB4R1 deficiencies do not affect the development of colitis in a model of adoptive T cell transfer

(A) Experimental layout: C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from spleen and lymph nodes of WT, *Timp1*$^{-/-}$ or *Ltb4r1*$^{-/-}$ mice. Mice were sacrificed upon development of clinical signs of inflammation (week 8). **(B)** Pathology score as assessed by histology scoring of the colon and cecum. **(C)** LPLs from the colon were isolated for flow cytometric analysis. Frequency and numbers of the indicated cytokine expressing cells are shown. Bars represent the mean ±SEM and each point represents an individual mouse. One experiment, n=5-6 per group.

3.2.6 TIMP1 and LTB4R1 deficiency does not affect *in vitro* Th17 differentiation

With our *in vivo* models we did not observe a reduction in the inflammatory potential of Th17 cells in TIMP1 or LTB4R1 deficient mice. We therefore set-up an *in vitro* system to assess if *Timp1* and *Ltb4r1* deficiency in $CD4^+$ T cells would affect their response to IL-23. Naïve FACS sorted naïve $CD4^+$ T cells isolated from spleens and lymph nodes of WT, *Il23r*$^{-/-}$, *Ltb4r1*$^{-/-}$ and *Timp1*$^{-/-}$ mice were plated in Th17 polarizing conditions with or without IL-23 (Fig. 3.12a). After 5 days of differentiation the cells were collected for gene and protein expression. qPCR analysis measuring the expression of Th17 associated genes revealed how Th17 conditioning media induced expression of *Il17a*, *Il17f* and *Il22* in cells from all genotypes, indicating an effective polarization to Th17 cells, but no difference in the level of expression between WT and KO mice was observed (Fig. 3.12b). IL-23 treatment further enhanced the expression of cytokines like *Il17a* and *Il22* at comparable levels in WT, *Ltb4r1*$^{-/-}$ and *Timp1*$^{-/-}$ mice (Fig. 3.12b). As expected, treatment with IL-23 did not affect the gene expression of *Il23r*$^{-/-}$ cells. When tested for protein expression of cytokines by flow cytometric analysis, we obtained similar results. Th17 conditioning media induced IL-17A expression in all genotypes and at similar levels between them, but IL-23 did not enhance expression (Fig. 3.12c). The expression of other pro inflammatory cytokines like IFN-γ and TNF- α was not affected by TIMP1 or LTB4R1 deficiency (Fig. 3.12c).

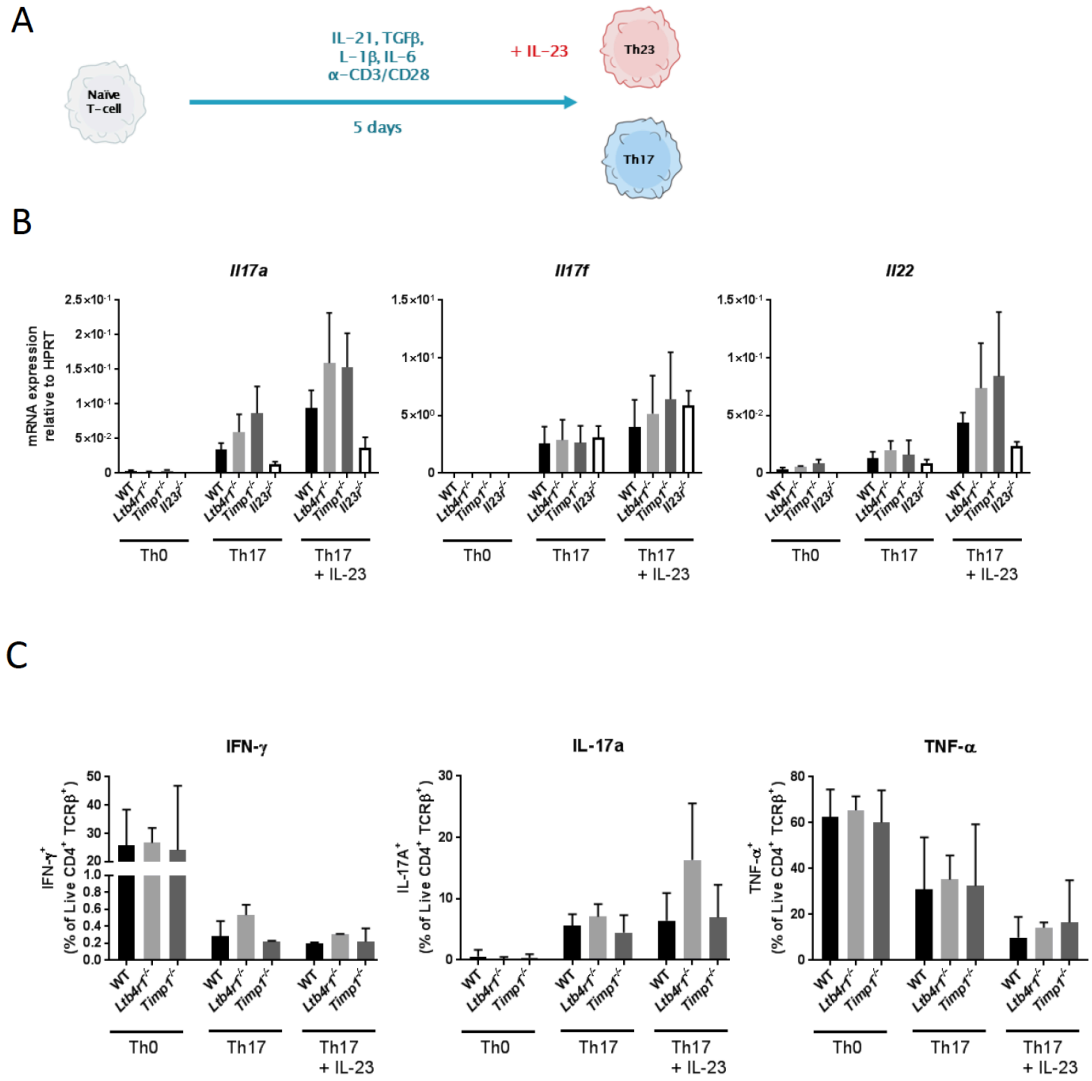


Figure 3.12: TIMP1 and LTB4R1 deficiencies do not impair the *in vitro* differentiation of Th17 cells

(A) WT, *Timp1*$^{-/-}$ and *Ltb4r1*$^{-/-}$ naïve CD4^{+} T cells isolated from spleens and lymph were cultured in Th17 conditioning medium with or without IL-23 for 5 days. **(B)** mRNA expression of the indicated genes was assessed by qPCR analysis. **(C)** Frequency of cytokine expressing T cells as assessed by flow cytometric analysis. Bars represent ±SEM. Data shown are representative of two independent experiments. n=2 per group

3.3 Discussion

In this chapter we have studied the molecular signature that is induced by IL-23 in Th17 cells, and tested the pathogenic potential of selected molecules in models of intestinal inflammation. Previous work in our lab identified a list of genes and proteins that, in *in vitro* differentiated Th17 cells, are upregulated when cultured in presence of IL-23. By combining high-throughput proteomic and transcriptomic analysis, a list of candidate molecules was selected for further validation both *in vitro* and *in vivo*. Three genes were identified as ideal targets for further investigation: *Timp1*, *Ltb4r1* and *Smox*. With an *in vitro* system we validated that the expression of these genes is IL-23 dependent in Th17 cells. In addition to that, they are expressed in IL-23R^{+}CD4^{+} intestinal T cells and are induced upon intestinal inflammation triggered by *C. rodentium* infection, further suggesting an involvement in the development of IL-23 dependent Th17 responses.

TIMP1 is one of the molecules for which we tested the pathogenic potential in mouse models of colitis. Several studies suggested an involvement of this MMP inhibitor in IL-23 driven Th17 responses. In fact, TIMP1 has been implicated in the development of EAE, as mice receiving adoptive transfer of *Timp1*$^{-/-}$ autoreactive Th17 cells are partially protected from disease [332]. TIMP1 is also associated with inflammation in IBD, as higher levels of expression were detected in the inflamed tissue of patients with both CD and UC [319, 320]. In addition, *Timp1* expression in *in vitro* differentiated Th17 cells is controlled by STAT3, the same transcription factor downstream of IL-23R signalling, further suggesting a possible IL-23-dependent induction [332]. However, in our study we did not detect a difference in the severity of pathology when we compared WT and *Timp1*$^{-/-}$ mice in three different models of

colitis. This could be possibly explained by redundancy in the biological roles of TIMP1, where other TIMPs could overcome the absence of TIMP1. TIMP1 shares structural and functional properties with other TIMPs, especially TIMP3: these two proteins have about 40% identical structure and are glycoproteins with complex sugar chains [333]. They also share affinity for the same target proteins such as the metalloproteinases MMP-9 and ADAM10 [334]. In contrast, TIMP1 and TIMP3 have opposing functions in regulating apoptosis, as TIMP1 inhibits while TIMP3 induces apoptosis in various cancer cell lines [335]. We could then speculate that TIMP3 partially compensates the function of TIMP1 in the context of TIMP1 deficiency.

Another limitation of our system is the fact that we utilized *Timp1* full knock-out mice, as mice with T cell specific *Timp1* genetic ablation were not available. TIMP1 is a ubiquitously expressed protein, therefore studying a cell-specific response in a full knock-out mice can be challenging and can lead to confounding results. In order to overcome this limitation, we adoptively transferred naïve *Timp1*$^{-/-}$ T cells in *Rag1*$^{-/-}$ host mice, a useful system to study T cell specific genetic ablations. However, TIMP1 deficient T cells mounted a Th17 response that was comparable to that of WT transferred T cells. Others have used a similar strategy to study the role of TIMP1 specifically in autoreactive Th17 responses in the central nervous system [332], and showed how TIMP1 confers pathogenicity to Th17 cells. The discrepancy in the results, can be explained by the highly context and tissue dependent nature of Th17 responses. Th17 cells are indeed associated with both homeostasis-promoting and pathogenic responses, and the same IL-17 have opposing effects in different IL-23 driven pathologies such as psoriasis, where it contributes to pathology [336], and IBD, where its blocking leads to enhanced disease [259]. Therefore, TIMP1 might

have a role in Th17 associated diseases like EAE, but its role in colitis seems dispensable for the development of pathology.

Similar conclusions can be drawn on *Ltb4r1* and its role as a potential regulator of Th17 pathogenicity. Although our *in vivo* and *in vitro* validation experiments confirmed how *Ltb4r1* expression is controlled by IL-23 in Th17 cells, these results did not reflect an actual involvement in intestinal inflammation when we tested *Ltb4r1*$^{-/-}$ mice in multiple models of colitis. As with *Timp1*, using an *Ltb4r1* full knockout mouse was necessary, as T cell specific knockout mice are not commercially available. Since multiple immune cells, including cell types involved in the pathogenesis of IBD like eosinophils and neutrophils, express *Ltb4r1* [324], this system was not flawless and not the optimal *in vivo* setting for studying Th17 specific responses. Using a transwell system, LTB4R1 has been shown to mediate migration in *in vitro* differentiated Th17 cells [325] but with LTB4R1 ablation, we did not detect a difference in the accumulation of T cells in the inflamed tissue, indicating an intact migration potential of *Ltb4r1*$^{-/-}$ T cells. It cannot be excluded that in *Ltb4r1*$^{-/-}$ mice a compensatory effect of LTB4R2 balances the absence of LTB4R1, masking the specific role of LTB4R1 in T cell responses. LTB4R2 is indeed expressed in T cells, however the two receptors have very different affinity to LTB4, with LTB4R1 having high affinity and LTB4R2 low affinity [337]. In contrast, LTB4R2 has high affinity to another ligand, 12-hydroxypeptadecatrienoic acid (12-HHT), that does not activate LTB4R1.Despite the structural similarities, the two receptors have different patterns of activation. It is therefore unlikely that LTB4R2 could fully compensate the absence of LTB4R1 in *Ltb4r1*$^{-/-}$ mice.

The selection of *Timp1* and *Ltb4r1* as targets to be tested in murine models of colitis was based on previously published results and by the availability of knock-out mice. However, other potential targets were considered in this project and could be investigated further in future studies. Several reports support the hypothesis that *Smox* could be crucial in intestinal Th17 responses. *Smox* is a gene that encodes for spermine oxidase, an enzyme that catalyses the oxidation of spermine with the production of spermidine, hydrogen peroxide and aldehyde 3-aminopropanal [338]. This enzyme plays a central role in the highly regulated catabolism and homeostasis of polyamines, which is mandatory for cellular life [339]. SMOX activity as a source of reactive oxygen species suggested a role of this molecule in inflammatory diseases [340]: SMOX expression is upregulated in the intestine of UC patients, and it correlates with severity of disease [341]. In addition to that, it has been shown to be expressed by *in vitro* generated Th17 and its expression is further enhanced by IL-23 treatment [342]. These data are in line with the results of our high-throughput screening and suggested that *Smox* could be involved in the IL-23 dependent Th17 pathologic response that characterizes IBD. However, at the time of designing this project there were no mouse strains with *Smox* deficiency available, making it a challenging molecule to study in *in vivo* models of colitis. The absence of efficient tools to study its activity *in vivo* could be overcome by utilizing specific inhibitors [181] or by developing T cell-specific or time-specific knock-out mice, in order to reduce the disruptive effect of SMOX deficiency on polyamines homeostasis and limit its ablation in selected tissues and cells. In a recent publication, a SMOX deficient mouse was described and revealed its role in regulating endoplasmic reticulum stress response [343].

A few considerations can also be observed regarding the design of the target-mining experiments that started this project. With a combination of transcriptomic and proteomic analysis of *in vitro* differentiated Th17 cells, we generated a list of molecules that are expressed by Th17 cells and are induced by IL-23. The generation of datasets from *in vitro* cultured cells is an approach that has been adopted by other studies aiming to identify molecules that confer pathogenicity to Th17 cells [204]. In the study by Lee *et al.* [205] microarray analysis on *in vitro* differentiated Th17 cells revealed a transcriptional signature of pathogenic Th17 cells and described the pathogenic potential of Tbet, one of the molecules that was enriched in *in vitro* generated Th17 cells, in a model of EAE. However, one of the most influential studies in the field, by Guablomme *et al.* [206], adopted a different and more reliable strategy by analysing the transcriptome of both *in vitro* and *in vivo* generated Th17 cells to identify regulators of Th17 pathogenicity. This was certainly a more relevant approach, as only molecules that were expressed by both *in vitro* and *in vivo* generated pathogenic Th17 cells were further tested in murine models of disease. This study revealed how four genes, *Gpr65*, *Plzp*, *Toso*, and *Cd5l*, are critical in inducing pathogenic Th17 responses in the CNS. By contrast, our screening approach was solely based on *in vitro* generated Th17 cells. This was a caveat, as the preliminary results obtained *in vitro* did not translate in *in vivo* models of colitis. A future approach could be to isolate Th17 cells from the inflamed mucosa of mice with active colitis, such as *Rag1*$^{-/-}$ mice adoptively transferred with naïve T cells, and perform transcriptome analysis on both the *ex vivo* isolated cells and *in vitro* generated Th17 cells. The combination of *in vivo* and *in vitro* generated datasets would allow the selection of targets to bring forward in the characterization with more confidence.

4 Chapter 4: Treg cells control type 2 immune responses at barrier sites through DGAT1

4.1 Introduction

Barrier sites, such as the colon and skin, are unique microenvironments where the immune system is in constant contact with the outside world. At these barrier sites, immune cells are exposed to stressors and stimuli including the microbiota, dietary components, metabolites, and other environmental factors that can affect their development and function. Some of these stressors, such as the microbiota, are common to all barrier-sites while others are organ specific, like UV light in the skin and food antigens in the intestine. Therefore, tissue-specific adaptation is necessary for the immune system to maintain immunological homeostasis and host protection, balancing resistance to harmful pathogens, and tolerance to the resident microbiota and innocuous environmental factors [111]. RNA sequencing techniques have allowed the identification of specific molecular signatures in immune cells at barrier sites, including that of effector T cells and Tregs. T cells, as they progress in their development to fully active immune cells, leave secondary lymphoid organs in a primed state, and acquire their activated phenotype when in the peripheral tissue [344]. The adaptation to different tissues encompasses organ-specific phenotypes and functions. This is has been described in tissue resident Tregs, as studies in different organs have revealed unique tissue-specific functions. For example, a seminal study in the field revealed how Tregs in the adipose tissue express PPARγ, a master transcription factor of adipocytes, they acquire specific metabolic functions and can control insulin resistance [137, 345]. Other studies described the tissue adaptation of skin Tregs, that promote hair growth by stimulating stem cell renewal [346] and can

facilitate tissue repair [347]. Intestinal Tregs have the specific function of regulating immunoglobulin A production in Peyer's patches [348] and induce tolerance to food antigens [349]. Therefore, it is of great interest to identify and describe the phenotype that characterizes tissue specific T cells.

Several studies have identified the genes that are critical for T cell responses at barrier sites by comparing the transcriptomic signature of T cells isolated from colon, skin and the respective draining lymph nodes [100, 144, 350]. Previous work in our lab has investigated tissue specific transcriptomic signatures in Tregs isolated from the colon and skin. By comparing differentially upregulated transcripts in Tregs isolated from the colon and mesenteric lymph node, *Schiering et al*. [144] identified a set of genes that are enriched in colonic Tregs (Fig. 4.1a). *Dgat1*, which encodes for the enzyme diacylglycerol O-acyltransferase 1 (DGAT1), was revealed to be one of the transcripts that were specifically enriched in Tregs isolated from the colon. Interestingly, this gene was also identified as highly expressed in skin Tregs: in a microarray dataset generated in our lab, the transcriptome of skin resident Tregs was compared to the transcriptome of Tregs form skin draining lymph nodes, and *Dgat1* was one of the top 30 upregulated genes in the skin Treg compartment (Fig. 4.1b). Furthermore, in the previous chapter we have shown that treatment of Th17 cells with IL-23 induce expression of *Dgat1* in vitro (Fig. 3.1d), suggesting a possible role of this enzyme in both Treg and Th17 responses.

DGAT1 has been mainly studied for its function as a fatty acid metabolism enzyme, while its role in immune function is yet to be fully determined. Its expression has been reported to be ubiquitous, with high levels in the adipose tissue, liver, skeletal

muscle and intestine [351]. DGAT1 is involved in the pathway that converts fatty acids into triglycerides (TG) by covalently joining fatty acid acyl-CoA and diacylglycerol (DG) into TG [351]. TG synthesis is an essential function of the cell, as storage of TG serves for energy utilization and synthesis of membrane lipids [352]. Of note, TG synthesis is not exclusive to DGAT1, as another DGAT protein, DGAT2, catalyses the same reaction [353]. However, DGAT1 has other non-shared functions. In fact, DGAT1 has been shown to be involved in retinoic acid (RA) homeostasis through the esterification of the RA precursor, retinol (vitamin A), into RA's main storage form retinyl ester [354]. RA is a molecule that is present in high concentration in organs such as the skin and the intestine, and it is known to promote the differentiation of Treg cells while inhibiting Th17 cell responses [355, 356]. A recent report by *Graham et al.* [357] linked the activity of DGAT1 to Th17 and Treg responses, by demonstrating how DGAT1 regulates bioavailability of retinoic acid. This suggests that DGAT1 could be involved in regulating T cell responses.

DGAT1 has been associated with several metabolic disorders. Studies on *Dgat1*$^{-/-}$ mice have suggested DGAT1 as a possible therapeutic target for metabolic disease, as DGAT1 deficient mice are resistant to diet induced obesity [358] and protected from insulin resistance [359]. DGAT1 inhibitors have therefore been developed for the treatment of obesity [360], although patients involved in clinical trials with such inhibitors have experienced side effects like nausea and diarrhea [361, 362], suggesting a crucial role of DGAT1 in intestinal homeostasis. In support of this, mutations in the *DGAT1* human gene have been linked to congenital diarrheal disorders [363, 364]. Taken together, these observations support the hypothesis that DGAT1 has a key function in Th17 and Tregs at barrier sites.

Therefore, the aims of this chapter are:

- Investigate the function of DGAT1 in effector Th17 responses
- Investigate the function of DGAT1 in Treg responses at barrier sites

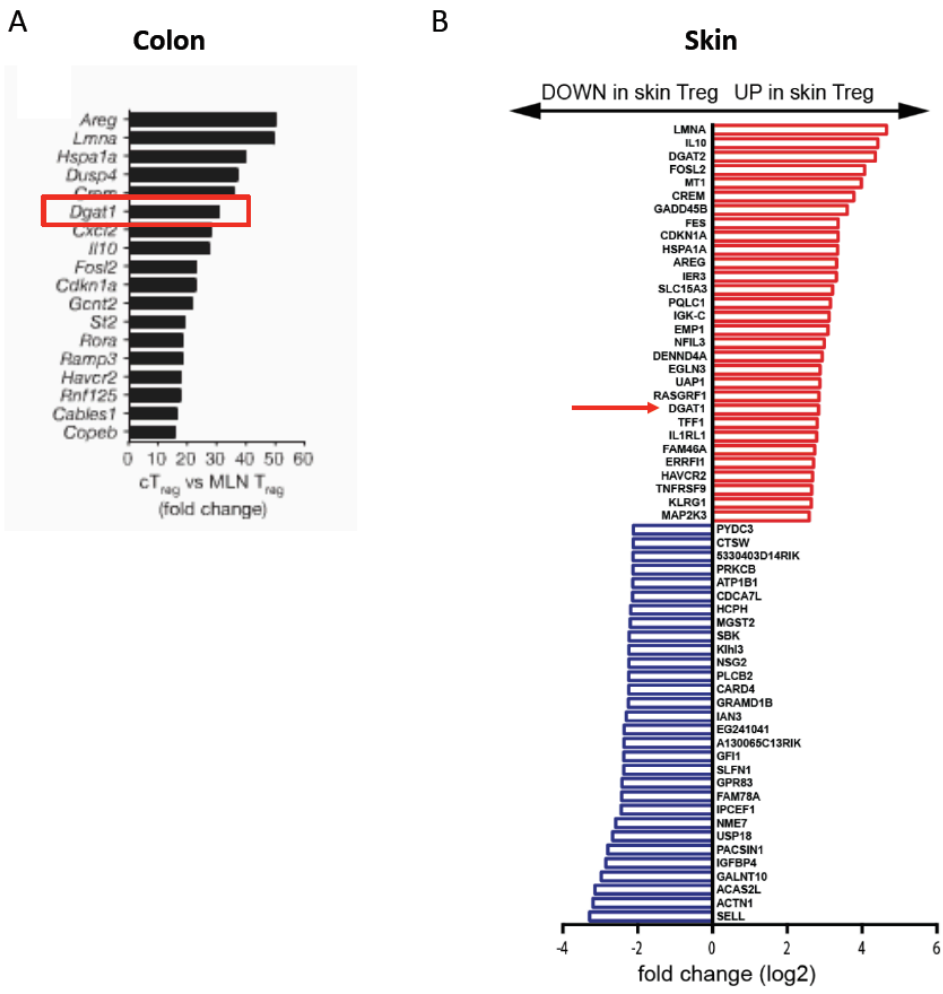


Figure 4.1: *Dgat1* is highly expressed in Tregs at barrier sites

(A) Top differentially upregulated transcripts in colonic Treg versus mLN Treg cells [144]. **(B)** Cervical lymph nodes (CLN) and skin from the ears of steady state *Foxp3*^{gfp} mice were harvested and CD45^{+}CD4^{+}TCRβ^{+}GFP^{+} cells sorted. RNA was extracted, amplified and gene expression assessed by microarray (Illumina Wg6 v2.0 Expression Bead Chips). Graph depicts the 30 most upregulated (red) and

downregulated (blue) genes between CLN and skin Tregs ranked according to fold change (*Data kindly provided by Krista Stockenhuber*).

4.2 Results

4.2.1 *Dgat1* is highly expressed by T cells at barrier sites

Previous transcriptomic data from our lab revealed that *Dgat1* is expressed by effector and regulatory T cells and it is enriched at barrier sites. To further confirm this by qPCR analysis, we compared expression of *Dgat1* in different T cell subsets isolated from the colon and secondary lymphoid organs. Colons, spleens and mLNs were collected from steady state C57BL/6 mice and leukocytes were isolated. Cells were then FACS sorted into $CD4^+TCR\beta^+CD25^-CD44^{low}CD62L^{high}$ naïve T cells, $CD4^+TCR\beta^+CD25^+$ Treg cells, $CD4^+TCR\beta^+CD25^-CD44^{high}CD62L^{low}$ effector T cells and $CD4^-TCR\beta^+CD8^+$ T cell populations and mRNA isolated for gene expression analysis by qPCR. Expression of *Dgat1* was higher in the colon than in SLOs (spleen and lymph nodes) for all T cell subsets analysed (Fig. 4.2a), confirming an enrichment of this gene in colonic T cells.

Given expression of *Dgat1* by T cells *in vivo*, we wanted to compare *Dgat1* expression in different T helper cell subset *in vitro*. Naïve T cells were FACS sorted from spleen and lymph nodes of C57BL/6 mice and cultured under Th0, Th1, Th2, Treg, Th17 and Th17+IL-23 (Th23) conditions as described in the Methods section. *Dgat1* expression was induced in Th1 differentiated cells, while Th2 cells showed a similar level of expression to Th0 cells. In line with previous results (Fig 3.1a), *Dgat1* was highly expressed in Th17 compared to Th0 and its expression was further enhanced with IL-23 treatment (Fig. 4.2b), suggesting a possible involvement of this

gene in IL-23 dependent T cell-driven inflammatory responses. In contrast with *in vivo* findings, *in vitro* differentiated Treg cells did not induce expression of *Dgat1,* as its expression was comparable to that of Th0 undifferentiated cells.

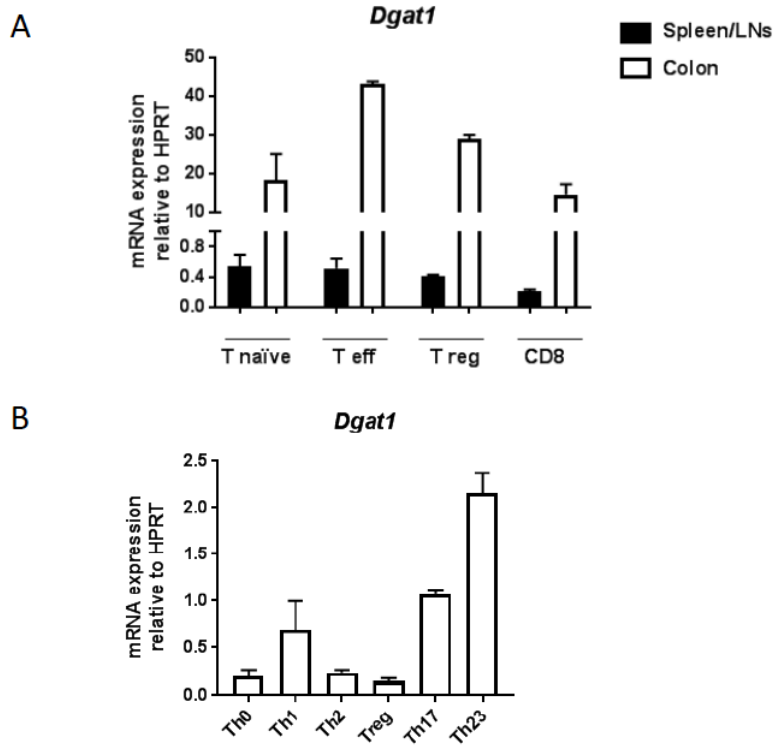


Figure 4.2: *Dgat1* expression in intestinal and *in vivo* differentiated T cells

(A) Spleen, lymph nodes and colon were harvested from steady state B57BL/6 and $CD4^{+}TCR\beta^{+}CD25^{-}CD44^{low}CD62L^{high}$ naïve T cells, $CD4^{+}TCR\beta^{+}CD25^{+}$ Treg cells, $CD4^{+}TCR\beta^{+}CD25^{-}CD44^{high}CD62L^{low}$ effector T cells and $CD4^{-}TCR\beta^{+}CD8^{+}$ T were FACS sorted. mRNA expression of *Dgat1* was assessed by qPCR analysis. One experiment, two biological replicates per group (three mice per replicate were pooled). **(B)** FACS sorted Naïve T-cells from spleen and lymph nodes of C57BL/6 mice were cultured under Th0, Th1, Th2, Treg, Th17 and Th23 conditions as described in the Methods section. mRNA expression of *Dgat1* was assessed by qPCR

analysis. Data shown are representative of two independent experiments. n=2 per group.

4.2.2 Characterization of the *CD4^{Cre}xDgat1$^{fl/fl}$* mice

Increased expression of *Dgat1* by CD4$^+$ T cells in the colon and skin led us to investigate whether DGAT1 plays a functional role in CD4$^+$ T cells at barrier sites. To study the role of DGAT1 in CD4$^+$ T cells *in vivo*, a conditional knock-out mouse strain was generated whereby *CD4^{Cre}* mice were crossed with *Dgat1$^{fl/fl}$* mice. Here, with a Cre/loxP recombination system *Dgat1* is specifically excised in every cell expressing CD4. T cell development occurs in the thymus, where T cells go through several stages of maturation and the surface molecules CD4 and CD8 are expressed [365]. In their earliest stage of maturation, T cells are termed "double negative" (DN), as they do not express either CD4 or CD8, while later in their development they acquire expression of both CD4 an CD8 in the "double positive" (DP) stage. Finally, terminally differentiated fully mature T cells express either CD4 or CD8 [365]. Upon development T cells migrate to secondary lymphoid organs, such as the spleen, and peripheral organs, like the colon, to exert their immune function [365].

To validate effective excision of *Dgat1* in CD4$^+$ cells, we FACS sorted different T cell subsets from the spleen, lymph nodes and thymus of *CD4^{Cre}xDgat1$^{fl/f}$* mice and *Dgat1$^{fl/f}$* (WT) mice. T cells were FACS sorted into naïve CD4$^+$ T cells, effector CD4$^+$ T cells, CD4$^+$CD25$^+$ Tregs and CD8$^+$ T cells (as in Fig.4.2b) form spleen and colon, and CD4$^+$, CD8$^+$, DN and DP T cells from the thymus. This setup allowed us to track the excision of *Dgat1* from the T cell compartment during their development in the thymus and in their mature/active stage in the spleen and colon.

Expression of *Dgat1* in all CD4$^+$ subsets isolated from *CD4^{Cre}xDgat1$^{fl/f}$* mice was undetected or below threshold (Ct>30), confirming ablation of this gene (Fig 4.3a).

The residual signal in $CD4^+$ cells from $CD4^{Cre}xDgat1^{fl/fl}$ mice is possibly due to a contamination of $CD4^-$ cells, where *Dgat1* is not ablated. $CD8^+$ cells sorted from all organs were deficient in *Dgat1* expression, suggesting that *Dgat1* is ablated at the DP stage of thymic development (Fig 4.3a). In contrast, DN cells sorted from the thymus of $CD4^{Cre}xDgat1^{fl/f}$ mice expressed *Dgat1*, likely due to CD4 not being expressed at this immature developmental stage (Fig 4.3a).

The function of DGAT1partially overlaps with that of DGAT2 [353]. It is possible that genetic ablation of *Dgat1* could trigger increased expression of *Dgat2* as a compensation mechanism. However, expression of *Dgat2* in T cells from the spleen, colon and thymus was similar between WT and $Dgat1^{CD4}$ cells (Fig 4.3b), arguing against compensation by DGAT2 in T cells.

After validating ablation of *Dgat1 in* $CD4^{Cre}xDgat1^{fl/f}$ mice, these mice were characterized for their $CD4^+$ T cell response *in vivo*. WT and $CD4^{Cre}xDgat1^{fl/fl}$ mice were sacrificed and leukocytes isolated from the colon lamina propria. Flow cytometric analysis showed a comparable frequency and number of $CD4^+$ $TCR\beta^+$ T cells (Fig. 4.4). Similar frequencies of $CD44^{low}CD62L^{high}$ naïve T cells between WT and $CD4^{Cre}xDgat1^{fl/fl}$ mice were also observed (Fig 4.4). Frequencies of $Foxp3^+$ Tregs, $GATA3^+Foxp3^+$ Tregs and $ROR\gamma^+Foxp3^+$ Tregs were similar between the two genotypes (Fig. 4.4). The frequency of $GATA3^+$ Th2 and $ROR\gamma^+$ Th17 cells was also comparable between WT and $CD4^{Cre}xDgat1^{fl/fl}$ mice (Fig 4.4). This suggested that at steady state, *Dgat1* ablation does not affect the proportion of T helper subsets in the colon. However, it is possible that T cell function may be altered by *Dgat1* ablation. The expression of cytokines by colonic $CD4^+$ T cells was then assessed by flow cytometric analysis. The frequency of IFN-γ, TNF-α and IL-13 was comparable

between WT and *Dgat1*^{CD4} T cells (Fig. 4.5). A significant increase in the frequency and numbers of IL-17A and IL-17F producing T cells was observed (Fig 4.5), suggesting that *Dgat1* may restrain production of such cytokines in the colon. However, this finding only partially replicated in a second experiment and would need additional studies to be confirmed. Expression of general inflammatory cytokines in the total tissue of WT and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice was also tested by qPCR analysis. The levels of mRNA expression of cytokines like *Tnf*, *Ifng*, *Il10* and *Csf2* were comparable between the two genotypes (Fig. 4.6). Similarly, expression of *Reg3g*, and *Reg3b,* anti-microbial protein [304] expressed by epithelial cells [366] and known to be downstream effectors of Th17 responses [81], was similar between WT and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice (Fig. 4.6). These results suggest that *Dgat1* may have a role in controlling Th17 responses in the intestine.

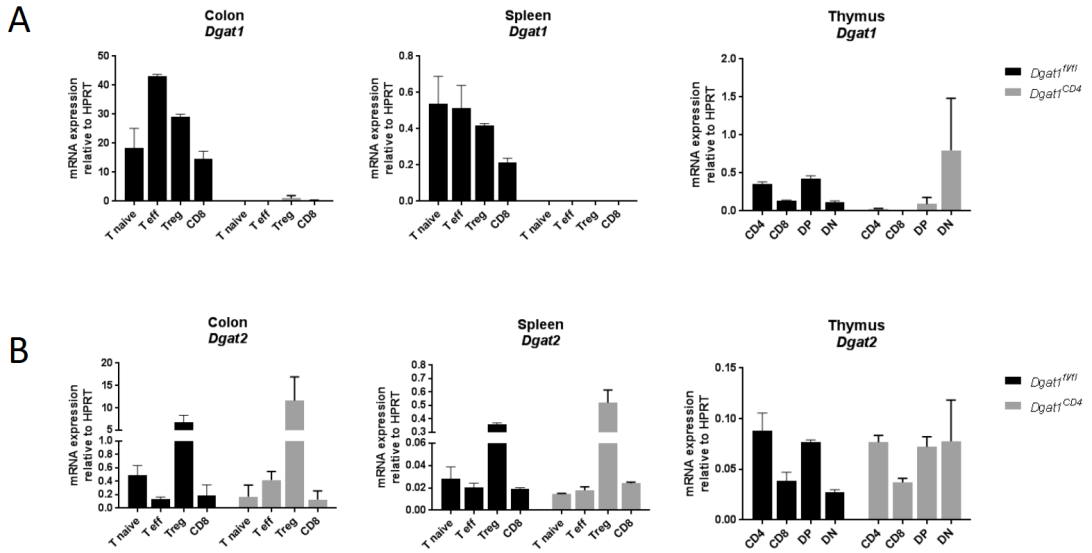


Figure 4.3: Validation of *Dgat1* excision in the *CD4*^{Cre}*xDgat1*$^{fl/fl}$ conditional knock-out mice

Colon, spleen and thymus were harvested from *Dgat1*$^{fl/fl}$ and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1*$^{\Delta CD4}$) mice. CD4^{+}TCRβ^{+}CD25^{-}CD44^{low}CD62L^{high} naïve T cells, CD4^{+} effector

T cells, $CD4^+TCR\beta^+CD25^+$ Tregs and $TCR\beta^+$ $CD8^+$ T cells were FACS sorted form spleen and colon, while $TCR\beta^+CD4^+$ T cells (CD4), $TCR\beta^+CD8^+$ T cells (CD8), $TCR\beta^+CD4^-$ $CD8^-$ double negative T cells (DN) and $TCR\beta^+CD4^+CD8^+$ double positive T cells (DP) were FACS sorted from the thymus. **(A)** *Dgat1* and **(B)** *Dgat2* mRNA expression was assessed by qPCR analysis. One experiment, n=2 per group.

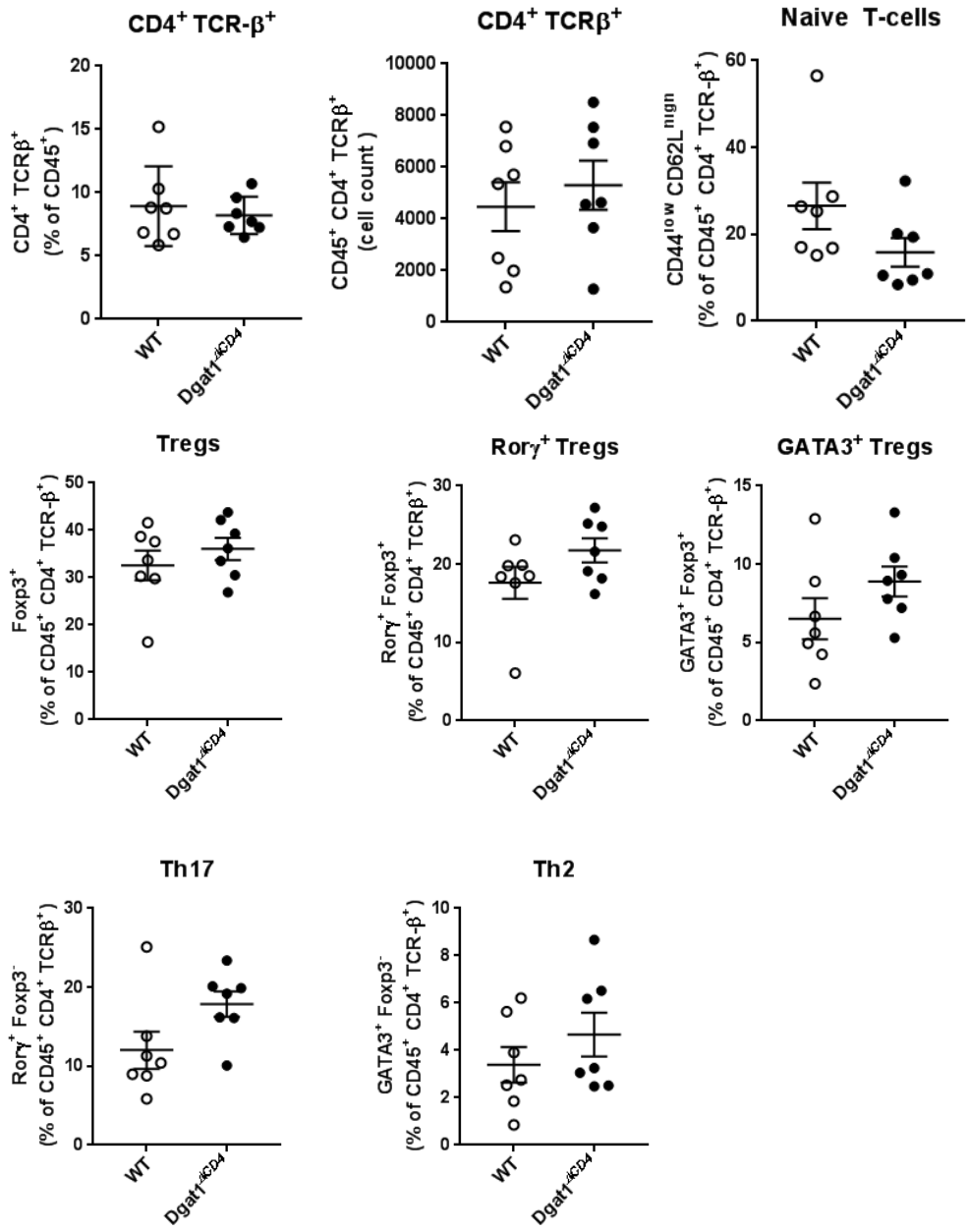


Figure 4.4: Characterization of the *CD4^{Cre}xDgat1$^{fl/fl}$* mice

Colons from steady state *Dgat1*$^{fl/fl}$ and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice were harvested and LPLs isolated for flow cytometric analysis. Frequency and numbers of the indicated T cell populations are represented. Bars represent the mean ±SEM and each point represents an individual mouse. Data shown are representative of two independent experiments. n=6-7 per group.

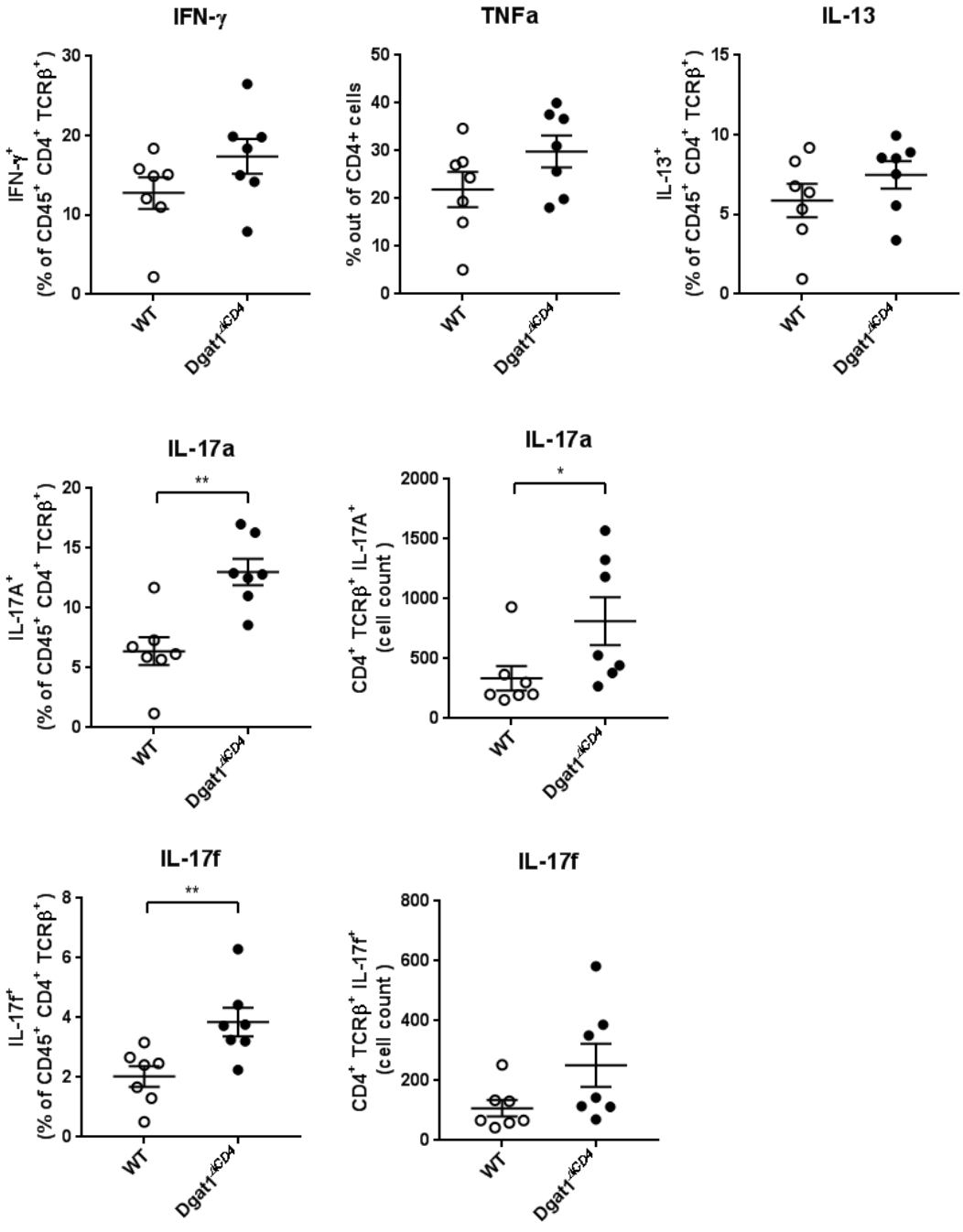


Figure 4.5: Colonic T cells from *CD4^{Cre}xDgat1$^{fl/fl}$* mice produce higher levels of IL-17 cytokines

Colon from steady state *Dgat1$^{fl/fl}$* and *CD4^{Cre}xDgat1$^{fl/fl}$* mice was harvested and LPLs isolated. After stimulation with PMA/Ionomicin for 3.5 hours, cells were stained and analysed by flow cytometry. Frequency and numbers of cytokine expressing T cells are represented. Bars represent the mean ±SEM and each point represents an individual mouse. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05, **p<0.01. Data shown are representative of two independent experiments. n=6-7 per group.

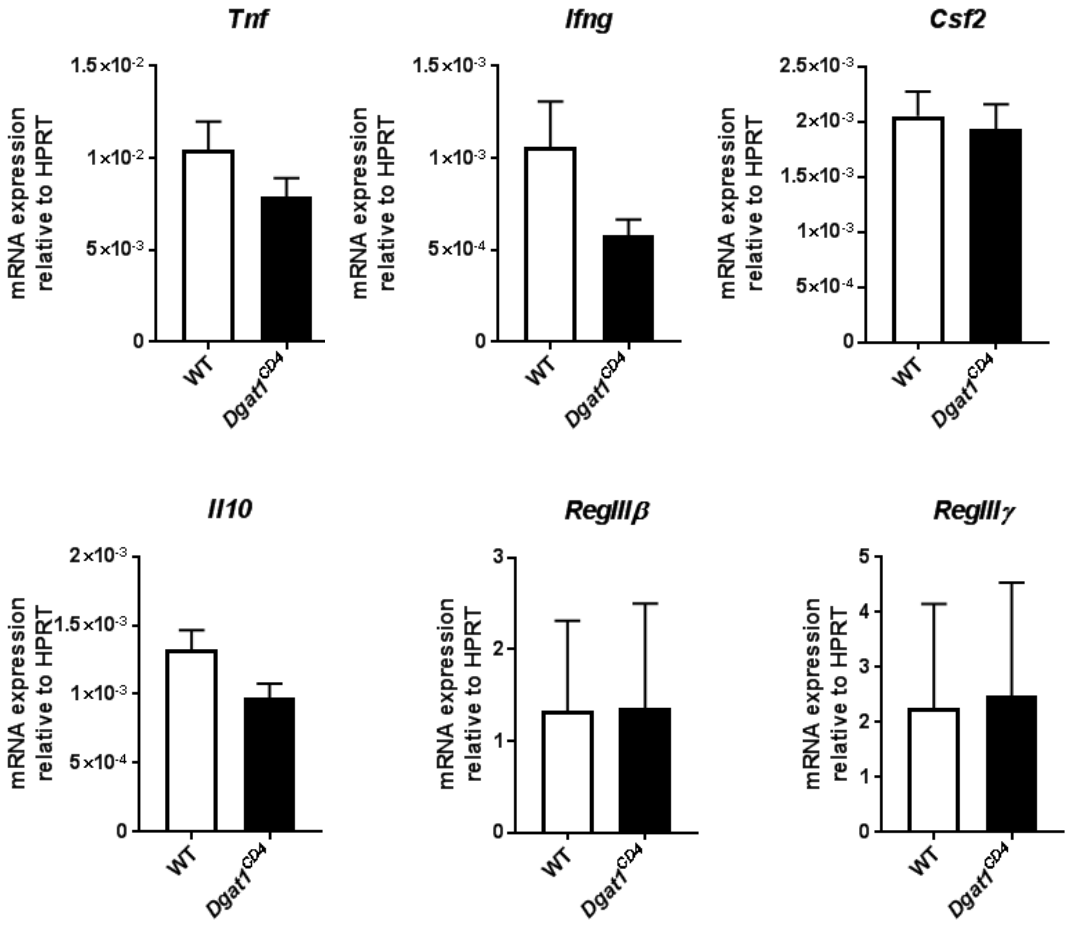


Figure 4.6: Immune profiling of the colon of *CD4^{Cre}xDgat1$^{fl/fl}$* mice

Colonic tissue was collected from *Dgat1*$^{fl/fl}$ and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice and mRNA isolated. mRNA expression levels of selected cytokines was assessed by qPCR analysis. Bars represent the mean ±SEM and each point represents an individual mouse. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05. Data shown are representative of two independent experiments. n=6-7 per group.

4.2.3 The role of *Dgat1* in the T cell response of adoptive T cell transfer colitis

In the previous paragraph a higher proportion of IL-17A and IL-17F producing CD4$^+$ T cells were observed in the colon of steady state *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice compared to WT mice, suggesting a possible involvement of DGAT1 in controlling Th17 responses (Fig. 4.5). To test the effect of *Dgat1* ablation in Th17 responses in the context of intestinal inflammation, we performed an adoptive T cell transfer model of colitis. Adoptive T cell transfer is a well-described model of T cell induced colitis, characterized by expression of high levels of inflammatory cytokines like TNF-α, IFN-γ, IL-17A and IL-17F. This model is considered a Th1/Th17 inflammation model as both TNF-α and IFN-γ drive the pathogenic response [367, 368] while concomitant neutralization of IL-17A and IL-17F was shown to be protective in this model [218]. To test the role of *Dgat1* in T cell dependent colitis, CD45RB^{high}CD4$^+$ naïve T cells were FACS sorted from spleens and lymph nodes of WT and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice and *Rag1*$^{-/-}$ recipient mice were adoptively transferred with either WT or *CD4*^{Cre}*xDgat1*$^{fl/fl}$ naïve T cells (Fig 4.7a). At the onset of intestinal inflammation (7 weeks) mice were sacrificed. Mice from both groups did not show signs of wasting disease, a classic sign of pathology in T cell transfer [86], as indicated by the stable body weight throughout the course of the experiment (Fig 4.7b). However, pathology

was evident when colonoscopy was performed on the mice, with both groups showing thickening of the epithelium, change in the vascular pattern and loose consistency of the stool, although no difference in the pathology score was observed between WT and *Dgat1*^{CD4} transferred mice (Fig 4.7c). To assess the effect of *Dgat1* on the T cell response, we performed flow cytometric analysis on the immune cells isolated from the colon lamina propria. WT and *Dgat1*^{CD4} transferred mice showed a similar accumulation of CD4^{+}TCRβ^{+} T cells in the colon. Frequency and number of RORγ^{+} Th17 cells were also comparable between the two groups, in accordance with the similar level of disease (Fig 4.8a). Frequency and numbers of CD4^{+} T cells producing TNF-α, IFN-γ, IL-17A and GM-CSF were also comparable between *Rag1*$^{-/-}$ mice transferred with either WT or *Dgat1*^{CD4} T cells (Fig. 4.8b). Production of IL-17F was not detectable. Similar results were obtained when markers of inflammation in the whole tissue of the colitic mice was assessed by qPCR analysis. Cytokines like *Ifng*, *Tnf* and *Csf2* as well as Th17 associated cytokines *Il17a* and *Il17f*, revealed an increased expression in colitic mice compared to non-transferred *Rag1*$^{-/-}$ mice, but no differential expression was detected when comparing WT and *Dgat1*^{CD4} cells transferred mice (Fig. 4.9).

These results indicate that DGAT1 does not play a role in the Th17 response of the T cell transfer model of colitis. However, it cannot be excluded that genetic ablation of *Dgat1* in the CD4^{+} compartment could have an impact on other models of T cell mediated disease.

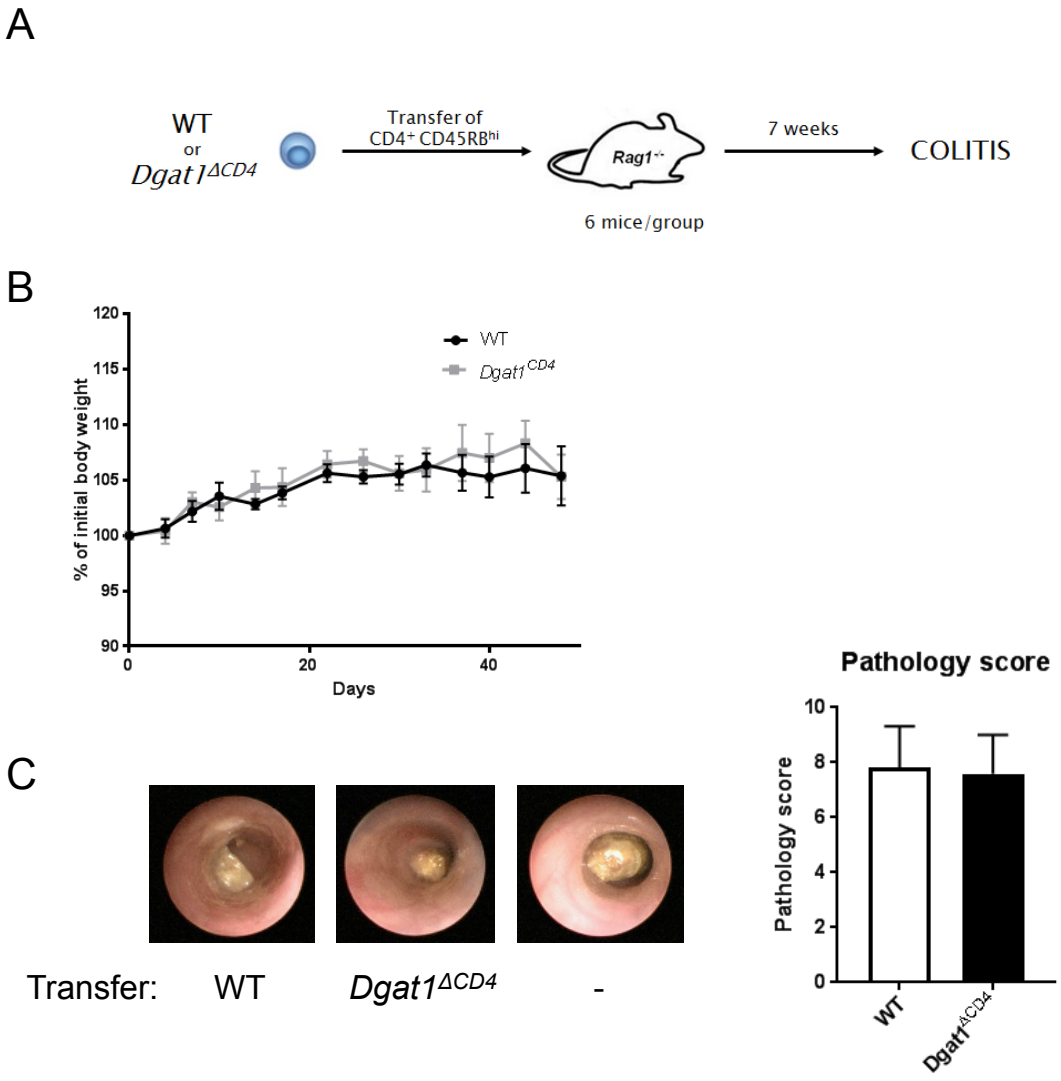


Figure 7: comparing WT and *Dgat1* deficient T cells in T cell transfer colitis

(A) Experiment layout: C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells isolated from spleen and lymph nodes of *Dgat1*$^{fl/fl}$ (WT) or *CD4*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1*$^{\Delta CD4}$) mice. Mice were sacrificed upon development of clinical signs of inflammation (week 8). **(B)** Percentage of initial body weight (g), shown as mean of the group with SD. **(C)** Pathology score based on the MEICS system [306]. Data shown are representative of two independent experiments. n=8 per group.

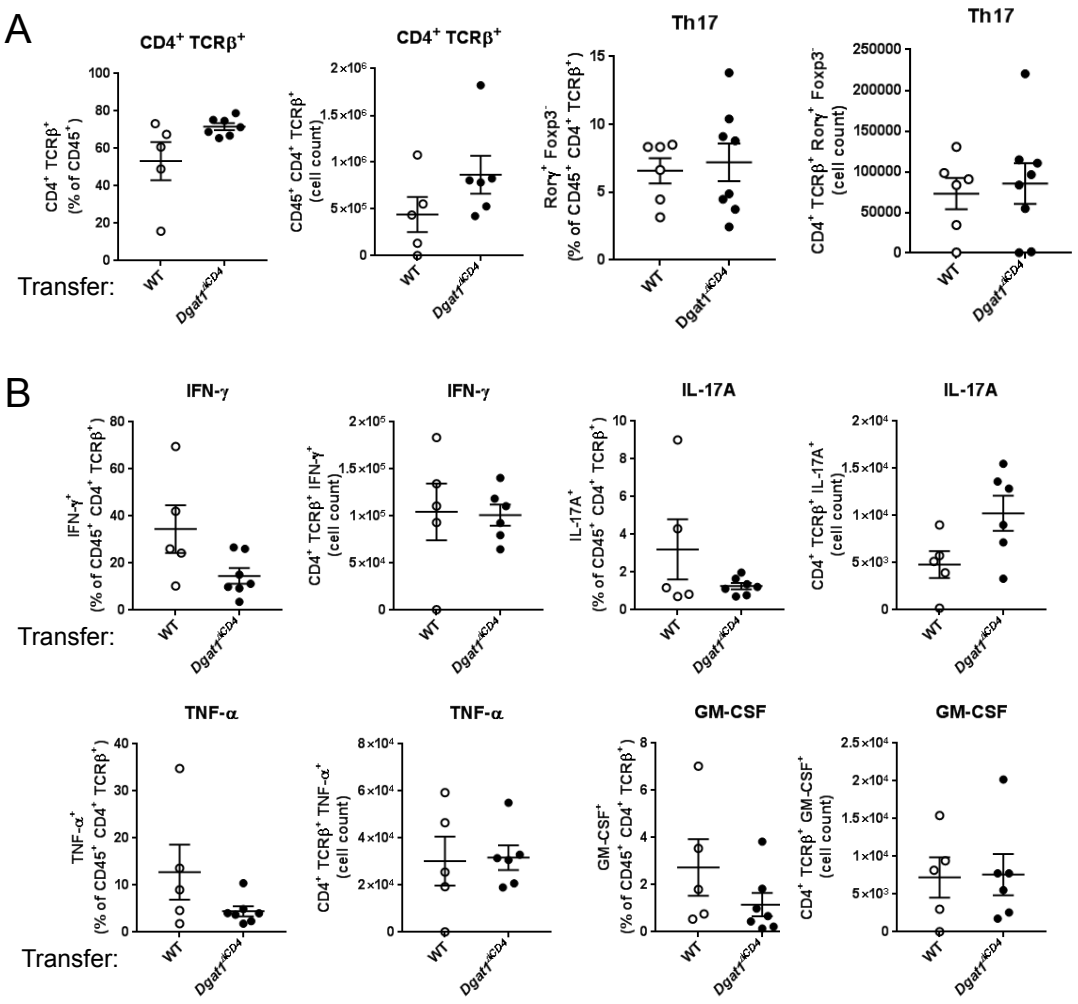


Figure 4.8: *Dgat1* deficiency does not affect the T cell response in T cell transfer colitis

Leukocytes isolated from the colon of T cell transferred mice were analysed by flow cytometry. **(A)** Frequency and numbers of the indicated T cell populations are represented. **(B)** After stimulation with PMA/Ionomicin for 3.5 hours, cells were stained and analysed by flow cytometry for cytokine expression. Frequency and numbers of cytokine expressing T cells are represented. Bars represent the mean ±SEM and each point represents an individual mouse. Data shown are representative of two independent experiments. n=8 per group.

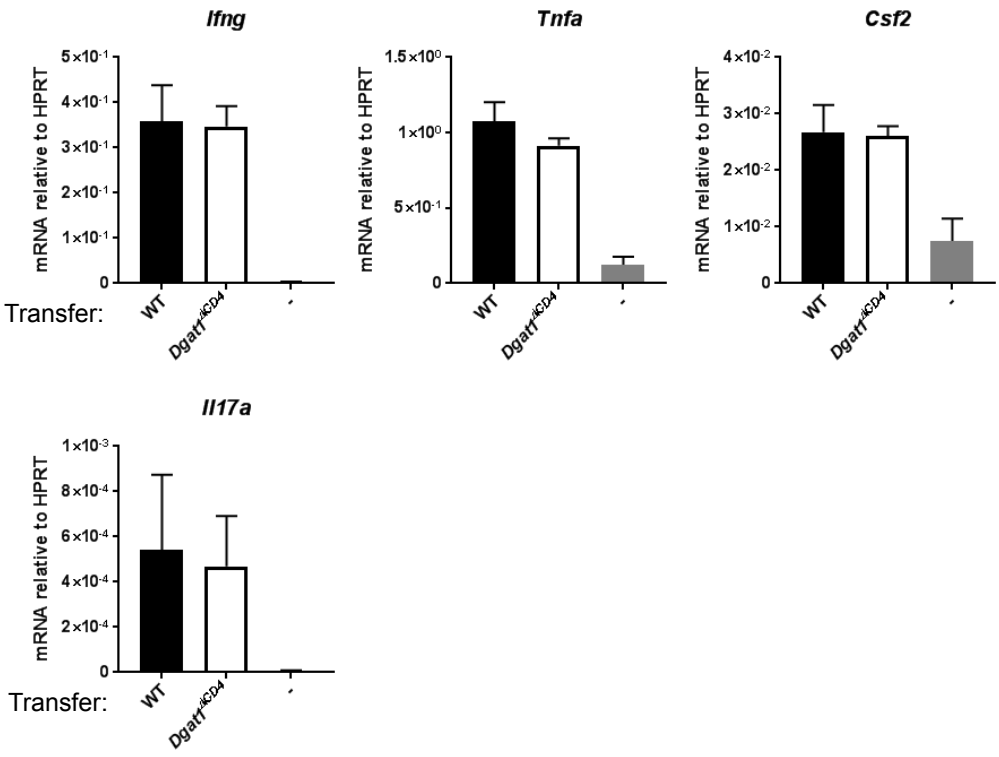


Figure 4.9: *Dgat1* deficiency does not affect the intestinal cytokine profile of T cell transferred mice

Colonic tissue was collected from *Rag1*$^{-/-}$ mice and from mice transferred with *Dgat1*$^{fl/fl}$ and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ T cells. mRNA was isolated and expression levels of selected cytokines was assessed by qPCR analysis. Bars represent the mean ±SEM and each point represents an individual mouse. Data shown are representative of two independent experiments. n=8 per group.

4.2.4 *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice have a type 2 signature

In Figures 4.1 and Fig 4.2 we showed how *Dgat1* is expressed by Foxp3^{+} Tregs, especially at barrier sites like the colon and the skin. To study the role of *Dgat1* specifically in Tregs, a conditional mouse strain was generated by crossing *Foxp3*^{Cre} with *Dgat1*$^{fl/fl}$ mice. In the resulting *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mouse strain *Dgat1* is genetically excised in all Foxp3 expressing cells. This mouse strain was then

characterized by comparing the T cell compartment of *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ and *Dgat1*$^{fl/fl}$ (WT) mice. Flow cytometric analysis showed a similar frequency of CD4^{+}TCRβ^{+}Foxp3^{+} Tregs in the colon of WT and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice, although we observed a trend towards a higher number of Treg cells in *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice (Fig 4.10a). When looking at Treg subsets, we observed an increase of frequency and numbers of GATA3^{+}Foxp3^{+} Tregs (Fig 4.10a). This subset of Tregs is crucial for protection against aberrant immune responses in the intestine, and have been associated with type 2-like tissue repair responses [99]. Both WT and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice showed comparable frequency of GATA3^{+} Th2 cells (Fig 4.10a). In contrast, the frequency and numbers of total Tregs, GATA3^{+}Foxp3^{+} Tregs, and Th2 cells in the small intestine were comparable between WT and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice (Fig. 4.10b). To assess if the increase in GATA3^{+} Tregs induced downstream effects, we performed qPCR analysis on mRNA isolated from colonic tissue. Interestingly, we observed a trend toward production of Th2 associated cytokines in the colon of *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$: the expression of *Il4*, *Il5*, *Il13* and *Tslp* was higher in *Foxp3*^{Cre}*xDgat1*$^{fl/lf}$ then in WT mice, although not statistically significant (Fig. 4.11a). These data suggested that ablation of *Dgat1* in the Treg compartment leads to a skewing of the colonic immune compartment towards a type 2 response. Interestingly, there were no apparent signs of pathology in the intestine, as shown by the H&E histological analysis of the colon (Fig. 4.11b).

Next, we investigated Th2 responses at other barrier sites. In the skin, we observed an increased frequency of total CD4^{+}TCRβ^{+}Foxp3^{+} Tregs and of the GATA3^{+} Treg subset in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice compared to WT mice, while the frequency of Th2 cells was comparable between the two groups (Fig. 4.12a). In the lungs, the frequency

of total Foxp3^{+} Treg cells, GATA3^{+} Tregs and Th2 cells were significantly higher in *Foxp3^{Cre}xDgat1$^{fl//fl}$* mice than WT (Fig 4.12b). As we observed a skewing towards type 2 immunity in multiple organs, we tested if this resulted in systemic manifestations. IgE is an immunoglobulin isotype associated with type 2 inflammatory responses like allergic asthma, atopic dermatitis and helminthic infections [173]. IgE is produced by plasma cells and binds the FcεRI expressed by basophils, eosinophils and antigen presenting cells, mediating their activation [177]. Therefore, we compared total IgE levels in the serum of WT and *Foxp3^{Cre}xDgat1$^{fl//fl}$* by ELISA and observed a significant increase in concentration in the serum of *Foxp3^{Cre}xDgat1$^{fl//fl}$* mice (Fig. 4.12c).

The genetic ablation of *Dgat1* specifically in Foxp3^{+} Tregs leads to a skewing of the immune system towards Th2-like responses at barrier sites. Our data suggest that *Dgat1* expression in Treg cells has a role in restraining type 2 immune responses.

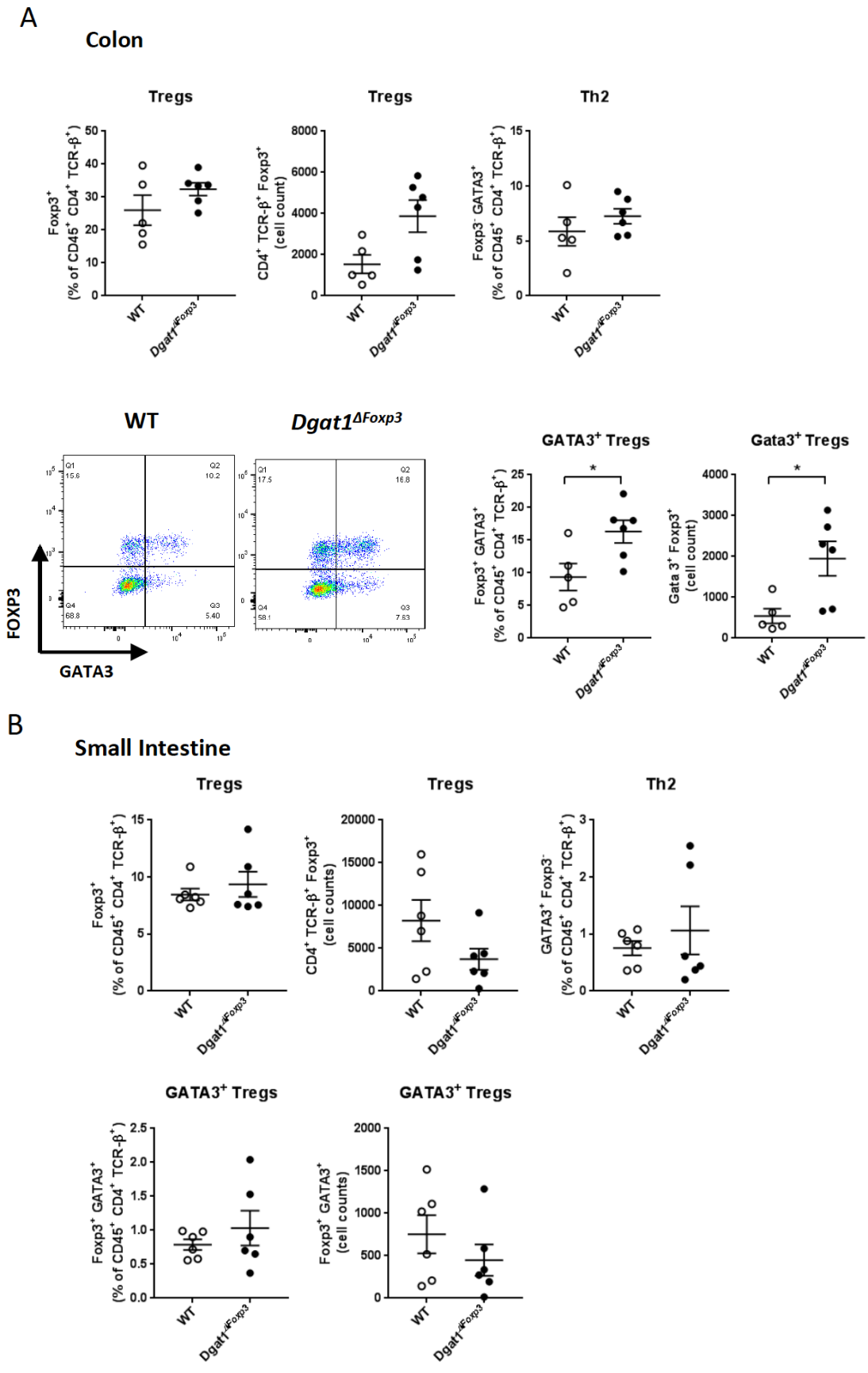

A
Colon
Tregs
Tregs
Th2
Foxp3+ (% of CD45+ CD4+ TCR-β+)
CD4+ TCR-β+ Foxp3+ (cell count)
Foxp3- GATA3+ (% of CD45+ CD4+ TCR-β+)
WT
Dgat1ΔFoxp3
WT
Dgat1ΔFoxp3
FOXP3
GATA3
GATA3+ Tregs
Gata3+ Tregs
Foxp3+ GATA3+ (% of CD45+ CD4+ TCR-β+)
Gata 3+ Foxp3+ (cell count)
B
Small Intestine
Tregs
Tregs
Th2
Foxp3+ (% of CD45+ CD4+ TCR-β+)
CD4+ TCR-β+ Foxp3+ (cell counts)
GATA3+ Foxp3- (% of CD45+ CD4+ TCR-β+)
GATA3+ Tregs
GATA3+ Tregs
Foxp3+ GATA3+ (% of CD45+ CD4+ TCR-β+)
Foxp3+ GATA3+ (cell counts)

Figure 4.10: *Foxp3^{Cre}xDgat1$^{fl/fl}$* mice have a higher proportion of GATA3^{+} Tregs than WT mice in the colon

Colon **(A)** and small intestine **(B)** from steady state 6-9 weeks old *Dgat1$^{fl/fl}$* (WT) and *Foxp3^{Cre}xDgat1$^{fl/fl}$* (*Dgat1Δ^{Foxp3}*) mice was harvested and LPLs isolated for flow cytometric analysis. Frequency and numbers of the indicated T cell populations are represented. A representative flow cytometry plot is shown (A, bottom left). Bars represent the mean ±SEM and each point represents an individual mouse. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05 Data shown are representative of three independent experiments. n=5-6 per group.

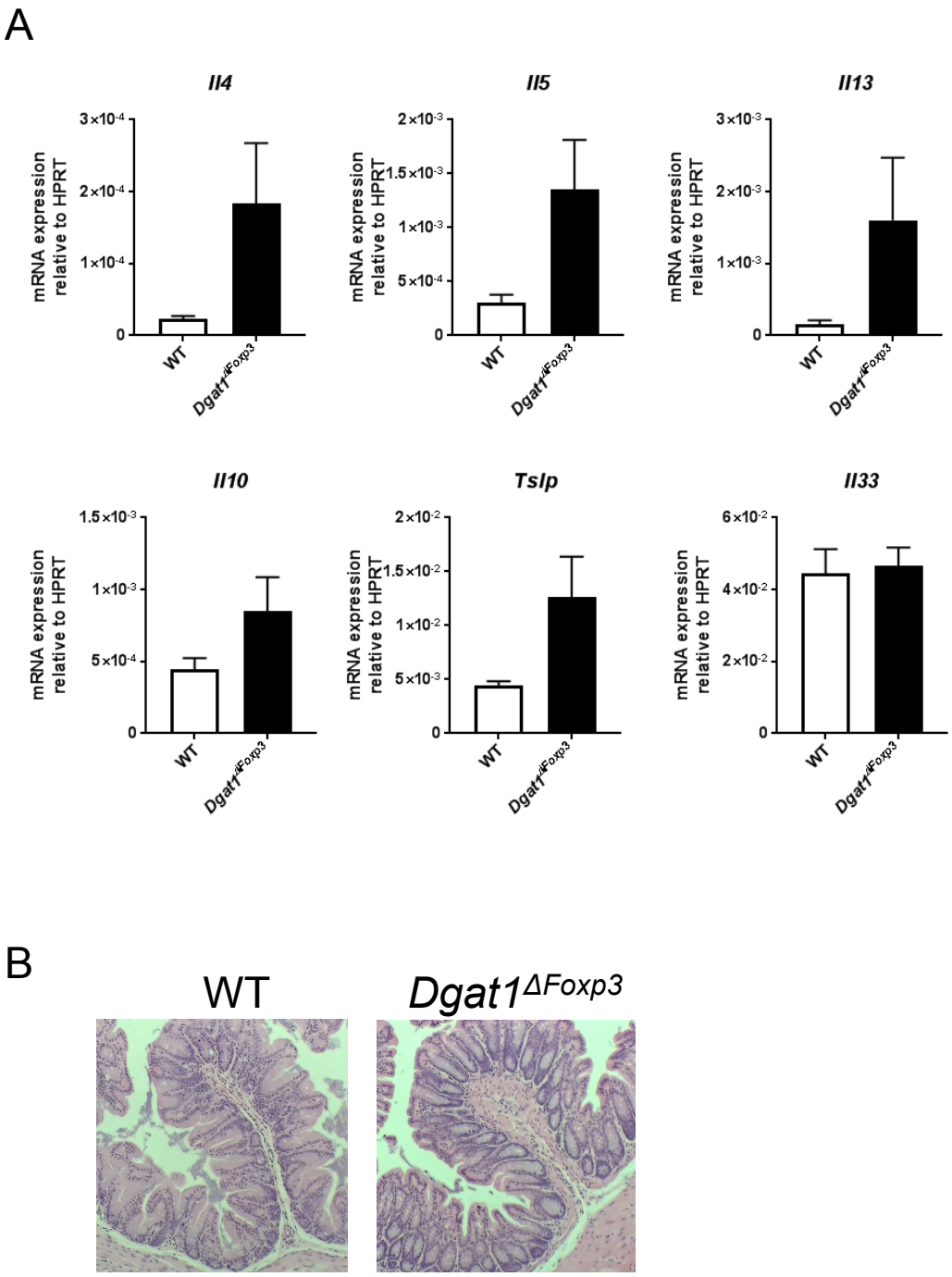


Figure 4.11: *Dgat1* deficiency in Tregs induces a non-pathologic type 2 skewing in the colon

Colon from steady state 6-9 weeks old *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice was harvested. **(A)** mRNA was extracted and expression levels of selected cytokines was assessed by qPCR analysis. Bars represent the mean ±SEM.

(B) Representative H&E staining of sections of the colon comparing *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice. Data shown are representative of two independent experiments. n=5-6 per group.

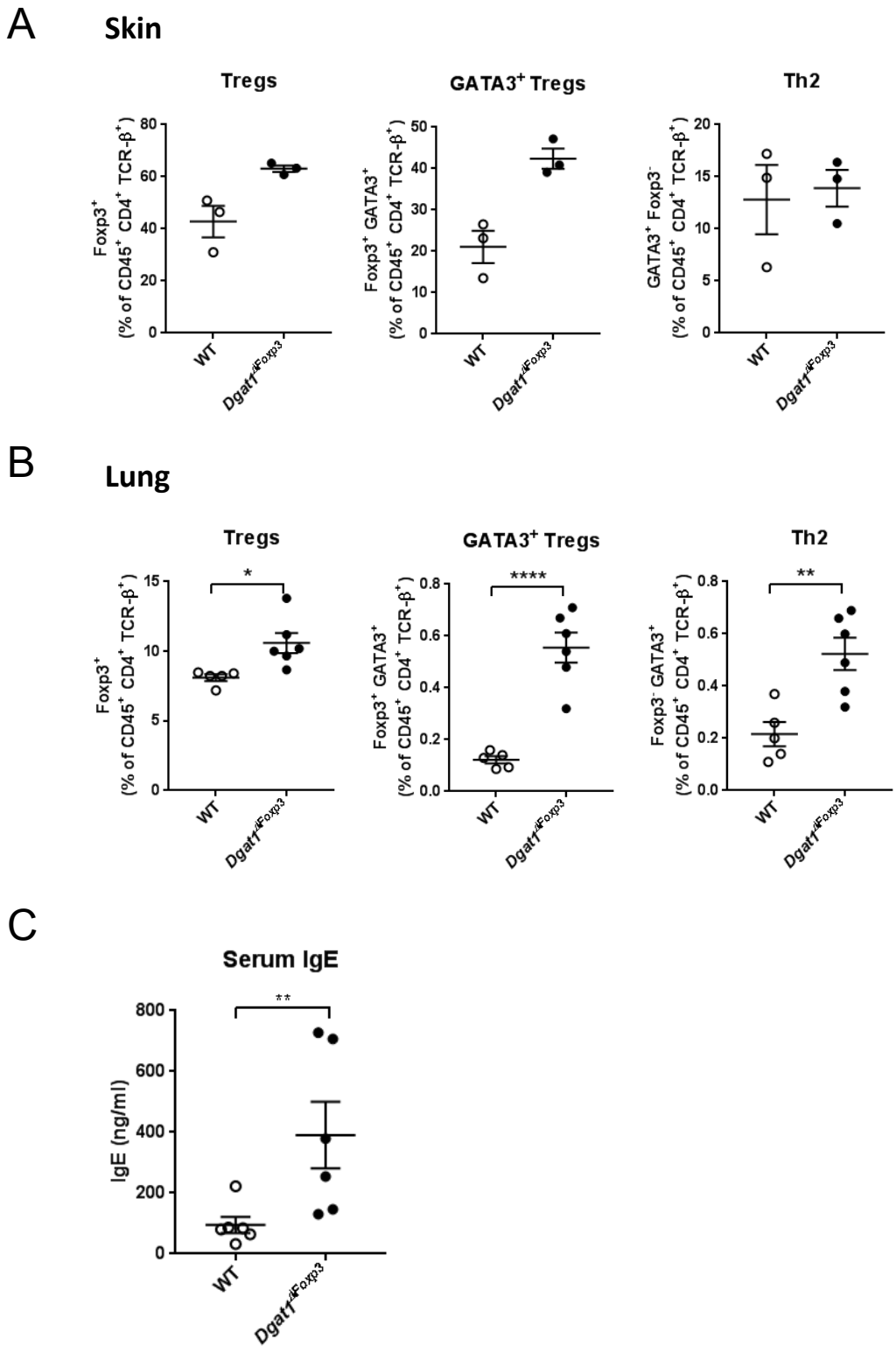


Figure 4.12: *Dgat1* deficiency in Tregs induces hyperglobulinemia E and a type 2 skewing in the skin and lung.

Lungs and skin from the ears of steady state 6-9 weeks old *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice were harvested and digested to obtain a single cell solution for flow cytometric analysis. Frequency of the indicated T cell populations from skin **(A)** and lungs **(B)** are shown. **(C)** ELISA measuring the concentration of IgE in the serum of *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice. Bars represent the mean ±SEM. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. **p*<0.05, ***p*<0.01, *****p*<0.0001. Data shown are representative of three independent experiments. n=5-6 per group.

4.2.5 Type 2 skewing in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice is enhanced with age

Foxp3^{Cre}*xDgat1*$^{fl//fl}$ mice have a skewing towards Th2 immunity due to the deletion of the *Dgat1* gene in Foxp3^{+} T regulatory cells. The mice analysed were adult 6-9 weeks old, and did not show apparent signs of pathology, despite having high levels of serum IgE, a common trait of active type 2 immune responses like food allergy [369], asthma [370] and atopic dermatitis [371]. DGAT1 has been shown to protect adipocytes from lipid-induced ER stress and associated inflammation, as it prevents toxic accumulation of fatty acids by catalysing their esterification to triglycerides [372]. Therefore, we hypothesized that *Dgat1* might have a similar function in Tregs and prevent cellular stress in a fatty acid rich environment, such as aged mice, where fatty acid-rich adipose tissue accumulates with age. We looked at older (10-11 months) *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice in order to evaluate if the absence of *Dgat1* in Tregs would result in an enhanced Th2 skewing and associated pathology. H&E histological analysis from the colon of WT and *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice did not show any sign of inflammation or pathology (Fig. 4.13a). In line with previous findings (Fig. 4.12c),

serum IgE levels were higher in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ compared to WT mice (Fig. 4.13b). Also, aged *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ had a higher concentration of serum IgE than 6 weeks old *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice. We then looked at the immune cell composition of the intestine by flow cytometric analysis. In both the colon and small intestine, the frequency and numbers of GATA3^{+} Treg cells was higher in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ than in WT mice (Fig 4.14a and Fig 4.14b). Interestingly, in the colon and small intestine of aged *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ we observed a higher frequency and number of eosinophils (Fig. 4.14a,b), a common trait of Th2 elicited immune responses [373]. qPCR analysis from whole tissue samples of both colon and small intestine showed a tendency to higher production of the Th2 associated cytokines IL-5 and IL-13 in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice when compared to WT (Fig. 4.14c),

In the skin of aged *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice we observed a significantly higher frequency of GATA3^{+} Treg cells when compared to WT mice (Fig. 4.15a), while in the lung both frequency and numbers of GATA3^{+} Treg cells were significantly higher in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ than WT mice (Fig. 4.15b). These results confirm our finding that *Dgat1* deficiency in Treg cells results in a higher frequency and number of GATA3^{+}Foxp3^{+} Tregs at barrier sites, and this leads to a systemic type 2 response characterized by increased expression of IgE that is enhanced with age. This provides further evidence that *Dgat1* ablation in Treg cells skews the immune system towards a type 2 response.

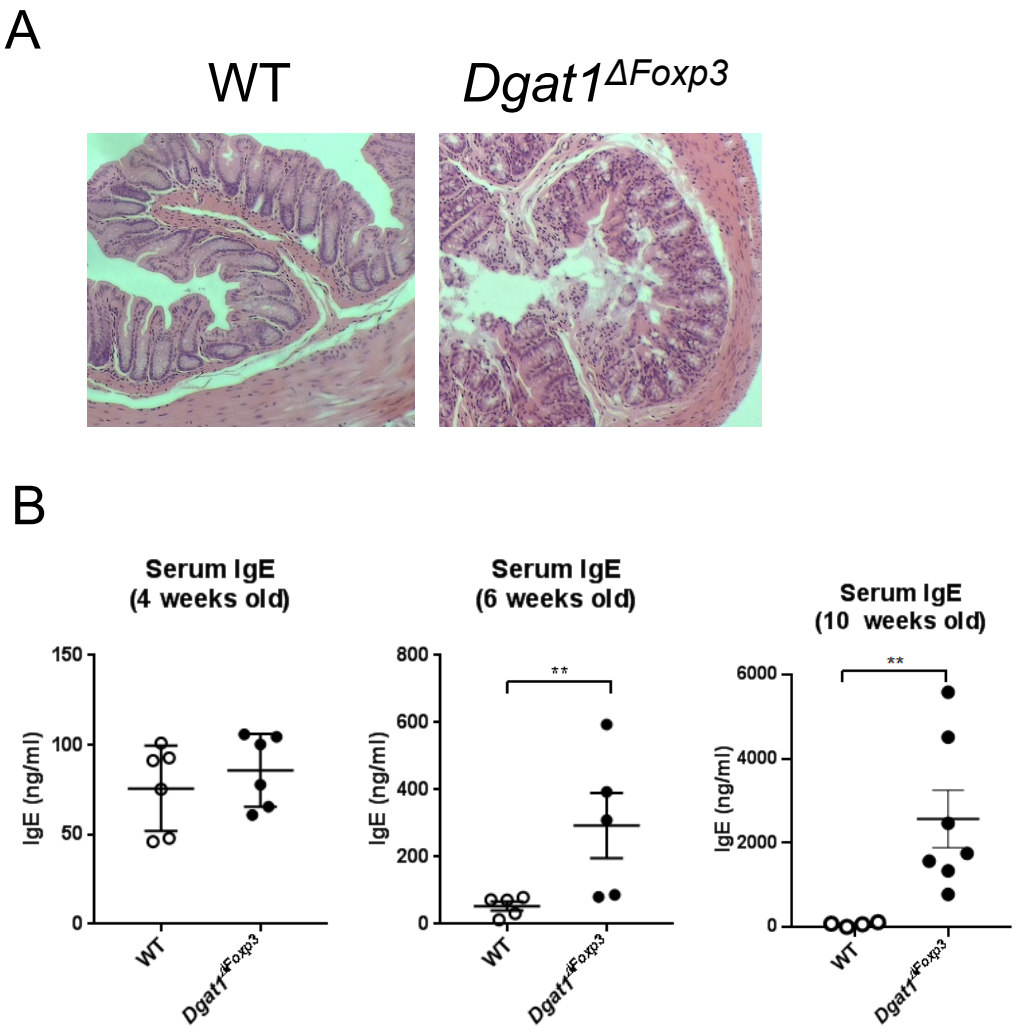


Figure 4.13: Hyperglobulinemia E in *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ is heightened with age without association of intestinal pathology

10-11 months old *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) steady state mice were sacrificed. Colon and serum were collected for analysis. **(A)** Representative H&E staining of sections of the colon comparing *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice. **(B)** ELISA measuring the concentration of IgE in the serum of *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice at different ages. Bars represent the mean ±SEM. Statistical significance was determined using

unpaired Mann-Whitney test, using GraphPad Prism. $^{**}p<0.01$. Data shown are representative of two independent experiments. n=4-8 per group.

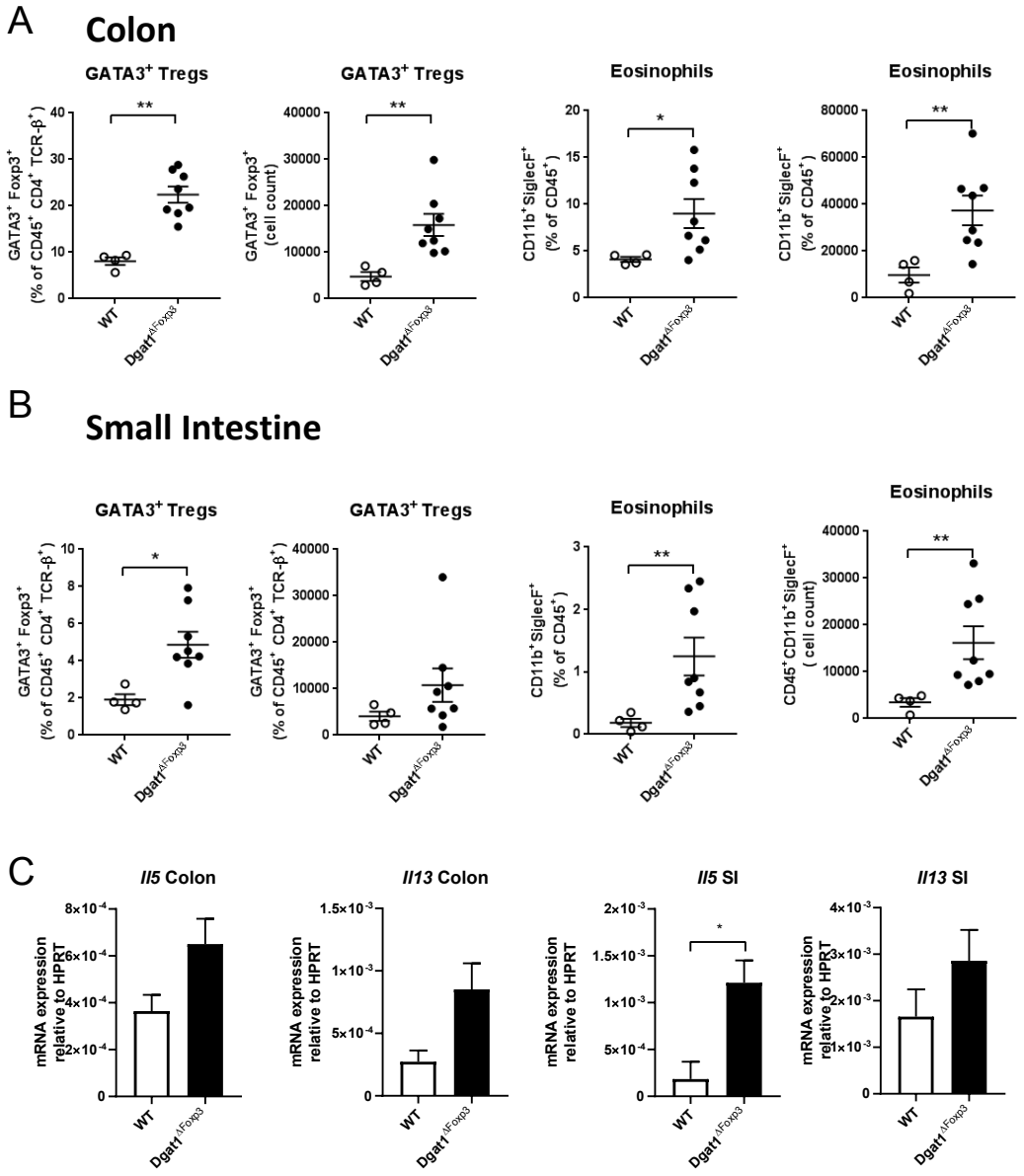


Figure 4.14: aged *Foxp3^{Cre}xDgat1$^{fl/fl}$* display a type 2 skewing with accumulation of eosinophils in the intestine

Colon and small intestine was harvested from 10-11 months old *Dgat1$^{fl/fl}$* (WT) and *Foxp3^{Cre}xDgat1$^{fl/fl}$* (*Dgat1Δ^{Foxp3}*) steady state mice. LPLs were isolated for flow cytometric analysis. Frequency and numbers of the indicated immune cells from the colon **(A)** and small intestine **(B)** are shown. **(C)** mRNA was extracted from colon

and small intestine and expression levels of selected cytokines was assessed by qPCR analysis. Bars represent the mean ±SEM. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05, **p<0.01. Data shown are representative of two independent experiments. n=4-8 per group.

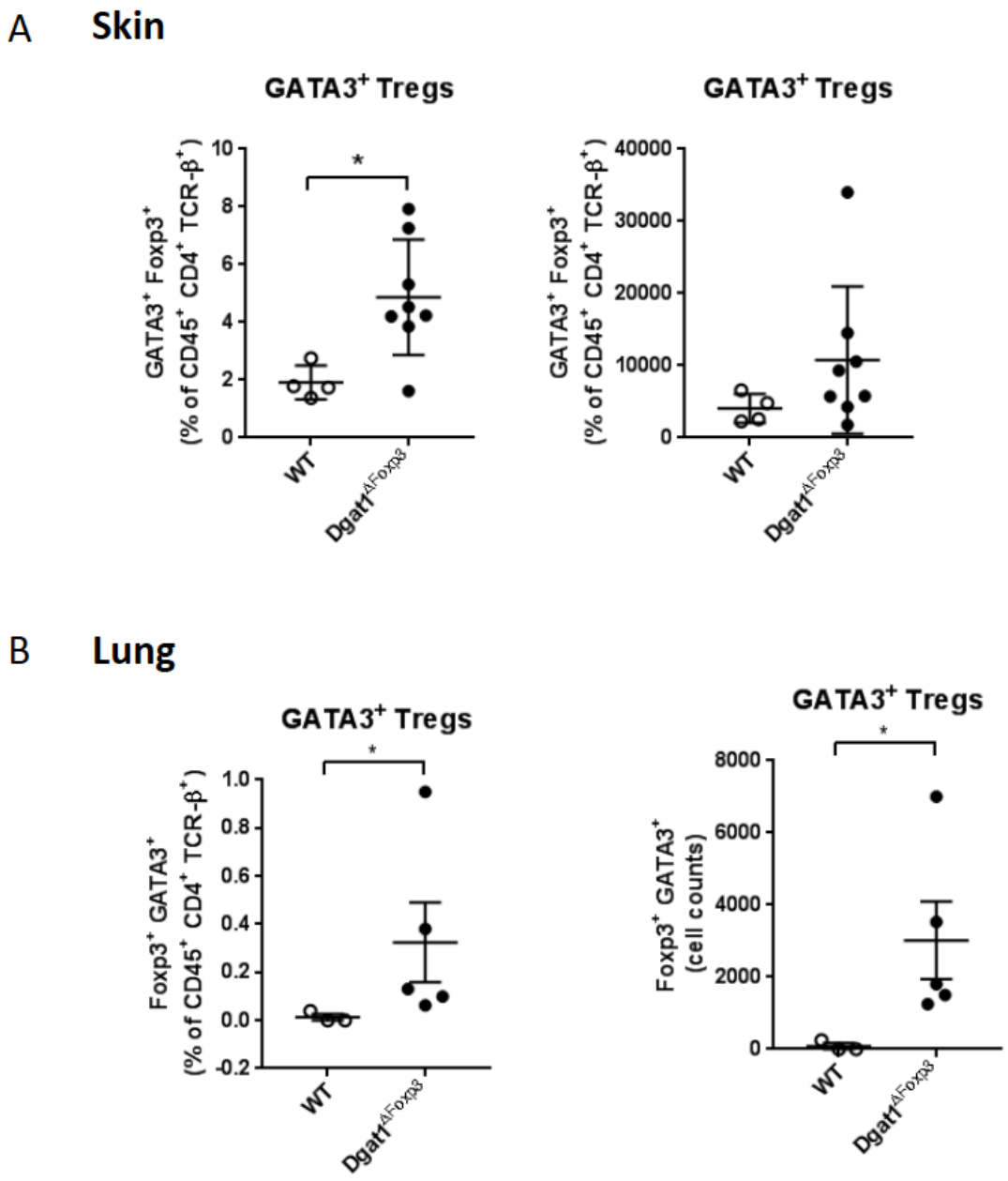


Figure 4.15: aged *Foxp3^{Cre}xDgat1$^{fl/fl}$* display increased frequency of GATA3^{+} Tregs in the skin and lung

Lungs and skin from the ears of steady state 10-11 months old *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice were harvested and digested to obtain a single cell solution for flow cytometric analysis. Frequency and numbers of the indicated T cell populations from skin **(A)** and lungs **(B)** are shown. Bars represent the mean ±SEM and each point is an individual mouse. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. **p*<0.05. Data shown are representative of two independent experiments. n=4-8 per group.

4.2.6 *Dgat1* deficiency in CD4 expressing cells do not induce a type 2 immune skewing

We have so far demonstrated that *Dgat1* deficiency in Foxp3$^+$ Tregs induce a skewing of the immune system towards a non-pathogenic type 2 immune response at barrier sites, with accumulation of GATA3$^+$ Tregs, expression of Th2 cytokines and high concentration of IgE in the serum. Most importantly, this phenotype seems to be age dependent, as hyperglobulinemia E is exacerbated in aged *Foxp3*^{Cre}*xDgat1*$^{fl/lf}$ mice. We showed in Fig. 4.4a how genetic ablation of *Dgat1* in the total CD4$^+$ population does not lead to a higher frequency of GATA3$^+$ Tregs or Th2 cells, and that the immune system is not skewed to a type 2 immune response. However, as we found that the type 2 phenotype in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice was enhanced in aged mice, we tested if *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice developed a similar immune skewing with age. Analysis by ELISA, showed that aged *CD4*^{Cre}*xDgat1*$^{fl//fl}$ mice have a concentration of IgE in the serum that is comparable to age matched WT mice (Fig. 4.16a). Along these lines, the frequency and number of GATA3$^+$Foxp3$^+$ Tregs in the colon, skin and lung was comparable between *CD4*^{Cre}*xDgat1*$^{fl//fl}$ and WT mice (Fig. 4.16b). These results indicate that deletion of *Dgat1* in the CD4$^+$ and CD8$^+$ T cell compartment does

not lead to the same effect observed in *Foxp3*^{Cre}*xDgat1*$^{fl/lf}$ mice, suggesting that the type 2 skewing observed in *Foxp3*^{Cre}*xDgat1*$^{fl/lf}$ mice is a result of a Treg specific mechanism.

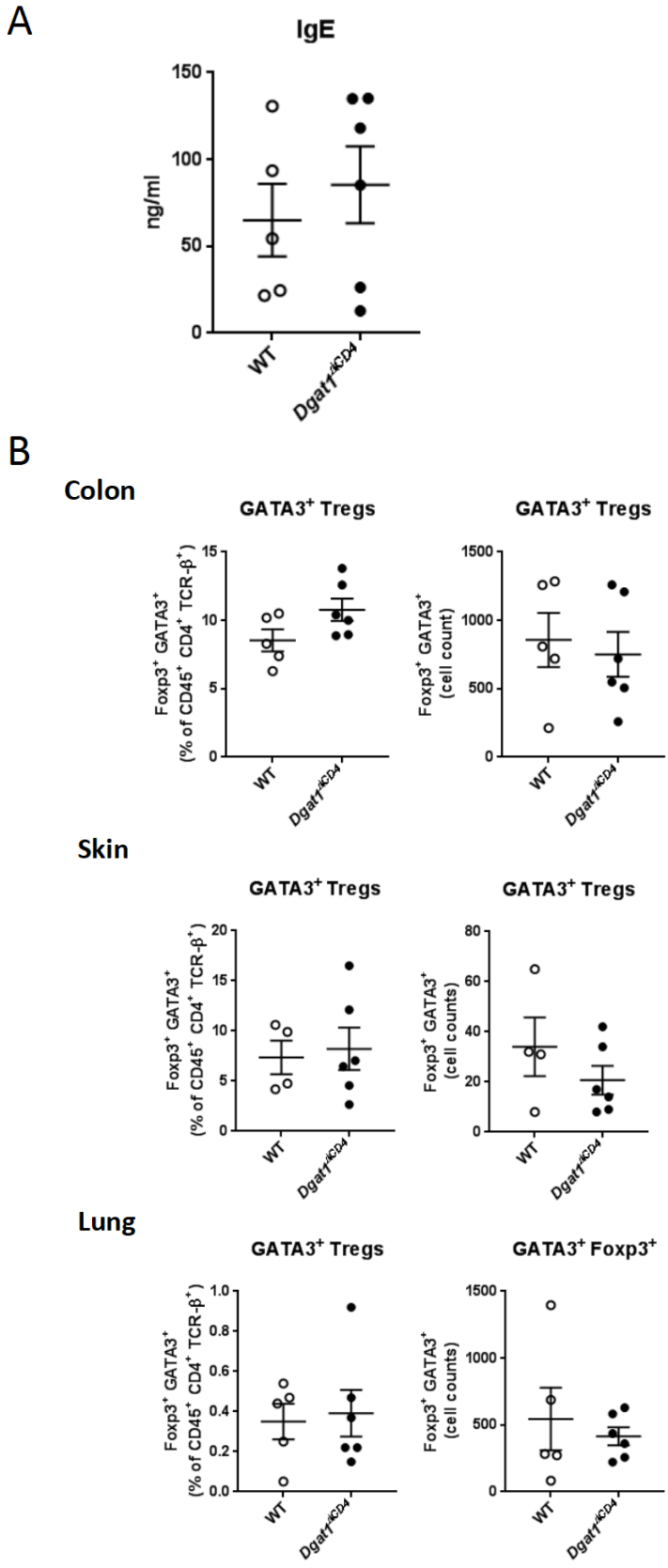


Figure 4.16: *Dgat1* deficiency in total CD4$^+$ cells of aged mice does not induce type 2 skewing

10-11 months old *Dgat1*$^{fl/fl}$ and *CD4*^{Cre}*xDgat1*$^{fl/fl}$ steady state mice were sacrificed for tissue collection and analysis. **(A)** ELISA measuring the concentration of serum IgE. **(B)** Colon (top), skin from the ears (middle) and lungs (bottom) were digested to obtain a single cell solution for flow cytometric analysis. Frequency and numbers of the indicated T cell population are shown. Bars represent the mean ±SEM and each point is an individual mouse. Data shown are representative of two independent experiments. n=5-6 per group.

4.2.7 *Dgat1* deficiency in Treg cells enhances the effects of calcipotriol treatment

Foxp3^{Cre}*xDgat1*$^{fl//fl}$ mice present several features that are commonly associated with Th2-driven pathologies, such as elevated concentrations of IgE in the serum [173], increased expression of Th2 cytokines [41] and accumulation of eosinophils [373]. However, *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice do not show apparent signs of pathology compared to WT mice. To assess if *Dgat1* deficiency in Foxp3^{+} Tregs affects the development of type 2 immune responses, we moved on to test the *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice in a model of type 2 inflammation. The skin is one of the barrier sites where *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ display a type 2 skewing. One of the most common Th2 associated skin inflammatory disorder is atopic dermatitis, affecting between 10 and 20% of the general population [374], and characterized by skin lesions with high expression of Th2 cytokines like IL-4, IL-5 [375] and IL-13 [376], hyperglobulinemia E and eosinophilia [377]. Topical application of the vitamin D3 analogue calcipotriol has been used as a mouse model of atopic dermatitis [378]: treatment with the vitamin D receptor agonist induces production of TSLP in epidermal keratinocytes that in turn induce expression of Th2 cytokines and production of IgE, resembling the skin

lesions typical of atopic dermatitis patients [305]. Therefore, *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ and WT mice were treated on the skin of both ears with calcipotriol and sacrificed after 10 days (Fig. 4.17a). Disease progression can be tracked by measuring ear thickness, which is indicative of inflammatory cell infiltration. Both *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ and WT mice developed an inflammatory response to the topical application of calcipotriol, however no difference in the intensity of the disease was observed, as shown by the comparable percentage increase in ear thickness between WT and *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice (Fig. 4.17b). Calcipotriol treatment induces hypergloublinemia E in mice [305]. A significantly higher concentration of serum IgE was observed in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ compared to WT mice treated with calcipotriol (Fig 4.17c). However, frequencies of total $CD4^+TCR\beta^+Foxp3^+$ Tregs, $Foxp3^+GATA3^+$ Tregs and $GATA3^+$ Th2 cells were unchanged upon inflammation and similar between WT and *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice (Fig 4.17d). When we looked at IgE coated immune cells, we observed a significantly increased frequency and number of $CD45^+$ IgE^+ cells in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice when compared to WT (Fig. 4.17a). $CD45^+$ cells from calcipotriol treated *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice also showed a higher intensity of IgE signal, as shown by MFI (Fig. 4.18a), indicating increased per/cell coating with IgE upon inflammation. The increase in frequency and numbers of IgE coated cells from *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice was observed in both untreated and calcipotriol treated mice (Fig 4.18a). This data suggest that the high production of IgE in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice might have a direct effect on the innate immune compartment. In fact, IgE is known to bind to the FcεRI and activate innate immune cells like mast cells and basophils in allergic inflammation and atopic dermatitis [379]. Indeed, in this disease model we observed an increased frequency and number of IgE^+ coated basophils in *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ mice (Fig 4.18b). We also looked for IgE coated mast cells, but did not observe a

differential frequency or number between *Foxp3*^{Cre}*xDgat1*$^{fl//fl}$ and WT treated mice (Fig 4.18b). However, it was interesting to observe that *Foxp3CrexDgat1*$^{fl//fl}$ vehicle treated mice had higher frequency and number of IgE coated mast cells when compared to WT counterparts (Fig 4.18b), suggesting that the hyperglobulinemia E that occurs in the setting of *Dgat1* deficiency in Tregs could induce an active state in mast cells even in unchallenged mice.

As previously reported [305], calcipotriol treatment induced expression of type 2 associated cytokines, but no differential expression of *Tslp*, *Il4*, *Il5*, *Il13*, *Il33* or *Il10* was detected between *Foxp3CrexDgat1*$^{fl//fl}$ and WT mice (Fig. 4.19).

With this experiments we have shown how *Dgat1* deficiency in Treg cells affects the immune response of an atopic dermatitis model, although without exacerbating disease severity.

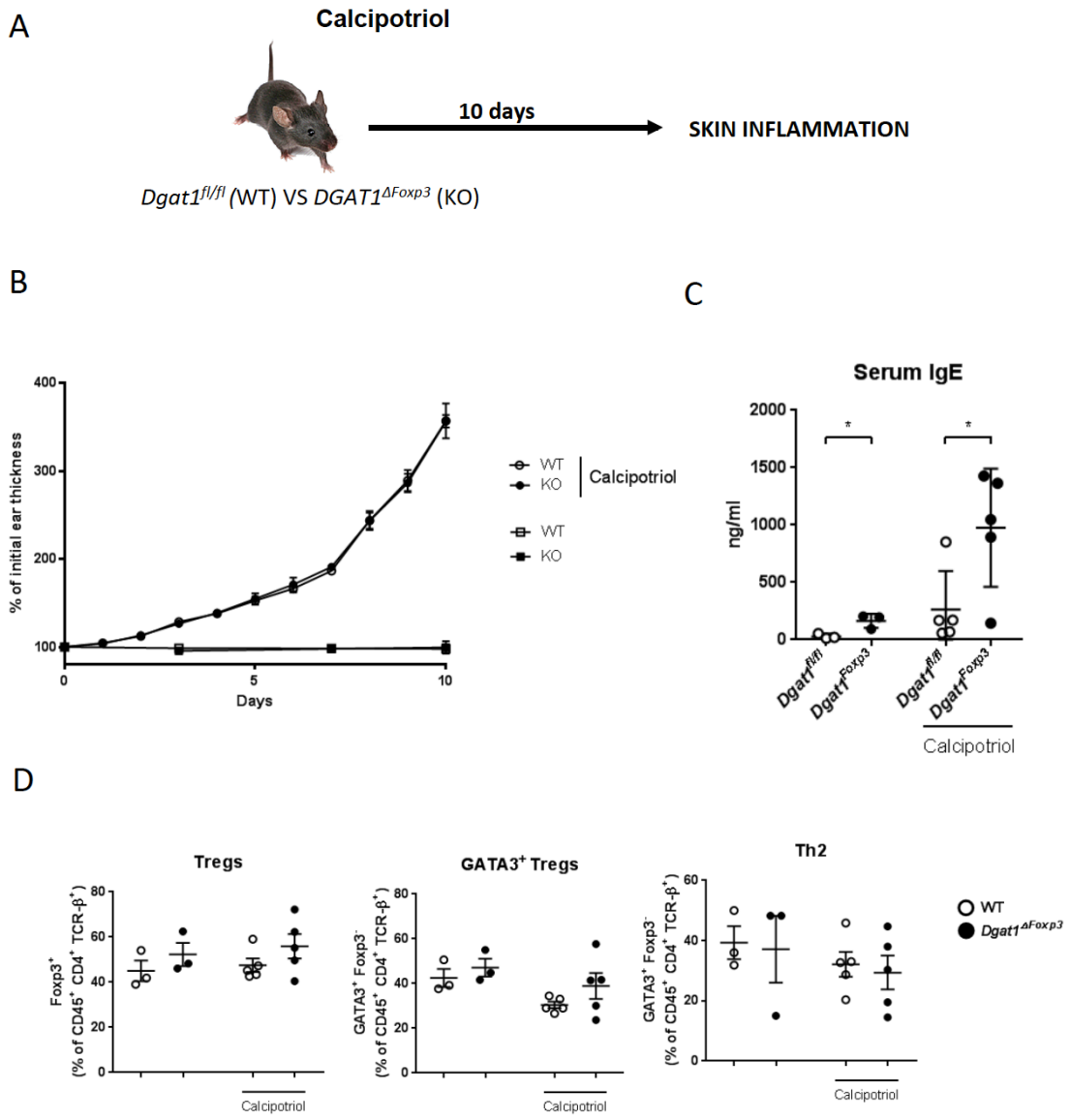


Figure 4.17: Topical application of calcipotriol induce comparable skin inflammation in WT and *Foxp3CrexDgat1*$^{fl//fl}$ mice

(A) Experiment layout: *Dgat1*$^{fl/fl}$ (WT) and *Foxp3CrexDgat1*$^{fl//fl}$ (KO) mice were treated with calcipotriol or vehicle for 10 days on the skin of both ears. **(B)** Inflammation of the skin was assessed by measuring the increase in thickness of the ears (shown as percentage of initial thickness at day 0). **(C)** ELISA measuring the concentration of IgE in the serum of *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice. **(D)** Flow cytometric analysis of skin immune cells. Frequency of

the indicated cell population is shown. Bars represent the mean ±SEM. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05. One experiment. n=4-5 per group.

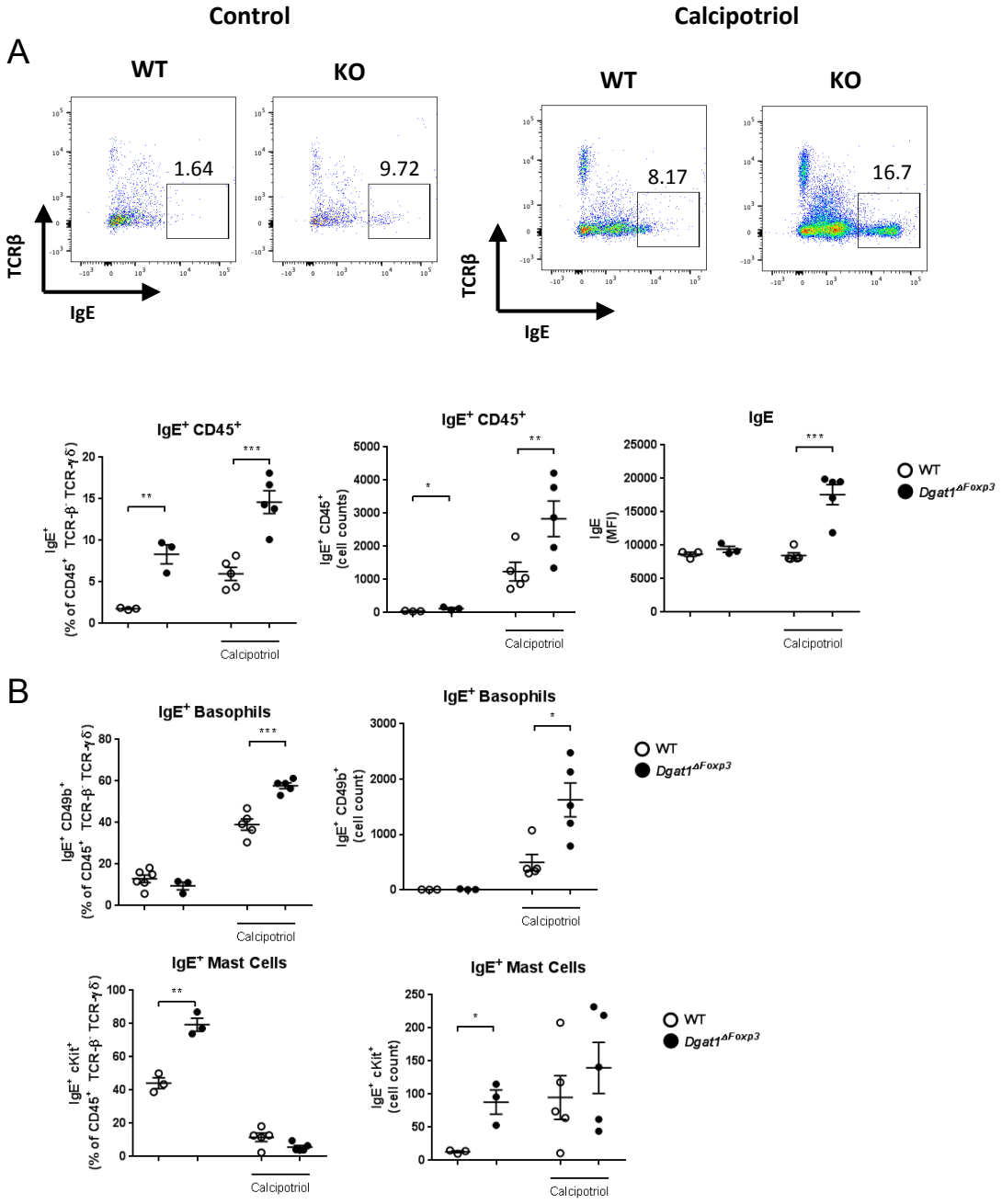


Figure 4.18: *Foxp3CrexDgat1*$^{fl/fl}$ calcipotriol treated mice display a higher proportion of IgE coated immune cells

Skin from the ears of steady state and calcipotriol treated *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice was harvested for flow cytometric analysis.

(A) and **(B)** Frequency and numbers of the indicated immune cell population are shown. Bars represent the mean ±SEM and each point is an individual mouse. A representative flow cytometry plot is also shown (top). Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. **p*<0.05, ***p*<0.01, ****p*<0.001. One experiment, n=4-5 per group.

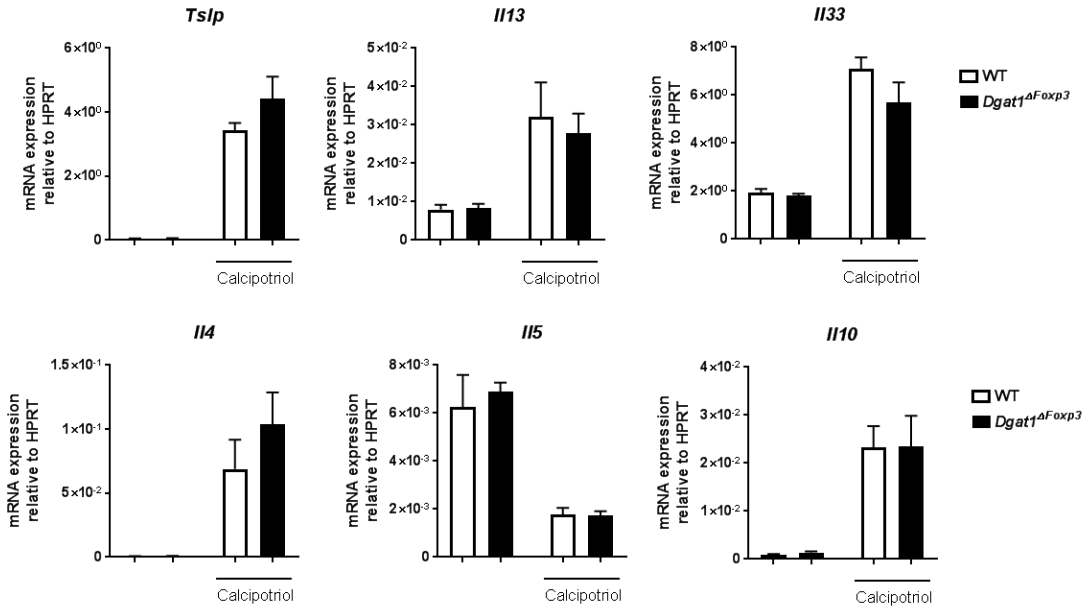


Figure 19: Calcipotriol treatment does not induce a differential expression of Th2 cytokines in *CD4*^{Cre}*xDgat1*$^{fl/fl}$ compared to WT

Skin from the ears of steady state and calcipotriol treated *Dgat1*$^{fl/fl}$ (WT) and *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ (*Dgat1Δ*^{Foxp3}) mice was harvested for gene expression analysis. mRNA was extracted and the expression of selected cytokines measured by qPCR analysis. Bars represent the mean ±SEM. One experiment, n=4-5 per group.

4.3 Discussion

In this chapter we have identified a novel function for DGAT1 in the Treg control of type 2 immune responses at barrier sites. Confirming preliminary data, we showed that the expression of *Dgat1* is higher at barrier sites than in secondary lymphoid organs. Specifically, we demonstrated how *Dgat1* is upregulated by $CD4^+$ effector and naïve T cells, $CD8^+$ T cells and $CD4^+$ Tregs in the colon, a finding that further suggested a role of DGAT1 in T cell responses.

Expression of *Dgat1*, a gene that encodes for an enzyme involved in fatty acids metabolism [352] is upregulated by T cells in organs like the skin and the intestine, which are lipid rich environments. In the skin, lipids contribute to provide a water resistant barrier to the epidermis: sebaceous glands and keratinocytes are the main source of lipids [380], with free fatty acids being the most abundant form of lipids [381]. The intestinal mucosa is also a fat-rich tissue, as it is the site where lipids from the diet are absorbed. In non-adipocyte cells like lymphocytes, exposure to high doses of FAs can lead to cellular stress and ultimately to damage and apoptosis, a process called lipotoxicity, as they have a limited capacity to convert fatty acids into TG [382]. Adipocytes are protected from fatty acid induced stress by DGAT1, which has been shown to avoid excessive FAs accumulation and ER stress by mediating the esterification of FAs into TG [372]. This protective mechanism is conserved in non-adipocytes, as in an *in vivo* model of lipotoxic cardiomyopathy, DGAT1 overexpression in cardiomyocytes increased FAs metabolism and reduced apoptosis [383]. Therefore, we can speculate that T cells, when in a lipid rich environment, upregulate the expression of DGAT1 as a protective mechanism to avoid cellular stress and damage. This could be assessed by culturing T cells *in vitro* with a mix of FAs and test if FAs induce *Dgat1* expression. Also, comparing the viability of WT

and *Dgat1* deficient T cells cultured with increasing concentrations of FAs could reveal a potential protective role of DGAT1 in T cells.

In order to understand how DGAT1 affects T cell responses at barrier sites, we generated a conditional mouse strain, the $CD4^{Cre}xDgat1^{fl/fl}$ mice, where *Dgat1* is selectively excised in CD4 expressing cells. However, as both $CD4^+$ and $CD8^+$ T cells during their development in the thymus co-express CD4 and CD8 [365], we detected *Dgat1* excision in both T cell subsets. As $CD4^+$ T cells are our main focus of study, this is a limitation of our *in vivo* system, since we cannot exclude that *Dgat1* deficiency in $CD8^+$ T cells could have an effect on the overall T cell response.
Characterization by flow cytometric analysis showed similar frequencies of T helper cells in the intestine of WT and $CD4^{Cre}xDgat1^{fl/lf}$ mice, indicating that in a homeostatic condition *Dgat1* deficiency does not affect T cell maintenance. However, *Dgat1* deficient $CD4^+$ T cells showed an increase in production of IL-17F and IL-17A. It has to be noted that this result was only partially confirmed in a repeat of this experiment, possibly due to the fact that the mice from the second experiment were housed in a different room in our facility, and microbiota changes might have affected the Th17 response. The increase in production of Th17 cytokines suggested that DGAT1 expression might have a potential role in restraining intestinal inflammation. In fact, IL-17F is produced by IL-23 responsive Th17 cells [56] and is thought to contribute to the development of IBD [76, 384]. Therefore, through an unknown mechanism, DGAT1 might function as a modulator of Th17 responses in the intestine. In order to test this hypothesis, and in light of the high expression level of *Dgat1* in effector $CD4^+$ T cells at barrier sites, we tested the role of DGAT1 in mediating immune responses in the intestine. We set up an adoptive T cell transfer

model of colitis, which allowed us to selectively transfer naïve $CD4^+$ T cells from WT or $CD4^{Cre}xDgat1^{fllflf}$ mice. With this model we were able to study the development of T cell dependent pathology, comparing WT and *Dgat1* deficient T cells, while excluding the possible confounding effect of *Dgat1* deficient $CD8^+$ T cells, as the adoptively transferred cells are purity-checked FACS sorted $CD4^+$ T cells. Although both the transfer of WT and *Dgat1* deficient T cells induced colitis, we did not observe a differential level of pathology between the two groups nor detected differences in the immune cell compartment. This could mean that DGAT1 expression in $CD4^+$ T cells is not necessary for inducing inflammation in the intestine. However, in adoptive T cell transfer the organs which naïve $CD4^+$ T cells are isolated from are spleens and lymph nodes, where, as we have shown in this study, expression of *Dgat1* is low in the T cell compartment. It is possible that transferred naïve T cells upregulate *Dgat1* expression upon colonizing the intestine, but we cannot exclude that the levels of *Dgat1* in the transferred cells remained low throughout the experiment, even when acquiring an effector phenotype. This could be tested by comparing the starting splenic naïve $CD4^+$ T cell population with $CD4^+$ T cells FACS sorted from the inflamed intestine and compare the expression of *Dgat1* in the two groups. To confidently exclude a role of DGAT1 in T cell mediated colitis, other studies on models of disease should be considered.

In $Foxp3^{Cre}xDgat1^{fl/fl}$ adult mice, we observed a skewing of the immune system towards type-2 immunity, characterized by accumulation of $GATA3^+$ Tregs at barrier sites (skin, lung and intestine), increased expression of Th2 cytokines and higher concentration of serum IgE than in WT mice. The type 2 skewing observed in $Foxp3^{Cre}xDgat1^{fl/fl}$ mice is an age dependent phenomenon, as aged $Foxp3^{Cre}xDgat1^{fl/fl}$

mice displayed an enhanced phenotype with marked accumulation of GATA3^{+} Tregs and eosinophils at barrier sites and a much higher concentration of serum IgE than 6-9 week old mice. Interestingly, the skewing of the immune system towards a type-2 response does not come with pathology, as *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice did not show any sign of disease. Although further studies are needed to clarify the mechanism by which DGAT1 controls type 2 immunity, we can speculate that its role in protecting from lipid-induced cellular-stress might be involved. In fact, studies on oxidative stress in human T cells revealed how in a cellular stress-inducing environment, CD4^{+} T cells acquire a Th2 phenotype and upregulate the type 2 cytokines IL-5 and IL-13 [385]. We can then hypothesize the type-2 switch could be a consequence of the stress response resulting from the accumulation of fatty acids in *Dgat1* deficient Tregs. In turn, the increased expression of Th2 cytokines might explain the infiltration of eosinophils, known to be mediated by IL-5 [386], and the increased expression of immunoglobulin E.

Whether DGAT1 deficiency has a cell intrinsic effect and induces a transdifferentiation of Tregs toward a Th2 phenotype is yet to be determined. Tregs are plastic cells and multiple studies report how in certain conditions Tregs can acquire T helper features, including Th2 [387, 388]. In the study by Noval Rivas *et al.* [388], mice with enhanced IL-4R signalling lost Treg-mediated oral tolerance to ovalbumin and developed allergic disease. This was due to a failure in the regulatory activity of Tregs that underwent a Th2-like reprogramming with acquired expression of GATA3 and IL-4. Similarly to our results, this study showed how the transdifferentiation of Tregs toward Th2-like cells can lead to loss of tolerance and promotes IgE responses. Tracking Foxp3 expression by generating a lineage-tracing

Dgat1 deficient mouse strain would allow us to understand if Dgat1 deficiency induces a reprogramming of Tregs into Th2-like cells.

Several studies have demonstrated the fundamental role of Treg cells in maintaining immune tolerance to specific antigens at barrier sites [111, 389]. Tregs can modulate allergic responses directly by acting on B cells and promoting the expression of IgG over IgE antibodies [184]. Tregs can achieve regulation of IgE responses also by inducing tolerogenic DCs or by suppressing the production of Th2 cells, both mechanism that are CTLA-4 dependent [117, 119]. Therefore, the heightened production of IgE in *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice is possibly due to a failure of Treg cells to induce tolerance to antigens that in normal conditions would not drive an IgE response. However, what antigen this IgE response is raised against is yet to be determined: as we detect a type 2 skewing in multiple peripheral organs (skin, lung, intestine) the specific antigen could be of environmental origin (microbiome, food or airborne) or a self-antigen. The continuous exposure to a specific antigen, with concomitant impaired tolerance due to *Dgat1* deficiency in Treg cells, provides a possible explanation to the age related enhancement of the observed phenotype. It is possible that the lost tolerance in *Dgat1* deficient mice leads to a progressively stronger type 2 response that sustains itself in a positive feedback loop. Therefore, *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ aged mice display a more pronounced skewing to type 2 immunity than adult mice as a consequence of continuous antigen experience.

Interestingly, the skewing of the immune system to a type 2 response was not detected in aged *CD4*^{Cre}*xDgat1*$^{fl/fl}$ mice, where all CD4^{+} T cells, including Foxp3^{+} Tregs, are *Dgat1* deficient. A possible explanation to this finding is that in *CD4*^{Cre}*xDgat1*$^{fl/lf}$

mice, non-Foxp3 expressing $CD4^+$ T might need expression of DGAT1 to mount a Th2 response. Therefore, although *Dgat1* deficiency in $Foxp3^+$ Tregs impairs immune tolerance, the concomitant deficiency of *Dgat1* in the whole $CD4^+$ compartment does not result in a type 2 response. It would be interesting to test this hypothesis in a model of type 2 inflammation: if expression of DGAT1 is needed for Th2 responses, we can speculate that in a model of antigen induced hypersensitivity, like OVA induced food allergy [390], the type 2 response should be lower in $CD4^{Cre}xDgat1^{fl/fl}$ than in WT mice.

As discussed above, the type 2 response observed in $Foxp3^{Cre}xDgat1^{fl/fl}$ mice does not lead to tissue damage or overt pathology, despite showing several features of Th2 associated inflammatory disorders like eosinophilia, hyperglobulinemia E and expression of Th2 cytokines. In order to study the role of DGAT1 in Tregs in the context of disease, we set up a model of atopic dermatitis, a skin inflammatory disorder characterized by a deranged Th2 immune response, by treating WT and $Foxp3^{Cre}xDgat1^{fl/fl}$ with calcipotriol, a vitamin D analogue [378]. Both WT and $Foxp3^{Cre}xDgat1^{fl/fl}$ mice developed disease when compared to untreated controls, but no difference in the pathology score was observed between the two treated groups. Despite the similar level of pathology, $Foxp3^{Cre}xDgat1^{fl/fl}$ mice showed a marked increase in concentration of serum IgE and accumulation of IgE coated innate cells in the skin when compared to WT mice. The increased frequency and numbers of IgE coated basophils, suggested an increased activation of the innate immune system, as IgEs bind to FcεRI expressing innate cells to induce their activation [379]. As a matter of fact, immunoglobulinemia E has been shown to be contributing to pathology in atopic dermatitis. In fact, promising results have been obtained on atopic dermatitis

patients treated with Omalizumab, a humanized monoclonal antibody targeting the high-affinity Fc receptor of IgE [391]. In light of this, it was unexpected to observe that, although calcipotriol treated *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ mice display a pronounced IgE response, this does not result in enhanced pathology. This might be due to the fact that the calcipotriol treatment regimen used in this experiment was already saturating the inflammatory capacity of the immune system, therefore masking potential differences in the pathogenic potential of *Dgat1* deficient Tregs. It would be interesting to test the role of *Dgat1* in Tregs on mice challenged with a sub-optimal calcipotriol treatment that might reveal a difference in pathology between *Foxp3*^{Cre}*xDgat1*$^{fl/fl}$ and WT mice.

With this study we have unveiled a new aspect of Treg induced immune tolerance at barrier sites. We have demonstrated that Tregs control Th2 like immunity and IgE production through DGAT1, and showed that *Dgat1* deficiency in Tregs leads to the development of a type 2 immune response. However, the specific mechanism through which Tregs exert their immunomodulatory function in this context is yet to be determined. Comparing the transcriptome of WT and *Dgat1* deficient Tregs isolated from the skin, lung and intestine, might reveal a set of genes that are differentially expressed in KO cells, therefore unveiling molecules that contribute to the tolerogenic potential of barrier sites Tregs.

5 Chapter 5: The effect of IL-23 on intestinal Th2 cells

5.1 Introduction

Epidemiological studies have described a distinct geographical distribution of IBD cases, with a higher prevalence in Western countries than in the developing world [392]. In addition, since 1990 the incidence of IBD has been increasing in newly industrialized countries in Africa, Asia and south America [393], suggesting that the adoption of higher hygienic standards, a consequence of economic and social development, led to higher likelihood of developing chronic inflammatory disorders like IBD. A causative link is yet to be determined, but it has been hypothesized that modern hygiene practises reduce the exposure to microbes and parasites, and this can affect the development of a fully functional immune system [394]. These epidemiologic observations led to the hypothesis that infection with intestinal parasites could protect from the development of IBD and has been proposed as a possible treatment [395]. Parasite infections induce a protective immune response in the host that can impair the worm development from a larval stage and affect its survival, inducing worm expulsion [396]. This immune response is characterized by the induction of Th2 associated cytokines such as IL-4, IL-5, IL-13 [397] as well as T cell- and innate cells derived IL-10 [398], that in turn can promote the emergence of alternatively activated macrophages [174]. Parasite driven Th2 responses are known to modulate concomitant immune responses: patients infected with *Schistosoma mansoni* mount an attenuated Th1 response to tetanus toxoid immunization [399]. This has led to the hypothesis that parasite infections could protect from the development of IBD by inhibiting pathogenic Th1/Th17 responses [400]. This

hypothesis is also based on findings from animal models of intestinal inflammation. Indeed, exposure to eggs from *Schistosoma mansoni*, induced a Th2 protective response that ameliorated disease in a TNBS-induced mouse model of colitis [401]. Studies suggest how this beneficial effect on colitis symptoms is dependent on Th2 cytokines like IL-13 [402]. Infection with helminths as a possible therapy for active IBD has been trialled in patients. A randomized controlled trial tested the effect of *Trichuris suis* infection in IBD patients, showing amelioration of clinical symptoms and improvement of the Disease Activity Index [403]. However, repeated infections were needed to reach clinical efficacy and concerns were raised about possible side effects like persistent infection and migration to other tissue sites [404].

Th17 and Th2 responses are reciprocally regulated, and this has been shown in mouse models of asthma [405] in models of colitis [406] and parasitic infections [407]. Several *in vitro* studies revealed how type 2 cytokines like IL-13 [406] and IL-4 [408] can suppress Th17 differentiation. This has been demonstrated in vivo too, in fact IL-13 was shown to inhibit IL-17 expression in a mouse model of house dust mite induced asthma [409] Furthermore, IL-13 attenuates colitis in *Il10*$^{-/-}$ mice through its inhibitory effect on Th1 and Th17 responses in the intestine [406]. However, little is known about IL-23 and the development of Th2 mediated immune responses. One study by Peng *et al.* [410] demonstrated that IL-23R-deficient mice have reduced inflammation and decreased levels of Th2 cytokines when compared to WT mice in a model of allergic airway inflammation. This study also showed that in a transgenic mouse overexpressing *Il23r* in T cells, expression of Th2 cytokines is enhanced upon intranasal OVA challenge. Nonetheless, it does not provide evidence of a direct effect

of IL-23 on Th2 cells in a physiological setting, nor investigate this axis in intestinal inflammation.

Previous work in our lab identified IL-23 as a pivotal cytokine in the development of colitis due to its direct effect on promoting Th17 responses [268]. The study by Schiering *et al.* [144] investigated the opposing effects of IL-33 and IL-23 on the emergence of Tregs in the context of colitis. That study described how IL-33 acts on the promotion of $Foxp3^+$ iTreg differentiation both *in vitro* and *in vivo* through direct effects on T cells. This promoting effect of IL-33 was inhibited by IL-23, providing a mechanistic link for the known role of IL-23 in restraining Treg cell generation [238]. With the aim of identifying genes that are intrinsically regulated by IL-23, they used an adoptive T cell transfer model of colitis. The transfer of naïve T cells into $Rag1^{-/-}$ mice drives a T cell dependent form of colitis and is therefore a powerful system to study T cell induced inflammation. Although transfer of $Il23r^{-/-}$ naïve T cells only induces mild pathology, the transfer of a mix of WT and $Il23r^{-/-}$ T cells leads to a severe form of colitis, as the presence of IL-23R sufficient T cells overcomes the necessity for a cell-intrinsic IL-23R signalling for T cell activation in the intestine [268]. By transferring a 1:1 mixture of WT ($CD45.1^+$) and $Il23r^{-/-}$ ($CD45.1^-$) naïve T cells, isolated from spleens and lymph nodes of donor mice, this study allowed to compare WT and $Il23r^{-/-}$ T cells isolated from an inflamed setting (Fig 5.1a). The mice were sacrificed when inflammation of the colon was detected (~8 weeks) and colonic $TCR\beta^+CD4^+$T cells FACS-sorted for mRNA extraction. Whole transcriptome gene expression analysis identified a Th2 signature in $Il23r^{-/-}$ T cells: the type 2 cytokines *Il4, Il5, Il13,* the transcription factors *Gata3* and *maf* and the receptor for IL-33 *Il1rl1* were among 104 genes that were significantly upregulated in $Il23r^{-/-}$ T

cells (Fig 5.1b). This indicated that IL-23 acts as a negative regulator of Th2 differentiation in the intestine *in vivo* in a cell-intrinsic manner. This study did not measure increased production of type 2 cytokines by *Il23r*$^{-/-}$ T cells at the protein level and did not investigate the physiological relevance of IL-23 signalling on Th2 cell development. Therefore, it remains to be determined how IL-23 regulates Th2 responses.

In this Chapter we studied how the differentiation and effector function of Th2 cells is affected by IL-23 in the intestine. We hypothesised that IL-23R-deficient T cells would skew towards a Th2 phenotype that could ameliorate colitis symptoms in mouse models of disease. Therefore the aims of this chapter are:

- To describe the effects of absence of IL-23 signalling on intestinal Th2 cells.
- To assess if an enhanced Th2 response ameliorates disease in mouse models of colitis.
- To determine if IL-33 and IL-23 have opposing effects on the *in vitro* differentiation of Th2 cells

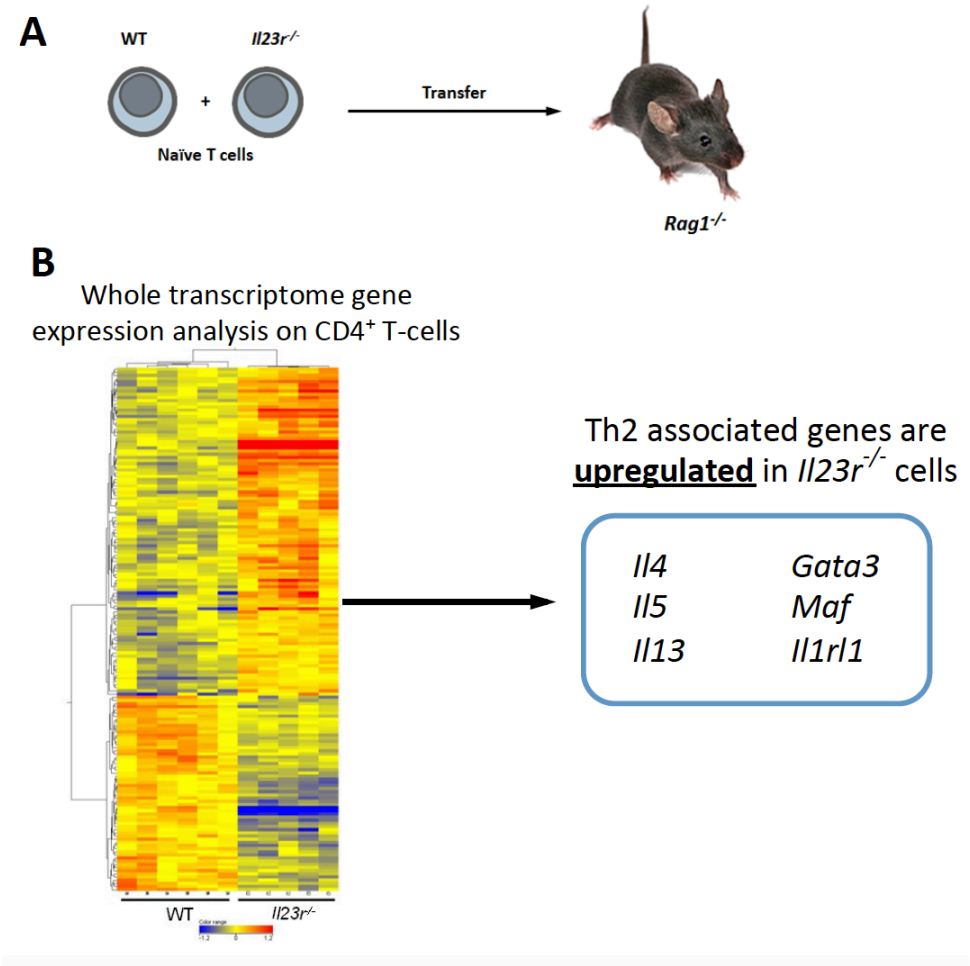


Figure 5.1: *Il-23r*$^{-/-}$ T cells have a Th2 signature

(A) C57BL/6 *Rag1*$^{-/-}$ mice were transferred i.p. with a 1:1 mixture of WT (CD45.1^{+}) and *Il23r*$^{-/-}$ (CD45.2^{+}) naïve CD4^{+}CD45RB^{hi} T cells. Upon development of colitis, mice were sacrificed and leukocytes from the colon lamina propria isolated. **(B)** TCR-β^{+}CD4^{+} WT or *Il23r*$^{-/-}$ T cells were sort-purified and RNA extracted for whole transcriptome gene expression analysis performed using the Illumina Bead Array platform. Two-dimensional cluster analysis (hierarchical, Pearson uncentred, average linkage, unsupervised) of the 168 genes whose expression was significantly affected in the comparison of *Il23r*$^{-/-}$ versus wild-type CD4^{+} T cells is shown. Th2 associated genes are highlighted. Data from Schiering *et al.* [144].

5.2 Results

5.2.1 IL-23 has a cell-intrinsic effect on the expression of Th2 cytokines

To validate the finding that IL-23 controls the expression of Th2 associated genes in intestinal $CD4^+$ T cells, we used a T cell transfer model of colitis and transferred WT and *Il23r*$^{-/-}$ naïve T cells isolated from spleen and lymph nodes into *Rag1*$^{-/-}$ mice. We set up three different experimental groups of mice: the first group of *Rag1*$^{-/-}$ mice was transferred with WT naïve $CD4^+TCR\text{-}\beta^+$ T cells, the second group with *Il23r*$^{-/-}$ naïve T cells and the third group with a 1:1 mix of WT ($CD45.1^+$) and *Il23r*$^{-/-}$ ($CD45.2^+$) naïve T cells (Fig. 5.2a). By comparing the phenotype of $CD4^+$ T cells isolated from mice of the first two groups, we investigated the effect of IL-23 on the overall immune response and how this affected T cells. However, comparing just these two experimental groups would not have answered the question if IL-23 directly regulates the T cell response or via an indirect pathways; thus the need of a co-transfer group. The co-transfer of WT and *Il23r*$^{-/-}$ naïve $CD4^+$ T cells has been shown to lead to T cell infiltration in the lamina propria of the intestine and cause severe colitis comparable to a single transfer with WT naïve T cells only [268]. Therefore, this approach is an ideal system to isolate *Il23r*$^{-/-}$ T cells from an inflamed environment, and, by comparing their phenotype to WT T cells, identify cell-intrinsic effects of IL-23 on this cells. Mice were sacrificed when inflammation of the colon was detected (~8 weeks) and Flow cytometric analysis was performed on immune cells isolated from the colon. Mice from all groups showed reconstitution with viable T cells, and in the co-transfer system $CD45.1^+$ and $CD45.2^+$ cells showed similar frequencies among the $CD4^+$ compartment (Fig. 5.2b).

In agreement with previously published data [144], ST2 resulted to be regulated by IL-23 in a cell-intrinsic way, as it showed higher expression on *Il23r*$^{-/-}$ $CD4^+$ T cells in

the co-transfer setting (Fig. 5.3a). This could have been due to a higher frequency of Tregs, as IL-23 negatively regulates Treg cells, however frequency and number of Foxp3 expressing cells was comparable between the two genotypes in the co-transfer setting. In contrast, in the co-transfer setting, *Il23r*$^{-/-}$ cells showed a higher frequency and number of GATA3^{+}Foxp3^{-} Th2 cells, while frequency of RORγt expressing Th17 cells was significantly lower with absolute numbers only showing a trend in reduction in *Il23r*$^{-/-}$ cells (Fig. 5.3a). In the single transfer groups, when comparing mice transferred with WT or *Il23r*$^{-/-}$ cells, no significant difference was observed in the frequency and numbers of the different T cell subsets, although a trend in reduction of RORγt expressing Th17 cells and increase of GATA3^{+} Foxp3^{-} Th2 cells was noticed in the *Il23r*$^{-/-}$ single transfer condition. We then looked at the expression of Th2 associated cytokines in both the single transfer and co-transfer settings. Interestingly, in the absence of IL-23R, IL-13 and IL-5 expressing cells were significantly higher in both frequency and absolute numbers (Fig. 5.3b). This was true for both the single transferred and co-transferred *Il23r*$^{-/-}$ cells, indicating a cell-intrinsic inhibitory effect of IL-23 on the production of such cytokines. IL-23 signalling seemed to inhibit the expression of IL-4 too, as a tendency towards higher expression was detected in *Il23r*$^{-/-}$ cells. Taken altogether these results suggest a novel *in vivo* function of IL-23 that, while promoting inflammation, suppresses Th2 cells differentiation and cytokines production.

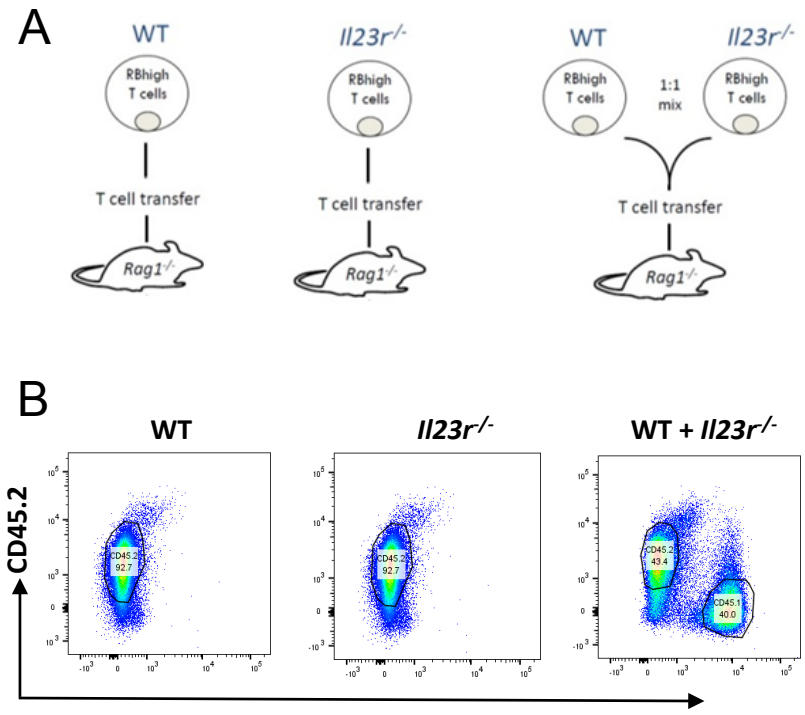


Figure 5.2: Adoptive transfer of WT and *Il23*$^{-/-}$ naïve T cells in *Rag1*$^{-/-}$ mice.

(A) Experiment layout: C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with 4x10^5 WT CD4$^+$CD25$^-$CD45RB^{hi} T cells or 4x10^5 *Il23r*$^{-/-}$ CD4$^+$CD25$^-$CD45RB^{hi} T cells or a 1:1 mixture of 2x10^5 WT (CD45.1$^+$) and 2x10^5 *Il23r*$^{-/-}$ (CD45.2$^+$) CD4$^+$CD25$^-$CD45RB^{hi} T cells. Mice were sacrificed upon development of clinical signs of inflammation (week 8). **(B)** LPLs were collected and analysed by flow cytometry. Representative flow cytometry plots for single transferred and co-transferred mice is shown comparing Live, CD4$^+$, TCR-β$^+$, CD45.1$^+$ or CD45.2$^+$ cells. One experiment, n=6 per group.

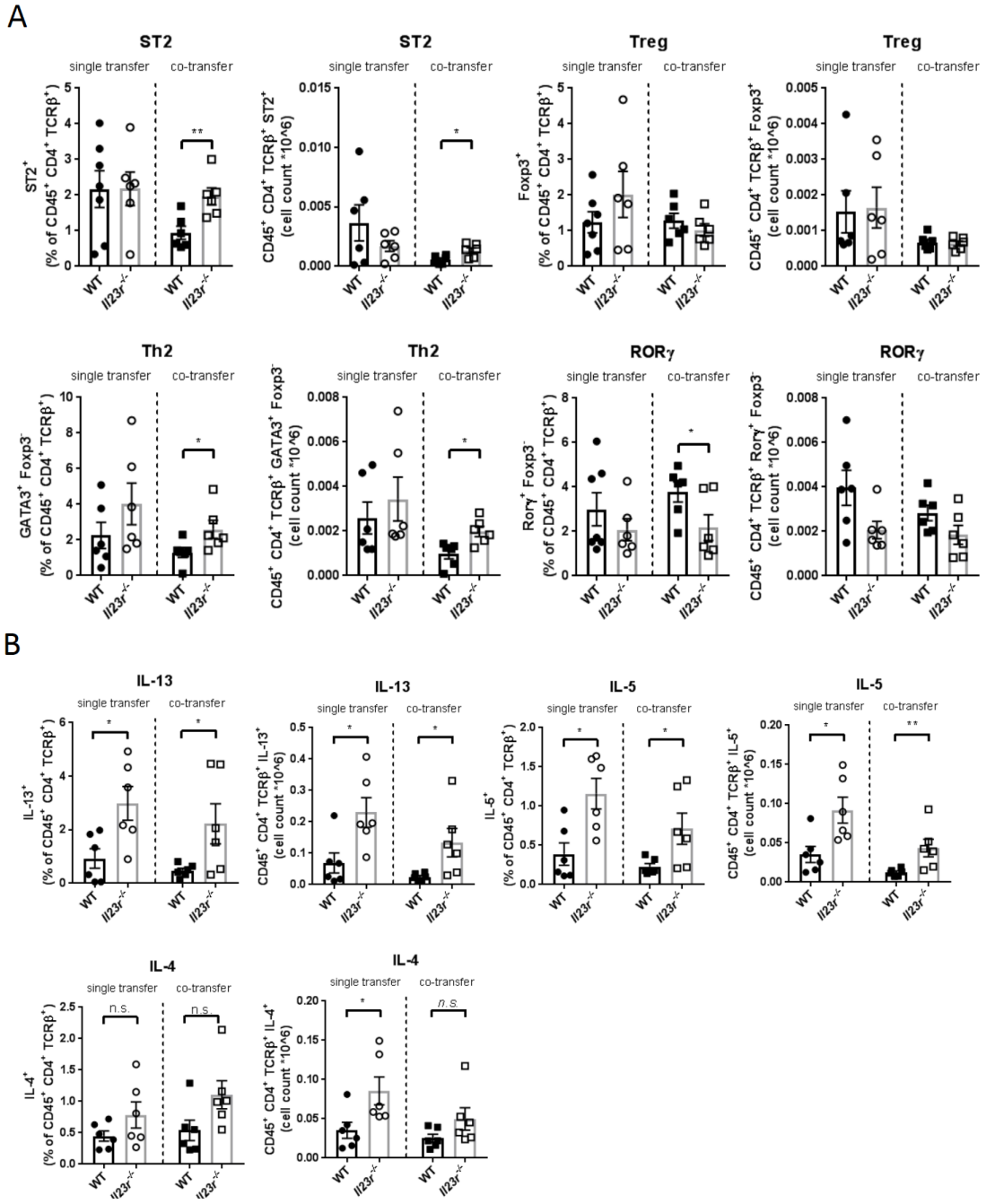


Figure 5.3: IL-23 controls the expression of Th2 cytokines in a cell-intrinsic manner.

Leukocytes isolated from the colon of transferred mice were analysed by flow cytometry. **(A)** Expression of the indicated molecules by $CD4^+$ T cells was determined. Each graph compares WT and *Il23r*$^{-/-}$ T cells from single transferred (left) or from co-transferred mice (right). Frequency and absolute numbers of cells are shown. **(B)** Frequency and absolute numbers of cytokine expressing $CD4^+$ T cells

from the colon are graphed. Bars represent the mean ±SEM and each point represents an individual mouse.

Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05, **p<0.01. One experiment, n=6 per group.

5.2.2 The effect of IL-23 and IL-33 on in vitro generated Th2 cells

One of the Th2 genes described in Fig. 5.1 to be controlled by IL-23 signalling in intestinal T cells is *Il1r1l*, the receptor for IL-33 or ST2 [144]. IL-33 and IL-23 have been showed to have opposing effects on Tregs both *in vivo* and *in vitro* [144], with IL-23 having an inhibitory effect and IL-33 promoting Treg stability and function. ST2 is known to be expressed by Th2 cells and it has been shown that IL-33 treatment promotes the expression of IL-5 and IL-13 by *in vitro* differentiated Th2 cells [411]. In vivo studies showed how IL-33 has an impact on *in vivo* type 2 effector functions, as it promotes T cell migration [412] and induces Th2 cytokine production. [413]. In contrast to our preliminary data (Fig. 5.1), one study reported IL-23 as a positive regulator of type 2 responses, as in a mouse model of allergic airway inflammation IL-23R deficient mice have reduced inflammation, although this study do not provide evidence of a direct effect of IL-23 on T cells [410]. Therefore we decided to generate a system to study the effect of IL-23 and IL-33 on *in vitro* differentiation of Th2 cells. In order to optimise Th2 polarising conditions, we performed a titration of IL-4, the cytokine driving type 2 differentiation. Naïve $CD4^+$ T cells were sorted from the spleen and lymph nodes of C57BL/6 mice and stimulated with anti-CD28 and anti-CD3 antibodies, IL-2 and increasing doses of recombinant IL-4, from 0.01 ng/ml up to 10 ng/ml, for 5 days. Expression of the canonical Th2 transcription factors and cytokines were measured by qPCR and flow cytometric analysis to assess Th2

differentiation. Expression of the Th2 associated genes *Gata3* and *maf* showed a dose dependent increase in expression that plateaued at 1 ng/ml of rIL-4 treatment (Fig. 5.4a). In contrast, flow cytometric analysis showed that the highest level of GATA3 protein expression was detected at 10 ng/ml, suggesting a possible post-transcriptional regulation dependent on IL-4. We also measured expression of genes encoding type 2 cytokines. Expression of *Il4, Il5* and *Il13* was increased with increasing IL-4 concentrations, with the highest gene expression observed for *Il4* and *Il5* at the top IL-4 concentration (10 ng/ml), while for *Il13* this was detected at 1ng/ml IL-4 (Fig 5.4a).

Based on these results, we chose 1ng/ml rIL-4 for subsequent experiments. This was to generate a sub-optimal Th2 cell population without saturating the differentiation potential of the cultured T cells and allow us to further push the differentiation with IL-33. Treatment with rIL-33 has been shown to induce Th2 cytokines production *in vitro* [411]. Therefore, we tried to recapitulate the effect of IL-33 on the *in vitro* differentiation of Th2 cells in our system. Stimulation of naïve $CD4^+$ T cells with both IL-4 and IL-33 resulted in significantly increased gene expression of *Il5* and *Il13* compared to IL-4 only stimulation, while the increase in *Il4* expression was not statistically significant (Fig 5.5a). As a negative control, we tested the expression of Th2 genes in both Th1 and Th0 conditions, where no expression of type 2 cytokines was detected. In contrast, IL-33 did not enhance *Gata3* expression. Also, the expression of the receptor for IL-33 *Il1rl1* resulted to be unaffected by treatment with IL-33 and was considerably lower then in Th1 cells, which have been reported to express ST2 [414]. The effect of IL-33 on cytokine production was also observed by flow cytometric analysis. The frequency of IL-5 producing cells was significantly increased in cells treated with both IL-4 and IL-33 (Fig 5.5b). However, IL-33 did not

increase production of IL-4 and IL-13 (Fig 5.5b). We then tested if IL-23 had an inhibitory effect on Th2 polarization. At both mRNA and protein level, addition of IL-23 to the Th2 polarizing conditions resulted in similar expression of Th2 cytokines compared to IL-4 only and IL-4 + IL-33 stimulation (Fig 5.5a and Fig5.5b). These results indicate that IL-23 has no inhibitory effect on *in vitro* Th2 polarization.

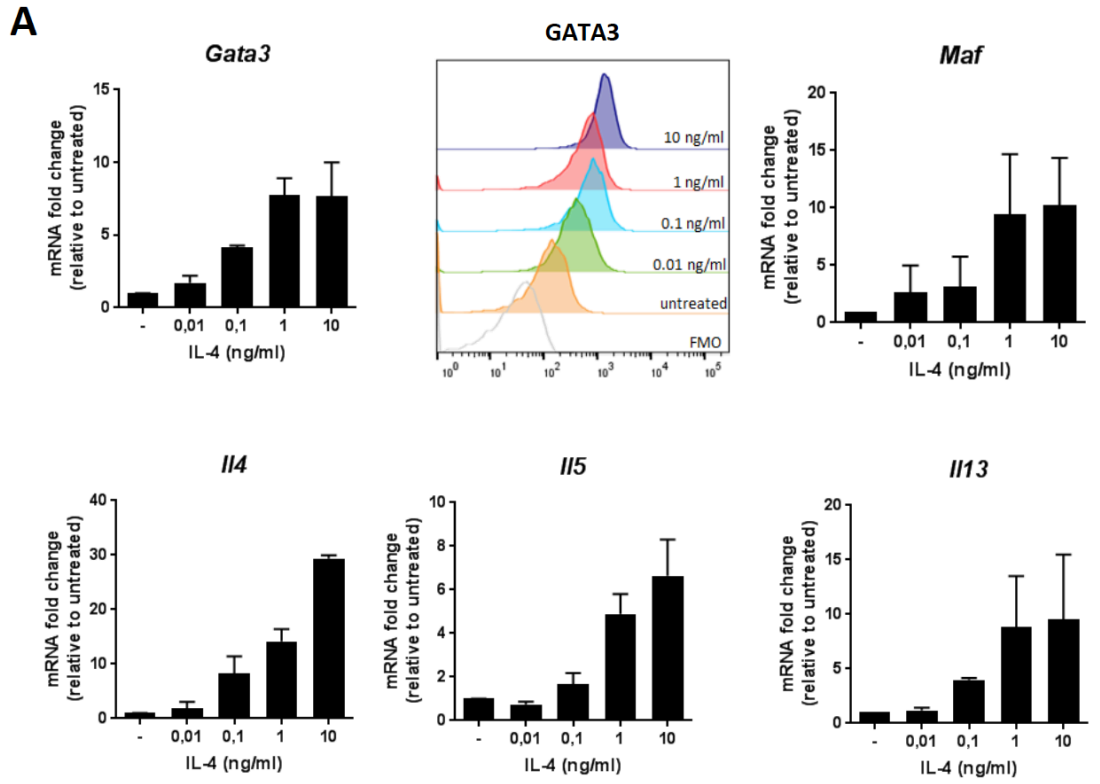


Figure 5.4: Optimization of *in vitro* Th2 cells differentiation.

(A) IL-4 titration: FACS sorted naïve $CD4^+$ T cells from C57BL/6J mice were plated in RPMI with αCD3, αCD28, IL-2 and treated with increasing doses of IL-4 (from 0.01ng/ml to 10 ng/ml) for 5 days. Differentiation was assessed by measuring mRNA expression of Th2 markers (shown as mean, represented as $2^{-\Delta\Delta Ct}$ relative to *Hprt*) and by flow cytometry (histogram). Graphs are representative of 2 independent experiments (n=2/group). Bars represent mean ±SEM.

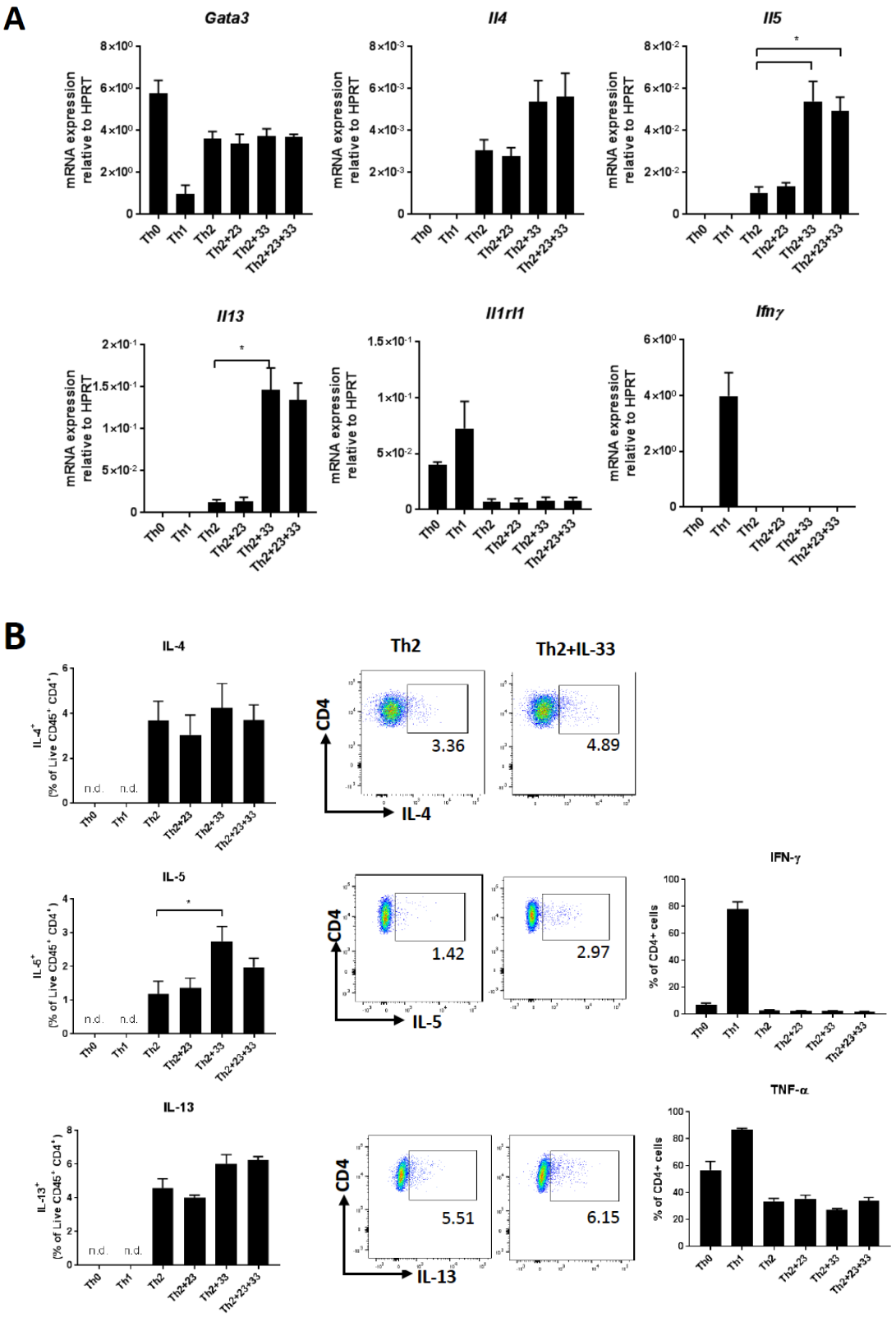


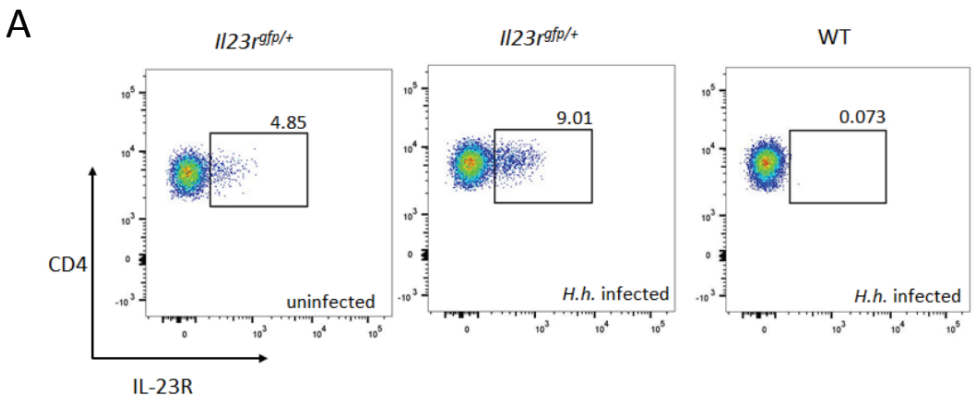
Figure 5.5: *In vitro* differentiation of Th2 cells is enhanced by IL-33 but not affected by IL-23 treatment.

FACS sorted naïve $CD4^+$ T cells differentiated to Th2 cells by IL-4 treatment only (1ng/ml) or with a combination of IL-4 and IL-33, or IL-4 IL-33 (10ng/ml) and IL-23 (20ng/ml). As a negative control, undifferentiated T cells (Th0) or Th1 cells differentiated with IFN-γ (10ng/ml) were analysed. **(A)** Expression of Th2 genes was measured by qPCR. **(B)** Flow cytometry analysis: percentage of cytokine positive cells of total $CD4^+$ cells is shown (left panel) with representative plots (right panel).
Data shown are representative of 3 independent experiments (n=2-4/ group). Statistical significance was determined using unpaired non-parametric One-way ANOVA, using GraphPad Prism.
Bars represent mean ±SEM. *$p<0.05$.

5.2.3 *Helicobacter hepaticus* colonization and the IL-23-Th2 axis

The T cell transfer model of colitis is a widespread and established model to investigate T cell specific mechanisms [85, 86]. However, one caveat of this model is the absence of adaptive immune cell populations in *Rag1*$^{-/-}$ mice. The absence of T cells and B cells in *Rag1*$^{-/-}$ mice leads the immune system to develop differently from a WT mouse and causes severe immunodeficiencies [415]. In order to test the effect of IL-23 on Th2 cells in a fully immunocompetent mouse, we investigated the IL-23-Th2 axis in mice colonized with the bacterial pathobiont *Helicobacter hepaticus*. In WT mice, colonization with *H. hepaticus* induces a host-protective immune response that is characterized by high expression of IL-10 by Treg cells [36, 275]. *H. hepaticus* colonization also induces expression of the IL-23 subunit IL-23a in WT mice [212] suggesting an involvement of the IL-23 signalling pathway in the response to the bacteria. In addition to that, *H. hepaticus* elicited models of colitis have been shown to be dependent on IL-23R expression on T cells [416]. Therefore we hypothesized

that *H. hepaticus* colonization would promote the expression of the IL-23R on $CD4^+$ cells. To test this we utilised *Il-23r*$^{gfp/+}$ mice, which contain a GFP reporter for the IL-23R protein. *Il-23r*$^{gfp/+}$ mice were infected with *H. hepaticus* and sacrificed 7 days after infection. Flow cytometry analysis showed that uninfected $CD4^+$ cells express the IL-23R (GFP), but upon *H. hepaticus* colonization the expression is enhanced (Fig 5.6a). As a negative control for GFP expression, WT infected mice were also analysed. Our finding that *H. hepaticus* colonization leads to increased frequency of $CD4^+$ T cells expressing IL-23R and previous data showing increased expression of *Il23p19* during *H. hepaticus* colonization [212] suggests that infection with this pathobiont may trigger IL-23 signalling pathways in IL-23 responsive cells. Therefore, we investigated the IL-23-Th2 axis in *H. hepaticus* colonized mice. WT and *Il23r*$^{-/-}$ mice were colonised with *H. hepaticus* and sacrificed 7 and 14 days later. Flow cytometric analysis showed that the frequency of $CD4^+IL\text{-}10^+$ cells was increased in the colon of WT mice 7 and 14 days post infection (Fig. 5.6b), in line with previous reports [275]. However, no difference in frequencies of $CD4^+IL\text{-}10^+$ cells was observed between WT and *Il23r*$^{-/-}$ mice (Fig. 5.6b). IL-4 expression followed a similar pattern, with an increase at 7 days and no difference between WT and *Il-23r*$^{-/-}$ cells. Interestingly, although not induced by *H. hepaticus* colonization in WT mice, IL-5 and IL-13 production were increased in *Il23r*$^{-/-}$ mice during *H. hepaticus* colonization (Fig. 5.6b). This suggests that IL-23 signalling in the context of *H. hepaticus* colonization may restrain production of Th2 cytokines by $CD4^+$ T cells.


A
Il23r gfp/+
Il23r gfp/+
WT
4.85
9.01
0.073
CD4
uninfected
H.h. infected
H.h. infected
IL-23R

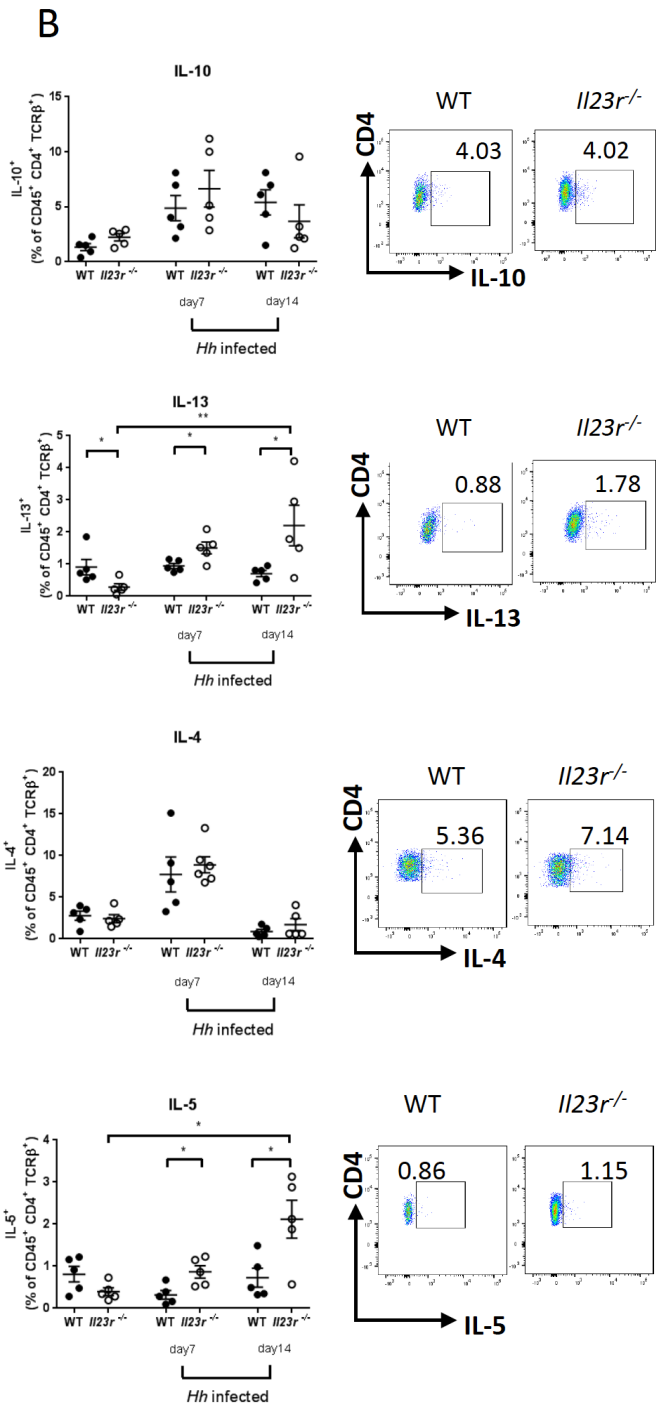

B
IL-10
WT
Il23r -/-
4.03
4.02
CD4
IL-10
day7
day14
Hh infected
IL-13
0.88
1.78
IL-13
IL-4
5.36
7.14
IL-4
IL-5
0.86
1.15
IL-5

Figure 5.6: *H. hepaticus* colonization drives Th2 cytokines expression in the absence of IL-23R signalling.

WT, *Il23r*$^{gfp/+}$ and *Il23r*$^{-/-}$ mice were infected with 1*10^{8} CFU of *H hepaticus* by oral gavage for two consecutive days. Lamina propria leukocytes were isolated one week and two weeks after infection. **(A)** Expression of IL-23R was assessed on WT and *Il23r*$^{gfp/+}$ mice by flow cytometry on total CD4^{+} T cells a week after infection. Data shown are representative of two independent experiments. N=2-3 per group **(B)** Leukocytes from WT and *Il23r*$^{-/-}$ mice were analysed by flow cytometry one week and two weeks post infection: frequency of cytokine expressing CD4^{+} is shown with representative FACS plots. Bars represent the mean ±SEM and each point represents an individual mouse. Statistical significance was determined using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05, **p<0.01. Data shown are representative of two independent experiments. N=6-7 per group.

5.2.4 Th2 cells and IL-23 in the *H. hepaticus* + αIL-10R model of colitis

In WT mice, infection with *H. hepaticus* and concomitant treatment with an anti-IL-10R blocking antibody drives inflammation of the colon and cecum causing severe colitis [417]. In this model, IL-23R signalling is necessary for development of disease, as IL-23R-deficient mice develop significantly reduced intestinal inflammation compared to WT mice [40]. To test the effect of IL-23 on Th2 cells in the context of inflammation, WT and *Il23r*$^{-/-}$ mice were infected with *H. hepaticus* and concomitantly treated with an anti-IL-10R blocking antibody. Mice were sacrificed at the onset of disease (14 days). WT mice developed severe inflammation of both the colon and cecum, with infiltration of leukocytes and thickening of the epithelial barrier (Fig 5.7b). In contrast, pathology was significantly reduced in IL-23-deficient

mice (Fig 5.7c). The absence of IL-23 signalling induced a higher frequency of T cells to produce IL-10 in the colon but not in the draining lymph nodes, as shown by flow cytometric analysis (Fig. 5.8a and Fig 5.8b), indicating a tissue-restricted effect of IL-23 on intestinal T cells. By contrast, no difference in Th2 cytokine production was detected between WT and *Il23r*$^{-/-}$ mice: IL-4, IL-5 and IL-13 expression was comparable in WT and *Il23r*$^{-/-}$ mice in the colon (Fig. 5.8a). In the mesenteric lymph nodes, IL-5 and IL-13 expression was undetectable by flow cytometry, while frequency of IL-4 expressing T cells was not different between WT and *Il23r*$^{-/-}$ mice (Fig. 5.8b). We also tested mRNA expression of cytokines at tissue level in the colon. *Ifng* expression correlated with severity of pathology as it was increased upon inflammation and heightened in WT mice, with lower expression in *Il23r*$^{-/-}$ mice (Fig 5.8c). However, *Il23r*$^{-/-}$ mice showed a slight increase in *Il5* and *Il13* mRNA expression when compared to WT mice (Fig. 5.8c). It is interesting to note that expression of *Il5* at tissue level was lower in uninfected *Il23r*$^{-/-}$ mice compared to WT mice. This result, in contrast with our initial hypothesis, could be due to T cell independent effects. In fact, IL-5 can be expressed by other cells like innate lymphoid cells [418], a subset of immune cells known to express the IL-23R in the intestine [419]. These data indicate that in the *H. hepaticus* + anti IL-10R model of colitis, *Il23r*$^{-/-}$ intestinal T cells do not rely on Th2 cytokines for their protective function.

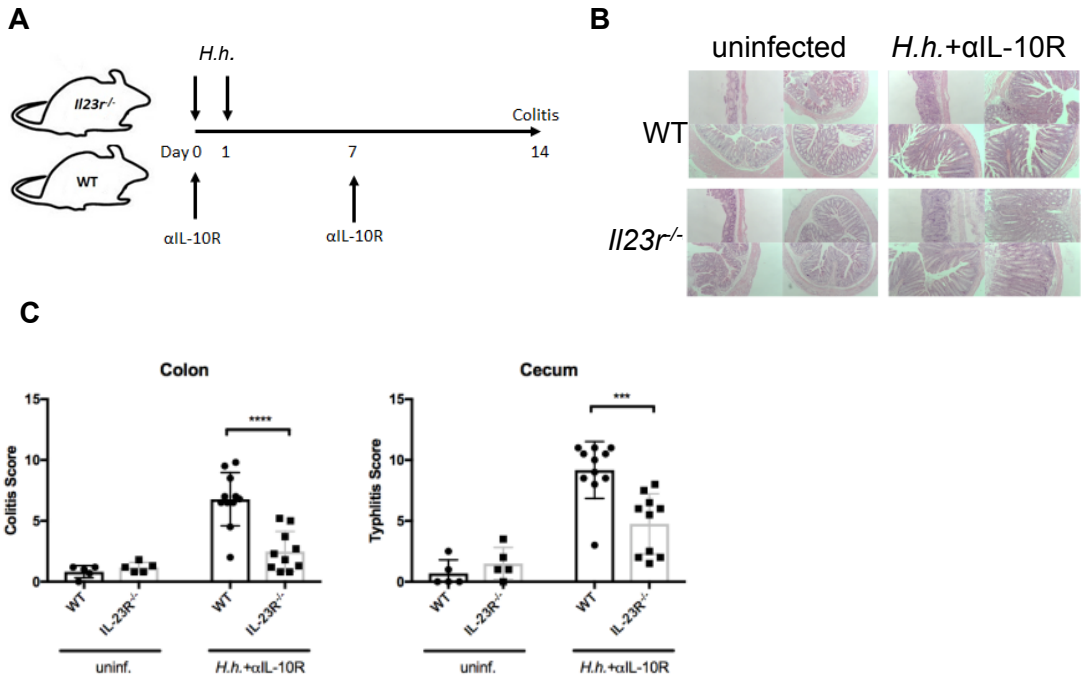
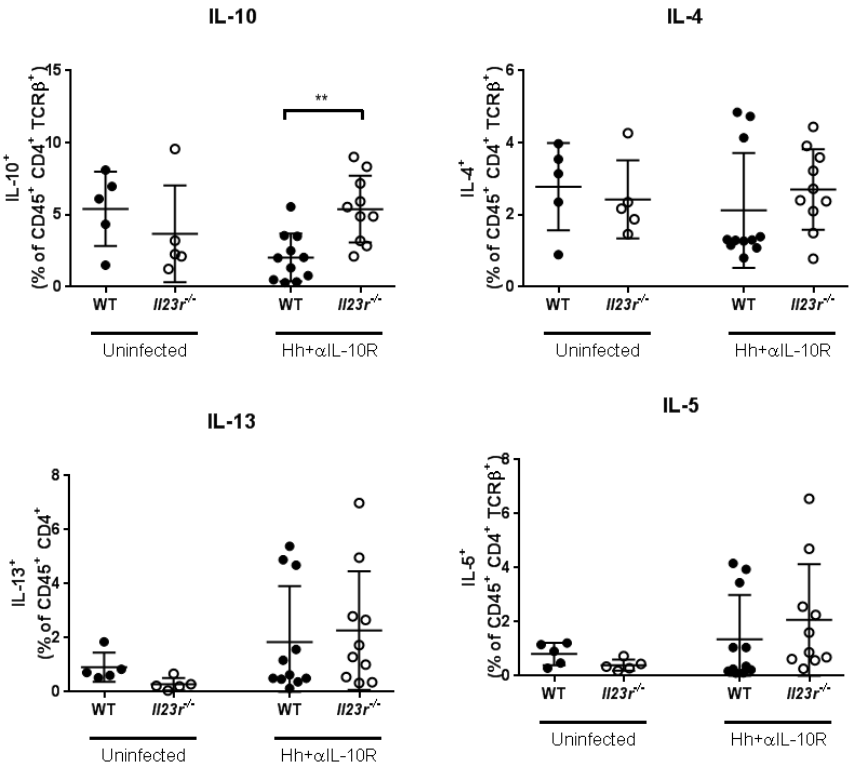


Figure 5.7: WT and *Il23r*$^{-/-}$ mice in the *H. hepaticus* + αIL-10R model of colitis.

(A) Experimental model. WT and *Il23r*$^{-/-}$ mice were infected with 1*10^{8} CFU of *H hepaticus* by oral gavage for two consecutive days. In addition, 1 mg of an anti-IL-10R blocking antibody was administered by i.p. injection once weekly starting on the day of infection. **(B)** H&E staining on caecum and three section of the colon showing inflamed and normal mucosae of infected and uninfected mice. **(C)** Pathology score as assessed by histology scoring of colon and cecum. Bars represent the mean ±SEM and each point represents an individual mouse. Data were analysed using unpaired Mann-Whitney test, using GraphPad Prism. *** P<0.001, **** P<0.0001. Data from two independent experiments were pooled. N=5-6 per group.

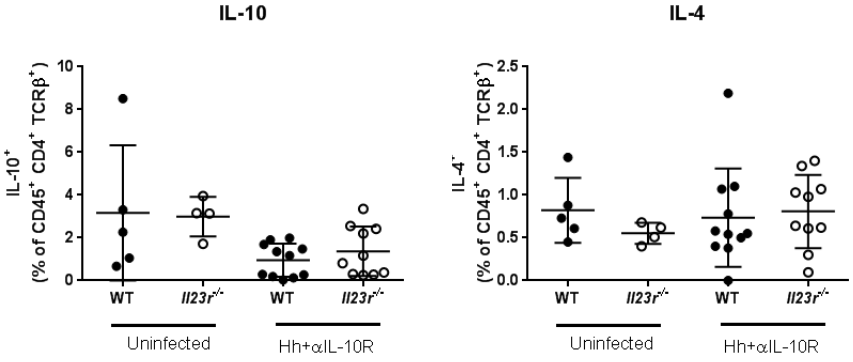
A

COLON



B

mLNs



C

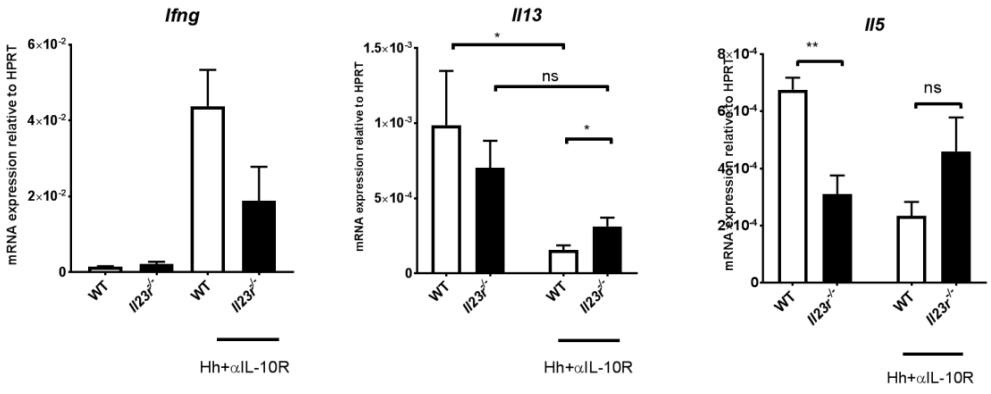


Figure 5.8: Effects of IL-23R signalling on T cells in the *H. hepaticus* + αIL-10R model of colitis.

(A) Leukocytes isolated from the colon of *H hepaticus* infected and uninfected mice were analysed by flow cytometry. Frequency of cytokine expressing $CD4^+$ T cells is shown. **(D)** Frequency of IL-4 and IL-10 expressing $CD4^+$ T cells isolated from the mesenteric lymph nodes are shown. **(E)** mRNA expression levels of selected cytokines on colonic total tissue, assessed by qPCR. Bars represent the mean ±SEM and each point represents an individual mouse. Data were analysed using unpaired Mann-Whitney test, using GraphPad Prism. * $P<0.05$, ** $P<0.01$. Data from two independent experiments were pooled. N=5-6 per group.

5.2.5 DSS induced colitis on WT and IL23R$^{-/-}$ mice

Our results so far suggested that in an inflamed immunocompetent mouse, IL-23R deficiency has minimal impact on Th2 cytokine production. The *H. hepaticus* + anti-IL-10R model involves blocking of IL-10, a relevant immunological signalling pathway of the intestine, that has been shown, in models of intestinal helminthic infection, to be essential for an efficient Th2 response [420]. Therefore adopted an inflammation model that would not need alteration of the cytokine response to develop. DSS induced colitis is a model of chemically induced colitis that is characterized by extensive epithelial barrier damage that drives inflammation and weight loss [421]. One study reports dependence of the IL-23R signalling pathway on development of disease, as *Il23r*$^{-/-}$ mice show a milder form colitis [422]. Considering the role of Th2 cells in orchestrating tissue repair by producing cytokines like IL-4, IL-5 and 13 that can induce alternative activation of macrophages and recruitment of

eosinophils [423], we tested whether *Il23r*$^{-/-}$ mice were protected from DSS colitis and investigate whether that protection was associated with a higher proportion of Th2 cells and cytokines. WT and *Il23r*$^{-/-}$ were treated with 2.5% DSS administered through the drinking water for five days. DSS was subsequently removed to avoid excessive ulceration of the colon lamina propria. The mice were sacrificed during inflammation (9 days) and colonic lamina propria leukocytes were isolated (Fig. 5.9a). Colon length and weight loss were recorded as readouts of disease [424]. Although both DSS-treated WT and *Il23r*$^{-/-}$ mice displayed shortening of the colon, no difference in colon length was observed between WT and *Il23r*$^{-/-}$ mice (Fig. 5.9c). Along these lines, inflamed *Il23r*$^{-/-}$ and WT mice showed a similar loss of body weight, indicating a comparable severity of disease (Fig. 5.9b). This result is in contrast with previous reports [422], although DSS colitis is a model highly dependant on environmental factors and its outcome can be affected by variables like differences in the microbiota and animal facilities [425].

Although we did not see a difference in pathology between WT and *Il23r*$^{-/-}$ mice, we tested if IL-23R deficient T cells skewed towards a Th2 phenotype during DSS colitis. *Il23r*$^{-/-}$ mice showed an increase of GATA3 expressing CD4^{+} T cells: flow cytometric analysis showed how the frequency of both GATA3^{+} Th2 and Foxp3^{+} GATA3^{+} Tregs were increased in the KO mice (Fig. 5.10a) In contrast, similar frequencies of RORγt expressing Th17 cells and Foxp3^{+} RORγt^{+} Tregs were observed in the colons of WT and *Il23r*$^{-/-}$ mice (Fig. 5.10a). These results suggested that CD4^{+} T cells in *Il23r*$^{-/-}$ mice skew specifically to a Th2 phenotype during DSS-induced colitis. Therefore, we next tested for production of Th2 cytokines in the colon. Production of IL-4, IL-5, IL-13, as well as IL-10, was comparable between *Il23r*$^{-/-}$ and

WT mice (Fig. 5.10b), suggesting that the higher frequency of GATA3 expressing cells did not result in an enhanced production of the classic Th2 cytokines.

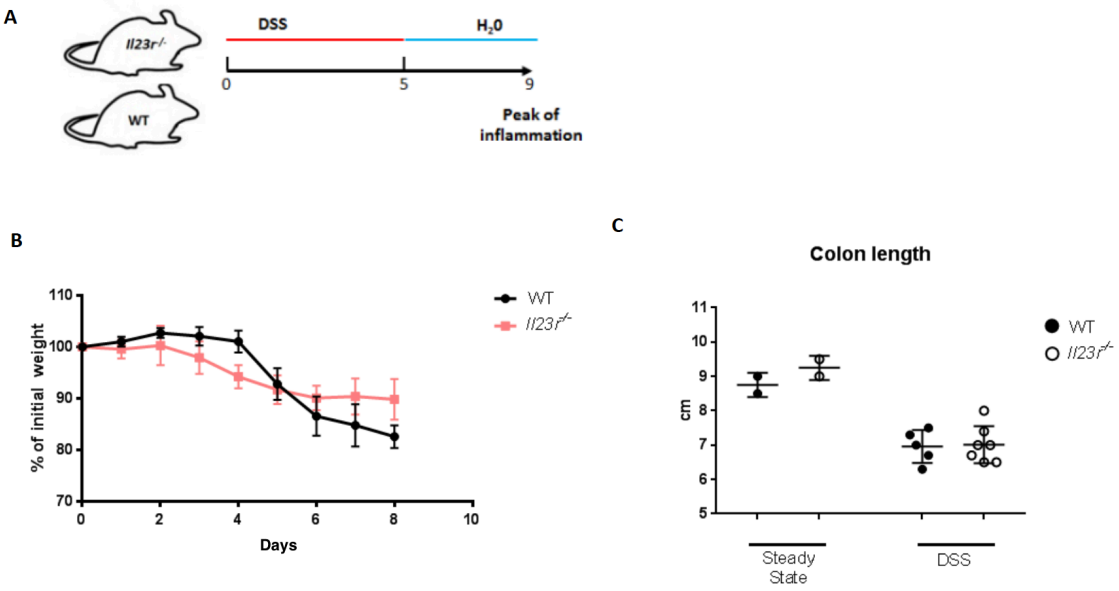


Figure 5.9: DSS induced colitis on WT and *Il23r*$^{-/-}$ mice

(A) Experimental model. WT and *Il23r*$^{-/-}$ mice were administered 2.5% DSS in the drinking water for 5 days. DSS was then withdrawn and replaced with normal water. The mice were sacrificed at day 9. **(B)** Percentage of initial body weight (g), shown as mean of the group with SD. **(C)** Colon length (cm): WT and *Il23r*$^{-/-}$ mice, treated or untreated with DSS are compared. Bars represent mean of group ±SEM. Data were analysed using unpaired Mann-Whitney test, using GraphPad Prism. One experiment, n=2 per steady state group, n=5-7 per inflamed group.

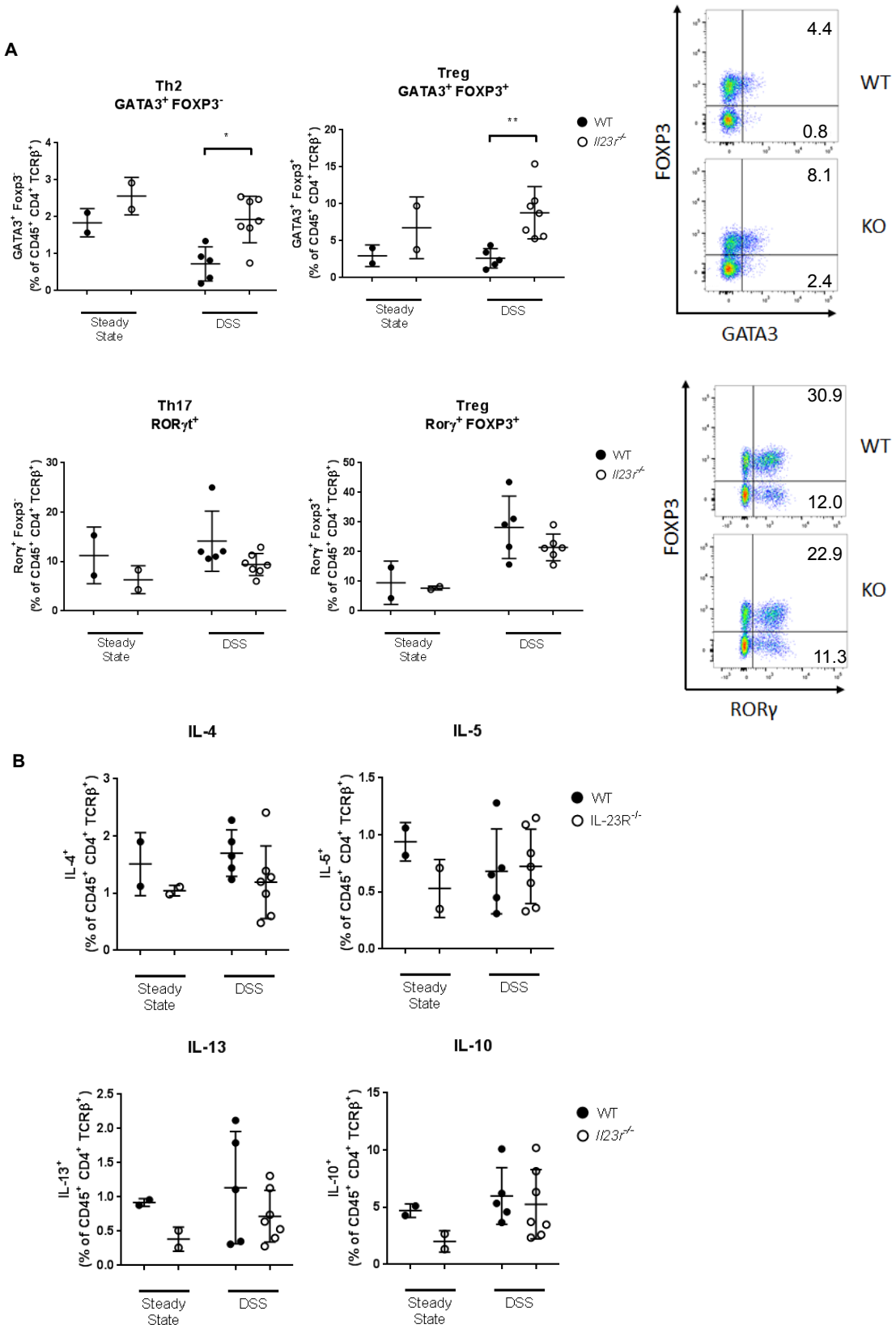


Figure 5.10: In DSS induced colitis *Il23r*$^{-/-}$ mice have a higher frequency of Th2 than WT mice.

Leukocytes from the colon of DSS treated and untreated mice were isolated and analysed by flow cytometry. **(A)** Frequencies of $CD4^+$ cells expressing T-helper cells markers (left). Representative flow cytometry plots are shown (right). **(E)** Frequency of cytokine producing T cells, assessed by FACS analysis and shown as percentage of all colonic $CD4^+$ cells. Bars represent mean of group ±SEM. Data were analysed using unpaired Mann-Whitney test, using GraphPad Prism. *p<0.05, **p<0.01.

5.3 Discussion

In this chapter we have investigated the role of IL-23 in shaping $CD4^+$ T cell responses in the intestine. Preliminary data generated in our lab [144] identified a Th2 signature in *Il23r*$^{-/-}$ T cells isolated from the inflamed colon of *Rag1*$^{-/-}$ mice adoptively transferred with naïve CD^+ T cells (Fig. 5.1a), suggesting that in active colitis, IL-23 suppresses a set of Th2 associated genes while promoting Th1/Th17 polarization. To confirm this hypothesis, we used different mouse models of intestinal inflammation and *in vitro* systems.

One of the main findings of our study came from an adoptive T cell transfer model of colitis. In Fig. 5.2 we investigated the cell-intrinsic effect of IL-23 on intestinal T cells, by transferring a 1:1 mix of WT and *Il23*$^{-/-}$ T cells to *Rag1*$^{-/-}$ mice. In line with our hypothesis, we observed a Th2 skewing of *Il23r*$^{-/-}$ T cells, with a higher expression of the transcription factor GATA3 and the cytokines IL-5 and IL-13 in *Il23r*$^{-/-}$ T cells when compared to WT CD^+ T cells. This finding confirms what observed by *Schiering et al.* [144], but is in contrast with another report [410] that describes an enhancing effect of IL-23 on Th2 responses in a model of allergic airway inflammation. However, that study used transgenic mice overexpressing *Il23r* on T cells, and it does not show evidence of a T cell intrinsic effect of IL-23 on the

production of Th2 cytokines. It is interesting to note how in our model IL-4 does not follow the same pattern as IL-5 and IL-13, as it is not significantly upregulated in *Il23r*$^{-/-}$ T cells compared to co-transferred WT. Independent expression of these three cytokines is common in Th2 cells, although they have been found to be regulated co-ordinately [426] via STAT6 and GATA3 [45]. However, it has been reported how in differentiated Th2 cells, IL-4 can still be produced when GATA3 is conditionally excised, while IL-5 and IL-13 need GATA3 expression for their continuous production [427] indicating a tied regulation of these two cytokines independent of IL-4. In support to this hypothesis is also the fact that the expression of ST2, the receptor for IL-33, is regulated by IL-23, as *Il23r*$^{-/-}$ adoptively transferred T cells show higher expression of both the gene and protein. It has been demonstrated that IL-33 promotes the expression of IL-5 and IL-13 [411], suggesting an inter-dependent regulation of these Th2 cytokines expression by IL-23 and IL-33. We can then speculate that IL-23 restrains the expression of IL-5 and IL-13, potentially through inhibition of ST2, while not affecting expression of IL-4.

To further test the effect of IL-23 on Th2 differentiation, we used *in vitro* T cell culture and various differentiation conditions. Others have tried to describe how IL-23 modulates Th2 differentiation *in vitro*. In contrast with our hypothesis, *Peng et al.* [410] show that differentiation of Th2 cells in co-culture with *Il23r*$^{-/-}$ irradiated antigen presenting cells (APCs) leads to a lower expression of Th2 cytokines when compared to cells co-cultured with WT APCs. As a matter of fact, this study does not provide convincing evidence of a direct effect of IL-23 on Th2 *in vitro* differentiation: when WT T cells are differentiated to Th2 *in vitro* with or without IL-23, only IL-13 expression show a mild IL-23 dependant enhancement, while other Th2 markers like

IL-4, IL-5 and GATA3 are unchanged, thus not providing a strong enough argument that IL-23 enhances Th2 differentiation directly.

As previously described [411, 413], IL-33 enhances the effector function of T cells cultured with IL-4 by increasing expression of cytokines and transcription factors associated with a Th2 phenotype. Although we were able to recapitulate this promoting effect of IL-33 on Th2 differentiation in our *in vitro* system, IL-23 did not show a detectable effect on Th2 genes expression, in contrast with our *in vivo* observations. A possible explanation is that *in vitro* cultures lack the immune environment of an *in vivo* system, some of which might be needed to effectively recapitulate the immunological process being studied. Also, in *in vitro* cultures, exogenous factors are provided at concentrations that might not be physiological. One limitation of our *in vitro* setup is the low expression level of Th2 cytokines, indicating a non-optimal Th2 differentiation. The rationale for using a sub-optimal concentration of IL-4 was that getting a full Th2 polarization would have saturated the differentiation potential of Th2 cells, making it impossible to further push Th2 differentiation with other stimuli like IL-33. We could hypothesize that to detect modulation of Th2 genes by IL-23 a full Th2 differentiation would be needed. Therefore, we cannot exclude that IL-23 modulates Th2 differentiation *in vitro*. It would be interesting to repeat those experiments with different conditions, trying to modulate a fully polarized Th2 cells with IL-23 *in vitro.*

As with our *in vitro* setting we did not manage to modulate the differentiation of Th2 cells with IL-23, we decided to further explore this axis *in vivo* in *Helicobacter hepaticus* infected mice. We showed that the infection with *H. hepaticus* induced expression of the IL-23R on the surface of intestinal T-cells, suggesting that upon

infection these cells are poised to respond to the bacteria, but are restrained by the host protective environment that characterizes *H. hepaticus* infections [275]. This is a well-known immunological network: IL-23 has a fundamental role in protecting against bacterial [75, 309], and mycotic infections [221], but a deranged IL-23-driven response can lead to inflammatory disorders [187], therefore Th17 responses have to be counteracted by regulatory feedback-loop provided by other immune cells like Tregs [428] and Th2 cells [409]. Indeed, in the absence of IL-23R signalling, *H. hepaticus* induced expression of Th2 cytokines IL-5 and IL-13 by T cells, confirming the hypothesis of a reciprocal regulation of IL-23-driven and Th2 immune responses. In line with our data (Fig 5.2), IL-4 was not differentially expressed in *Il23r*$^{-/-}$ mice when compared to WT, indicating that IL-23 co-regulates IL-5 and IL-13 independently of IL-4. If IL-5 and IL-13 contribute to the protective environment elicited upon *H. hepaticu*s infections yet to be determined. Administration of anti-IL-13 or anti-IL-5 blocking antibodies in *H. hepaticus* infected *Il23*$^{-/-}$ mice might reveal a role of these cytokines in maintaining intestinal homeostasis.

Studies using mouse models of colitis showed how a helminth-induced Th2 response can ameliorate the clinical symptoms of IBD [401] and that this beneficial effect is dependent on Th2 cytokines like IL-13 [402]. In addition, IL-13 has been shown to protect from experimental colitis in *Il10*$^{-/-}$ mice [406]. Therefore we tested if the increased expression of Th2 cytokines by *Il23r*$^{-/-}$ cells could contribute to protection in a model of *H. hepaticus* induced colitis. In the *H. hepaticus* + anti-IL-10R model of colitis *lL23r*$^{-/-}$ mice showed a milder form of colitis when compared to WT counterparts, as previously reported [40], but no difference in expression of Th2 cytokines was observed between *lL23r*$^{-/-}$ and WT mice. This indicates that in that

setting, despite IL-23R deficiency, T cells are not skewed towards a type-2 response, possibly because of the IL-10 signalling blockade. In fact, IL-10 can contribute to type 2 immunity [420] and might be necessary for triggering the production of Th2 cytokines in the intestine.

As Th2 cells are known to orchestrate tissue repair via production of cytokines that induce alternative activation of macrophages and recruitment of eosinophils [41, 429, 430], we tested the IL-23-Th2 axis in DSS colitis, a model of chemically induced intestinal inflammation characterized by extensive tissue damage. Even though preliminary, this experiment partially confirmed our results as IL-23R deficient mice had a higher frequency of GATA3 expressing Th2 cells when compared to WT, although no difference in cytokine expression was observed during inflammation. As this model is characterized by severe epithelial damage, it would be interesting to test the IL-23-Th2 axis at a later time point, where resolution of inflammation occurs [431]. We hypothesise that at that stage, in the absence of IL-23R signalling, T cells would express more type-2 cytokines and promote tissue repair and a faster resolution of the inflammatory response. Overall, with this project we have described a novel regulatory effect of IL-23 that, while promoting inflammation, restrains T cells from expressing type-2 cytokines in a cell-intrinsic manner. Further experiments would be needed to confirm this finding.

One limitation of this study is the absence of a type-2 inflammation model where we investigate the IL-23-Th2 axis; most of the *in vivo* experiments performed have used Th1/Th17 models of intestinal inflammation therefore it resulted challenging to detect a strong Th2 response. This poised some technical challenges too: since in the mouse models used the expression of Th2 cytokines is limited, we did not have a positive

control or reference for our antibody stainings. Crossing a cytokine-reporter mouse to our *Il23r*$^{-/-}$ strain would make a very useful tool for the purpose of this project.

It would be therefore interesting to investigate the IL-23-Th2 axis in model of type-2 immune response. Oxazolone induced colitis would be a good candidate model, as the intracolonic administration of this haptenating agent leads to a pathogenic Th2 inflammation of the intestine [432]. It is a commonly used model to study intestinal type 2 immune responses, however it does not completely fit for our purpose, as there is no report of an involvement of IL-23 in the development of inflammation. There are reports of asthma models where IL-23 and type 2 cytokines are concomitantly present and contribute to disease [433], like ovalbumin induced asthma [434], but no intestinal model with such characteristics has been described. It would be therefore interesting to test the IL-23-Th2 axis in a model of inflammation where both these immune components are needed for pathology.

We can conclude that the co-regulation of IL-23 and Th2 in the intestine is a complicated immunological network that still needs to be completely unravelled. Further studies on both mouse models and *in vitro* systems are needed to fully understand the interdependence of IL-23R signalling and Th2 cells that we have described in this chapter.

6 Chapter 6: General discussion

With this project we studied T cell responses at barrier sites and the factors that influence them. Barrier sites are unique microenvironments in constant contact with the outside world. Here, the immune system is exposed to stressors and environmental stimuli that can affect their phenotype and function. Some of these factors are shared to all barrier-sites while others are organ specific. Therefore, the immune system developed tissue-specific adaptations that are necessary for maintaining immunological homeostasis and host protection. In particular, T cell populations that reside at barrier tissues are extraordinary adaptable and display tissue specific-characteristics. The importance of studying T cell mediated inflammation at the site of interaction with the outside world lies in the fact that numerous inflammatory disorders originate from a deranged adaptive response that is often triggered by stimuli of environmental origin. There is strong evidence that diseases like atopic dermatitis, allergic asthma or IBD all have a contribution of T cells in their pathogenesis and understanding how these responses originate can help identify potential life-saving treatments.

One of the mediators of mucosal inflammation that received the most attention in recent years is IL-23. This cytokine is a major regulator of inflammatory responses at barrier sites, and it has profound effects on T cell responses. It is now widely accepted that the IL-23/Th17 axis has a central role in the development of colitis, as IL-23 responsive T cells produce a combination of cytokines that, if not tightly controlled, can lead to intestinal pathology [68]. Several pre-clinical studies have demonstrated the crucial involvement of IL-23 in the pathogenesis of IBD [64, 192, 201, 212].

These studies allowed the development of promising therapeutic strategies that target the IL-23 signalling to dampen the deranged inflammation that affects IBD patients. Blocking antibodies targeting the IL-12/IL-23 shared subunit IL-12p40 have been approved for moderate-to-severe CD [256] while selective anti-IL-23p19 antibodies, that specifically target IL-23, are being trialled in CD patients [257, 435]. Blocking of the IL-23 pathway has shown clinical and histologic improvement in IBD patients and will offer an alternative treatment to patients that do not respond to existing therapies like TNF blockade. However, therapies based on anti-cytokine drugs only seem to have beneficial clinical effects in certain subgroups of patients, reflecting the fact that the cytokine networks that control inflammation of the mucosa are complex and affected by multiple levels of regulation by environmental, genetic and immunological factors. In addition, the pathogenesis of colitis is likely to differ between patients, which explains why the targeting of a single cytokine is not effective in some cases. Therefore, studies focusing on unravelling the complex immunological networks that control mucosal inflammation are still needed.

With this aim in view, our project studied downstream effects of IL-23 on T cell responses at barrier sites. IL-23 affects T cell responses with two known mechanisms: it both promotes and sustains activation of Th17 cells while simultaneously inhibiting the regulatory potential of Treg cells, leading to an overall skewing of the adaptive immune system towards a pro-inflammatory response [209, 212, 238]. In this study we identified *Timp1* and *Ltb4r1* as IL-23 controlled genes and we tested their involvement in intestinal Th17 responses. Although the choice of *Timp1* and *Ltb4r1* was supported by *in vitro* and *in vivo* validations, the genetic ablation of these genes did not affect the Th17 response and the associated pathology in several IL-23-

induced murine models of colitis. Both these molecules were previously described as mediators of Th17 pathology in models of EAE [326, 332]. Our data highlight how Th17 responses are extremely variable and context dependent: TIMP1 and LTB4R1 are essential for autoimmune inflammation of the CNS, but are dispensable for intestinal inflammation. Further studies are therefore needed for the identification of the key molecules that confer pathogenicity to intestinal Th17 cells. The application of novel techniques such as single-cell sequencing on T cells isolated from the inflamed intestinal mucosa of IBD patients could reveal novel therapeutic targets for the development of more targeted therapies.

With this study we also described a new IL-23 effect on intestinal T cells, as IL-23 promotes Th17 activation while inhibiting Th2 differentiation. Several studies showed how Th17 and Th2 responses are reciprocally regulated in different settings, including asthma [405], colitis [406] and parasitic infections [407]. Microarray data generated in a previous study suggested that in colitis, the Th17/Th2 balance is controlled by IL-23. Indeed, in a T cell transfer model of colitis, we revealed how IL-23R deficient T cells have a higher expression of Th2 cytokines when compared to WT counterparts. Although further studies are needed to confirm these findings, these results suggest that IL-23, in addition to its known role in inhibiting Treg function, suppresses the emergence of Th2 cells in the intestine. With this mechanism IL-23 promotes the pro-inflammatory activity of Th17 cells and inhibits the potential counteracting effect of Th2 cells. Studying the IL-23-Th2 axis in the intestine could unravel the complex reciprocal regulation of Th17 and Th2 responses in inflammatory diseases. Therapies promoting Th2 over Th17 responses might be a possible strategy to ameliorate disease in IBD patients.

As the focus of this study was adaptive immune responses at barrier sites, with this project we also investigated the heterogeneity of Treg cells in peripheral tissues. In recent years, several studies revealed how at different barrier sites, unique populations of Tregs acquire tissue-specific phenotypes and functions [99]. With the aim of identifying molecules that are crucial for Treg function at barrier sites we described the role of DGAT1, a molecule involved in lipid metabolism whose expression is enriched in Tregs at barrier sites. Our main finding is a new role for barrier site Tregs in controlling type 2 immune responses through DGAT1. In particular, DGAT1 deficient Tregs lose the ability to regulate IgE production, leading to an uncontrolled IgE response. Further studies are needed to fully describe this immune checkpoint: the mechanism through which Tregs exert this novel regulatory function is yet to be determined, and revealing a new immunomodulatory function of Tregs on Th2 responses could elucidate how Tregs maintain homeostasis at barrier sites. The identification of what antigen triggers this deranged IgE response would be a major step forward on understanding the mechanism of suppression of intestinal Tregs. Studying the role of *Dgat1* in Treg biology and how a disruption of its function can alter immune responses could potentially shed light on the pathogenesis of type 2 inflammatory disorders like allergic responses or atopic dermatitis. In addition, how the deficiency in DGAT1 affects the metabolism of tissue Tregs was beyond the scope of this thesis, but our findings suggest that Treg cells at barrier sites display unique metabolic profiles that are intrinsically linked to the their function. Although the understanding of Tregs metabolic adaptation *in vivo* is still limited, *in vitro* studies have shown that the metabolic state of Treg cells is plastic and can adapt to different metabolic substrates [436]. Therefore, linking the dynamic metabolism that

characterize barrier site Tregs with their tissue-specific adaptations might reveal novel phenotypes and functions and help develop more targeted therapies.

7 References

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