

The Role of Interleukin 33 in Intestinal Homeostasis

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Abstract

IL-33 is a pleiotropic cytokine that orchestrates both innate and adaptive immunity. It is commonly associated with type 2 immune responses but recently expression of the IL-33 receptor, ST2, was reported on Treg cells found preferentially in non-lymphoid tissues, such as the visceral adipose tissue, muscle or colon. A crucial role of Tregs in maintaining intestinal homeostasis has been well described. However, little is known about the functional relevance of the ST2-expressing Treg population in the colon. Phenotypic and functional characterisation of Tregs in the gut revealed the presence of two distinct populations: $ST2^+/Gata3^+$ and $Ror\gamma t^+$ Tregs. Thymic-derived $ST2^+/Gata3^+$ Tregs showed a more activated phenotype and produced IL-10 under homeostatic conditions. Upon microbial challenge and colitis, $ST2^+/Gata3^+$ Tregs were decreased, while $Ror\gamma t^+$ Tregs expanded. Furthermore, *in vitro* experiments demonstrated that IL-33 directly induced activation of the Gata3 pathway in Tregs, which enhanced expression of Foxp3 and ST2. Additionally, amphiregulin was also induced in Tregs upon stimulation with IL-33. However, *in vivo*, IL-33 was dispensable for both the maintenance of Treg cells under homeostatic conditions and Treg function in *Helicobacter hepaticus*–driven colitis. Investigation of the negative regulators of IL-33 showed that IL-23 inhibited IL-33-mediated effects on Tregs. We also observed increased production of soluble ST2 by stromal cells during intestinal inflammation, which likely contributed to the reduction of IL-33 bioavailability. Finally, a systematic analysis of the cellular source of IL-33 revealed that $PDGFR\alpha^+$ stromal cells located in the T cell zone of secondary and tertiary lymphoid tissues were a major IL-33-producing cell population in the gut.

Collectively, our findings suggest that signals received by the stromal compartment upon cell injury may trigger a specific phenotype of Tregs with a repair capacity, and thus, promote intestinal homeostasis. These findings improve our understanding of tissue-resident Tregs and open an exciting avenue to explore heterocellular signalling between stromal cells and Tregs.

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Abbreviations

AD	Atopic Dermatitis
AHR	Airway Hyper-Reactivity
AMP	Antimicrobial Peptide
APC	Antigen-Presenting Cell
Areg	Amphiregulin
ASF	Altered Schaedler Flora
BAL	Bronchoalveolar Lavage
CD	Cluster of Differentiation
cfu	Colony-Forming Units
ChIP	Chromatin Immunoprecipitation
CNS	Conserved Noncoding Sequence
DAMP	Damage-Associated Molecular Pattern
DAPI	4',6-Diamidino-2-Phenylindole
DC	Dendritic Cell
DSS	Dextran Sulfate Sodium
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FC	Fold Change
FMO	Fluorescence Minus One
Foxp3	Forkhead Box P3
FRC	Fibroblastic Reticular Cell
GALT	Gut-Associated Lymphoid Tissue
GF	Germ Free
GFP	Green Fluorescent Protein
GI	Gastrointestinal
gMFI	Geometric Mean Fluorescent Intensity

GVHD	Graft-Versus-Host Disease
GWAS	Genome-Wide Association Studies
HEV	High Endothelial Venule
i.p.	Intraperitoneal
i.v.	Intravenous
IBD	Inflammatory Bowel Disease
IEC	Intestinal Epithelial Cell
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IL-1RAcP	Interleukin 1 Receptor Accessory Protein
ILC	Innate Lymphoid Cell
ILF	Isolated Lymphoid Follicle
iNKT	Invariant Natural Killer T Cell
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-Linked Syndrome
iTreg	Induced Treg
KO	Knockout
LCMV	Lymphocytic Choriomeningitis Virus
LN	Lymph Node
LP	Lamina Propria
LPS	Lipopolysaccharide
M cell	Microfold Cell
mAb	Monoclonal Antibody
MAMP	Microbe-Associated Molecular Pattern
MEICS	Murine Endoscopic Index of Colitis Severity
MHC	Major Histocompatibility Complex
mLN	Mesenteric Lymph Node
MNP	Mononuclear Phagocyte
MyD88	Myeloid Differentiation Primary Response Gene 88
NK	Natural Killer Cell

nTreg	Natural Treg
OD	Optical Density
PDGFR	Platelet-Derived Growth Factor Receptor
PRR	Pattern Recognition Receptor
PSA	Polysaccharide A
pTreg	Peripheral Treg
RA	Retinoic Acid
RIN	RNA Integrity Number
Rorγt	RAR-related orphan receptor-γt
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SCFA	Short Chain Fatty Acid
SD	Standard Deviation
SEM	Standard Error of the Mean
SFB	Segmented Filamentous Bacteria
SIGIRR	Single Ig IL-1-Related Receptor
SILT	Solitary Isolated Lymphoid Tissue
SNP	Single-Nucleotide Polymorphism
SPF	Specific Pathogen-Free
sST2	Soluble ST2
STAT	Signal Transducer and Activator of Transcription
TCR	T Cell Receptor
Tfh	Follicular Helper T Cell
TGF	Transforming Growth Factor
Th	Helper T Cell
TLR	Toll-Like Receptor
TNBS	Trinitrobenzene Sulfonic Acid
TNF	Tumour Necrosis Factor
Treg	Regulatory T Cell
TSLP	Thymic Stromal Lymphopoietin
tTreg	Thymic Treg
UC	Ulcerative Colitis

US	Unstimulated
VAT	Visceral Adipose Tissue
WT	Wild Type

Chapter 1. General introduction

The immune system represents a network of cells, tissues and organs that co-ordinate responses against a wide range of pathogens and protect the host from infections. It can also act as a powerful tool against abnormal cells or tissue, such as tumours. While the robust response of the immune system is critical for the host's protection, hyperactive responses towards external antigens as well as self-molecules are a signature of many immune diseases. These include allergies, inflammatory conditions as well as autoimmune disorders, in which the immune system attacks normal host tissue.

1.1 The intestinal immune system

The intestine is probably the most challenging site for the immune system, where immune cells have to remain tolerant to a plethora of dietary and commensal bacteria-derived antigens, yet mount an effective immune response against invading pathogens, for which the intestine is a common site of entry. Dysregulated immune responses towards bacterial antigens in the intestine in a genetically susceptible host are a hallmark of inflammatory bowel disease (IBD) (Maloy and Powrie, 2011).

1.1.1 The anatomy of the intestines

The intestine is one of the largest immune compartments. However, it is not uniform and exhibits functional differences along its length, which determine its architecture and cellular composition. While food digestion is the main role of the small intestine,

the colon is the primary site of water absorption and the niche for commensal bacteria colonisation. To increase the efficiency of digestion, the surface area of the small intestine is increased by finger-like projections known as villi. By contrast, villi are absent from the large intestine. The intestines can be divided into distinct cellular compartments: the epithelium, lamina propria (LP), a thin muscle layer of the muscularis mucosae, the submucosal layer of connective tissue, and another thick muscle layer (Fig. 1.1). Most of the immune cells can be found in the epithelium, LP and the underlining muscularis mucosae. While these sites represent the effector sites of the intestinal immune system, the organised lymphoid tissues of Peyer's patches, caecal and colon patches, solitary isolated lymphoid tissues (SILTs), and the draining lymph nodes comprise sites where immune responses are initiated. Together these structures are referred to as the gut-associated lymphoid tissue (GALT) (Hamada et al., 2002, Mowat, 2003, 2014, Buettner and Lochner, 2016).

1.1.2 Intestinal microbiota

The intestine contains a diverse community of microorganisms, including protozoa, fungi, viruses, archaea and the most prevalent group, bacteria. Collectively these microorganisms are termed the gut microbiota. The total amount and diversity of bacteria increase down the length of the GI tract, and it is estimated that a remarkable number of 10^{14} bacteria colonises the human colon (Sender et al., 2016). The diversity of bacterial species in the colon is enormous, with over 1000 different species detected (Human Microbiome Project, 2012). Firmicutes and Bacteroidetes are the two most abundant phyla in a healthy human colon, followed by Actinobacteria and Proteobacteria (D'Argenio and Salvatore, 2015). Commensal bacteria have been shown

to be an integral part of the mammalian body, and they bring numerous benefits to the host. These include breakdown, absorption and storage of nutrients, preventing the growth of pathogenic bacteria, regulation of gut maturation and integrity as well as modulation of immune responses (reviewed in Ivanov and Honda (2012)). Therefore, the dynamic relationship between microbiota and the host is crucial for maintaining intestinal homeostasis; however, perturbations to the microbiota-host interactions may lead to dysbiosis, which has been suggested to play an important role in the pathogenesis of multiple diseases, including IBDs (Hansen, 2015). Recent technical advances have largely improved our knowledge of microbiota-host interactions providing tools to study individual bacterial species and more complex communities, specific metabolites, bacterial genes and others (Rooks and Garrett, 2016). These include improvements not only in methods of culturing bacteria *in vitro* but also more powerful sequencing methods and bioinformatics tools. Key studies investigating the role of the intestinal microbiota in maintaining intestinal homeostasis are highlighted in the relevant sections of this thesis.

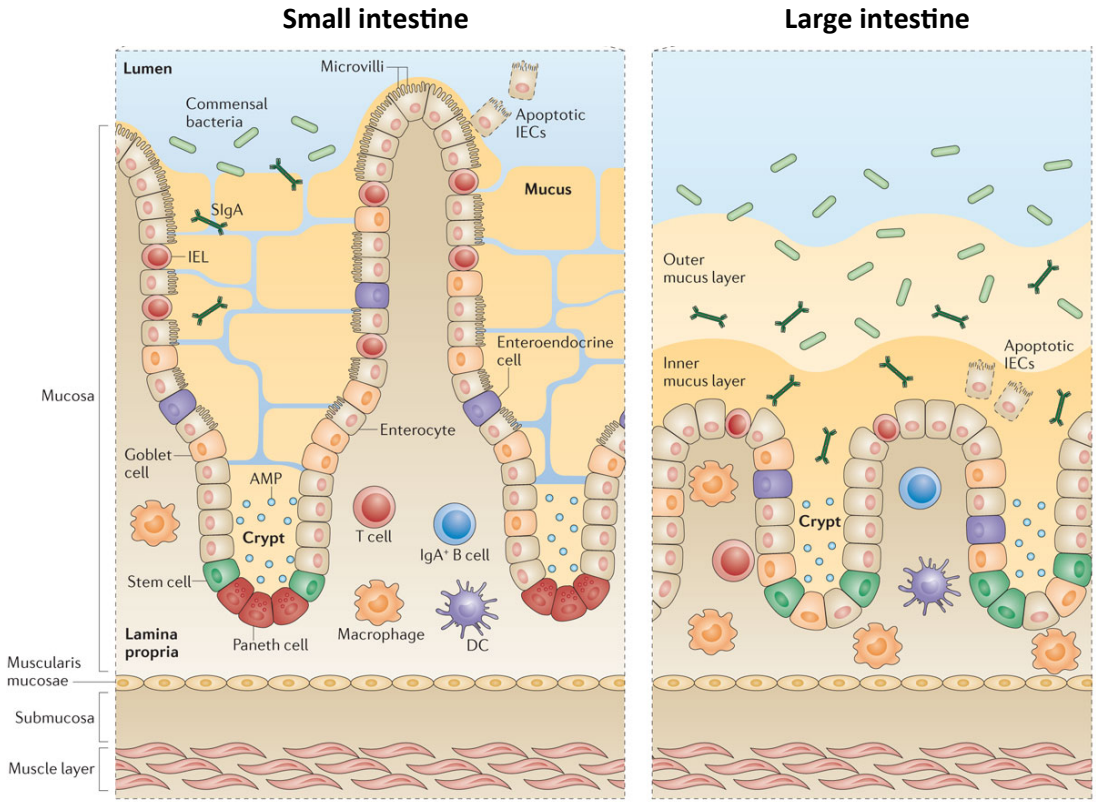


Figure 1.1. The intestinal immune system.

A schematic diagram representing the architecture of the intestines and major components of the intestinal immune system.

A mucus layer overlies a single-cell barrier of epithelial cells, which together compartmentalise intestinal commensal bacteria. Numerous different immune cell types can be found in the intestinal lamina propria, under which lies the muscle layer primarily consisting of non-haematopoietic cells.

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1.1.3 The epithelial barrier

Since the intestinal commensal bacteria are essential for the maturation of GALT and fine-tuning of immune responses, the host evolved a range of adaptations to compartmentalise the microbiota and restrain reactivity towards them. Only a single-cell layer of the epithelia separates the microbiota from the colonic LP, which creates a permissive environment for the mutualistic relationship between the host and the commensal bacteria to develop.

A single cell layer of the columnar epithelium forms a physical barrier against the luminal contents. The crucial role of intestinal epithelial cells (IECs) in segregating host and microbes was demonstrated in an early study, in which a mouse line with a defective epithelium induced by expression of a dominant negative N-cadherin was used. These animals developed intestinal inflammation, which was likely due to the entry of luminal bacteria into the LP (Hermiston and Gordon, 1995). Increased bacterial translocation into the colonic LP resulting from epithelial barrier abnormalities has also been suggested to contribute to the pathology of IBD, showing that an intact barrier is essential to prevent bacterial entry (Mankertz and Schulzke, 2007). Multiple different cell types with specialised functions constitute the epithelial cell barrier and thus, contribute to the maintenance of intestinal homeostasis. For instance, goblet cells secrete gel-forming mucins – mainly highly glycosylated proteins – that form a thick layer of mucus. This mucus layer extends 150 µm away from the epithelium, and prevents close contact between the bacteria and host cells. While the inner layer of the mucus is very dense and devoid of bacteria in healthy individuals, the outer layer is looser, forming a niche for commensal bacteria to reside (Johansson et

al., 2008). The thickness of the mucus layer is very important as shown by increased susceptibility to inflammation in *Citrobacter rodentium*-induced colitis upon thinning of the inner mucus layer due to antibiotic exposure (Wlodarska et al., 2011). Mice deficient in the dominant mucus structural component Muc2 developed intestinal inflammation and cancer, which was also likely due to the direct contact of intestinal bacteria with host cells (Johansson et al., 2008). Additionally, IBD patients have a significantly thinner mucus layer in the involved tissue compared to their non-involved tissue or healthy controls (Fyderek et al., 2009), although it remains unclear whether this is a primary or secondary effect.

In contrast to the colon, in the small intestine antimicrobial peptides (AMPs) play a fundamental role in segregating luminal contents from the host's tissue. AMPs are secreted by Paneth cells, which represent another specialised cell type within the intestinal epithelium that is found close to the base of the crypts. AMPs include defensins, cathelicidins, C-type lectins, such as the regenerating islet-derived protein 3-α (RegIIIα, or the mouse ortholog RegIIIγ (Cash et al., 2006)), and enzymes with antimicrobial properties (reviewed in Muniz et al. (2012)). A defective function of Paneth cells has been shown to markedly increase penetration by both commensal and pathogenic bacteria (Vaishnava et al., 2008).

The ability of the IECs to sense the intestinal microbiota is critical for the maintenance of intestinal homeostasis, and to this end, IECs express a range of germ-line-encoded pattern recognition receptors (PRRs). PRRs include highly conserved Toll-like receptors (TLRs), nuclear oligomerisation domain (NOD)-like receptors (NLRs) and RIG-I-like

receptors (RLRs). PRRs recognise microbe-associated molecular patterns (MAMPs), which include a wide range of microbe-derived molecules (e.g. lipopolysaccharide, peptidoglycans, flagella, microbial nucleic acids), as well as damage-associated molecular patterns (DAMPs), which are released upon cell damage or stress (Seong and Matzinger, 2004, Abreu, 2010, Broquet et al., 2011, Elinav et al., 2013). PRRs expression by IECs has been proven essential for the epithelial homeostasis in a number of studies, in which mice deficient in PPRs or their signalling components displayed bacterial dysbiosis, and developed intestinal inflammation (Rakoff-Nahoum et al., 2004, Vijay-Kumar et al., 2007, Vaishnava et al., 2008, 2010, Slack et al., 2009). While PRRs expressed by IECs are important in sensing the microbiota, exacerbated immune response towards bacterial antigens could lead to chronic inflammation. Therefore, mechanisms exist, which maintain hyporesponsive PRR signalling, such as abundant expression of negative regulators of PRR-dependent pro-inflammatory signalling, for example, single Ig IL-1-related receptor (SIGIRR) (Xiao et al., 2007). The differentially regulated anatomical location of TLRs also contributes to hyporesponses within the intestine. For instance, while apical exposure of IECs to TLR9 ligands resulted in a tolerogenic response, basolateral exposure led to NF-κB activation (Lee et al., 2006).

Microfold cells (M cells) represent another specialised lineage of IECs. They are primarily found in the follicle-associated epithelium that overlies lymphoid structures, such as Peyer's and caecal patches, or the isolated lymphoid follicles (ILFs) (Lorenz and Newberry, 2004, Mabbott et al., 2013). M cells sample microbial antigens from the luminal contents, which are then delivered via transcytosis across the epithelium to

various immune cell populations, including antigen-presenting cells (APCs). A similar mechanism of luminal antigen delivery has also been recently proposed for goblet cells in the small intestine (McDole et al., 2012).

1.2 The intestinal innate immune system

1.2.1 Mononuclear phagocytes

To maintain intestinal homeostasis in the presence of millions of bacteria colonising the lumen of the intestine, tolerogenic immune responses are dominant in the colonic LP. The described IECs can produce immune mediators, including transforming growth factor beta (TGF-β) and thymic stromal lymphopoietin (TSLP), which promote differentiation of APCs with tolerogenic properties that in turn can skew adaptive immune responses to an anti-inflammatory profile (Rimoldi et al., 2005, Zeuthen et al., 2008). Mononuclear phagocytes (MNPs) represent a very heterogeneous population in the colonic LP. Two main subtypes can be identified under homeostatic conditions based on the expression of cluster of differentiation (CD) 103 and CX3 chemokine receptor 1 (CX_3CR1): the $CD11c^+CD103^+CX_3CR1^-$ dendritic cells (DCs) and the $CD11c^{low}CX_3CR1^{hi}$ intestinal resident macrophages (Bogunovic et al., 2009, Varol et al., 2009). They both can uptake, process and present antigens on major histocompatibility complex class I or II (MHCI and MHCII) molecules, yet differ in their function and migratory capacity.

Following uptake of the antigen $CD103^+$ DCs migrate to the mesenteric lymph nodes (mLN) and the Peyer's patches, where they influence the differentiation of naïve $CD4^+$

T cells and the secretion of immunoglobulin A (IgA) by B cells (Macpherson and Uhr, 2004, Schulz et al., 2009b). These migratory DCs also induce expression of homing receptors, such as CCR9 and $\alpha_4\beta_7$, on the surface of $CD4^+$ T cells allowing them to traffic back to the intestinal LP (Mora et al., 2003, Johansson-Lindbom et al., 2005). Importantly, $CD103^+$ DCs are potent inducers of forkhead box $P3^+$ (Foxp3) regulatory T cells (Tregs) via production of TGF-β and retinoic acid (RA) (Coombes et al., 2007, Sun et al., 2007).

The tolerogenic environment of the mucosa is also maintained by CX_3CR1^+ macrophages, which produce high amounts of interleukin 10 (IL-10), suppress effector responses and promote Treg survival and function (Murai et al., 2009, Hadis et al., 2011, Kayama et al., 2012). IL-10 is also important for the macrophages phenotype since a targeted deletion of IL-10 receptor (IL-10R) on $CXCR3^+$ macrophages results in the development of spontaneous colitis (Shouval et al., 2014, Zigmond et al., 2014). In contrast to the migratory $CD103^+$ DCs, monocyte-derived $CXCR3^+$ macrophages reside in the colonic LP close to IECs (Niess et al., 2005, Schulz et al., 2009b). This places them in a strategic position to sample antigens by formation of transepithelial dendrites that can reach the luminal contents and also to effectively clear pathogens by phagocytosis (Rescigno et al., 2001, Niess et al., 2005).

1.2.2 Innate lymphoid cells

Innate lymphoid cells (ILCs) are a recently described class of lineage marker-negative innate immune cells that play an important role in both tissue development and immunity. They can be classified into three main populations, similar to $CD4^+$ helper T

(Th) cells (described below), based on the expression of specific transcription factors and production of cytokines. Group 1, 2 and 3 ILCs (ILC1s, ILC2s, ILC3s) express T-bet, Gata3 and Rorγt and produce IL-12/18, IL-5 /13 and IL-17/22, respectively (Spits et al., 2013). They are potent amplifiers of both innate and adaptive immune responses, including the following intestine-specific functions. Firstly, lymphoid tissue-inducer (LTi) cells, which are dependent on the expression of RAR-related orphan receptor-γt (Rorγt) and belong to ILC3s, are critical for the development of lymphoid structures of the GALT (Sun et al., 2000, Eberl and Littman, 2003, van de Pavert and Mebius, 2010, Spits et al., 2013). Secondly, ILC3s in the intestine promote epithelial barrier integrity, for instance through IL-22 production (Sonnenberg et al., 2011b, 2012), and constitute a first line of defence against pathogens, for example, *C. rodentium* (Satoh-Takayama et al., 2008, 2009, Sonnenberg et al., 2011b). ILC3s are also involved in disease-driving responses as was demonstrated in experimental models of colitis and human IBD (Buonocore et al., 2010, Geremia et al., 2011, Pearson et al., 2016). Additionally, a role of ILC2s in the intestinal repair upon intestinal inflammation has been highlighted recently (Monticelli et al., 2015).

1.2.3 Other innate cells

Other innate cells are also abundant in the intestinal mucosa, for instance, eosinophils and mast cells (Mishra et al., 1999, Yu and Perdue, 2001, Al-Haddad and Riddell, 2005, Ramsay et al., 2010). Both immune cell populations contribute to mucosal homeostasis and repair processes in the steady state environment of the intestine. For instance, eosinophils promote plasma cells to switch to IgA production (Chu et al., 2014), while

mast cells produce factors enhancing epithelial barrier integrity (Groschwitz et al., 2009).

1.3 The intestinal adaptive immune system

1.3.1 B cells

B cells represent an important axis in mucosal homeostasis, primarily via production of secretory IgA. B cells can undergo IgA class switching both in the secondary lymphoid tissues and within the colonic LP (Cerutti, 2008). Secretory IgA is highly concentrated in the mucus layer, complementing the physical and biochemical barriers established by the mucus layer and the AMPs. Secreted IgA can bind bacteria glycans non-specifically (Mestecky and Russell, 2009) or in a microbe-specific fashion to facilitate fine-tuning of immune responses (Hapfelmeier et al., 2010). Interestingly, it has been shown that intestinal bacteria highly coated with IgA represent species with the most colitogenic potential, suggesting that the B cell-mediated immune responses are preferentially directed at disease-driving bacteria (Palm et al., 2014).

1.3.2 T cells

The intestine contains multiple specialised T cell populations, including a wide array of unconventional T cells, which are especially abundant among epithelial enterocytes, and are collectively known as intraepithelial lymphocytes (IELs). These mainly include T cell receptor (TCR)$\gamma\sigma^+$ T cells, CD8$\alpha\alpha^+$ cells, and double negative T cells that neither express CD4 nor CD8. Invariant T cells, such as mucosal-associated invariant T cells (MAIT cells) and invariant natural killer T cells (iNKT cells) can also be found in the colonic mucosa. They all contribute to the maintenance of intestinal homeostasis, and

play a role in inflammatory responses (reviewed in Hayday and Gibbons (2008), Cheroutre et al. (2011)); however, they are outside of the scope of this thesis and are not discussed in more detail.

1.3.2.1 $CD4^+$ T cell development and activation

T cells derive from haematopoietic stem cells. In both neonatal and adult mice, T cell progenitors migrate from the bone marrow into the thymus, where they undergo maturation and acquire antigen specificity. This involves generation of the α- and β-chains of the TCR and expression of the co-receptors CD4 or CD8 (reviewed in Germain (2002)). The rearrangement of TCR genes is one of the key steps involved in cells committing to the T cell lineage. The recombination activating genes 1 and 2 (Rag1 and 2) are critical in this process, and both Rag1- and Rag2-deficient mice have no lymphocytes (Mombaerts et al., 1992, Shinkai et al., 1993). Rag1 and 2 enzymes regulate the V(D)J gene recombination producing an impressive number of TCRs with different specificity (reviewed in Schatz and Ji (2011)). A transient Rag1 and 2 gene expression mediates β-chain rearrangement, followed by the α-chain rearrangement. Subsequently, thymocytes undergo MHC restriction through a process called positive selection (reviewed in Klein et al. (2014)). Thymic cortical epithelial cells express both MHCI and MHCII molecules on their surface and present a wide range of self-peptides to the thymocytes. Most thymocytes are subject to death by neglect since they express TCRs unable to interact with the MHC molecules of the cortical epithelial cells. Thymocytes with TCRs able to recognise self-peptides in the context of MHC molecules are allowed to survive. Subsequently, the remaining thymocytes undergo a process of negative selection in the thymic medulla, where thymocytes expressing TCRs with high

affinity for the self-peptides are induced to undergo apoptosis (reviewed in Klein et al. (2014)). This process removes self-reactive T cells that, if not eliminated, could become potent inducers of autoimmune responses. This step represents the process of central tolerance. Negative selection is mediated by medullary thymic epithelial cells, which present to thymocytes a vast range of normally tissue-restricted antigens. This process is controlled by the transcription factor autoimmune regulator (AIRE) (Anderson et al., 2002), and its importance was demonstrated in patients that carry a mutated variant of *AIRE* and develop a severe autoimmune disease known as Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) (Finnish-German, 1997). Finally, the process of differentiation of T cells in the thymus gives rise to two distinct populations of immunocompetent T cells: MHCII-restricted $CD4^+$ T cells and MHCI-restricted $CD8^+$ T cells. Subsequently, T cells leave the thymus and migrate out into the blood, lymphoid organs and tissues throughout the body.

T cell activation and generation of an effective immune response require not only activation of the TCR by a corresponding peptide antigen but also the engagement of co-stimulatory molecules on APCs (reviewed in Smith-Garvin et al. (2009)). TCR activation alone results in anergy, which is one of the examples of peripheral tolerance (reviewed in Fathman and Lineberry (2007)). $CD4^+$ T cells express CD28 on their cell surface, which when bound by co-stimulatory molecules expressed by APCs, provides the second signal required for T cell activation. CD80 and CD86, also called B7-1 and B7-2 respectively, are expressed on APCs and bind to CD28. Other co-stimulatory molecule modules have also been reported, such as CD40-CD40L, ICOS-ICOS-L, OX40-OX40-L, GITR-GITR-L, CD2-CD48/CD45 (reviewed in Chen and Flies (2013)). The effector

activities of T cells may also be downregulated, and such a response is mediated by the engagement of co-inhibitory molecules. Examples of co-inhibitory molecules include CTLA-4, PD-1, and others (reviewed in Chen and Flies (2013)). The co-inhibitory molecules are particularly important for the function of Tregs, which constitute the principal mediator of peripheral tolerance and will be described in more detail below.

$CD4^+$ T helper (Th) cells are critical components within the effector immune response, responsible for host protection. The balance between different subsets of $CD4^+$ T cells is crucial. For example, when regulatory mechanisms are impaired or when pro-inflammatory pathways become dominant intestinal inflammation may develop (Maynard and Weaver, 2009). Importantly, the colonic mucosa of IBD patients is infiltrated by $CD4^+$ T cells suggesting that these cells are an important component of the intestinal immune system (Kaser et al., 2010b). Initially, $CD4^+$ T cells were divided into T-helper 1 (Th1) and T-helper 2 (Th2) cells specialised in immune responses against intracellular bacteria and viruses and parasites, respectively (Mosmann et al., 1986). Today it is well appreciated that while polarised immune responses are critical for fighting specific pathogens, the Th lineages are not definitive and many more subsets of Th cells have been defined. Here, the major Th cell subsets will be briefly described, followed by a comprehensive description of the Treg literature (Fig. 1.2).

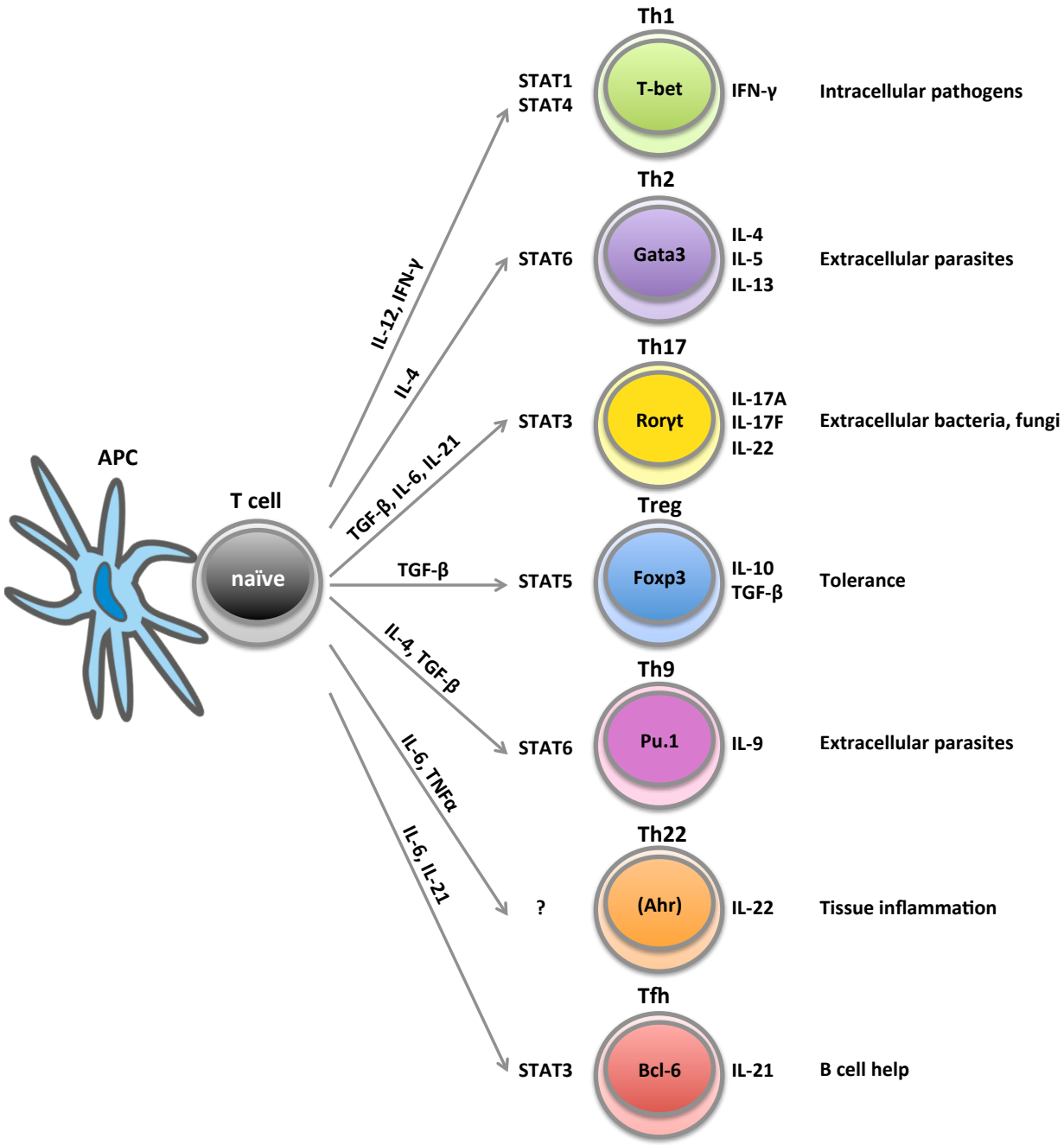


Figure 1.2. Differentiation of CD4$^+$ T cell subsets.

Upon antigen uptake, processing and presentation, an APC primes a naïve CD4$^+$ T cell and in the presence of polarising cytokines promotes differentiation of distinct Th cell subsets via activation of signal transducer and activator of transcription (STAT) proteins and lineage defining transcription factors, which drive the production of signature cytokines. Each Th cell subset is specialised in effector function. Studies on signalling pathways activated in Th22 cells are still under investigation.

While T cell differentiation is presented here in a linear fashion, it is important to emphasize that these processes are not as straightforward and exhibit a range of plasticity, for example, cells may co-express other transcription factors and produce different cytokines.

APC – antigen-presenting cell, Th – helper CD4$^+$ T cell, Tfh – follicular helper T cell

1.3.2.2 Th1 cells

Th1 cells are particularly important in mediating effector responses against intracellular bacteria and viruses, and orchestrate cellular immunity by promoting macrophage, NK cell and cytotoxic $CD8^+$ T cell activities (Murphy et al., 2000). Th1 cells are usually characterised by the expression of the T-box transcription factor T-bet and production of high amounts of interferon (IFN)-γ. Their transcriptional profile is driven by signal transducer and activator of transcription 1 (STAT1) and STAT4 in response to IFN-γ and IL-12, respectively, resulting in the induction of T-bet (Lighvani et al., 2001, Afkarian et al., 2002). This, in turn, increases expression of IL-12R and IFN-γ (Schulz et al., 2009a, Yang et al., 2007b). IL-18 can further synergise with IL-12 to promote IFN-γ production independent of TCR activation (Yoshimoto et al., 1998). Additionally, another STAT1-activating cytokine, IL-27, can also induce T-bet and IL-12R expression in $CD4^+$ T cells (Takeda et al., 2003).

IFN-γ-producing $CD4^+$ T cells are increased in the colonic mucosa of CD patients (Fuss et al., 1996), and consistently, $CD4^+CD45RB^{hi}$ T cells transferred into SCID mice produced large amounts of IFN-γ contributing to intestinal inflammation (Powrie et al., 1994b). While blockade of IFN-γ led to amelioration of the disease, T-cell-derived IFN-γ might not be the critical driver of the disease (Simpson et al., 1998). Additionally, STAT4-deficient $CD4^+$ T cells and blockade of IL-12 resulted in a milder disease (Simpson et al., 1998, Wirtz et al., 1999). However, studies of $CD4^+$ T cells lacking T-bet in a T cell transfer setting have provided conflicting results. While Neurath et al. (2002) showed that T-bet-deficient $CD4^+$ T cells failed to induce colitis, two more recent studies did not replicate these results (Krausgruber et al., 2016, Zimmermann et al.,

2016), and Krausgruber et al. (2016) suggested that T-bet is a modulator of IL-23-driven responses rather than a driver of disease pathogenesis.

1.3.2.3 Th2 cells

Th2 cells are involved in host defence against gastrointestinal nematodes, and in mediating allergic reactions. They are central in coordinating humoral immune responses, inducing B-cell antibody class switching, and attracting innate cells, such as eosinophils and mast cells (Luckheeram et al., 2012). Th2 cells are characterised by the expression of Gata3, and IL-4, IL-5 and IL-13 cytokines (Zhang et al., 1997, Zheng and Flavell, 1997). Their differentiation is driven by IL-4, which signals through STAT6 and induces Gata3 transcription factor expression (Ansel et al., 2006). STAT3-signalling cytokines, such as IL-6 and IL-21, can synergise with STAT6 to promote the Th2 signature (Stritesky et al., 2011), while TSLP, IL-25 and IL-33 can enhance Th2 cytokine production (Paul and Zhu, 2010).

In contrast to Th1 and Th17 (described below) $CD4^+$ T cells, Th2 cells are not abundant in the absence of a relevant microbial driver such as a parasite infection (Artis et al., 2005, Maynard and Weaver, 2009). Although originally increased amounts of IL-5 and IL-13 were identified in T cells isolated from the colonic mucosa of ulcerative colitis (UC) patients, later it was appreciated that iNKT cells could have been the primary source of these cytokines (Fuss et al., 1996, Fuss et al., 2004).

There is a close relationship between Th1 and Th2 cells: T-bet directly inhibits Gata3 (Usui et al., 2006), while Gata3 prevents the production of IFN-γ (Yagi et al., 2010). A

balance between these two major cell lineages is critical as demonstrated, for example, by T-bet-deficient mice that develop severe airway inflammation (Finotto et al., 2002). While for years most of the research has focused on either Th1 or Th2-polarised responses, Th17 cells were identified as another major player in the coordination of immune responses, particularly important in mucosal immune responses (Cua et al., 2003).

1.3.2.4 Th17 cells

Th17 cells are specialised in defence against extracellular bacteria and fungi, but their role in the pathogenesis of several autoimmune diseases has also been emphasised in recent years (Weaver et al., 2007). They can be defined by expression of Rorγt and production of primarily IL-17A, IL-17F, IL-21 and IL-22 (Ivanov et al., 2007). On one hand, these effector cytokines induce pro-inflammatory responses, for instance by chemoattracting neutrophils; on the other hand, they promote epithelial barrier integrity and AMP secretion (Maloy and Kullberg, 2008, Guglani and Khader, 2010). Th17 differentiation is promoted by TGF-β together with STAT3-activating cytokines, such as IL-6 or IL-21, which promote induction of Rorγt (Veldhoen et al., 2006). Rorγt drives the Th17 transcriptional programme and production of IL-17 (Ivanov et al., 2006, Zhou et al., 2007, Manel et al., 2008, Yang et al., 2008, Ciofani et al., 2012). Other combinations of cytokines have also been shown to induce Th17 differentiation *in vitro*, including IL-1β+IL-6+IL-23 or TGF-β3+IL-6+IL-23 (Chung et al., 2009, Ghoreschi et al., 2010, Lee et al., 2012). TGF-β, which acts via SMAD-dependent signalling, represses expression of cytokines characteristic of other Th lineages, namely IFN-γ, IL-2 and IL-4 that would otherwise inhibit Th17 differentiation (Takimoto et al., 2010).

Rorγt-deficient and STAT3-deficient mice have significantly reduced numbers of Th17 cells highlighting the important role of these transcription factors in controlling the Th17 cell fate (Ivanov et al., 2006, Harris et al., 2007, Yang et al., 2007a, 2008). IL-23 was originally thought to be essential for Th17 cell differentiation. However, now it is well established that IL-23 is dispensable for Th17 cell differentiation, and instead reinforces Th17 responses and endows Th17 cells with a pathogenic potential (Ghoreschi et al., 2010, McGeachy et al., 2009, Langrish et al., 2005, Awasthi et al., 2009, McGeachy et al., 2007). Additionally, in the last years it has been discovered that Th17 cells can also express IL-10. While Rorγt^{+}IL-17A^{+}IFN-γ^{+} Th17 cells can drive pathogenic responses, Rorγt^{+}IL-17A^{+}IL-10^{+} Th17 cells likely represent a non-pathogenic subtype (Zielinski et al., 2012, Lee et al., 2012). However, the specificity and function of these different subtypes of Th17 cells remain to be elucidated.

Following the discovery of IL-23 and its important role in driving Th17 responses, Th17 cells have been recognised as important drivers of intestinal inflammation. Increased production of IL-17 cytokines has been noted in the colonic mucosa of IBD patients (Kobayashi et al., 2008, Rovedatti et al., 2009). Studies of mice deficient in the IL-23p19 subunit specific to IL-23, in contrast to p40, which is shared by IL-12, demonstrated that IL-23 is required for the development of colitis in multiple experimental settings (Hue et al., 2006, Uhlig et al., 2006b, Yen et al., 2006, Ahern et al., 2010). Moreover, transfer of Rorγt-deficient T cells led to milder colitis compared to wild type T cells (Leppkes et al., 2009). Interestingly, transfer of IL-17A-deficient or IL-17F-deficient T cells alone did not ameliorate colitis suggesting redundancy between these cytokines (Leppkes et al., 2009).

While TGF-β is required for Th17 cell differentiation, it also induces the expression of Foxp3 and thus, promotes the Treg lineage (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006). This reciprocal relationship between the two $CD4^+$ T cell subsets appears to be an important determinant of intestinal homeostasis.

1.3.2.5 Other non-regulatory $CD4^+$ T cells subsets

The collection of $CD4^+$ Th cell subsets has broadened in the last years, and new subsets dependent on different combinations of cytokines and distinct transcription factors for their development have been described. These include Th9, Th22 and follicular Th (Tfh) cells.

Th9 and Th22 cells are named according to the most abundant cytokine they produce, IL-9 and IL-22, respectively. Th9 cells are closely related to Th2 cells and are similarly specialised in the defence against extracellular parasites. Their differentiation is driven by IL-4 and TGF-β resulting in the induction of STAT6 and the transcription factor Pu.1 (reviewed in Kaplan et al. (2015)).

IL-22 production can be induced in a wide range of T cells. $CD4^+$ T cells, which express IL-22 but no IFN-γ, IL-4 or IL-17A are termed Th22 cells. They can be differentiated in the presence of IL-6 and tumor necrosis factor (TNF)-α, and they express aryl hydrocarbon receptor (AHR) transcription factor, which likely maintains IL-22 production. Th22 cells express CCR4 and CCR10 chemokine receptors, and therefore, are likely to play a central role in the skin, contributing to host defence against pathogens and in promoting skin repair (reviewed in Sonnenberg et al. (2011a)).

Tfh cells play a vital role in the development of humoral immunity providing support for B cells. Tfh cells take an active part in B cell activation and affinity maturation and are essential for germinal centre formation. They are induced in the presence of STAT3-activating cytokines, IL-6 and IL-21 that lead to transcription of the master transcriptional regulator of the Tfh cell lineage, Bcl-6. Tfh cells produce large amounts of IL-21 and express CXCR5, which promotes their migration to the germinal centres, where they promote differentiation of B cells (reviewed in Crotty (2014)).

1.3.2.6 $CD4^+Foxp3^+$ regulatory T cells

Treg cell function is to maintain immune tolerance and prevent unnecessary inflammatory responses by modulating both the innate and adaptive immune responses. Balanced control of immune responses by Tregs is central to preventing autoimmune diseases, maintaining homeostasis at barrier sites and limiting effector responses in transplantation settings. However, the same immunosuppressive function of Tregs may have negative consequences in other contexts. For instance, Tregs may also contribute to establishing chronic infection and promoting tumour progression. Thus, Tregs influence the outcome of a wide range of immune responses, and tight regulation of their development and function is critical.

Tregs are characterised by the expression of high amounts of CD25 – the IL-2Rα chain, the Foxp3 transcription factor and production of immunosuppressive cytokines like IL-10 and TGF-β. The initial observations that neonatal thymectomy performed in 3-day old mice resulted in T-cell mediated tissue destruction, which could be rescued by adoptive transfer of cells isolated from the thymus or spleen, suggested the presence

of cells with immunosuppressive characteristics (Sakaguchi et al., 1982, 1995, Asano et al., 1996). Additional experiments uncovered a population of $CD4^+$ T cells with an antigen-experienced phenotype that could suppress effector responses of naïve $CD4^+CD45RB^{high}$ T cells transferred into immunodeficient animals (Powrie and Mason, 1990, Morrissey et al., 1993). These results suggested that the described subset of cells could not only regulate responses to self-antigens but also to dietary and microbial antigens in the gut. These cells were subsequently described as $CD4^+CD25^+$ T cells and shown to prevent inflammatory responses in multiple settings, including in thymectomised mice, transplant rejection as well as intestinal inflammation (Sakaguchi et al. (1995), reviewed in Shevach (2000)).

Later studies demonstrated that the expression of Foxp3 endows Tregs with their immunosuppressive phenotype and is indispensable for their function (Fontenot et al., 2003, Hori et al., 2003, Khattri et al., 2003). A loss-of-function variant of the human *FOXP3* gene results in the development of the Immunodysregulation Polyendocrinopathy Enteropathy X-linked Syndrome (IPEX), which manifests with multiorgan inflammation (Bennett et al., 2001, Wildin et al., 2001). Scurfy mice, which also carry a mutation in the *Foxp3* gene, develop a lymphoproliferative disease that parallels IPEX patient symptoms (Brunkow et al., 2001, Wildin et al., 2001). The non-redundant requirement for Foxp3 expression in Tregs has been demonstrated in multiple studies. For example, ectopic expression of Foxp3 converted naïve $CD4^+$ T cells into cells with Treg phenotype and function. This was demonstrated by their ability to efficiently prevent the development of colitis in a T cell transfer setting (Hori et al., 2003).

Treg development

A majority of Foxp3$^+$ Tregs develop in the thymus (tTregs) (Itoh et al., 1999). However, Foxp3 expression can also be induced in naïve CD4$^+$ T cells in the periphery (pTregs). CD25$^+$CD4$^+$CD8$^-$ Tregs differentiate in the thymus from CD4$^-$CD8$^-$ thymocytes upon presentation of the self-peptide by thymic stromal cells in the context of MHCII and additional co-stimulatory signals via CD28 (Itoh et al., 1999). Both the abundance of self-peptides and the affinity of the TCR for the peptide govern Treg development (Jordan et al., 2001, Kawahata et al., 2002, van Santen et al., 2004). In the periphery, naïve CD4$^+$ T cells can differentiate into Foxp3-expressing Tregs and acquire immunosuppressive function in the presence of IL-2 and TGF-β (Chen et al., 2003, Kretschmer et al., 2005). The intestinal environment harbours a substantial proportion of pTregs, and indeed, oral tolerance appears to be promoted by pTregs induced in the GALT. This process is supported by retinoic acid, a metabolite of vitamin A, produced by CD103$^+$ DCs (Benson et al., 2007, Coombes et al., 2007, Sun et al., 2007). By contrast, IL-6 has been shown to hinder TGF-β-mediated conversion of naïve T cells into Foxp3$^+$ Tregs (Bettelli et al., 2006), and instead promoted Th17 cell differentiation (Bettelli et al., 2006, Veldhoen et al., 2006).

Once differentiated, tTregs and pTregs are not easy to distinguish. Multiple studies attempted to identify differentially expressed markers between these two populations. However, to date, only the transcription factor Helios and a surface marker Neuropilin 1 (Nrp-1) have been suggested to preferentially mark tTregs (Thornton et al., 2010, Weiss et al., 2012, Yadav et al., 2012). Yet the reliability of Helios and Nrp-1 as tTreg markers has been questioned over the recent years

precluding their ultimate use as definite markers of the tTreg population (Gottschalk et al., 2012, Singh et al., 2015, Szurek et al., 2015).

There is evidence that tTregs and pTregs have non-redundant roles in the control of immune responses (Haribhai et al., 2011). However, lack of reliable differentiating markers of tTregs and pTregs has hindered studies of the functional differences between these two populations. Nevertheless, genetic deletion of specific conserved noncoding sequences (CNSs), which constitute regulatory regions of the *Foxp3* gene locus, have provided some insight. CNS3 and CNS1 have been shown to be of particular relevance for the development of tTregs and pTregs, respectively. Consistently, CNS3-deficient mice had a selective reduction in the tTreg compartment, whereas CNS1 deletion resulted in preferential ablation of pTreg induction at barrier sites (Tone et al., 2008, Zheng et al., 2010). Additionally, CNS1 deficiency resulted in the development of Th2-driven inflammation in the intestine and lungs, suggesting that pTregs may be especially important for limiting effector responses in mucosal tissues (Zheng et al., 2010).

Transcriptional control of Tregs

Extensive studies of the molecular mechanisms controlling Treg development have been performed. Both TCR- and cytokine-mediated signalling are critical for the induction and maintenance of Foxp3 expression in Tregs. A two-step model has been suggested, whereby TCR activation and co-stimulatory molecule engagement open the *Foxp3* locus and induce cytokine responsiveness followed by signals delivered by STAT5-activating cytokines that promote Treg differentiation (Burchill et al., 2008, Lio

and Hsieh, 2008). TCR stimulation at the Treg precursor stage leads to activation of multiple signalling modules, including factors such as TAK1, Bcl10, CARMA1, PKCθ, and IKK-β, which results in the activation of the NF-κB pathway (Schmidt-Supprian et al., 2003, 2004, Wan et al., 2006, Gupta et al., 2008, Barnes et al., 2009, Medoff et al., 2009, Molinero et al., 2009). The importance of this pathway in thymic Treg generation was demonstrated in Rag-deficient TCR-transgenic mice, in which normally Tregs do not develop. Genetically enforced activation of the NF-κB pathway resulted in Treg generation independent of TCR signalling (Long et al., 2009). The NF-κB family member c-Rel binds to the CNS3 of the *Foxp3* locus, which is particularly important for the induction of Foxp3 expression (Isomura et al., 2009, Long et al., 2009, Zheng et al., 2010). Upon TCR activation several other transcription factors are recruited to the *Foxp3* promoter including AP1, CREB, NFAT, AML1/Runx1, and ATF, and promote its gene expression (Kim and Leonard, 2007, Tone et al., 2008, Kitoh et al., 2009, Rudra et al., 2009). Foxo proteins have also been shown to regulate Treg differentiation with Foxo1 and Foxo3 binding sites found in the *Foxp3* promoter (Harada et al., 2010, Kerdiles et al., 2010, Ouyang et al., 2010). By contrast, activation of the AKT–mTOR pathway due to continued TCR activation inhibits Treg differentiation (Haxhinasto et al., 2008, Sauer et al., 2008, Delgoffe et al., 2009). Furthermore, in the periphery upon TCR engagement, TGF-β induces binding of Smad3 and NFAT to the CNS1 enhancer, thereby promoting *Foxp3* expression (Marson et al., 2007, Tone et al., 2008). Consistently, retinoic acid, which promotes Foxp3 expression by conventional $CD4^+$ T cells, has been suggested to enhance Smad3 binding to CNS1 (Xu et al., 2010). Additionally, butyrate, one of the abundant short-chain fatty acids (SCFAs) found in the

intestine, can also promote pTreg differentiation by enhancing acetylation of CNS1 (Furusawa et al., 2013).

STAT5-activating cytokines, primarily IL-2 but also IL-7 and IL-15, are key to Foxp3$^+$ Treg differentiation (Vang et al., 2008). TCR activation upregulates CD25 expression and thus, allows binding of IL-2 that subsequently activates STAT5, which binds directly to the *Foxp3* locus (Burchill et al., 2008, Lio and Hsieh, 2008). STAT5-deficient mice lack Tregs, demonstrating a non-redundant role for STAT5 signalling in Treg generation (Burchill et al., 2007, Yao et al., 2007). Additionally, IL-2-induced signalling has been shown to contribute to the demethylation of the CNS2 of the *Foxp3* locus, which is critical for the sustained stability of mature Foxp3$^+$ Tregs particularly during cell division (Zheng et al., 2010, Feng et al., 2014, Li et al., 2014b). Demethylation at the CNS2 together with Cbf-β-Runx1 binding allows Foxp3 to promote its stable expression by binding to CNS2 (Zheng et al., 2010). Notably, genetic deletion of CNS2 resulted in a loss of Foxp3 expression by Tregs upon exposure to pro-inflammatory stimuli, demonstrating the essential function of the CNS2 regulatory region in Foxp3 stability (Zheng et al., 2010, Feng et al., 2014, Li et al., 2014b).

While Foxp3 is a master regulator of the Treg lineage, it does not act alone, and its expression alone is not sufficient to induce an immunosuppressive phenotype. For example, Foxp3 was detected in conventional CD4$^+$ T cells upon activation without the associated regulatory phenotype (Miyao et al., 2012). By contrast, T cells that did not express functional Foxp3, due to green fluorescent protein (GFP) insertion, still displayed some features of the Treg lineage (Gavin et al., 2007, Lin et al., 2007). These

observations can be explained by recent studies that investigated the binding partners of Foxp3 that take an active part in defining the regulatory function of Tregs, collectively referred to as the Foxp3 interactome (Fu et al., 2012, Rudra et al., 2012). Rudra et al. purified Foxp3 complexes and using a proteomic approach identified more than 350 partners of Foxp3, including direct targets of Foxp3, such as Gata3 or STAT3 (Rudra et al., 2012). Fu et al., on the other hand, used a computational approach to identify five transcription factors (Eos, IRF4, Satb1, Lef1 and GATA-1) that when co-expressed with Foxp3 elicited a Treg phenotype (Fu et al., 2012). Both studies highlighted the importance of positive and negative feedback loops co-regulated by Foxp3, its binding partners and chromatin modulators that collectively determine the stability of the Treg lineage (Hori, 2012).

Mechanisms of suppression

Multiple mechanisms of action of Tregs have been described that can occur in both cell-dependent and -independent fashions. Tregs are able to suppress immune responses of various immune cell types, including but not limited to naïve and memory $CD4^+$ T cells, $CD8^+$ T cells, NK and NKT cells, B cells and DCs (Shevach, 2009). While the initial activation of Tregs is dependent on antigen stimulation, the effector mechanisms of suppression mediated by Tregs do not require further antigen signals and thus, facilitate a wider range of Treg-controlled immunosuppression.

Firstly, Tregs inhibit effector immune responses in a cell-contact-dependent way due to increased expression of inhibitory molecules on their cell surface. Engagement of these molecules can directly modulate the effector cells or alternatively induce an

immunosuppressive phenotype in APCs (Shevach, 2009). CTLA-4, GITR, LAG-3, ICOS, PD-1 and OX-40 are among the surface markers enriched on Foxp3$^+$ Tregs that have been shown to promote their regulatory function or survival (McHugh et al., 2002, Shimizu et al., 2002, Herman et al., 2004, Totsuka et al., 2005, Liang et al., 2008, Wing et al., 2008, Griseri et al., 2010). CTLA-4, which binds to CD80 and CD86 on APCs, is particularly worth highlighting. Mice with a selective deletion of CTLA-4 in Foxp3$^+$ Tregs develop a fatal systemic autoimmune disorder (Wing et al., 2008). Additionally, blocking CTLA-4 with a monoclonal antibody (mAb) abrogated Treg-mediated suppression of colitis in a T cell transfer model (Read et al., 2006). Furthermore, *in vitro* experiments indicated that CTLA-4-deficient Tregs were not able to inhibit upregulation of co-stimulatory molecules on DCs, and thus, did not promote the immunosuppressive phenotype (Onishi et al., 2008).

Secondly, Foxp3$^+$ Tregs are a rich source of immunosuppressive cytokines, such as IL-10, TGF-β or IL-35. Characterisation of mice with conditional deletion of IL-10 in Foxp3-expressing Treg cells demonstrated that IL-10 production by Tregs is particularly important at mucosal sites, such as the intestine (Rubtsov et al., 2008). Furthermore, control of established colitis and experimental autoimmune encephalomyelitis was mediated by Treg-derived locally produced IL-10 (McGeachy et al., 2005, Uhlig et al., 2006a). IL-10 has also been suggested to promote Foxp3 expression under inflammatory conditions, and acted in an autocrine manner via a STAT3-dependent mechanism to promote its own production (Murai et al., 2009, Chaudhry et al., 2011). Importantly, other hematopoietic cells also produce IL-10. For example, whereas Foxp3$^+$ Tregs are the major T cell source of IL-10 in the colon, CD4$^+$Foxp3$^-$IL-10$^+$ cells,

also known as Tr1 cells, are abundant in the small intestine, and have been shown to promote intestinal homeostasis (Maynard et al., 2007, Huber et al., 2011). TGF-β is another broadly immunosuppressive cytokine, which is enriched in the intestines. TGF-β-deficient mice suffer from a lymphoproliferative autoimmune disorder resulting in death within the first few weeks of life (Shull et al., 1992). This observation may be explained by studies, which showed that the ability to respond to TGF-β by the effector T cells was critical for Treg-mediated prevention of inflammation (Fahlen et al., 2005). However, it is worth noting that Tregs, expressing a dominant-negative form of the TGF-βRII, also prevented colitis, suggesting it is dispensable for their function (Fahlen et al., 2005). Furthermore, the role of TGF-β in promoting Foxp3 expression by binding to CNS2 has been well described (Chen et al., 2003). IL-35 is an IL-12 family immunosuppressive cytokine specifically enriched in $Foxp3^+$ Tregs (Collison et al., 2007). It consists of two subunits, IL-12α and IL-27β, which are encoded by *Il12a* and *Ebi3*, with *Ebi3* directly regulated by Foxp3. Ebi3- and Il12a-deficient Tregs displayed reduced regulatory activity both *in vitro* and *in vivo*, highlighting production of IL-35 as another Treg mechanism of immunosuppression (Collison et al., 2007). Galectin-1, a member of the family of beta-galactoside-binding proteins, which is upregulated in Tregs upon TCR activation, could also inhibit effector responses (Garin et al., 2007). Additionally, activated Tregs have been shown to express a co-inhibitory molecule TIGIT. Upon engagement, TIGIT induced secretion of fibrinogen-like protein 2, which hampered effector T cell proliferation (Joller et al., 2014).

Thirdly, metabolic disruption has also been highlighted as a mechanism, through which Tregs downregulate effector responses. For example, Tregs express high amounts of

CD25, and therefore, compete with effector T cells for survival factor availability (Pandiyan et al., 2007). Additionally, Tregs are also enriched in CD39 and CD73 endonuclease expression (Kobie et al., 2006, Deaglio et al., 2007). These two endonucleases mediate generation of extracellular adenosine, which inhibits effector responses in T cells, when bound to the A2A receptor (Bono et al., 2015).

Finally, it has also been suggested that Tregs have a cytotoxic activity, and may produce granzyme A and B resulting in cytolysis of T cells or APCs (Grossman et al., 2004, Gondek et al., 2005, Zhao et al., 2006).

Environmental adaptation of Tregs

Recently it has been appreciated that Tregs localised in non-lymphoid tissues, in contrast to their lymphoid counterparts, have developed distinct adaptations that provide them with tools to control both immune and non-immune processes in a context-dependent manner. Despite exposure to distinct environments, Tregs found in non-lymphoid tissues share certain characteristics (Panduro et al., 2016). For example, they have been shown to express a TCR repertoire distinct from their lymphoid counterparts and from conventional $CD4^+$ T cells isolated from the same tissue (Feuerer et al., 2009). Tissue Tregs also exhibit a differential transcriptional profile with obvious enrichment of transcripts encoding effector molecules, chemokines and their receptors, and immunosuppressive cytokines such as IL-10 (Feuerer et al., 2009, Burzyn et al., 2013, Schiering et al., 2014).

Tregs residing in non-lymphoid tissues have been also associated with numerous physiological functions. For instance, specific ablation of visceral adipose tissue (VAT) Tregs by targeting peroxisome proliferator-activated receptor gamma (PPAR-γ), which controls the development of the VAT Treg transcriptional programme, resulted in exacerbation of VAT inflammation and a decline in metabolic indices related to glucose metabolism (Cipolletta et al., 2012). Additionally, VAT Tregs have been suggested to control metabolic responses to thermogenic changes (Medrikova et al., 2015). Tregs have also been found to localise to the skeletal muscle following both acute and chronic injury. These Tregs, characterised by a distinct phenotype, were crucial for muscle regeneration, a process that was partially mediated by production of amphiregulin (Burzyn et al., 2013). Notably, Tregs found in the lung have also recently been reported to produce amphiregulin, which had a non-redundant role in tissue repair following acute influenza infection (Arpaia et al., 2015). Intestinal Tregs also exhibit some characteristics that distinguish them from Tregs found in lymphoid tissues and extra-intestinal non-lymphoid sites. These will be discussed in more detail in Chapter 3.

Furthermore, Tregs can also enhance their ability to control inflammatory responses through co-expression of transcription factors typically associated with the differentiation program of effector Th cells, such as T-bet, Gata3, IRF4 and STAT3 (Liston and Gray, 2014). For instance, T-bet expression by Tregs regulated expression of CXCR3 by promoting homing of Tregs to sites of Th1-driven inflammation (Koch et al., 2009). Similarly, a specific deletion of IRF4 in Foxp3-expressing cells was critical for the control of Th2 responses and mice lacking IRF4 expression in Tregs displayed

typical immunopathology mediated by deregulated humoral immunity (Zheng et al., 2009). Furthermore, STAT3 expression by Tregs was required for the control of Th17-mediated intestinal inflammation (Chaudhry et al., 2009). The described studies showed that deletion of the 'master transcriptional regulators' of Th cell differentiation in Tregs led to subset-specific deregulation of effector responses. This suggests that the ability of Treg cells to adapt to their surrounding inflammatory milieu is critical in preventing the development of immunopathology.

Treg plasticity and instability

The ability of Tregs to acquire expression of transcription factors associated with other $CD4^+$ T cells subsets represents one of the examples of T cell plasticity, whereby $CD4^+$ T cell can adapt to the external environment and exhibit phenotypic plasticity. Tregs that convert to effector phenotype and acquire pro-inflammatory cytokine expression have also been previously described. Loss of Foxp3 by Tregs is often associated with a marked pro-inflammatory milieu induced by severe or chronic inflammation, or lymphopenic conditions. For example, lethal infection with *Toxoplasma gondii* led to a decrease in $Foxp3^+$ Tregs and expression of T-bet and IFN-γ (Oldenhove et al., 2009). Additionally, up to 50% of purified $Foxp3^+$ Tregs upon transfer into lymphocyte-deficient hosts lost expression of Foxp3 and other Treg signature molecules. These Tregs also upregulated production of significant amounts of effector cytokines, such as IFN- γ and IL-17 (Duarte et al., 2009, Komatsu et al., 2009, Yurchenko et al., 2012). Furthermore, in healthy lymphocyte-sufficient mice about 20% of $CD4^+$ T cells were identified as exFoxp3 cells using transgenic mouse lines (Zhou et al., 2009, Miyao et al., 2012). These exFoxp3 cells produced effector cytokines characteristic of different $CD4^+$

Th cell lineages. Notably, both exFopx3 cells and Foxp3$^+$ cells, which produced pro-inflammatory cytokines, were detected in various experimental models and human diseases (reviewed in DuPage and Bluestone (2016)). For example, exFoxp3 cells could promote disease development in a mouse model of diabetes and multiple sclerosis (Zhou et al., 2009, Bailey-Bucktrout et al., 2013).

Understanding of Treg adaptations, which developed to effectively suppress effector responses under inflammatory conditions, is key to developing Tregs as immunotherapies. This is especially relevant in the context of graft-versus-host disease (GVHD) or autoimmune conditions, which are associated with an increased inflammatory environment. Additionally, characterising factors controlling Foxp3 stability and understanding functional implications of the loss of Foxp3 under specific conditions will provide further insights into the pathogenesis of autoimmune and inflammatory diseases.

1.3.3 Commensal bacteria guide CD4$^+$ T cell differentiation

The microbiota shapes the intestinal immune system, and its composition is a major factor in the differentiation of CD4$^+$ T cells in the gut. Mice bred in germ-free (GF) conditions have an underdeveloped mucosal immune system, including reduced numbers of CD4$^+$ T cells, that can be reversed by colonisation with commensal bacteria (Macpherson and Harris, 2004). Th17 cells and Foxp3-expressing Tregs appear to be the two CD4$^+$ T cell subsets that are most sensitive to variations in the intestinal microbiota composition. Th17 cells are most abundant in the small intestinal LP, with lower frequencies recorded in the colonic LP (Ivanov et al., 2006, 2008). By contrast,

Tregs are most abundant in the large intestine (Uhlig et al., 2006a, Atarashi et al., 2011, Kim et al., 2016). This $CD4^+$ T cell subset compartmentalisation may be caused by differences in the composition of the intestinal microbiota between the small and large intestine. Presence of bacteria is essential for Th17 and Treg development as demonstrated in the studies of GF mice. Mice devoid of bacteria had almost undetectable numbers of IL-17^+ Th17 cells in the intestines (Ivanov et al., 2006, 2008), and $Foxp3^+$ Tregs were drastically reduced in the colonic LP (Atarashi et al., 2011, Geuking et al., 2011, Kim et al., 2016). Interestingly, Treg numbers in the small intestinal LP either did not change or were increased in GF mice (Atarashi et al., 2011, Kim et al., 2016). This observation could be explained by a recent study by Kim et al., which showed that Tregs in the small intestinal LP were primarily induced by dietary antigens (Kim et al., 2016).

Th17 cell abundance has been shown to vary extensively between animal facilities despite an identical genetic background of the mice (Ivanov et al., 2008, 2009). Induction of Th17 cell differentiation correlated with the presence of segmented filamentous bacteria (SFB) (Ivanov et al., 2009), identified as a unique member of the order of the Clostridiales with a highly reduced genome (Sczesnak et al., 2011). SFB preferentially colonises the terminal ileum and has been found in close contact with the intestinal epithelium, where it activates a unique transcriptional programme (Ivanov et al., 2009). Gene analysis indicated that SFB induced serum amyloid A protein, one of the acute phase response mediators, that when tested in *in vitro* co-cultures, could act on DCs to induce Th17 cell differentiation (Ivanov et al., 2009). Although SFB appears to play an important immunomodulatory role in mice, their

presence has not been confirmed in the studies of the human microbiome (Sczesnak et al., 2011). However, a recent report demonstrated that adhesion to IECs was critical for effective Th17 cell induction by SFB (Atarashi et al., 2015). This was replicated for other adhesive bacteria, such as *C. rodentium* and *Escherichia coli* O157 (Atarashi et al., 2015). Notably, a number of bacteria, which were able to robustly induce Th17 cells, isolated from the faeces of UC patients were also adhesive strains. These results suggest that the IEC-adhesiveness might be a key feature of bacterial strains capable of potent Th17 cell induction (Atarashi et al., 2015).

Many reports exist of specific bacterial strains able to promote induction, accumulation and function of Tregs in the intestine (Tanoue et al., 2016). pTregs are likely to be primarily responsive to the microbial-derived signals since recent studies showed that GF mice harboured almost exclusively a population of $Nrp1^{+}Helios^{+}Foxp3^{+}$ Tregs in their colons, and antibiotic treatment depleted mainly $Nrp1^{-}Helios^{-}Foxp3^{+}$ Tregs (Ohnmacht et al., 2015, Sefik et al., 2015, Yang et al., 2016a). Recently, Sefik et al. showed that a broad range of bacterial species not restricted to a single phylum (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria) were able to promote Treg differentiation in the colonic LP upon mono-colonisation (Sefik et al., 2015). Previously, certain species of spore-forming *Clostridia*, belonging to clusters IV, XIVa, and XVIII, have been highlighted to promote accumulation of Tregs in the colon (Atarashi et al., 2011, 2013). Inoculation of a mixture of strains of *Clostridia* isolated from both conventionally bred mice and healthy humans could potently induce Tregs (Atarashi et al., 2011, 2013). Colonisation with these particular strains of *Clostridia* led to expansion of predominantly $Ror\gamma t^{+}$ Tregs (Atarashi et al., 2013, Ohnmacht et al.,

2015, Sefik et al., 2015, Yang et al., 2016a), and upregulated expression of immunosuppressive markers, including IL-10, CTLA-4 and ICOS (Atarashi et al., 2011, 2013). Since mice colonised with *Clostridia* displayed higher amounts of two anti-inflammatory mediators, TGF-β and Indoleamine 2,3-Dioxygenase Enzyme (IDO), in the epithelium, it has been suggested that *Clostridia* may promote a tolerogenic DC phenotype, and thus, conversion of naïve T cells into Foxp3-expressing Tregs (Atarashi et al., 2011). Furthermore, species of *Lactobacillus*, including *Lactobacillus reuteri* or *Lactobacillus murinus*, and *Bifidobacteria* could also increase the number of Tregs in the gut (Karimi et al., 2009, Round and Mazmanian, 2010, Tang et al., 2015). While the mechanism of Treg induction by *Lactobacillus* has not been well studied yet, the process mediated by *Bacteroides fragilis* has been defined. *B. fragilis* promoted a robust increase in IL-10-producing $Foxp3^+$ Tregs in the colonic LP (Round and Mazmanian, 2010). This process was mediated by polysaccharide A (PSA) in a TLR-2-dependent fashion. This was demonstrated in mice colonised with a mutant strain of *B. fragilis* that lacks PSA expression, which exhibited comparable amounts of IL-10 to GF mice (Round and Mazmanian, 2010, Round et al., 2011). In contrast to the requirement for TLR signalling by *B. fragilis*-derived PSA, boosted Treg induction in response to *Clostridia* colonisation was not impaired in MyD88-deficient mice suggesting that different mechanisms may exist to control Tregs in the intestine (Atarashi et al., 2011). SCFAs, such as butyrate, which represent the end products of fermentation of dietary fibers by commensal bacteria, have also been shown to promote Treg number and function in the colon (Arpaia et al., 2013, Furusawa et al., 2013, Smith et al., 2013). In line with these observations, analysis of the *Clostridia* genome revealed multiple genes that could be involved in the biosynthesis of SCFAs

(Narushima et al., 2014). Finally, a more complex experimental setting, in which mice were colonised with a small consortium of bacterial species, known as the altered Schaedler flora (ASF), revealed that the microbiota induced both pro-inflammatory Th17 cells and anti-inflammatory Tregs (Geuking et al., 2011). The production of IL-10 by $CD4^+$ T cells induced by ASF colonisation was central to effective control of the Th17-mediated pro-inflammatory responses (Geuking et al., 2011).

The above studies suggest that the composition of the intestinal microbiota is an important determining factor that modulates the balance between Th17 and Treg cells in the intestine, and therefore, influences the intestinal immune response. Indeed, experiments in rodents demonstrated that modifying the composition of the microbiota might promote protective or pathological immune responses. For example, Geuking et al. showed that Treg increases due to ASF colonisation were central to maintaining intestinal homeostasis after challenge with Dextran Sulfate Sodium (DSS) (Geuking et al., 2011). Mice unable to respond to the bacterial signals developed intestinal pathology and Th17 effector responses. Colonisation with *Clostridia* also resulted in protective effects upon both DSS- and Oxazolone-induced colitis in adult life with attenuated pathology observed in both models compared to conventional specific pathogen free (SPF)-bred mice (Atarashi et al., 2011, 2013). Another study showed that *Clostridia* also played an important role in maintaining epithelial barrier integrity and contributed to protection against food allergen sensitisation (Stefka et al., 2014). Additionally, SCFAs administration has been shown to attenuate disease in a T cell transfer model of colitis dependent on the expression of Ffar2, an SCFA receptor, by the transferred Tregs (Smith et al., 2013). Furthermore, the protective role of the

microbiota has also been shown in the context of *B. fragilis* (Mazmanian et al., 2008, Round and Mazmanian, 2010). The *B. fragilis*-derived PSA molecule protected animals from experimental colitis induced by *Helicobacter hepaticus*. This was due to the ability of PSA to downregulate IL-17 production and promote IL-10 production by $CD4^+$ T cells (Mazmanian et al., 2008).

Importantly, microbiota-induced Th17 cells have also been ascribed a protective function in the mucosa. When mice were colonized with microbiota containing SFB, their resistance to *C. rodentium* infection was significantly enhanced (Ivanov et al., 2009). These animals exhibited ameliorated intestinal pathology but also reduced colonisation by the pathogen. Production of Th17 cytokines, such as IL-17 and IL-22, induced by SFB could account for this effect (Ivanov et al., 2009). Also, direct competition between SFB and *C. rodentium* for the intestinal niche may contribute to the reduced colonisation by the pathogen. However, it is worth noting that Th17 responses elicited by colonisation with SFB may also contribute to pathogenic processes. For instance, genetically predisposed K/BxN mice, when infected with SFB, develop accelerated and more severe arthritis compared to mice free of SFB (Wu et al., 2010). Gut-reactive Th17 cells have also been implicated in the pathogenesis of inflammatory diseases affecting extra-intestinal sites, including encephalomyelitis and uveitis (Berer et al., 2011, Horai et al., 2015). By contrast, SFB was protective in the NOD mouse strain predisposed to diabetes (Kriegel et al., 2011). Together these results demonstrate that microbiota-host interactions do not only affect the intestinal environment but also contribute to systemic immune responses, and are highly context-dependent.

Although rodent studies employing gnotobiotic mice, both GF and mono-colonised, have provided insights into the interactions between the components of the intestinal microbiota and immune cells, they represent an artificial environment. In humans, a wide range of environmental factors, including unlimited exposure to pathogens, is likely to complicate these interactions. Nevertheless, the experimental evidence and also findings from humans indicate that the intestinal microbiota may play a key role in pathogenesis of disease.

1.4 Inflammatory bowel diseases

IBDs are chronic relapsing inflammatory disorders of the GI tract. Crohn's disease (CD) and ulcerative colitis (UC) are two most common forms of IBD. While CD can affect any part of the GI tract from mouth to anus, UC is more restricted to the distal parts. Clinically, patients suffer from ulceration, oedema and hemorrhage, while histological analysis demonstrates immune cell infiltration, goblet cell depletion, crypt abscesses as well as distortion of mucosal glands (Hendrickson et al., 2002, Abraham and Cho, 2009). Additionally, while UC is usually confined to the mucosa, in CD the inflammation may be transmural reaching all the way to the serosa. IBD is most commonly diagnosed in patients between the second and fourth decades of life, and it is estimated that 2.5-3 million people are affected in Europe, with the incidence numbers rising in the developing world (Burisch et al., 2013). IBDs represent complex diseases with a combination of genetic, environmental, microbial and immune factors likely playing a role in the pathogenesis. While the aetiology is still not fully understood, it is generally accepted that the chronic inflammation observed in IBD patients develops as

a result of deregulated immune response directed against the intestinal flora in genetically susceptible hosts (de Souza and Fiocchi, 2016).

Genome-wide association studies (GWAS) have identified over 200 genetic loci linked to IBD (Jostins et al., 2012, Liu et al., 2015). The majority of susceptibility loci identified by the GWAS influences the genetic susceptibility of both forms of IBD, thus, it is likely that many of the pathways underlying the pathogenesis of CD and UC are shared.

Inappropriate immune reactivity towards the intestinal contents and microbiota dysbiosis can be observed in IBD patients. Antibodies against microbial antigens, including *Escherichia coli* outer membrane porin C and *Saccharomyces cerevisiae*, could be detected in the serum of CD patients (Mow et al., 2004). Multiple studies have also reported that bacterial communities in both CD and UC patients were less heterogeneous and exhibited higher numbers of mucosa-associated bacteria compared to healthy controls (Chassaing and Darfeuille-Michaud, 2011). For example, reduction in Bacteroidetes and Firmicutes bacteria was reported in faecal samples from IBD patients (Frank et al., 2007, Mondot et al., 2011). Both phyla comprise bacterial species, such as *Bacteroides fragilis* or *Faecalibacterium prausnitzii*, that have been previously shown to have protective and anti-inflammatory effects (Mazmanian et al., 2005, Sokol et al., 2008).

Further support for the role of microbiota in the pathogenesis of IBD comes from animal studies. Most experimental models of colitis have an attenuated phenotype in GF animals (Strober et al., 2002), and some genetically susceptible mouse strains

require triggering microbiota for the induction of the disease, for instance, IL-10-deficient mice (Sellon et al., 1998). However, it is not clear whether microbiota dysbiosis is the cause or the result of the inflammatory responses in the intestine. Of note, studies in rodents showed that faecal contents of colitic mice could induce colitis upon transfer to healthy animals suggesting that unbalanced microbiota can contribute to the pathogenesis of IBD (Garrett et al., 2010, Elinav et al., 2011).

A defective epithelial barrier as well as bacterial sensing and handling are likely to act in concert with microbial triggers to cause IBD. Increased barrier permeability has been reported in CD patients (Bjarnason et al., 1983), and consistently, reduced levels of AMPs, including both α-defensins and β-defensins were detected in CD (Wehkamp et al., 2003, 2005). Additionally, mice lacking mucin 2 protein, which is the major component of the mucus layer, developed spontaneous colitis (Van der Sluis et al., 2006). Furthermore, GWAS have established a strong link between defective bacterial handling and IBD by identifying *NOD2*, *ATG16L1* and *IRGM* variants as strongly associated with IBD susceptibility (de Lange and Barrett, 2015). NOD2 is essential for bacterial recognition, and its impaired function could lead to defective bacterial clearance, and thus, pro-inflammatory responses. NOD2 also mediates induction of autophagy, which has a role in both bacterial clearance and modulation of immune responses (Shaw et al., 2011). Defective clearing of bacteria caused by the *NOD2*, *ATG16L1* and *IRGM* variants, which are all involved in autophagy, may be responsible for the perpetuation of chronic inflammation, linking microbial triggers to deregulated effector immune responses (de Souza and Fiocchi, 2016). Additionally, *NOD2* mutations may be a cause of reduced intestinal barrier defences since it is also

indirectly involved in AMP production (Wehkamp et al., 2005). Additionally, the endoplasmic reticulum (ER) stress pathway has been highlighted by GWAS, with the ER stress response *XBP1* variant associated with higher susceptibility to IBD (Kaser et al., 2008). Also, increased ER stress levels, which are also regulated by both microbial and pro-inflammatory stimuli, were detected in the epithelium of IBD patients. Thus, highlighting an additional factor in microbial-immune interactions that may be defective in patients with IBD (Kaser et al., 2010a).

The role of the immune system in the development of IBDs is indisputable. Multiple lines of support come both from genetic studies, which identified many genes involved in the immune response function (e.g. *IL23R*, *IL10*, *STAT3*), and experimental models of colitis, in which mutations in cytokines, cell surface receptors and transcription factors related to immunity (e.g. IL-10, CTLA-4, Foxp3) result in intestinal pathology. On one hand, exacerbated pro-inflammatory responses are an important feature of IBD. Specifically, the IL-23-Th17 axis appears to represent a major driver of pathology in IBD. Firstly, GWAS identified multiple polymorphisms in the *IL23R* locus associated with higher risks of both forms of IBD (Duerr et al., 2006), and subsequently, other variants in genes associated with Th17 type of responses were identified (*JAK2*, *STAT3*, *RORC*, *IL17A*, *IL21*) (de Lange and Barrett, 2015). Secondly, the critical role of IL-23 in driving intestinal pathology has been demonstrated in both innate and T cell-dependent models of colitis (Ahern et al., 2008). On the other hand, defects in negative regulatory pathways have been highlighted in the context of IBD development, too. For example, mutations in *IL10R* have been associated with severe early-onset IBD, suggesting that a breakdown of intestinal homeostasis due to

defective immunosuppressive mechanisms might underlie IBD pathogenesis (Glocker et al., 2009). Therefore, both clinical and experimental evidence suggest that not only aberrant effector responses but also reduced immunoregulatory responses contribute to the pathogenesis of IBD, highlighting the need for more targeted therapies.

IBDs are not curable, however, the discovery that an anti-TNF mAb could block the disease in CD patients improved the treatment options for IBD patients dramatically (Targan et al., 1997). While anti-TNF antibodies have been shown to be very effective at dampening immune responses, they are associated with numerous side effects, including increased risk to secondary infections. Additionally, approximately a third of IBD patients are refractory to anti-TNF therapy (Peyrin-Biroulet, 2010). Recent advances in the understanding of the inflammatory pathways involved in IBD pathogenesis resulted in many clinical trials performed to test the efficacy of more specific biological-based therapies (Chan and Ng, 2016). For instance, a mAb to the p40 subunit of interleukin-12 and interleukin-23 appears to be effective in CD patients with refractory disease for whom other treatments have failed (Mannon et al., 2004, Sandborn et al., 2012). As mentioned, although it is well established that Tregs are critical for the maintenance of intestinal homeostasis, their role in IBD has not been well defined. A treatment protocol employing Tregs could provide a method of inducing tolerance avoiding the side effects associated with immunosuppression of therapies targeting pro-inflammatory cytokines (Lord, 2015). However, while Tregs may represent an attractive axis for treatment, so far the difficulty to generate large numbers of stable Tregs as well as uncertainty about the sustained therapeutic benefit

of a Treg-based treatment have hampered progress in this area (Salas and Panes, 2015).

1.5 Murine models of IBD

Studies of various models of intestinal inflammation in rodents have substantially improved our knowledge of mechanisms regulating intestinal homeostasis, and its breakdown. While not a single model of intestinal inflammation can recapitulate all the clinical features of IBD, a wide range of murine models of colitis have been developed that resemble aspects of the pathology observed in IBD patients (reviewed in Kiesler et al. (2015)). They can be broadly divided into models, in which the intestinal inflammation is induced by chemicals disrupting the epithelial barrier (e.g. DSS), genetic defects leading to immune dysregulation (e.g. IL-10-deficiency, TCRα-deficiency, IL-7 overexpression), or infection, which perturbs the host-microbiota axis (e.g. *C. rodentium*). The experimental setting can be either acute or chronic, allowing one to preferentially dissect innate responses or a wider range of both innate and adaptive immune responses. In this thesis, a model of *Helicobacter hepaticus* infection with IL-10 blockade was used as a model of intestinal inflammation and is described in more detail below.

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

Helicobacter hepaticus is a microaerobic gram-negative spiral bacterium first isolated from the livers of A/JCr mice suffering from chronic hepatitis (Fox et al., 1994). It was also shown to colonise the caecum and colon of infected animals. While *H. hepaticus* can chronically colonise wild type mice, it induces chronic typhlitis, colitis, and rectal

prolapse only in immunodeficient strains (Ward et al., 1996, Foltz et al., 1998, Maloy et al., 2003). Infection with *H. hepaticus* also leads to typhlocolitis in IL-10-deficient mice (Berg et al., 1996, Burich et al., 2001), and this can be reproduced when IL-10R signalling is blocked by administration of a blocking anti-IL-10R antibody (Kullberg et al., 2006). The pathology is the most pronounced in the caecum and proximal colon – the primary sites of *H.hepaticus* colonisation. Histologically, extensive epithelial cell hyperplasia, goblet cell depletion and dense leukocyte infiltrates can be observed as well as in more severe cases crypt abscesses, submucosal immune cell infiltration and oedema. Administration of a blocking anti-IL-10R antibody alone to wild type mice does not induce intestinal inflammation (Kullberg et al., 2006), indicating that the endogenous microbiota is not sufficient to promote colitis even with deficiency in IL-10 signalling. These studies also showed that infection with *H. hepaticus* can induce both *H.hepaticus*-specific regulatory $CD4^+CD25^+$ and $CD4^+CD25^-$ IL-10-expressing cells, validating the critical role of IL-10 in the control of *H.hepaticus*-induced colitis (Kullberg et al., 2002).

H.hepaticus-driven intestinal inflammation in the presence of IL-10R blockade induces activation of a mixed Th1/Th17 response. T cells isolated from mLN and re-stimulated *in vitro* with a soluble *H. hepaticus* antigen produced elevated amounts of both IFN-γ and IL-17 (Kullberg et al., 2006). In support of a role for Th1 cells in disease pathogenesis, pathology was reduced in IFN-γ-deficient animals. Furthermore, this colitis model is IL-23-dependent (Kullberg et al., 2006), and IL-23 produced by $CD11c^+$ mononuclear phagocytes is essential to trigger the disease (Arnold et al., 2016).

A particular plus of this model of intestinal inflammation is that it allows analysis of the role of the immune system in the development of colitis in immuno-competent mice in a lymphocyte-replete setting. Indeed, identified drivers of inflammation reflect features of human IBD. It is particularly useful to study intestinal microbiota interactions as well as the induction of tolerogenic responses. Specific mechanisms may also be studied in isolation using transgenic mice or pharmacological inhibitors.

1.6 Interleukin 33

1.6.1 IL-33 a member of the IL-1 family of cytokines

IL-33 is a member of the IL-1 family of cytokines, which consists of 11 members. These include the originally discovered four cytokines IL-1α, IL-1β, the IL-1 antagonist IL-1Ra and IL-18, and newer members: the antagonist of IL-36α IL-36Ra, IL-36α, IL-36β and IL-36γ (encoded by three distinct genes), IL-37, IL-38 and IL-33 (Garlanda et al., 2013a). IL-1 family cytokines play a central role in the regulation of inflammatory responses induced by innate immune cells (Sims and Smith, 2010). However, they can also modulate the adaptive arm of the immune system by skewing $CD4^+$ T cell differentiation (Guo et al., 2009). It is likely that IL-1 family cytokines arose due to duplication of an ancestral gene (Sims and Smith, 2010) since almost all IL-1 family member-encoding genes cluster on human chromosome 2, with the exception of IL-18 and IL-33, whose genes can be found on chromosomes 11 and 9, respectively (Nicklin et al., 2002, Taylor et al., 2002). The structure of the proteins is also common: 12 β-strands arranged in a β-trefoil fold at the core, which is indispensable for their function (Gosavi et al., 2008). IL-1 family cytokines also share a signalling module consisting of

the adaptor myeloid differentiation primary response protein 88 (MyD88), the kinases IL-1R-associated kinases (IRAKs), and the TNF receptor-associated factor (TRAF) ubiquitin ligases that become activated upon binding to a TIR-domain-containing receptor (O'Neill and Bowie, 2007).

1.6.2 IL-33 as a nuclear factor

IL-33 was discovered in 2005 as the ligand for the orphan receptor ST2 (suppression of tumorigenicity 2), also known as IL-1RL1, T1 or IL-33R (Schmitz et al., 2005). While human IL-33 is 270 amino acids long, the murine equivalent is 266 amino acids long. The C-terminus region ($IL\text{-}33_{112\text{-}270}$) folds into the β-trefoil characteristic of IL-1 family cytokines and is indispensable for the function of IL-33 as a cytokine (Lingel et al., 2009, Liu et al., 2013). By contrast, the N-terminal domain ($IL\text{-}33_{1\text{-}65}$) is required and also sufficient for the nuclear localisation of IL-33 (Carriere et al., 2007). The full-length 30kDa IL-33 protein is associated with the chromatin in the nuclei *in vivo*, and was originally described as a nuclear protein enriched in high endothelial venules (HEVs), and therefore, named nuclear factor from HEV (NF-HEV) (Baekkevold et al., 2003). Structurally, IL-33 binds to the acidic pocket formed by the H2A-H2B dimer at the surface of nucleosomes, promoting nucleosome-nucleosome interactions, and therefore, regulating chromatin accessibility (Roussel et al., 2008). Functionally, the role of IL-33 as a nuclear factor is not clear. However, it has been suggested to act as a transcriptional repressor, and it could form a complex with a potent transcriptional repressor, the histone methyltransferase SUV39H1 (Carriere et al., 2007, Shao et al., 2014). Two research groups observed IL-33 interactions with NF-κB, either forming a complex with p65 to dampen pro-inflammatory responses or binding to the p65

promoter to modulate endothelial cell activation (Ali et al., 2011, Choi et al., 2012). Nevertheless, the functional relevance of these findings remains to be established *in vivo*. Although the function of IL-33 in gene regulation *in vivo* is enigmatic, its nuclear localisation is critical as demonstrated by experiments where the N-terminal domain was deleted resulting in constitutive release of IL-33. This led to ST2-dependent lethal inflammation (Bessa et al., 2014).

1.6.3 The release of IL-33

IL-33, in addition to its nuclear functions, has potent immune activity when released. Like the majority of other IL-1 family cytokines, IL-33 does not contain a signal peptide, and therefore, is not processed via the conventional ER-Golgi release route. IL-33 can be released either as a full-length form or cleaved, depending on the conditions (Fig. 1.3). It has been suggested to act as an alarmin, a molecule released upon cell damage to attract immune cells to the site of tissue injury (Cayrol and Girard, 2014). This function was also attributed to another IL-1 family cytokine, IL-1α, as well as high-mobility group box 1 protein (HMGB1) (Bertheloot and Latz, 2016). Originally it was proposed that IL-33 required processing by caspase 1 to generate the mature biologically active form (Schmitz et al., 2005), similar to IL-1β or IL-18 (Garlanda et al., 2013a). However, later it was established that full-length IL-33 has biological activity (Cayrol and Girard, 2009, Luthi et al., 2009, Talabot-Ayer et al., 2009). Furthermore, caspase 1 cleavage within the IL-1-like domain led to its inactivation, rather than activation (Cayrol and Girard, 2009). Further studies showed that IL-33 is cleaved by apoptosis-associated caspases, caspase 3 and 7, generating biologically inactive fragments (Cayrol and Girard, 2009, Luthi et al., 2009, Ali et al., 2011). These studies

confirm the role of IL-33 as an alarmin: upon cell damage or necrosis processing of IL-33 is not possible, and a full-length bioactive form of IL-33 is released; however, upon controlled cell death apoptotic caspases 3 and 7 inactivate IL-33, preventing its downstream pro-inflammatory effects. Consistent with this, the full-length bioactive IL-33 form is released upon cellular necrosis (Cayrol and Girard, 2009, Luthi et al., 2009) as well as upon damage after CNS injury by oligodendrocytes (Gadani et al., 2015). Mechanical stress could also trigger the release of biologically active IL-33 without associated cell death (Kakkar et al., 2012, Chen et al., 2015).

1.6.4 Regulation of IL-33 activity

The activity of IL-33 can also be increased, between 10- to 30-fold, in the presence of pro-inflammatory cellular infiltrates. For example, neutrophil-derived serine proteases cathepsin G and elastase as well as chymase and tryptase produced by mast cells can cleave IL-33, which yields mature forms with a truncated N-terminal domain (Lefrancais et al., 2012, 2014). Importantly, the cleaved forms were detected *in vivo* during allergic inflammation, and mast cell-cleaved IL-33 could enhance ILC2 activation *ex vivo* (Lefrancais et al., 2014). While the inflammatory milieu promotes IL-33 maturation, mechanisms limiting IL-33 bioactivity have also been described. In the extracellular environment, the activity of IL-33 was rapidly terminated due to a change in conformation as a result of oxidation of cysteines, which led to disulfide bond formation in the IL-1-like domain (Cohen et al., 2015). Such an inactivated form of IL-33 could be detected in the lungs of both asthmatic patients and in a murine model of allergy. A role in the inactivation of alarmins, including IL-33, by mast cell-derived chymase was also proposed (Roy et al., 2014).

The IL-1 family of cytokines also includes antagonists that provide an additional axis of negative regulation. While IL-1Ra inhibits binding of IL-1α and IL-1β to their receptor IL-1R, IL-18 binding protein (IL-18BP) prevents binding of IL-18 to IL-18R, soluble ST2 (sST2) has been shown to act as a decoy receptor for IL-33 (Garlanda et al., 2013b). Soluble and membrane-bound forms are produced by alternative promoter splicing and sST2 lacks the transmembrane and cytoplasmic domains (Gachter et al., 1996, Iwahana et al., 1999). IL-33-induced NF-κB pathway activation and downstream Th2 type cytokine production were indeed inhibited in the presence of sST2, demonstrating its antagonistic nature (Hayakawa et al., 2007). Overall, IL-33 bioactivity is tightly regulated: while the full-length form released upon necrosis or cell damage is bioactive, it can be further matured by pro-inflammatory mediators. However, the activity of IL-33 can also be rapidly terminated either upon oxidation or when bound by its decoy receptor.

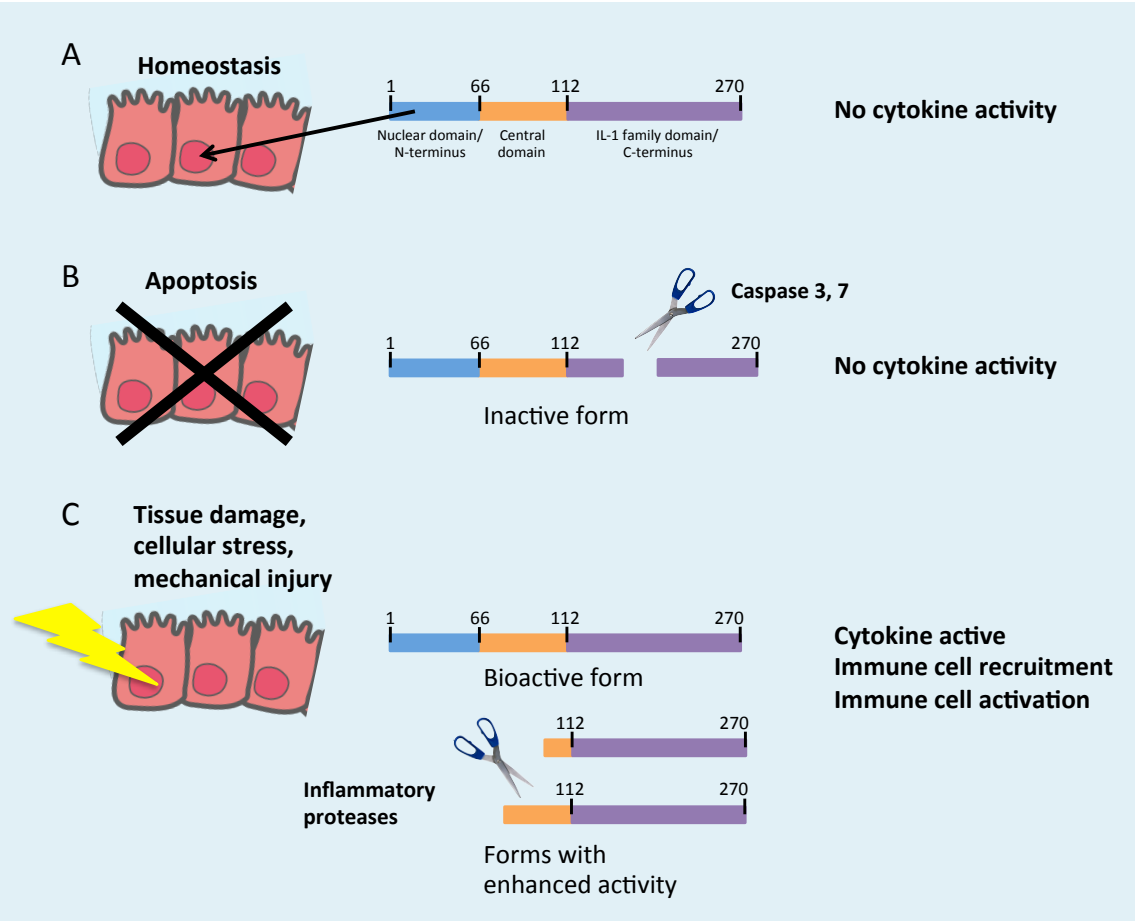


Figure 1.3. IL-33 processing.

IL-33 has three domains: N-terminus domain indispensable for its nuclear location, central domain and C-terminus IL-1-like family domain. Different forms of IL-33 have been identified. (A) Under homeostatic conditions, a full-length IL-33 resides in the nuclei of cells. (B) Upon programmed cell death, or apoptosis, IL-33 is cleaved by apoptosis-associated caspases 3 and 7 within the IL-1 family domain, and is released into extracellular space in an inactive form. This form has no cytokine activity and therefore does not elicit an immune response. (C) Upon tissue damage, cellular or mechanical stress, IL-33 acts as an alarm signal. A full-length IL-33 is released into extracellular space. This form can be further cleaved by inflammatory proteases to produce forms with 10-30 fold higher bioactivity. These forms when bound to ST2 mediate cytokine-specific activities of IL-33 such as immune cell recruitment, immune cell activation and others.

For specific references see sections 1.6.2-4.

1.6.5 IL-33 signalling

IL-33 binds to a heterodimeric receptor composed of high-affinity ST2 and low-affinity IL-1R accessory protein (IL-1RAcP) co-receptor (Lingel et al., 2009) (Fig. 1.4). IL-33 binding to ST2 induces a conformational change, which allows the recruitment of IL-1RAcP. This represents a shared mechanism among other IL-1 family members with the specific receptor displaying molecular flexibility, enabling it to form a heterodimer with the universal IL-1RAcP, which exhibits a rigid confirmation (Liu et al., 2013). The requirement for IL-1RAcP in IL-33 signalling was demonstrated in IL-1RAcP-deficient mice, which failed to respond to IL-33 administration (Chackerian et al., 2007). Upon binding, the intracellular TIR domains of ST2 and IL-1RAcP are brought together, and the adaptor protein MyD88, the kinases IRAK1 and IRAK4, and TRAF6 are subsequently recruited (reviewed in Martin (2013)). This, in turn, leads to activation of NF-κB and the mitogen-activated protein (MAP) kinases p38, JNK, and ERK1/2 (Schmitz et al., 2005).

Additionally, the receptor tyrosine kinase c-kit has been suggested to regulate IL-33 signalling in mast cells, suggesting cell-specific adaptations (Drube et al., 2010). C-kit was found to be associated with IL-1RAcP in mast cells, and its ligand stem cell factor was required for IL-33-induced cytokine production. The requirement for c-kit or other receptor tyrosine kinase has not been investigated in other cells. Furthermore, the IL-33-ST2-IL-1RAcP complex interactions can be negatively regulated by SIGIRR, which disrupts the dimerisation of ST2 and IL-1RAcP. SIGIRR was found to form a complex with ST2 *in vitro* and inhibited IL-33-mediated signalling (Bulek et al., 2009). In line with this finding, SIGIRR-deficient mice displayed more marked Th2 responses upon IL-33

administration compared to wild type mice. They also exhibited more enhanced Th2 responses in a model of asthma (Bulek et al., 2009). Ubiquitin ligases have also been implicated in the modulation of ST2, and thus, IL-33 signalling. FBXL19, an E3 ubiquitin ligase, could bind to ST2, poly-ubiquinate it and target it for proteasomal degradation (Zhao et al., 2012).

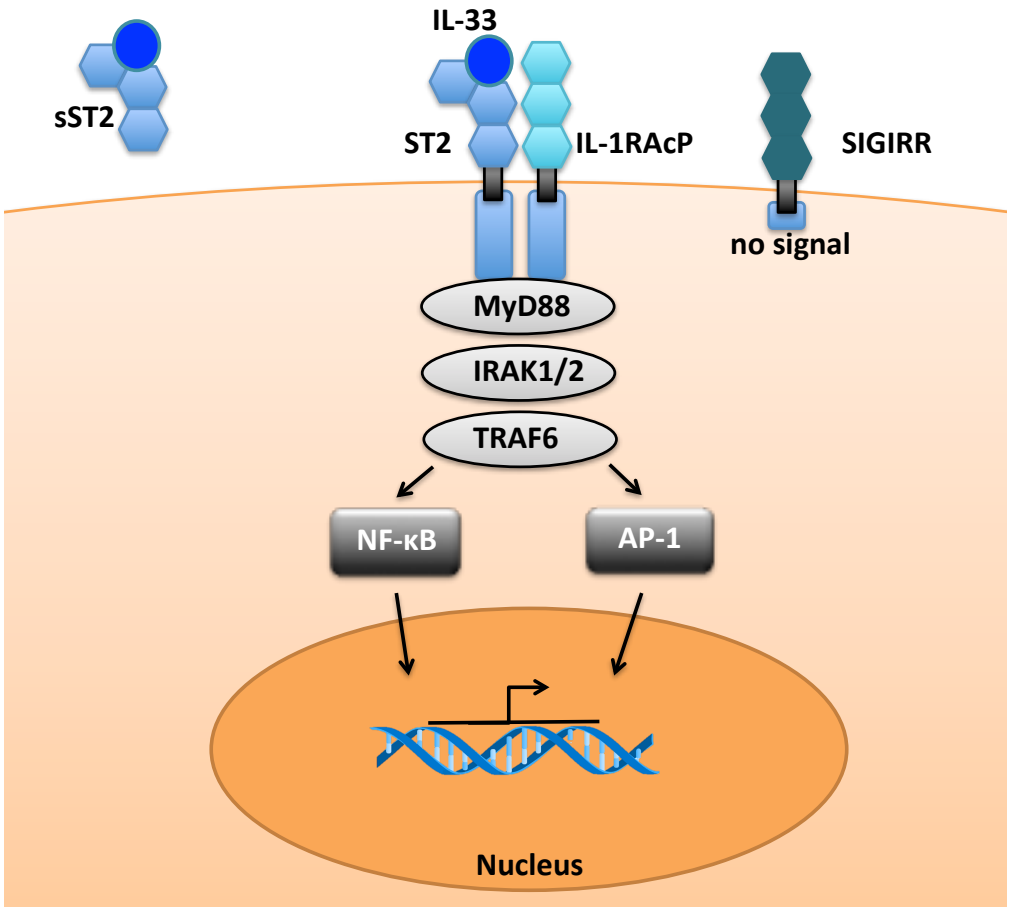


Figure 1.4. IL-33 signalling.

IL-33 binds to ST2 leading to IL-1RAcP recruitment. A signalling cascade is initiated that involves MyD88, IRAKs and TRAF6 resulting in the activation of NF-κB and AP-1 transcription factors, which translocate to the nucleus and promote transcription of genes encoding effector mediators, such as *Il5* and *Il13*. IL-33 can be negatively regulated by the decoy receptor sST2 and SIGIRR, which inhibit downstream signalling when associated with IL-1RAcP.

IL-1RAcP – IL-1 receptor accessory protein, IRAK - Interleukin-1 receptor-associated kinase, TRAF6 – tumour necrosis factor receptor-associated factor 6, NF-κB – nuclear factor κB, AP-1 – activator protein 1, sST2 – soluble ST2, SIGIRR - single Ig IL-1-related receptor.

1.6.6 IL-33 in disease

Like other IL-1 family cytokines, IL-33 is a potent amplifier of both innate and adaptive immune responses. Originally it was described as an inducer of type 2 immune responses (Schmitz et al., 2005). IL-33 stimulation selectively induced IL-5 and IL-13 production in Th2-polarised cells *in vitro*. Splenomegaly characterised by an increase in mononuclear cells and preferentially eosinophils as well as enhanced antibody production was observed upon IL-33 administration *in vivo.* It also resulted in increased mucus production at mucosal surfaces and the cellular infiltrate was evident in several organs (Schmitz et al., 2005). This is further supported by studies in a knock-in transgenic mouse, in which bioactive IL-33 is constitutively released due to a deletion of the N-terminal nuclear domain (Bessa et al., 2014). These mice succumbed to fatal multiorgan failure mediated by highly pro-inflammatory innate immune cell infiltration. Consistent with these findings, ST2 is expressed on a variety of immune cells involved in mediating type 2 immune responses: Th2 cells (Lohning et al., 1998, Xu et al., 1998), mast cells (Allakhverdi et al., 2007, Morita et al., 2015), basophils (Smithgall et al., 2008, Suzukawa et al., 2008), eosinophils (Cherry et al., 2008, Pecaric-Petkovic et al., 2009) and ILC2s (Monticelli et al., 2011, Salimi et al., 2013, Monticelli et al., 2015). However, since the original report describing IL-33 and its function, it has become apparent that IL-33 is involved in a wider range of immune responses. For example, the role of IL-33 in defence mechanisms against intracellular pathogens has been indicated. In line with this, ST2 is also expressed on Th1 cells (Baumann et al., 2015), $CD8^+$ T cells (Yang et al., 2011, Bonilla et al., 2012, Gao et al., 2015), NK T cells and NK cells (Smithgall et al., 2008, Bourgeois et al., 2009, 2011, Gao et al., 2015).

APCs, including both macrophages and DCs (Turnquist et al., 2008, 2010, Rank et al., 2009, Matta et al., 2014), can also upregulate ST2 on their surface.

Notably, IL-33- and ST2-deficient animals do not develop any obvious abnormalities under steady state conditions. Rather, the role of IL-33 becomes evident when animals are challenged with infectious or inflammatory stimuli (Hoshino et al., 1999, Senn et al., 2000, Townsend et al., 2000, Oboki et al., 2010, Louten et al., 2011, Pichery et al., 2012). Below, the role of IL-33 in a range of diseases is briefly described.

IL-33 and infections

Since type 2 immune responses are central in mediating extracellular intestinal worm expulsion, it is not surprising that the first report of the role of IL-33 in host defence was in a nematode infection (Humphreys et al., 2008). Following *Trichuris muris* infection, resistant BALB/c mice displayed increased amounts of *Il33* mRNA. Additionally, administration of IL-33 improved resistance to the worm in a susceptible strain of AKR mice, resulting in complete expulsion of the worms. This effect was likely driven by type 2 immune responses as demonstrated by elevated levels of IL-4, IL-13 and IL-9, and decreased amounts of IFN-γ, TNF-α and IL-17 (Humphreys et al., 2008). IL-33 is readily produced in response to helminth infection. It induces goblet cell proliferation and mucin production, which are critical for worm expulsion (Maizels et al., 2009). Specifically IL-33 has been shown to act together with IL-25 on ILC2s in the early phase of *Nippostrongylus brasiliensis* infection, and mediated its clearance by IL-33/IL-25-induced production of IL-13 by ILC2s (Moro et al., 2010, Neill et al., 2010, Price et al., 2010, Hung et al., 2013). Infection with intestinal nematodes also results in

the manifestation of the pathology in the lung, which is characterised by pronounced pulmonary eosinophilia (Maizels et al., 2009). Yasuda et al. demonstrated that *Strongyloides venezuelensis* infection led to the production of IL-33 by alveolar epithelial cells, which induced IL-5 and IL-13 (Yasuda et al., 2012). This resulted in the eosinophil recruitment and mucus production in the lung, which was reduced in IL-33-deficient mice. Similar to other studies, ILC2s appeared to be the target cell type of IL-33-mediated effects since lymphocyte-deficient mice displayed a similar phenotype to wild type controls (Yasuda et al., 2012). Interestingly, it has been suggested that worms could directly induce epithelial cell necrosis leading to the release of bioactive IL-33 (Andronicos et al., 2012). However, this mechanism remains to be confirmed *in vivo*.

IL-33 is also involved in anti-viral responses. $CD8^+$ T cell responses are key drivers of anti-viral immunity, and IL-33 has been shown to directly promote $CD8^+$ T cells with potent cytotoxic activity (CTLs) (Bonilla et al., 2012). Upon lymphocytic choriomeningitis virus (LCMV) infection, IL-33 was upregulated in the spleen. Enhanced clonal expansion and effector function of CTLs, which were required for the control of the virus, were also observed. ST2-deficient mice mounted reduced anti-LCMV responses, while administration of exogenous IL-33 could boost the anti-viral defence (Bonilla et al., 2012). IL-33 also showed enhancing effects on highly activated effector Th1 cells during LCMV infection (Baumann et al., 2015). Similarly, treatment with IL-33 resulted in a reduced viral load and associated pathology upon infection with Coxsackievirus B (Sesti-Costa et al., 2013). In that study, the authors could show two distinct functions of IL-33: firstly, IL-33 bound to ST2 expressed on $CD8^+$ T cells and NK

cells increasing IFN-γ and TNF-α production that directly reduced viral replication; secondly, IL-33 induced IL-4 production by mast cells, which promoted alternative type M2 macrophages and Tregs that promoted resolution of inflammation in the tissue (Sesti-Costa et al., 2013). Furthermore, IL-33-dependent production of IL-6 and TNF-α by mast cells was also critical to protect mice from lethality upon cutaneous herpes simplex virus 2 infection (Aoki et al., 2013). By contrast, IL-33 contributed to disease exacerbation in respiratory syncytial virus infection, which could be ameliorated by administration of an anti-ST2 mAb leading to a reduced Th2-driven lung eosinophil infiltration (Walzl et al., 2001). Influenza A virus infection also led to induction of IL-33 in alveolar macrophages. This resulted, via ILC2-produced IL-13, in mucus secretion and muscle contraction, which in turn induced airway hyper-reactivity (AHR) (Chang et al., 2011). AHR represents a hallmark of asthma, and consistently, exacerbation of asthma upon rhinovirus infection was dependent on IL-33 (Jackson et al., 2014). While IL-33 may have deleterious effects on anti-viral responses in certain experimental models of virally-induced asthma, it was also important for ILC2-driven repair and improvement of lung function following influenza infection (Monticelli et al., 2011). It is, therefore, likely that the role of IL-33 in host defence against viruses is not only virus-specific but also depends on the phase of the infection.

Although less studied, the role of IL-33 in bacterial and fungal infections has also been reported (Hazlett et al., 2010, Nelson et al., 2011, Flaczyk et al., 2013, Piehler et al., 2013). For example, increased amounts of IL-33 were found in the peritoneal lavage fluid of septic mice and exogenous IL-33 administration boosted bacterial clearance through increased neutrophil recruitment (Alves-Filho et al., 2010).

IL-33 and inflammatory diseases

While IL-33 can promote potent immune responses against a wide range of pathogens, its role has also been emphasized in the development of inflammatory diseases, in particular respiratory and allergic diseases.

Respiratory and allergic diseases

Evidence of the functional relevance of IL-33 in the development of asthma comes from GWAS. Polymorphisms in both *IL33* and *IL1RL1*, which encodes ST2, were reproducibly identified as susceptibility loci for asthma (Moffatt et al., 2010, Grotenboer et al., 2013). Consistently, increased amounts of IL-33 were detected in the bronchoalveolar lavage (BAL) fluid of asthmatic patients, and correlated with disease severity (Prefontaine et al., 2009). Murine models of asthma have provided additional support for the role of IL-33 in disease pathogenesis. The authors of the original study on IL-33 reported that IL-33 administration induced epithelial cell hypertrophy in the lung, increased mucus production as well as eosinophilic infiltration, consistent with the fact that IL-33 is a potent inducer of Th2-associated cytokines (Schmitz et al., 2005). A majority of studies, in which the IL-33/ST2 axis was manipulated, demonstrated a pathological role for IL-33 in models of asthma. These studies highlighted eosinophils, ILC2s, M2 macrophages and Th2 cells as the primary targets of IL-33 signalling (Coyle et al., 1999, Kondo et al., 2008, Kurowska-Stolarska et al., 2008, 2009, Liu et al., 2009). However, reports that do not support the pathological role of IL-33 in the lung also exist (Hoshino et al., 1999, Townsend et al., 2000). These may reflect redundancy between IL-33 and other type 2 response inducers, such as IL-25 and TSLP. IL-33 appeared to be critical in promoting AHR, whereas other key

features of asthma, such as elevated amounts of type 2 cytokines or eosinophilic infiltration, could be rescued in the presence of IL-25, which together with IL-33 is essential for ILC2 development (Barlow et al., 2013). Interestingly, blockade of IL-33 signalling significantly improved the resolution of AHR following allergen-induced inflammation (Kearley et al., 2009). Furthermore, IL-33 has been shown to specifically promote airway remodelling in severe steroid-resistant asthma (Saglani et al., 2013). The importance of IL-33 in driving asthma pathogenesis is illustrated by current developments of an anti-ST2 mAb in the clinical setting for patients who have asthma (Janssen Research & Development, 2014).

The ability of IL-33 to exacerbate allergic inflammation is also evident in atopic dermatitis (AD) and rhinitis. Mice, which display enhanced IL-33 expression selectively in keratinocytes, developed spontaneous dermatitis (Imai et al., 2013). Activation of ILC2s, eosinophils and mast cells, which promoted the skin pathology, was observed in both this spontaneous model of AD and upon allergen exposure in murine skin (Imai et al., 2013, Salimi et al., 2013). IL-33 produced by the nasal epithelium was also central in driving nasal inflammation in an allergic model of rhinitis through promoting inflammatory mediator production by mast cells and basophils (Haenuki et al., 2012).

Cardiovascular diseases

A role for IL-33 in cardiovascular diseases is also worth highlighting since elevated serum levels of sST2 in acute myocardial infarction patients serve as a marker of poor prognosis in clinics (Shimpo et al., 2004). The cardio-protective role of IL-33 has been demonstrated in a mouse model of pressure overload-induced cardiac hypertrophy

(Sanada et al., 2007). Administration of IL-33 had both anti-hypertrophic and anti-fibrotic effects that led to improved animal survival. The anti-hypertrophic effects of IL-33 could be inhibited with sST2 *in vitro (Sanada et al., 2007)*. Another study recently found that IL-33 released by endothelial cells upon myocardial pressure overload promoted a beneficial systemic inflammatory response (Chen et al., 2015). Furthermore, atherosclerosis, which can often lead to myocardial infarction, has also been suggested to be modulated by IL-33 (Miller et al., 2008). IL-33 treatment significantly reduced atherosclerotic plaque development in susceptible ApoE-deficient mice on a high-fat diet. This was accompanied by a switch from a Th1 to Th2 type response. Additionally, IL-33 administration induced protective anti-oxidized low-density lipoprotein antibodies (Miller et al., 2008). Finally, another group showed that macrophage foam cell formation, which is the main feature of atherosclerotic plaques, was reduced in response to IL-33 treatment in the same ApoE model of murine atherosclerosis (McLaren et al., 2010). Altogether, results to date strongly indicate that IL-33 has a cardio-protective role.

IL-33 and other inflammatory diseases

As described, IL-33 can drive both pathological and protective responses depending on the disease and experimental setting. Studies to date argue for a positive role of IL-33 in the modulation of rheumatoid arthritis. Blocking IL-33 signalling with an sST2-Fc fusion protein or anti-ST2 antibody significantly attenuated disease pathology in a commonly used model of collagen-induced arthritis (Leung et al., 2004, Palmer et al., 2009). This was associated with a decrease in Th1-associated cytokines, reduced mast cell degranulation and decreased levels of anti-collagen antibodies, suggesting that IL-

33 may promote joint destruction in multiple different ways (Leung et al., 2004, Palmer et al., 2009, Xu et al., 2008).

The recent appreciation that IL-33 promoted a specific population of ST2-expressing Tregs in the VAT highlighted its potentially significant role in metabolic diseases, such as type 2 diabetes (Cipolletta et al., 2012, 2015). ST2-deficient mice fed a high-fat diet had reduced metabolic indices related to glucose metabolism (Miller et al., 2010). Consistently, administration of exogenous IL-33 to the genetically obese *ob*/*ob* strain of mice improved metabolic parameters, which resulted in reduced adiposity. This was associated with an increase in Th2 cells in the adipose tissue and a shift towards an M2 macrophage profile (Miller et al., 2010). IL-33 has also been found to increase caloric expenditure by promoting beiging of white adipose tissue mediated by ILC2s (Brestoff et al., 2015).

IL-33 and IBDs

Increased amounts of IL-33 and ST2 have also been reported in mucosal samples from both UC and CD patients, particularly in patients with active disease (Beltran et al., 2010, Kobori et al., 2010, Pastorelli et al., 2010, Seidelin et al., 2010, Sedhom et al., 2013, Waddell et al., 2015). A number of studies investigated the role of IL-33 in the development of intestinal inflammation in models of experimental colitis. This will be further discussed, and the role of IL-33 in the maintenance of intestinal homeostasis will be explored in Chapter 4 of this thesis.

IL-33 and repair

Early analysis of IL-33-deficient mice suggested a role for IL-33 in wound healing (Oboki et al., 2010). In line with this, recently it has been reported that IL-33 induced ILC2-mediated response was critical for wound closure upon cutaneous injury (Rak et al., 2016). Exogenous administration of IL-33 enhanced wound healing, while this process was significantly slowed down in IL-33-deficient mice, which could be explained by the observed decrease in the ILC2s. ILC2s have also been shown to promote repair in response to IL-33 in the context of influenza infection as well as intestinal inflammation (Monticelli et al., 2011, 2015). Amphiregulin, an EGF-like growth factor, produced by ILC2s upon IL-33 exposure, was central to the repair process. IL-33 can also contribute to the restoration of tissue homeostasis by promoting Tregs. Arpaia et al. suggested that Tregs were the key producers of amphiregulin upon influenza infection and indispensable for improving lung function and repair (Arpaia et al., 2015). Mice with a selective deletion of amphiregulin in $Foxp3^+$ T cells suffered from severe acute lung damage. Notably, amphiregulin-producing Tregs could be induced independently of TCR triggering by stimulation with two IL-1 family cytokine members, IL-18 and IL-33 (Arpaia et al., 2015). Furthermore, Tregs found in the muscle promoted tissue repair upon acute and chronic muscle injury, and this process was dependent on IL-33 (Burzyn et al., 2013, Villalta et al., 2014, Kuswanto et al., 2016). Finally, ST2-expressing Tregs can downregulate type 1 allo-immune responses that destroy tissues in the context of GVHD (Matta et al., 2016). Depletion of these Tregs led to increased lethality in a mouse model of acute GVHD.

While Tregs and ILC2s promoted by IL-33 at the site of tissue injury can mediate both resolution of inflammation and subsequent repair of the tissue, on-going stimulation can also be associated with development of fibrotic changes. Indeed, IL-33 has been linked to fibrosis in lung and liver (McHedlidze et al., 2013, Li et al., 2014a). Lung fibrosis induced by bleomycin was associated with the IL-33-induced expansion of ILC2s and M2 macrophages, which produced IL-13 and TGF-β1, which contributed to the fibrotic changes (Li et al., 2014a). IL-33 expression was also required for hepatic fibrosis development, which similarly promoted IL-13 production by ILC2s resulting in tissue remodelling and fibrosis (McHedlidze et al., 2013).

The role of IL-33 has also been studied in several other settings (reviewed in (Liew et al., 2010, 2016, Lopetuso et al., 2013). These include cancer, with multiple studies reporting increased amounts of IL-33 in tumour tissue (Mager et al., 2015, Maywald et al., 2015, Yang et al., 2016b); organ transplantation, where IL-33 was for the first time shown to promote Tregs and, thus, allograft survival (Turnquist et al., 2011); or injuries of the central nervous system, where IL-33 is likely to have a functional relevance since the brain has the highest amount of IL-33 among the investigated organs (Schmitz et al., 2005).

As discussed, IL-33 can exhibit diverse functions and modulate responses of a range of immune cells. Its roles vary depending on the investigated disease and the experimental setting. On one hand, in homeostasis, IL-33 is likely to exhibit a regulatory role as a nuclear factor. On the other hand, its release can be triggered by inflammatory insults or invading pathogens, and the resulting responses promote host

defence. IL-33 also contributes to exacerbations of allergic and asthmatic responses while exhibiting a protective role in most experimental models of arthritis, type 2 diabetes or cardiovascular diseases. IL-33 is also central in promoting tissue repair. While studies in the last decade contributed a wealth of information on the biology of IL-33, there are still many conflicting results, and the function of IL-33 remains unresolved in multiple disease settings. Although it is well established that IL-33-induced type 2 responses are critical for worm expulsion in the intestine, the mechanisms by which IL-33 promotes intestinal homeostasis and its role in the development of colitis are still unknown.

1.7 Aims of the thesis

It is well established that Tregs have a non-redundant function in mediating tolerance, and their presence is particularly important at mucosal surfaces, such as the intestine. Recent studies emphasised the important role of Tregs in non-lymphoid tissues and reported expression of ST2 on these Treg cell populations. However, whether IL-33 may modulate Tregs, and promote intestinal homeostasis via a Treg-mediated mechanism remains unknown. Furthermore, while studies of IL-33 in the context of inflammatory responses in the intestine have provided some evidence that IL-33 may be functionally relevant, its specific role remains to be elucidated. Complex regulation of IL-33 activity has also been described, thus, identifying modulators of IL-33 activity and its cellular source in the gut may provide a better understanding of the functions that IL-33 mediates in the gut. However, studies to date have not investigated this question in a comprehensive manner.

The aims of this thesis are:

1) To characterise Treg populations in the colon (discussed in Chapter 3)
2) To evaluate the role of IL-33 in the control of immune responses (discussed in Chapter 4)
3) To determine the expression of IL-33 in the murine colon (discussed in Chapter 5).

Chapter 2. Materials and Methods

2.1 Mice

Wild type C57BL/6, B6.Rag1$^{-/-}$, B6.SJL-Cd45.1, B6.Foxp3^{GFP}, B6.ST2$^{-/-}$, B6.IL18R1$^{-/-}$, B6.Foxp3^{hCD2}, B6.IL-10^{GFP}, B6.IL10$^{-/-}$, B6.IL10R$^{-/-}$, B6.MyD88$^{-/-}$, B6.IL-33$^{+/Cit}$, B6.IL-33$^{Cit/Cit}$, B6.PDGFRα^{eGFP} were bred and maintained under SPF conditions in accredited animal facilities at the University of Oxford, UK. B6.ST2$^{-/-}$ mice were obtained from P. Fallon (Trinity College Dublin, Dublin, Ireland), BALB/c IL-33$^{+/Cit}$ from A. McKenzie (University of Cambridge, Cambridge, UK), B6.IL18R1$^{-/-}$ from K. Maloy (University of Oxford, Oxford, UK), B6.IL10R$^{-/-}$ from R. Cornell (University of Oxford, Oxford, UK), B6.PDGFRα^{eGFP} from F. Watt (King's College, London, UK). BALB/c IL-33$^{+/Cit}$ were backcrossed to C57BL/6 background until the progeny was at least 99.9% C57BL/6. This was assessed by single-nucleotide polymorphism (SNP)-based genotyping by Charles River Laboratories, UK. B6.Foxp3^{hCD2} and B6.IL-10^{GFP} were crossed in-house to generate a B6.Foxp3^{hCD2}IL-10^{GFP} double-reporter mouse line.

Both females and males were used in the experiments. Mice were age- and sex-matched, and were at least 6 weeks old when used unless stated otherwise. 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* experiments

2.2.1 Recombinant IL-33 injections

Foxp3^{GFP} reporter mice were injected intraperitoneally (i.p.) daily with 1µg carrier-free recombinant IL-33 protein (Biolegend) for three consecutive days. Mice were sacrificed a day after the last injection and the spleens of the animals were harvested for flow cytometric analysis. Control mice were injected with Phosphate-Buffered Saline (PBS, Sigma-Aldrich).

2.2.2 *Helicobacter hepaticus*/anti-IL-10R induction of colitis

H. hepaticus NCI-Frederick isolate 1A (strain 51449) was grown on blood agar plates made with laked horse blood supplemented with trimethoprim (5µg/ml), vancomycin (10µg/ml) and polymyxin B (25U/ml) (TVP, all from Oxoid) under microaerophilic conditions (3-5% O_2, 10% CO_2, 10% H_2, balance N_2) in a CampyPak jar (Oxoid) at 37°. After 24 hours bacteria were transferred from the agar plates into liquid culture of Tryptic Soy Broth (TSB, Sigma-Aldrich) supplemented with 10% Fetal Calf serum (FCS, Sigma-Aldrich) and TVP antibiotics in vented Erlenmeyer flasks (Corning) and continued to grow under microaerophilic conditions at 37° in a shaking incubator at 200rpm. After 24 hours optical density (OD) of the culture was read at 600nm using a BioPhotometer (Eppendorf). Bacteria were then centrifuged at 3000rpm for 20 minutes. The viability of bacteria was assessed using a fluorescent Live/Dead assay (Molecular Probes) under a fluorescent microscope Olympus CKX41.

To induce colitis mice were infected with 1x10^8 colony-forming units (cfu) (equal to 1OD) *H. hepaticus* by oral gavage with a 22G curved needle on day 0 and day 1. Mice

also received 1mg of anti-IL-10R blocking antibody (clone 1B1.2, produced in-house) via an i.p. injection weekly from the start *of H. hepaticus* infection. Mice were sacrificed at the peak of inflammation, on day 14, unless stated otherwise.

2.2.3 Generation of bone marrow chimaeras

To determine the contribution of haematopoietic derived cells, bone marrow chimaeras were used. Firstly, $Rag1^{-/-}$ animals were irradiated twice with 550 RAD from a ^{60}Co source with a break of 8 hours. Then, the femurs and tibias of B6.SJL-Cd45.1 and $ST2^{-/-}$ mice were harvested, and flushed with PBS/0.1% Bovine Serum Albumin (BSA, Sigma-Aldrich) to recover the bone marrow cells. Red blood cells were removed by incubation with ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2-7.4, all from Sigma-Aldrich) for 3 minutes at room temperature followed by a wash with PBS/0.1% BSA. Cells were filtered through a 70µm strainer (Fisher Thermo Scientific) and resuspended in sterile PBS. $10x10^6$ B6.SJL-Cd45.1 or $10x10^6$ $ST2^{-/-}$ or $5x10^6$ B6.SJL-Cd45.1 together with $5x10^6$ $ST2^{-/-}$ cells were injected intravenously (i.v.) into irradiated $Rag1^{-/-}$ recipients. At 3-4 weeks post-irradiation mice were tested for reconstitution by sampling the blood from the tail vein (for isolation of leukocytes from the blood see section 2.3.2) and flow cytometric analysis (for flow cytometry procedure see section 2.4). Mice were used 10 weeks after the irradiation for further experiments.

2.2.4 Histological assessment

To determine the extent of the colonic disease, colons were cleaned in PBS and 1cm pieces were cut from the proximal, middle and distal colon. These 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, cleaned, placed on a blotting paper (Invitrogen) and fixed as above. Paraffin-embedded sections were cut into 4µm sections and stained with haematoxylin and eosin. Inflammation was scored in a blinded fashion by two researchers according to the scoring system outlined below (Fig. 2.1). The average of proximal, middle and distal colon scores was calculated to obtain the colitis score, while the caeca were scored separately. The images of the sections were acquired on an Olympus BX51 microscope.

A.	**EPITHELIUM**	**HYPERPLASIA and/or**	**GOBLET CELL DEPLETION**
	0	**None**	**None**
	1	**Mild (1.5x)**	**Mild (25%)**
	2	**Moderate (2-3x)**	**Marked (25-50%)**
	3	**Severe (>3x)**	**Substantial (>50%)**
B.	**INFLAMMATION IN LAMINA PROPRIA**		
	0	**None - few leucocytes**	
	1	**Mild - some increase in leucos at tips of crypts OR many lymphoid follicles**	
	2	**Moderate - marked infiltrate (notable broadening of crypt)**	
	3	**Severe - dense infiltrate throughout**	
C.	**AREA AFFECTED**	**(% of section)**	
	0	**None**	
	1	**up to 25%**	
	2	**25-50%**	
	3	**>50%**	
D.	**MARKERS OF SEVERE INFLAMMATION**		
	0	**None**	
	1	**Submucosal inflammation OR Few crypt abscesses (<5)**	
	2	**Submucosal inflammation AND Few Crypt Absecesses (<5)**	
	2	**Many crypt abscesses (<5) OR Extensive submucosal inflamm OR Crypt branching (*Hh)***	
	3	**Many crypt abscesses (<5) AND Extensive submucosal inflamm / Crypt branching (*Hh)***	
	3	**Ulceration OR Extensive fibrosis**	

Figure 2.1. The assessment criteria for the histological scoring of the colonic and caecal sections.

2.2.5 Endoscopic assessment

To track disease progression high resolution mouse endoscopy was performed as described in Becker et al. (2006). Briefly, mice were anaesthetised with 4% isofluorane gas mixture. The endoscope (HOPKINS Optik 64019BA, Karl Storz Aida Vet) was slowly inserted via the rectum and the colon was insufflated with air to facilitate entry of the endoscope. The endoscope was inserted along approximately 4 cm length, which corresponds to the region of the colon up to the first flexure. A high-resolution video was recorded during the procedure. Following endoscopic assessment mice were allowed to recover from anaesthesia and were imaged every two weeks for the duration of the experiment.

The assessment of the colitis severity was performed according to the criteria outlined in Table 2.1. Two independent researchers scored the recorded images in a blinded fashion.

		0	1	2	3
A	**Thickening of the colon**	Transparent	Moderate	Marked	Non-transparent
B	**Changes of the vascular pattern**	Normal	Moderate	Marked	Bleeding
C	**Fibrin visible**	None	Little	Marked	Extreme
D	**Granularity of the mucosal surface**	None	Moderate	Marked	Extreme
E	**Stool consistency**	Normal + solid	Still shaped	Unshaped	Spread

Table 2.1. The murine endoscopic index of colitis severity (MEICS).

2.3 Cell isolation

2.3.1 Isolation of leukocytes from the spleen and lymph nodes

Spleens and peripheral lymph nodes including superficial cervical, axillary, brachial, inguinal lymph nodes and mesenteric lymph nodes were harvested following the sacrifice of the mice. Organs were mashed through a 70µm strainer and washed with PBS/0.1% BSA/5mM EDTA (Sigma-Aldrich) and centrifuged at 1500rpm for 5 minutes. Splenic cells were additionally incubated with 500µl ACK lysis buffer for 3 minutes at room temperature to remove the red blood cells, followed by a wash with PBS/0.1% BSA/5mM EDTA. Single cell suspensions were resuspended in an appropriate volume of PBS/0.1% BSA/5mM EDTA for the subsequent analysis.

2.3.2 Isolation of leukocytes from the blood

Blood was collected via cardiac puncture into a tube containing 100µl 0.5M EDTA. Red blood cells were lysed by incubation with 300µl ACK lysis buffer for 3 minutes at room temperature. Cells were washed with PBS/0.1% BSA/5mM EDTA and resuspended in the same buffer for the subsequent analysis.

2.3.3 Isolation of leukocytes from the colon, caecum and small intestine

Colons, caeca and small intestines of the mice were removed from the animals following the sacrifice. Colons were cut open longitudinally, cleaned of the remaining faeces and cut into 1cm-long pieces. Caeca were cut open and cleaned before being cut into small pieces. Small intestines were cleaned of faeces, the Peyer's patches were cut out and disposed of, and excessive mucous was removed by extensive washing with PBS/0.1% BSA. The tissue was washed twice for 20-25 minutes in RPMI (Sigma-

Aldrich)/10% FCS/5mM EDTA at 37°C in a shaking incubator at 200rpm to remove the epithelial cells, followed by a short wash in RPMI/10% FCS/15mM Hepes (Sigma-Aldrich) to neutralise the activity of the EDTA. In the indicated experiments the epithelial cells were retained for analysis after an additional wash with PBS/0.1% BSA/5mM EDTA. The tissue was digested for an hour in the presence of 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 strainer, and the digestion was terminated by addition of ice-cold RPMI/10% FCS/5mM EDTA followed by centrifugation at 1500rpm for 5 minutes. A three-layer (30/40/70%) Percoll (GE Healthcare) density-gradient was used to enrich for the lymphocytes. Cells obtained from the digestion were layered in the 30% layer on top of the 40% and 70% layers, and centrifuged for 20 minutes at 1800rpm without application of the brake. The 40/70% Percoll interface was isolated and washed with PBS/0.1% BSA/5mM EDTA. Single cell suspensions were resuspended in a required volume of PBS/0.1% BSA/5mM EDTA for the subsequent analysis. Additionally, the 30/40% Percoll interface was used to recover stromal cells in the indicated experiments.

2.3.4 Isolation of leukocytes from the lung

All the lobes of lung were harvested and cut into small pieces. The 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 terminated by addition of ice-cold RPMI/10% FCS/5mM EDTA. The cells were centrifuged at 1500rpm for 5 minutes and single cell suspensions were

resuspended in an appropriate volume of PBS/0.1% BSA/5mM EDTA for the subsequent analysis.

2.3.5 Isolation of leukocytes from the skin

Both ears were harvested and cut into very small pieces. Tissue was digested in RPMI/0.1% BSA with 3mg/ml collagenase D (Roche) and 400µg/ml Liberase TL (Roche) for 80 minutes at 37°C in a shaking incubator at 200rpm. Digested tissue was passed through a needle to further disrupt the tissue and release cells. Cells were passed through a 70µm cell strainer, and the digestion was terminated by addition of ice-cold RPMI/10% FCS/5mM EDTA. Cells were centrifuged at 1500rpm for 5 minutes before being separated on a Percoll density-gradient as described in section 2.3.3. Cells from the 40/70% Percoll interface layer were recovered, washed, and resuspended in a required volume of PBS/0.1% BSA/5mM EDTA for the 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), 100 U/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 and cells were incubated for 3.5-4 hours at 37°C before further processing for flow cytometric analysis.

2.4.2 Surface staining

All staining and washing procedures were performed in PBS/0.1% BSA/5mM EDTA. All centrifugations were performed at 1500rpm for 5 minutes. For myeloid cell staining an initial 10-minute incubation with anti-CD16/CD32 (eBioscience) was performed to minimise any non-specific antibody binding. Appropriate antibody mixes were added together with the Fixable Viability Dye eFluor® 780 (eBioscience) and incubated for 20 minutes at 4°C in the dark followed by two washes.

When the anti-ST2 antibody was used in the primary antibody mix, cells were stained with streptavidin for additional 15 minutes at 4°C followed by two additional washes.

For the anti-IL-10R staining, firstly, the cells were stained with an anti-IL-10R antibody conjugated to phycoerythrin (PE) for 20 minutes, followed by two washes. Subsequently cells were incubated with a biotinylated anti-PE antibody for 10 minutes, followed by a wash and a 10-minute incubation with a PE-streptavidin. This was repeated three times to amplify the signal before the standard surface staining protocol was applied.

For surface-only stainings, cells were directly analysed on a flow cytometer.
All antibodies used can be found in Table 2.2.

2.4.3 Intracellular staining

Prior to the intracellular staining the surface staining protocol was performed as described in the previous section. Cells were then 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 ambient temperature, followed by a wash in 1X permeabilisation buffer. Antibody cocktail was prepared in the permeabilisation buffer and cells were incubated for 30 minutes at 4°C followed by two washes with the permeabilisation buffer to remove any residual antibodies. Cells were analysed on a flow cytometer immediately for the stainings including the transcription factors, or on the following day when cytokine staining was performed.

2.4.4 Flow cytometric analysis

Cells were acquired either on LSRII or Fortessa (BD Biosciences) using FACSDiva software (BD Biosciences). Compensation using single-coloured-stained CompBeads (BD Biosciences) was always performed prior to the sample acquisition. The analysis of the results was performed using FlowJo 10 (Tree Star).

2.4.5 Cell sorting

Single cells suspensions were obtained as described in section 2.3 and subsequently enriched using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (STEMCELL Technologies) according to the manufacturer's instructions. Cells were stained with appropriate antibody mixes for 20 minutes at 4°C in the dark, washed twice with PBS and resuspended in PBS/0.1% BSA/5mM EDTA and filtered prior to the sort. 4',6-diamidino-2-phenylindole (DAPI) was added for the viability assessment. Cells were sorted into PBS/10% FCS or directly into RLT for the microarray samples. The purity of the sorted cells was assessed by a flow cytometric analysis. Cells were sorted on FACS AriaIII (BD Biosciences) by Dr H. Ferry, P. Amjadi or J. Webber.

Specificity	Clone	Fluorochrome
Surface markers		
CD103	M290	PE, FITC
CD11b	M1/70	V500
CD11c	3.9	BV605
CD25	7D4	AF647, BV421
CD31	390	FITC
CD4	RM4-5	BV605
CD44	IM7	APC, AF700
CD45	30-F11	AF700, BV650
CD45.1	A20	BV510, PerCP-Cy5.5
CD45Rb	C363-16A	APC, PerCP-Cy5.5
CD62L	MEL-14	PE
CTLA-4	UC10-4F10-11	PE
Ep-CAM	G8.8	PE-Cy7
GITR	YGITR 765	FITC
hCD2	RPA-2.10	PE-Cy5
ICOS	C398.4A	FITC
IL-10R	1B1.3a	PE
IL-18Rα	112614	FITC
KLRG1	2FI/KLRG1	PerCP-Cy5.5
Ly6C	HK1.4	PE-Cy7
Ly6G	1A8	BV570, PerCP-Cy5.5
MHCII	M5/114.15.2	AF700
PD-1	RMP1-30	PE-Cy7
Podoplanin	8.1.1.	APC
Siglec-F	E50-2440	BV421
ST2	101001B	biotinylated
TCRβ	H57-597	BV510, PE-Cy7, APC
TIGIT	1G9	PE
Intracellular markers		
Foxp3	FJK-16s	PE-Cy5
Gata3	TWAJ	eFluor660
Helios	22F6	eFluor450, AF488
IFN-γ	XMG1.2	PE-Cy7
IL17A	TC11-18H10.1	FITC
Ki67	SolA15	PerCP-eFluor710
Rorγt	Q31-378	PE, BV421
T-bet	4B10	BV711
TNF	MP6-XT22	AF700

Table 2.2. List of antibodies used in flow cytometry and cell sorting experiments.

AF – Alexa Fluor®, BV – Brilliant Violet™, APC – Allophycocyanin, FITC - Fluorescein, PE – Phycoerythrin

2.5 *In vitro* cultures

2.5.1 Natural Treg (nTreg) cultures

FACS-sorted $CD4^+Foxp3^+$ cells from $Foxp3^{GFP}$ reporter mice were plated at a density of $2x10^5$ per well in flat-bottom plates (Corning) coated with anti-CD3 (5µg/ml, eBioscience) in RPMI-1640 media supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml each of penicillin and streptomycin. Anti-CD28 (2µg/ml, eBioscience), IL-2 (1U/ml, Peprotech), and, if applicable, IL-23 (20ng/ml, R&D) were added to the cultures. IL-33 (10ng/ml, Biolegend) was added after 24 hours and cells were harvested at the indicated time points. Cells were used for RT-qPCR or Western Blot analysis. In experiments, in which STAT3 inhibitor was used, STAT3 VI (10mM, Santa Cruz) was added 30 minutes before stimulation with the cytokines.

2.5.2 Induced Treg (iTreg) cultures

FACS-sorted $CD4^+CD44^{low}CD62L^+Foxp3^-$ naïve T cells from $Foxp3^{GFP}$ reporter mice were plated as described in section 2.5.1. in complete RPMI-1640 medium with anti-CD3 and anti-CD28. Additionally TGF-β1 (500pg/ml, R&D) was added to the cultures. Cells were cultured in presence of IL-33 (1ng/ml, Biolegend) and/or IL-23 (20ng/ml, R&D), or were acutely stimulated after 48 hours cells with IL-33 (10ng/ml, Biolegend). Cells were harvested for RT-qPCR or Western Blot analysis.

2.5.3 Colonic stromal cell cultures

Colons were processed as described in section 2.3.3. Cells were recovered from the 30/40% interface of the Percoll density gradient and washed with PBS/0.1% BSA/5mM EDTA. Cells were plated at $1x10^7$ cells/ml in complete DMEM media (Sigma-Aldrich)

supplemented with 40 mg/ml gentamycin (Sigma-Aldrich). After 24-48 hours non-adherent cells were removed by vigorous washing, and the media was replaced. Adherent stromal cells were cultured for 10 days until confluent. Cells were then plated at $1x10^6$ cells/ml and bacterial (*H. hepaticus*, *C. rodentium*, lipopolysaccharide (LPS)) or cytokine (IL-1β, IL-6, IL-17, IL-18, IL-21, IL-22, IL-23, IL-33, TNF-α) stimulations were performed for 24 hours, and the supernatants collected. Dr B. Owens performed stimulations of the colonic stromal cells.

2.5.4 Colon explant cultures

Murine colon was removed from the peritoneal cavity and a piece about 0.5cm in length was cut out from the middle of the colon. The weight of the tissue was recorded and the piece was incubated in complete RPMI-1640 medium for 24 hours at 37°C. Following incubation supernatants were collected and stored at -80°C for growth factor and cytokine analysis.

2.6 Analysis of gene expression

2.6.1 RNA extraction

Tissue was firstly homogenised in soft tissue homogenising CK14 tubes (Stretton Scientific) in 700µl RLT for 30s using Precellys24 homogeniser (Precellys). Cells were directly lysed in the RLT lysis buffer (Qiagen). RNA was extracted with the RNeasy Mini RNA extraction kit (Qiagen) according to the manufacturer's instructions inclusive of the DNase digestion step. RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific) and stored at -80°C.

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

cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with up to 2µg of RNA according to the manufacturer's instructions inclusive of the RNase Inhibitor (Applied Biosystems) in a final volume of 20µl. Synthesis was performed in a C1000 Touch™ Thermal Cycler (BIO-RAD) and cDNA was stored at -20°C.

The RT-qPCR reactions were performed using mouse TaqMan gene expression assays (Life Technologies) (details of probes can be found in Table 2.3) with 2µl of cDNA and 2X Precision Fast qPCR mastermix with ROX at a lower level (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 the analysis, for each sample the expression levels of the gene of interest were normalised to *Hprt* levels, and the gene expression was calculated using the $2^{-\Delta Ct}$ method.

To measure the gene expression levels of soluble and membrane-bound *St2* and the Foxp3/Gata3-and-Foxp3 target genes SYBR green PCR SensiMix (Quantace) was used with the following primers soluble *St2*: 5'-TCGAAATGAAAGTTCCAGCA-3' and 5'-TGTGTGAGGGACACTCCTTAC-3'; membrane-bound *St2*: 5'-AAGGCACACCATAAGGCTGA-3' and 5'-TCGTAGAGCTTGCCATCGTT-3'. Primers for the Foxp3/Gata3- and Foxp3-target genes were described previously in Rudra et al. (2012).

Gene	Taqman probe
Areg	Mm01354339_m1
Cxcr3	Mm99999054_s1
Dgat2	Mm00499536_m1
Foxp3	Mm00475162_m1
Gata3	Mm00484683_m1
Il10ra	Mm00434151_m1
Il1rl1	Mm00516117_m1
Il23a	Mm00518984_m1
Il33	Mm00505403_m1
Irg1	Mm01224532_m1
Slfn1	Mm00488306_m1

Table 2.3. Taqman probes used in the RT-qPCR assays.

2.6.3 Microarray sample preparation

FACS-sorted cells were lysed in the RLT lysis buffer and RNA was extracted using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. The quality of the RNA was tested using the Agilent RNA 6000 Pico Kit (Agilent Technologies) according to the manufacturer's protocol and analysed on the Agilent 2100 Bioanalyser (Agilent Technologies). Only samples with RNA integrity number (RIN) > 7 were used for subsequent amplification.

RNA was amplified using the TargetAmpT 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre, Illumina) according to the manufacturer's instructions. All reactions were performed in a C1000 Touch™ Thermal Cycler. Amplified Biotin-aRNA samples were quantified with the NanoDrop spectrophotometer and normalised quantities used for the microarray analysis. A test qPCR was performed as described in section 2.6.2 and only samples, in which *Hprt* and *Foxp3* could be reliably detected by qPCR, were used

for the whole transcriptomic analysis. Samples were stored at -80°C until further processing.

2.6.4 Microarray analysis

The amplified Biotin-aRNA samples were hybridised to MouseWG-6 v2.0 Expression BeadChip (Illumina) by the microarray core facility at the Wellcome Trust Centre for Human Genetics, Oxford, UK. The core facility provided the probe signal intensities, which were extracted using GenomeStudio software (version 1.9.0, Illumina).
The subsequent analysis was performed by Dr Nicholas Ilott, University of Oxford, UK. Only probes expressed significantly above background (detection p-value<0.05) were used for downstream analysis. Expression profiles were normalised using a variance stabilising transformation and quantile normalisation using the Lumi package from R/Bioconductor (Du et al., 2008). Differentially expressed probes were called using the empirical Bayes method implemented in LIMMA from R/Bioconductor (Ritchie et al., 2015). Significance was defined as a Benjamini-Hochberg adjusted p-value<0.05.

2.7 Chromatin Immunoprecipitation (ChIP)

$4x10^6$ FACS-sorted $CD4^+CD44^{low}CD62L^+Foxp3^-$ naïve T cells were cultured as described in section 2.5.2. in the presence of anti-CD3 (5 µg/ml), anti-CD28 (2 µg/ml), TGF-β1 (500pg/ml) together with or without IL-23 (20ng/ml). After 48 hours cells were stimulated with IL-33 (10ng/ml) for 45 minutes, washed with PBS and fixed with formaldehyde (Sigma-Aldrich) at a final concentration of 1% for 15 minutes at 37°C. Cells were lysed in sonication buffer (10mM Tris-HCl pH 8.0, 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Na-Deoxycholate, 0.5% Na-Lauroylsarcosine, all from Sigma-

Aldrich). Nuclear extracts pre-treated with protease inhibitors (Roche) were sonicated using a Bioruptor (Diagenode) to obtain 500 base-pair-long fragments (3*30 second cycles on the highest setting). An aliquot of the fragmented DNA was set aside at this point as the input DNA. The fragmented nuclear extracts were incubated with 3µg antibodies against Gata3 (L50-823, BD Pharmingen) or RNA polymerase II (sc-899, Santa Cruz Biotechnology) pre-mixed with protein G magnetic beads (30µl beads/sample, GE Healthcare) on a rotor at 4°C overnight. The immune-precipitated immuno-complexes were isolated using a magnet, washed extensively with a wash buffer (50mM Hepes-KOH pH 7.6, 500mM LiCl, 1mM EDTA, 1% NP-40, 0.7% deoxycholic acid, all from Sigma-Aldrich) and eluted with a Tris-EDTA (TE) buffer (Sigma-Aldrich) containing 2% SDS at 65°C overnight. In order to reverse the cross-linking between the DNA and the proteins, both the immuno-complexes and the control input DNA were incubated at 65°C overnight. The DNA was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The immune-precipitated DNA fragments were tested using SYBR Premix Ex Taq II master mix (Takara Bio) and the following primers: *St2* promoter: 5'-TGGCCTCCTTGGAAAGGCTTGGT-3' and 5'-AGTGCAGGAGGGGCATGGAGATGA-3'; *St2* enhancer: 5'-GCCAACCACAACAGCAGATGGGGAAA-3' and 5'-ACTGAGATCCTGCCTGGCTTCCCT-3'. Primers for quantification of Gata3 binding to *Foxp3* promoter and CNS regions were described previously in Zheng et al. (2010). The samples were assayed in duplicate on a Rotor-Gene 6000 (Corbett Research Ltd) and results were normalised to input DNA and data presented as fold enrichment relative to the unstimulated cells.

2.8 Analysis of protein expression

2.8.1 Cell lysate preparation and quantification

Cells obtained in *in vitro* stimulation assays were lysed in Mph Lysis Buffer (50mM Tris pH 8.0, 150mM NaCl, 10% Glycerol, 1% NP40, all from Sigma-Aldrich) supplemented with PMSF protease and phosphatase inhibitors (Thermo Fisher Scientific), and stored at -20°C. Protein concentration was quantified in duplicate using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. 2ul of each sample were assayed in duplicates. The absorbance was read at 540 nm by a spectrophotometric plate reader SPECTROstar Nano (BMG Labtech), and analysed using SPECTROstar Nano Data Analysis software (BMG Labtech).

2.8.2 Immunoblotting

Cell lysates containing equal protein amount were firstly mixed with Laemmli sample buffer (Sigma-Aldrich) and heated at 95°C for 5 minutes to denature the protein. Amersham ECL Rainbow Marker (GE Healthcare) was loaded on every gel in order to assess the size of the bands. Samples were separated by the NuPAGE SDS-PAGE Pre-Cast gels (Life Technologies) in 1X NuPAGE MOPS SDS Running Buffer (Life Technologies). This was followed by a wet transfer onto a PVDF membrane (Amersham) in 1X NuPAGE transfer buffer (Life Technologies) containing 20% methanol. Non-specific background binding was blocked with 5% BSA in TBST (50mM Tris, 150mM NaCl, 0.05% Tween 20, all from Sigma-Aldrich). Membranes were incubated with anti-phospho-Gata3 (ab61052, Abcam), anti-Gata3 (L50-823, BD Pharmingen), anti-ST2 (101001B, MD Bioproducts) or anti-actin (A5541, Sigma-Aldrich) antibodies at 4°C overnight. Following incubation membranes were washed

extensively in TBST and incubated for one hour at ambient temperature with horseradish peroxidase–conjugated secondary antibody (Thermo Fisher Scientific). Blots were detected with Pierce ECL Plus (Thermo Fisher Scientific) and developed using X-Ray FujiFilm (Fisher Scientific).

2.8.3 Enzyme-linked immunosorbent assays (ELISAs)

Cytokine secretion was quantified using specific ELISAs against IL-23 (eBioscience), IL-33 (eBioscience) or soluble ST2 (R&D) according to the manufacturer's instructions. 50µl of the supernatant samples were prepared as described in sections 2.5.3 and 2.5.4 and quantified in duplicate. The absorbance was read at 450 nm by a spectrophotometric plate reader SPECTROstar Nano, and the subsequent analysis was performed using SPECTROstar Nano Data Analysis software.

2.9 Imaging studies

2.9.1 Two-photon *ex vivo* microscopy

Organs for the *ex vivo* imaging were isolated from IL-33$^{+/Cit}$ reporter mice or C57BL/6 control mice directly before imaging and kept in PBS at ambient temperature until imaged. Approximately 1cmx1cm size sections were imaged for lung, caecum and colon. Colonic sections were imaged from two sides: the luminal and the muscularis mucosae. Images were collected using a 20X water dipping lens and the spectral detector of a Zeiss 780 upright microscope (Carl Zeiss).

2.9.2 Image analysis of the two-photon images

The *ex vivo* microscopy images were linearly unmixed using the Zen software (Carl Zeiss) to separate autofluorescence, collagen, mucus and citrine based on single colour controls. The images were analysed using Imaris 8 (Bitplane). Images were drift-corrected based on mucus or collagen signal. The analysis was performed by Dr Emily Thornton, University of Oxford, UK.

2.9.3 Immunofluorescent labelling

Organs were harvested and immediately embedded in OCT Compound (Tissue-Tek) and stored at -80°C. Colons were rolled into 'Swiss rolls' prior to embedding in OCT. 5-7µm sections were cut using a Cryostat (Leica) and collected onto Superfrost Plus Micro Slides (VWR) and stored at -20°C. If required, slides were allowed to thaw at ambient temperature before being washed with PBS to remove the OCT, and fixed for 5 minutes with 3.7% formalin. Blocking was performed with 10% rabbit/donkey serum (all from Sigma-Aldrich) containing Fc block (eBioscience) in TBS for 1 hour at room temperature. Primary antibody staining was performed in 10% rabbit/donkey serum over night at 4°C. Following incubation slides were washed extensively with TBS, and incubated with the secondary antibody (Alexa Fluor antibodies, Life Technologies) for one hour at ambient temperature in the dark. Slides were again washed, and mounted with ProLong Gold Antifade Mountant with DAPI (Life Technologies) and imaged on an Olympus BX51 microscope.

Primary antibodies used in the immunofluorescence experiments are listed in Table 2.4 and secondary antibodies are listed in Table 2.5.

Specificity	Clone	Host species
B220	RA3-6B2	rat
CD31	MEC 13.3	rat
CD4	4SM95	rat
E-cadherin	36/E-Cadherin	mouse
Foxp3	FJK-16s	rat
GFP	polyclonal	rabbit
IL-33	polyclonal	goat
αSMA	polyclonal	rabbit

Table 2.4. List of primary antibodies used in the immunofluorescence staining experiments.

Reactivity	Fluorochrome
rabbit anti-goat	AF488, AF555
donkey anti-goat	AF568
rabbit anti-mouse	AF555
donkey anti-rat	AF488

Table 2.5. List of secondary antibodies used in the immunofluorescence staining experiments.

AF – Alexa Fluor®

2.10 Statistical analysis

All statistical analysis was performed in Prism 6.0 (GraphPad).

The Mann Whitney U test or the Wilcoxon signed rank test was used to compare two unpaired or paired samples, respectively. For comparison of more than two groups either a repeated measure ANOVA followed by Bonferroni post-hoc test or a Kruskal-Wallis one-way analysis of variance with a Dunn's post-hoc test were performed. The microarray analysis is described in section 2.6.4.

Differences were considered statistically significant when $p \leq 0.05$. Significance is indicated according to the following: * $p \leq 0.05$,** $p \leq 0.01$,*** $p \leq 0.001$,**** $p \leq 0.0001$.

Chapter 3. Regulatory T cells in intestinal homeostasis

3.1 Introduction

Tregs are a subset of $CD4^+$ T cells that express the transcription factor Foxp3 and are critical for the regulation of effector responses. Tregs make up about 10% of all circulating $CD4^+$ T cells in both mice and humans; however, this percentage is higher in non-lymphoid tissues. In recent years the role of tissue Tregs not only as a conventional immunosuppressive cell but also a critical modulator of tissue function and homeostasis has been highlighted (Panduro et al., 2016). Studies to date have primarily focused on the characterisation of VAT Tregs (Feuerer et al., 2009), skeletal muscle Tregs (Burzyn et al., 2013) and Tregs residing in the colonic LP (Schiering et al., 2014, Ohnmacht et al., 2015, Sefik et al., 2015). Both immune and non-immune functions of extra-intestinal non-lymphoid-resident Tregs are listed in Chapter 1, whereas here, only colonic Tregs are described.

Tregs are particularly enriched in the colon. This most likely reflects the high density of microbial antigens at this site. Tregs constitute a crucial axis within the mucosal immune system by suppressing unnecessary innate and adaptive effector responses towards innocuous antigens. Their critical role in regulation of intestinal homeostasis has been demonstrated multiple times: splenic $CD4^+$ T cells depleted of all $CD25^+$ T

cells led to development of spontaneous multiorgan autoimmunity including intestinal inflammation upon transfer into BALB/c athymic nude (*nu/nu*) mice (Sakaguchi et al., 1995); $CD4^+CD25^+$ T cells were shown to prevent and even cure colitis (Powrie et al., 1993, Powrie et al., 1994a, Maloy et al., 2003, Mottet et al., 2003); Scurfy mice with a mutation in the *Foxp3* locus suffer from intestinal manifestations (Brunkow et al., 2001); and finally human IPEX patients also experience GI symptoms (Wildin et al., 2001). Colonic LP Tregs express high amounts of surface molecules and immunosuppressive cytokines (e.g. CTLA-4, ICOS, GITR, KLRG-1, CD103, OX40, Gpr15, IL-10, TGFβ). These molecules have been associated with Tregs survival and regulatory function by allowing them to maintain tolerance towards self and non-self antigens. Despite clear evidence of their suppressive function, the specificity of Tregs has remained enigmatic. TCR sequencing experiments have provided some indication that Tregs may react towards specific bacterial antigens (Lathrop et al., 2011, Cebula et al., 2013). Recently it has also been described that commensal bacteria modulate Tregs in the colon. For example, certain species of *Clostridia* (clusters IV, XIVa, XVIII) (Atarashi et al., 2011), *Lactobacillus* (Tang et al., 2015) and *Bacteroides* (Round et al., 2011) have been shown to induce Tregs in the colonic LP and enhance their regulatory function, in some instances through increasing Treg IL-10 production. Colonic Tregs also express SCFA receptors, which when bound by bacterial metabolic breakdown products, such as butyrate, promoted their function and survival (Smith et al., 2013).

Interestingly, it has been suggested that colonic Tregs induced by microbial stimuli primarily express Rorγt, the transcription factor typically associated with the Th17 lineage and known to play a key role in the pathogenesis of autoimmune diseases.

These Rorγt^{+} Tregs, depending on the experimental setting, have been shown to either limit Th2 (Ohnmacht et al., 2015) or Th1/Th17 (Sefik et al., 2015, Yang et al., 2016a) effector responses. These and other studies suggest that distinct populations of Tregs can be found in the colon and their function might be specialised and finely tuned in order to maximise the regulatory capacity of the colonic Treg population as a whole.

Our recent comparative transcriptomic analysis of Tregs isolated from the colonic LP and the draining mLNs showed a number of differences between these populations (Schiering et al., 2014). Amongst these, *Il1rl1*, which codes for ST2, the IL-33 receptor, was increased in the colonic Tregs. Interestingly, a similar observation was made in the studies of both VAT and muscle Tregs (Feuerer et al., 2009, Burzyn et al., 2013). Furthermore, Vasanthakumar *et al* (2015) showed that IL-33 signalling into VAT-resident Tregs is critical for both their development and survival. These observations suggest that ST2 expression could be an important tissue-specific adaptation of Tregs; however, its role in colonic Treg development and function remains unknown.

In this chapter we explore colonic LP Tregs using primarily flow cytometric analysis to describe differences between distinct populations, with a specific focus on the ST2-expressing Tregs. The main objectives of the chapter are:

- To characterise Tregs in the colon at steady state, specifically the $ST2^{+}$ population, in order to provide insight into how they promote tissue homeostasis, including
    - Determining their preferred location
    - Defining their transcriptional profile
    - Assessing their immunosuppressive potential
- To evaluate whether and how Treg populations in the colonic LP are altered in response to physiological and environmental changes, such as inflammation.

3.2 Results

3.2.1 ST2-expressing Tregs are enriched at tissue barrier sites.

Tregs constitute on average about 10% of $CD4^+$ T cells and can be found throughout the body. In the colonic LP this frequency is markedly higher and up to 30% of $CD4^+$ T cells may express Foxp3 (Izcue et al., 2009). We have previously shown that Tregs in the colon are specifically enriched in ST2 expression (Schiering et al., 2014), and here set out to investigate whether high ST2 expression is specifically restricted to the colon or is a broader tissue-specific feature.

ST2 expression in Tregs isolated from the blood, spleen, peripheral lymph nodes including superficial cervical, axillary, brachial and inguinal lymph nodes, mLNs, the lung, skin and colon was evaluated (Fig. 3.1 A). In line with previous reports (Schiering et al., 2014), less than 10% of Tregs found in the lymphoid tissues expressed ST2, and on average 12.8% Tregs were $ST2^+$ in the circulation. The frequency of $ST2^+$ Tregs was substantially increased in the non-lymphoid tissues such as the lung (mean of 24.2%), skin (45.4%) and colon (59.6%) (Fig. 3.1 B). Mean fluorescent intensity (MFI) measurements were used to evaluate the amount of ST2 protein expressed on a per cell basis among the Treg populations isolated from the various organs. The geometric MFI was also the highest among non-lymphoid tissue Tregs and Tregs with the highest levels of ST2 expression were observed in Tregs isolated from the skin and colon (Fig. 3.1 C).

In summary, $ST2^+$ Tregs are preferentially located in the non-lymphoid organs such as the lung, skin and colon, with highest proportion found in the colonic LP.

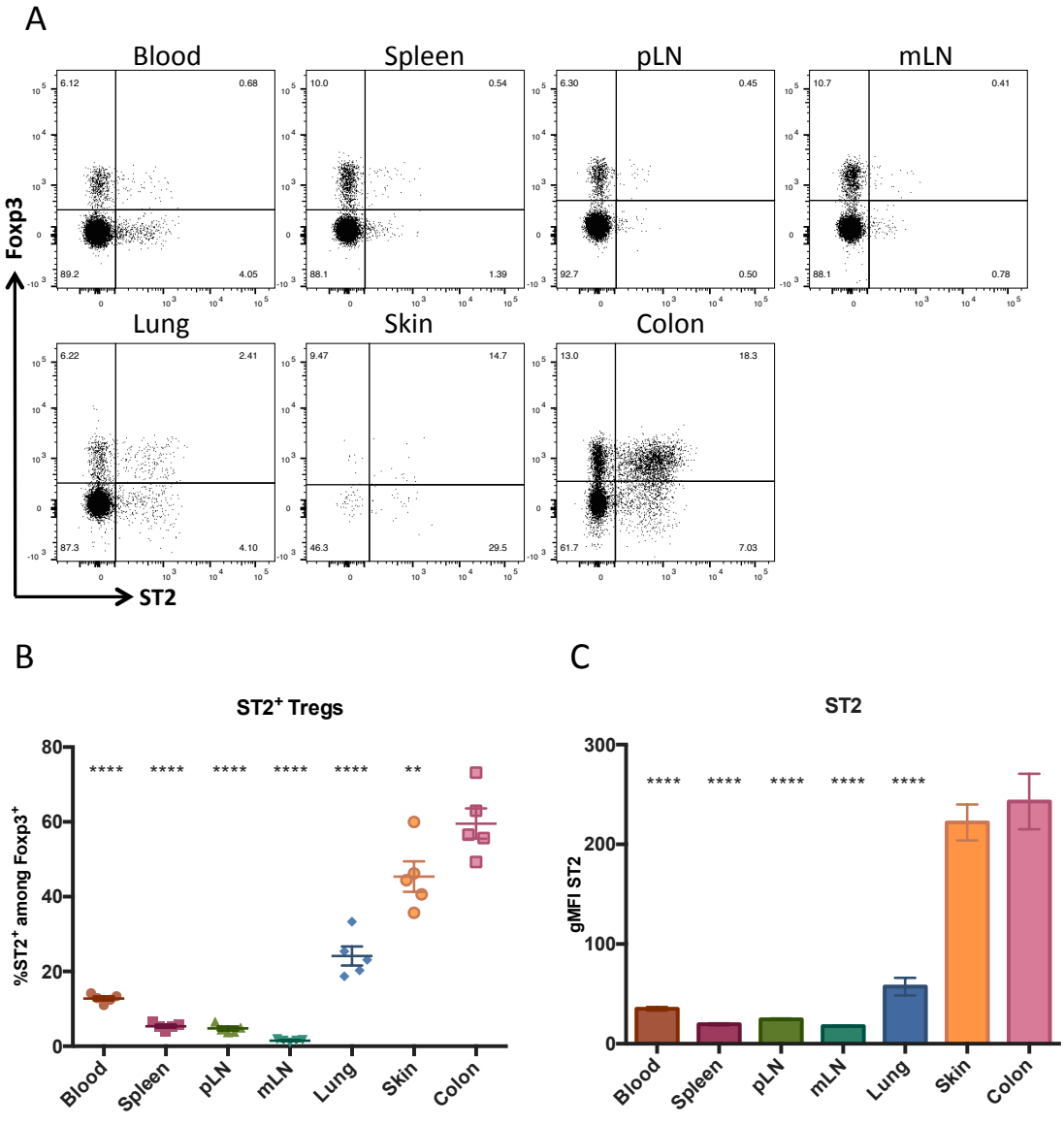


Figure 3.1. ST2-expressing Tregs are enriched at barrier sites, especially the colon.

C57BL/6 mice were sacrificed at steady state and single cell suspensions from the indicated organs were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing co-expression of ST2 and Foxp3. Gated on single live TCRβ$^+$CD4$^+$Foxp3$^+$ cells isolated from the indicated organs.
(B) Frequency of ST2$^+$ cells among TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs in the indicated organs.
(C) Geometric mean fluorescence intensity (gMFI) of ST2 among TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs in the indicated organs.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents results from a single experiment (n=5 mice). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test compared to the control group of the colon. ** $P \leq 0.01$, ****$P \leq 0.0001$.

3.2.2 The colon harbours the highest proportion of ST2$^+$ Tregs in the GI tract.

As the intestine is heterogeneous in architecture and function throughout its length we next assessed the frequency of Tregs along the length of the mouse intestine. The murine intestines comprise the small intestine (duodenum, jejunum and ileum), the caecum (equivalent of the human appendix), and the colon. The immune system of the small and large intestine has discrete functions, anatomy and immune cell composition, which are dictated in part by differences in dietary antigen and microbial load (Mowat and Agace, 2014). Therefore, we investigated Treg populations in these three different locations in the murine GI tract (Fig. 3.2). Due to low cell numbers it was not possible to divide the small intestine into its distinct parts, hence, it was processed as a whole.

As previously reported (Mowat and Agace, 2014), we observed a gradient of Foxp3-expressing CD4$^+$ T cells in the intestine of steady state mice with the highest proportion found in the colon (Fig. 3.2 A). Tregs expressing ST2 followed the same pattern, with the highest expression observed in the colon (Fig. 3.2 B). The frequency of ST2$^+$ Tregs found in the small intestine was on average 14%, which is similar to the Treg populations found in lymphoid organs and in circulation. Intermediate frequencies of both total Tregs and ST2$^+$ Tregs were found in the caecum, supporting that there is a gradient of Tregs along the murine GI tract (Fig. 3.2 A, B).

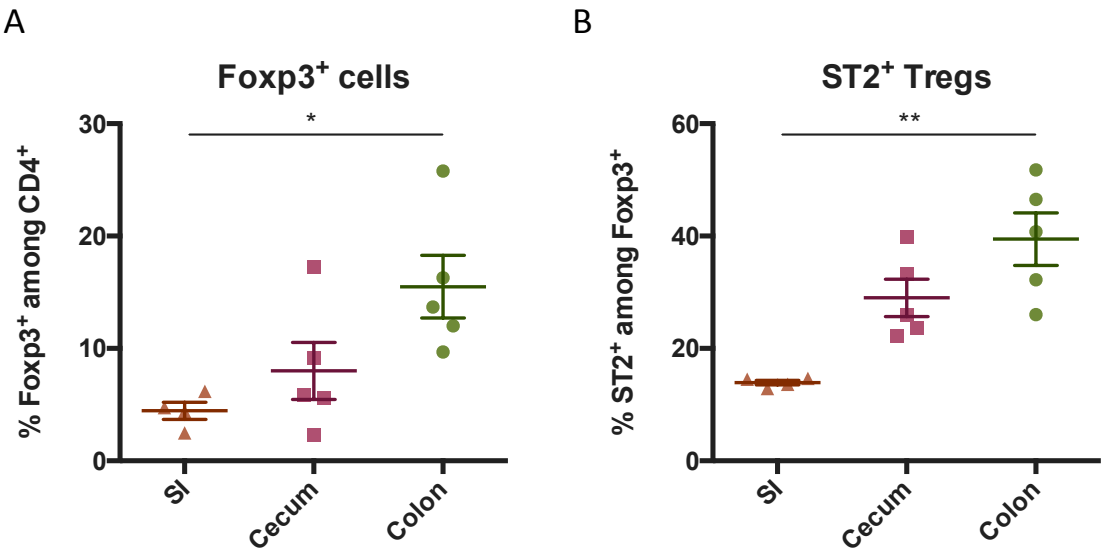


Figure 3.2. The frequency of ST2-expressing Tregs increases along the length of the GI tract.

C57BL/6 mice were sacrificed at steady state and single cell suspensions from the small intestine, caecum and colonic LP were stained for flow cytometric analysis.

(A) Frequency of $Foxp3^+$ Tregs among $TCR\beta^+CD4^+$ T cells in the indicated organs.
(B) Frequency of $ST2^+$ cells among $TCR\beta^+CD4^+Foxp3^+$ Tregs in the indicated organs.

Each point represents a single mouse and bars represent mean ±SEM.

The above staining was performed in 3 individual experiments with n≥4 mice. Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * P≤0.05, ** P ≤ 0.01.

3.2.3 Colonic ST2$^+$ Tregs also express Gata3 and Helios.

Since the transcription factors T-bet, Gata3 and Rorγt, which are primarily associated with Th1, Th2 and Th17 cells respectively, have been recently shown to be co-expressed by Foxp3-expressing Tregs (Koch et al., 2009, Wang et al., 2011, Wohlfert et al., 2011, Ohnmacht et al., 2015, Sefik et al., 2015, Yang et al., 2016a), we investigated whether ST2$^+$ colonic Tregs express any of these proteins.

Flow cytometric analysis of colonic Tregs showed that only Gata3 and Rorγt are expressed in the ST2$^+$ cells (Fig. 3.3 A). The vast majority, about 70%, of ST2$^+$ Tregs co-expressed the Gata3 transcription factor (Fig. 3.3 A, B). A small proportion of ST2$^+$ Tregs also expressed Rorγt. Of note, in our animal facility we have not observed any T-bet expression by CD4$^+$Foxp3$^+$ T cells under steady state or inflammatory conditions. Furthermore, we observed that Tregs isolated from either the spleen or the mLN expressed negligible amounts of both Gata3 and Rorγt (data not shown).

To establish whether ST2$^+$ Tregs are derived from the thymus or are peripherally induced, we performed flow cytometric analysis of Helios expression, which under steady state conditions has been shown to be constitutively expressed by tTregs but not pTregs or iTregs (Thornton et al., 2010).

Most ST2$^+$ Tregs and Gata3$^+$ Tregs stained positive for Helios (Fig. 3.3 C) suggesting that these populations of colonic Tregs are tTregs. By contrast, Rorγt$^+$ Tregs did not express Helios (Fig. 3.3 C), indicating that this population most likely represents pTregs.

In summary, we observed two distinct Treg populations in the colonic LP: Gata3^{+} and Rorγt^{+} Tregs, with most of the Gata3^{+} Tregs co-expressing ST2. In accordance with previously published data based on Helios expression in the colon (Schiering et al., 2014, Ohnmacht et al., 2015, Sefik et al., 2015) ST2^{+}/Gata3^{+} Tregs represent tTregs while Rorγt^{+} Tregs are pTregs.

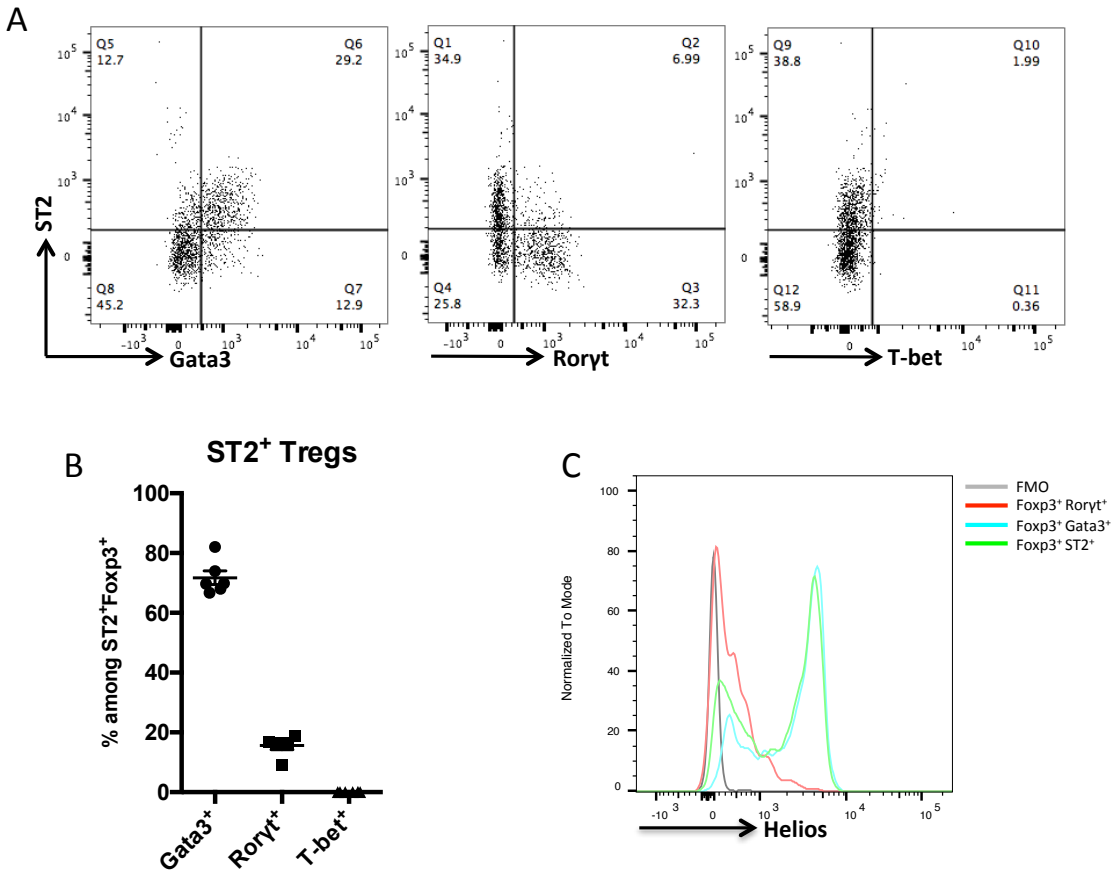


Figure 3.3. ST2-expressing colonic Tregs co-express Gata3 and are Helios$^+$.

C57BL/6 mice were sacrificed at steady state and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing co-expression of ST2 and CD4$^+$ Th cell lineage-defining transcription factors: Gata3, Rorγt and T-bet. Gated on single live TCRβ$^+$CD4$^+$Foxp3$^+$ cells.
(B) Frequency of Gata3$^+$, Rorγt$^+$ and T-bet$^+$ among ST2$^+$Foxp3$^+$ colonic Tregs.
(C) Flow cytometric analysis of Helios expression. Histogram depicts Helios expression in Rorγt$^+$ (red), Gata3$^+$ (blue) or ST2$^+$ (green) colonic Tregs. A grey line indicates an FMO for Helios.

Each point represents a single mouse and bars represent mean ±SEM (n=6 mice).

The data are representative of one of more than 10 experiments.

3.2.4 ST2$^+$/Gata3$^+$ Tregs are present in the colon at weaning.

As Gata3$^+$ and Rorγt$^+$ Tregs may represent different developmental states, we analysed these cell populations over time. Since ST2$^+$/Gata3$^+$ Tregs were largely Helios$^+$ and therefore likely thymus-derived, we hypothesised that these cells could be found in the murine colonic LP early in life. In contrast, we predicted that Rorγt$^+$Helios$^-$ Tregs, as previously demonstrated in GF and antibiotic-treated mice (Ohnmacht et al., 2015, Sefik et al., 2015), would only be induced following exposure to microbial antigens. At around day 20 post-birth the diet of laboratory mice transitions from mother's milk to solid food and the microbiota changes accordingly (Sefik et al., 2015). Therefore, we compared colonic Treg populations in mice at weaning (3 weeks old) and at 10 weeks of age under homeostatic conditions (Fig. 3.4).

A significant decrease in the frequency of ST2$^+$ and Gata3$^+$ Tregs was observed between 3 and 10 weeks of age in the murine colon (45.4% in 3W vs 32.7% in 10W and 25.1% in 3W vs 13.3% in 10W for ST2 and Gata3, respectively) (Fig 3.4 A, B, C). By contrast, this decrease was mirrored by an expansion of the Rorγt$^+$ Treg population (19.7% in 3W vs 45.5% in 10W) (Fig 3.4 A, B, C). This is consistent with the significant decrease in Helios$^+$ Tregs after weaning suggesting that at this point there is an increase in the frequency of peripherally induced Tregs (53.0% in 3W vs 34.5% in 10W) (Fig. 3.4 C). Of note, the total number of Tregs increased with age, which is consistent with the animals' increase in size (data not shown).

The 3.5W time point is included in the graphs in order to illustrate how robust the induction of Rorγt$^+$Helios$^-$ Tregs is once the animals are weaned and their exposure to

microbial stimuli is enhanced. There was an observable decrease in $ST2^+$ and $Gata3^+$ Tregs and concomitant increase in $Ror\gamma t^+$ Tregs in the colonic LP (Fig. 3.4 C). However, further experiments are required to confirm a link to the microbial exposure.

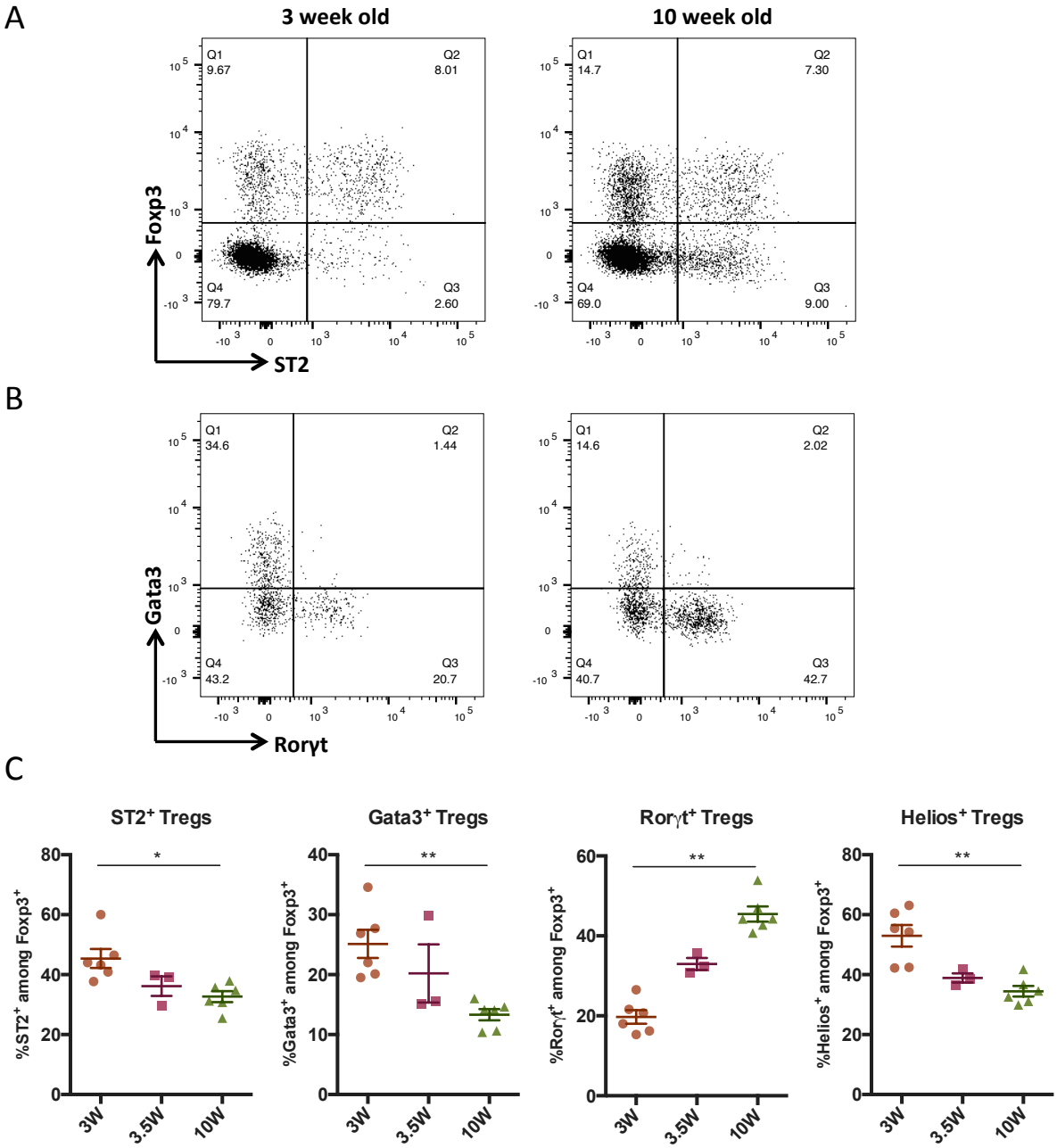


Figure 3.4. ST2$^+$/Gata3$^+$ Tregs are present in the colon at weaning.

C57BL/6 mice were sacrificed at steady state at 3, 3.5 or 10 weeks of age and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing co-expression of Foxp3 and ST2 in 3-week- and 10-week-old mice. Gated on single live TCRβ$^+$CD4$^+$.
(B) Representative flow cytometry plots showing co-expression of Gata3 and Rorγt$^+$ in 3-week- and 10-week-old mice. Gated on single live TCRβ$^+$CD4$^+$Foxp3$^+$.
(C) Frequency of ST2$^+$, Gata3$^+$, Rorγt$^+$ and Helios$^+$ among colonic TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents results of a single experiment (n=6 mice for 3 week and 10 week and n=3 mice for 3.5 week). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * $P \leq 0.05$, ** $P \leq 0.01$.

3.2.5 Colonic ST2$^+$/Gata3$^+$ Tregs display increased activation marker expression compared with Rorγt$^+$ Tregs.

Multiple surface markers have been associated with the suppressive function of Tregs. Since functional assays assessing the immunosuppressive function of colonic Tregs are not feasible due to very low cell numbers, the expression of a number of surface markers was investigated amongst colonic Tregs by flow cytometry. ST2$^+$, Gata3$^+$ and Rorγt$^+$ colonic Tregs were compared under steady state conditions.

Colonic LP Tregs displayed high IL-2Rα or CD25 as well as CD44 expression (Fig. 3.5) consistent with an effector memory phenotype. CD44^{hi}CD62L^{low}KLRG1$^+$CD103$^+$Foxp3$^+$ cells have been referred to as the mouse equivalent of human effector Tregs (Liston and Gray, 2014), and here interestingly only ST2$^+$ and Gata3$^+$ Tregs expressed high levels of KLRG1 while Rorγt$^+$ Tregs were completely negative for this activation marker (Fig. 3.5). ST2$^+$ and Gata3$^+$ Tregs also expressed higher amounts of CD103, PD-1, TIGIT and GITR than Rorγt$^+$ Tregs (Fig. 3.5). All these markers have been linked to Treg immunosuppressive function (Liston and Gray, 2014). In addition, all colonic Tregs expressed similar amounts of ICOS while Rorγt$^+$ Tregs had a higher expression of CTLA-4 (Fig. 3.5).

In summary, based on the investigated surface markers associated with Treg immunosuppressive activity, ST2$^+$/Gata3$^+$ Tregs display an increased activation marker expression compared to Rorγt$^+$ Tregs in the colonic LP.

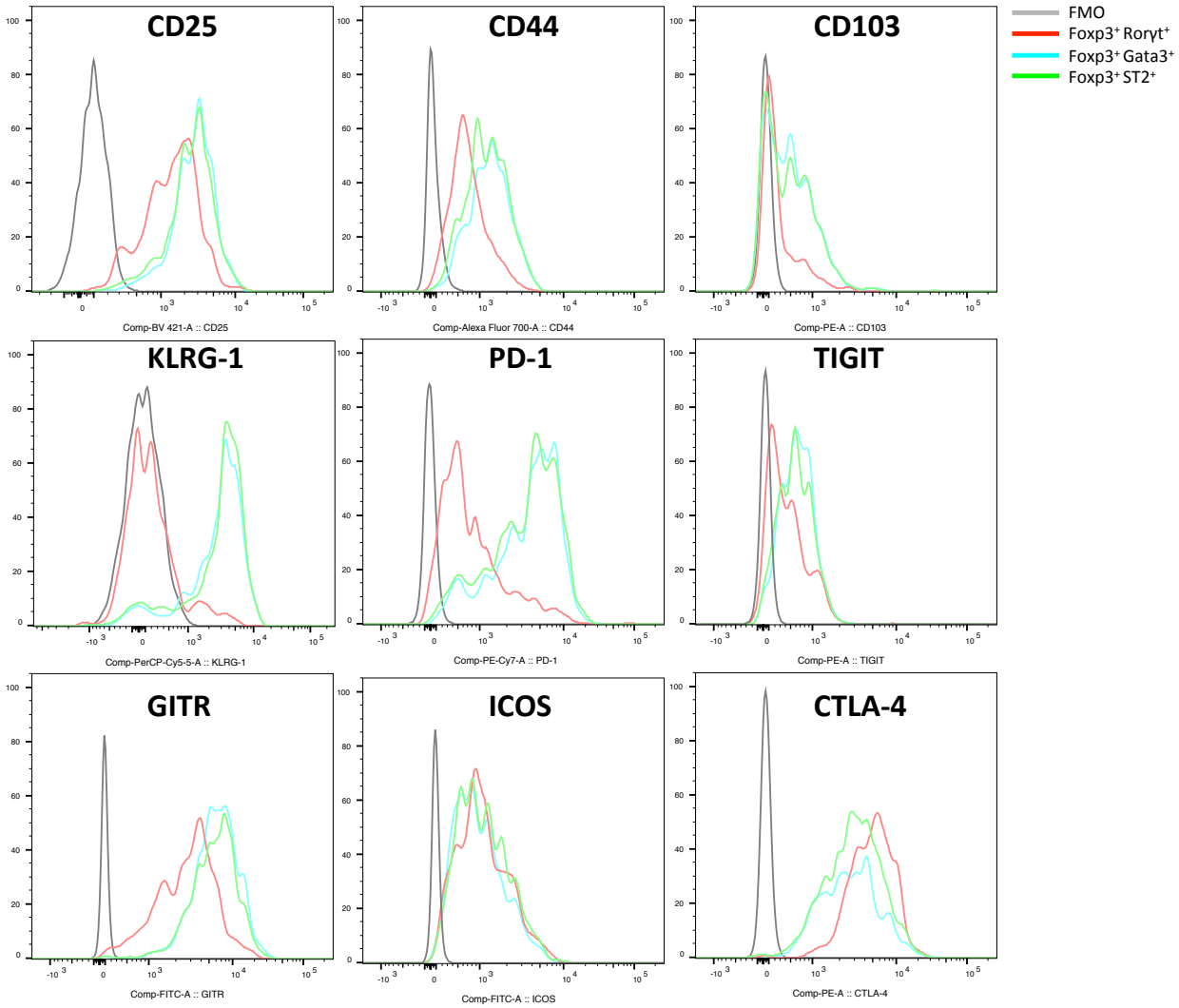


Figure 3.5. Colonic Gata3$^+$/ST2$^+$ Tregs display increased activation marker expression in contrast to Rorγt$^+$ Tregs.

C57BL/6 mice were sacrificed at steady state and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

Flow cytometric analysis of surface Treg cell activation markers. Histograms depict expression of indicated markers in Rorγt$^+$ (red), Gata3$^+$ (blue) or ST2$^+$ (green) Tregs. Grey line indicates FMOs for each marker. Gated on single live TCRβ$^+$CD4$^+$Foxp3$^+$ cells.

The staining was performed in more than 3 individual experiments.

3.2.6 A comparative transcriptomic analysis of colonic ST2$^+$ and ST2$^-$ Tregs.

There are multiple reports of Tregs found in non-lymphoid organs that have a distinct transcriptional profile compared to their lymphoid counterparts (Feuerer et al., 2009, Burzyn et al., 2013, Schiering et al., 2014, Cipolletta et al., 2015). To further understand ST2-expressing Tregs in the colon, a comparative transcriptomic analysis of colonic ST2$^+$ and ST2$^-$ Tregs was performed. Foxp3GFPKI mice (Bettelli et al., 2006) that have a bicistronic enhanced GFP reporter introduced into the endogenous *Foxp3* locus were used to isolate colonic Tregs. Viable TCRβ$^+$CD4$^+$GFP$^+$ST2$^-$ and TCRβ$^+$CD4$^+$GFP$^+$ST2$^+$ cells, referred to as ST2$^-$ and ST2$^+$ respectively, were FACS-sorted from the colonic LP of steady state Foxp3GFPKI mice according to a gating strategy depicted in Figure 3.6 A. 5-10 colons were pooled to obtain a pair of ST2$^-$ and ST2$^+$ experimental samples. Due to low numbers of the recovered colonic Tregs (between 2,000-20,000 cells per sample) the RNA of the colonic Tregs was amplified. Subsequently samples were analysed on an Illumina Mouse Bead Array platform. A detailed description of the processing of the samples and the bioinformatics analyses are described in Chapter 2.

A volcano plot was generated in order to visualise broad differences in gene expression between the ST2$^-$ and ST2$^+$ Tregs isolated from the colonic LP. Significantly (adjusted p ≤0.05) downregulated or upregulated genes after multiple testing corrections in ST2$^+$ Tregs are indicated in red (Fig. 3.6 B). Two vertical lines indicate a two-fold downregulation (log_2FC=-1) and upregulation (log_2FC=1) in the ST2$^+$ vs ST2$^-$ Tregs. Most genes, however, fall in-between the two vertical lines suggesting that the expression level changes were not large (Fig. 3.6 B). 7 probes were differentially expressed after multiple testing corrections (Table 3.1). Among the 7 genes with a

significant difference in expression between $ST2^-$ and $ST2^+$ populations there were two downregulated genes: *Cxcr3* and *Slfn1*, and 5 upregulated genes in $ST2^+$ Tregs: *Il1rl1*, *Dgat2*, *Irg1*, *Il10ra* and *Areg* (Fig. 3.6 C).

The most significantly upregulated gene in $ST2^+$ Tregs was *Il1rl1*, which encodes ST2. This confirmed that the results of the array are in line with the experimental design. Other upregulated genes included *Dgat2*, which encodes Diacylglycerol O-Acyltransferase 2 (DGAT2), one of the two DGAT enzymes involved in mammalian triglyceride metabolism, and is important for lipid synthesis and storage (Cases et al., 2001, Harris et al., 2011). Additionally, *Irg1,* which encodes Immune-responsive Gene 1 (IRG1) that catalyses itaconic acid production and has been implicated in antimicrobial function in macrophages (Michelucci et al., 2013), was upregulated in $ST2^+$ Tregs. Furthermore, IL-10Rα (encoded by *Il10ra*), which together with IL-10Rβ form the IL-10R expressed on many immune cells, including Tregs (Moore et al., 2001), was upregulated in $ST2^+$ Tregs. The role of IL-10 signalling in Tregs has been previously shown to be important for their survival and function (Murai et al., 2009, Chaudhry et al., 2011). Finally, *Areg* encoding amphiregulin was specifically upregulated in $ST2^+$ Tregs. Amphiregulin is an EGF-like growth factor, which like EGF binds to the EGFR, and plays a role in tissue remodelling and repair processes (Monticelli et al., 2011, Enomoto et al., 2009, Burzyn et al., 2013). Amphiregulin has also been implicated in Treg-mediated muscle repair (Burzyn et al., 2013).

Cxcr3, which is the most significantly downregulated gene in $ST2^+$ Tregs in the colon, encodes the chemokine receptor C-X-C Motif Chemokine Receptor 3 (CXCR3). Notably,

CXCR3 has been previously reported to be expressed on T-bet^{+} Tregs and required for the suppression of Th1-inflammatory responses (Koch et al., 2009). *Slfn1*, which encodes one of the Schlafen family members (Slfn1) and is involved in cell proliferation (Brady et al., 2005) was also downregulated in ST2^{+} Tregs; however, its role has not been studied in the context of immune cells (Mavrommatis et al., 2013).

RT-qPCR was performed on independently acquired and non-amplified RNA samples in order to validate differentially expressed genes between ST2^{-} and ST2^{+} colonic Tregs identified by the microarray analysis (Fig. 3.7). Six out of seven investigated genes were consistent with the whole transcriptional analysis, with three genes showing a significant result. The RT-qPCR analysis of *Il10ra* provided an inconclusive result, a result that will be further studied in section 3.2.8.

In summary, our comparative transcriptomic analysis did not reveal large differences between colonic ST2^{-} and ST2^{+} Tregs, and the significantly differentially expressed genes were involved in immunosuppression (*Il10ra*), immune-metabolism (*Dgat2* and *Irg1*) and repair processes (*Areg*).

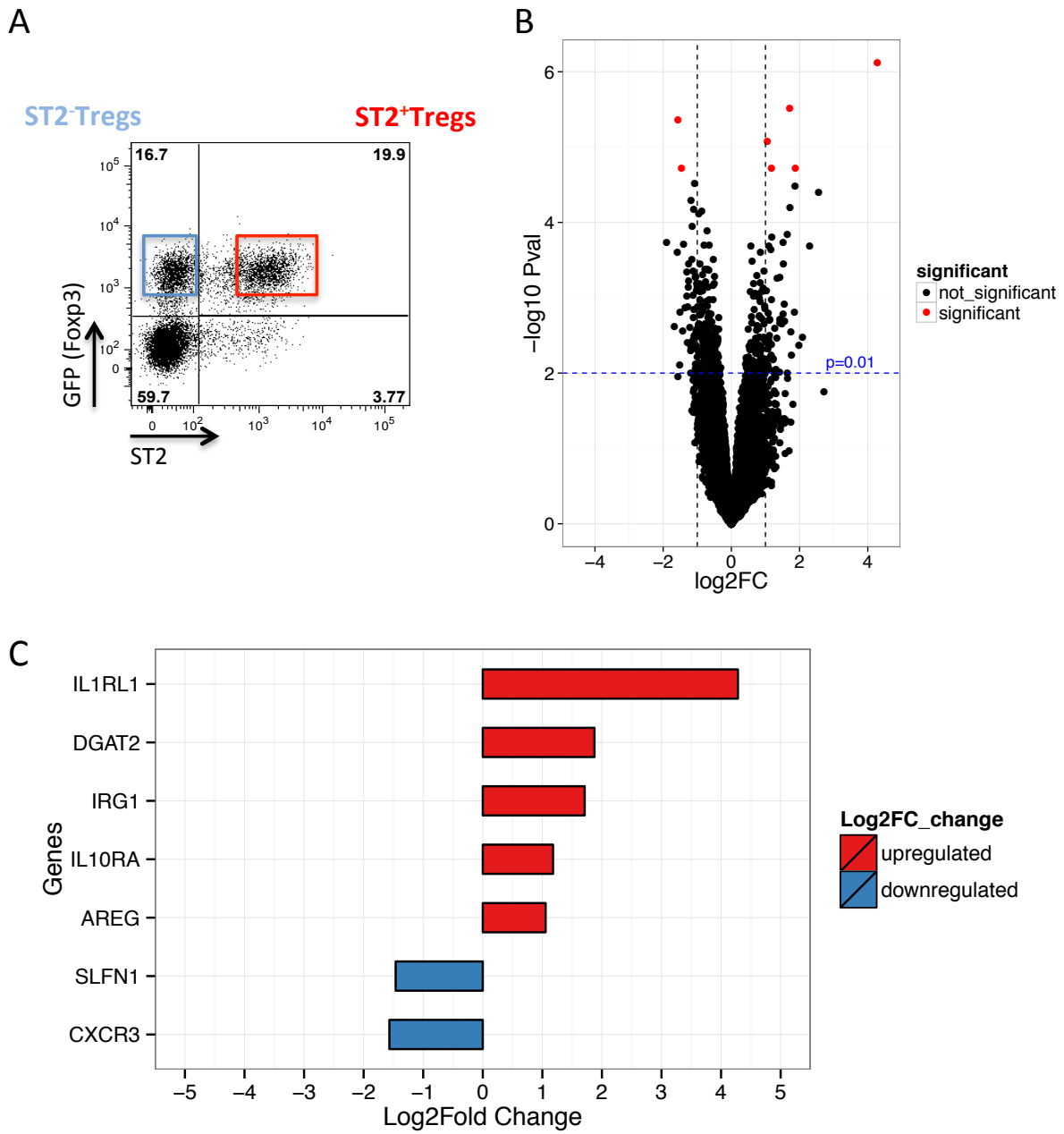


Figure 3.6. A comparative transcriptomic analysis of colonic ST2$^+$ and ST2$^-$ Tregs.

Foxp3GFPKI mice were sacrificed at steady state and cells were isolated from the colonic LP and caecum. Single live TCRβ$^+$CD4$^+$GFP$^+$ST2$^-$ and ST2$^+$ cells were FACS-sorted, RNA extracted and amplified. 5-10 colons and caeca were pooled for one sample of 2k-10k cells. Only samples with RIN>7 were amplified. Whole transcriptome expression analysis was performed using the Illumina MouseWG-6 V2 beadarray. Expression profiles were normalised using a variance stabilising transformation and quantile normalisation using the Lumi package from R/Bioconductor. Differentially expressed probes were called using the empirical Bayes method implemented in LIMMA from R/Bioconductor. Significance was defined as a Benjamini-Hochberg adjusted p-value 0.05.

(A) A schematic FACS plot showing the gating strategy for the cell sorting. Gated on single live TCRβ$^+$CD4$^+$ cells.
(B) A volcano plot showing fold change between ST2$^+$ and ST2$^-$ colonic Tregs (x-axis) vs nominal p-value (y-axis). Two vertical lines indicate 2-fold downregulation (left-side) and 2-fold upregulation (right-side) in ST2$^+$ Tregs. A blue horizontal line indicates nominal significance value p=0.01. Dots in red indicate genes that are significantly downregulated or upregulated after multiple testing corrections (n=6 pairs of ST2$^+$ and ST2$^-$ samples).
(C) A list of upregulated and downregulated genes in the ST2$^+$ vs ST2$^-$ Tregs with adjusted p-value ≤0.05. For more details on the genes see Table 3.1.

gene	full name of the protein	log2FC	AveExpr	P.Value	adj.P.Val
Il1rl1	Interleukin 1 receptor-like 1	4.283209241	10.45617044	7.62E-07	0.011002909
Dgat2	Diacylglycerol O-acyltransferase 2	1.875453481	9.357087097	1.90E-05	0.03963508
Irg1	Immune-responsive gene 1 protein	1.711106507	8.427616625	3.08E-06	0.021028236
Il10ra	Interleukin-10 receptor subunit alpha	1.179295322	9.983847989	1.92E-05	0.03963508
Areg	Amphiregulin	1.05534985	12.3451533	8.35E-06	0.030135281
Cxcr3	C-X-C chemokine receptor type 3	-1.567621805	9.731043236	4.37E-06	0.021028236
Slfn1	Schlafen 1	-1.462646891	8.360157371	1.91E-05	0.03963508

Table 3.1. Genes significantly upregulated or downregulated in colonic ST2$^+$ Tregs versus ST2$^-$ Tregs.

Log2FC – log2 of the fold change, AveExpr – average expression of the gene across the samples, P Value – nominal p value, adj. P Val – p value after multiple testing corrections.

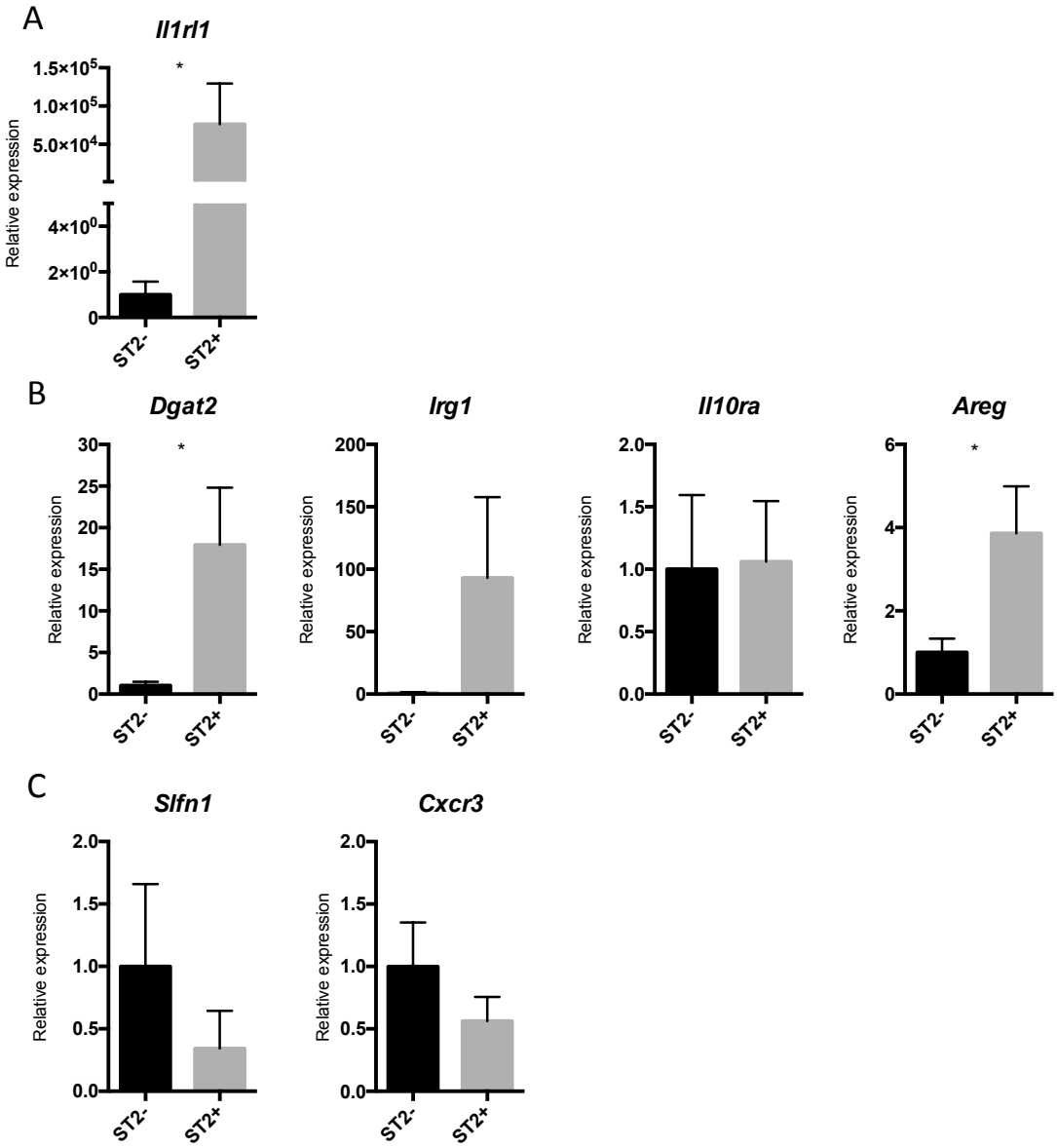


Figure 3.7. qPCR validation of differentially expressed genes in colonic ST2$^+$ versus ST2$^-$ Tregs.

Foxp3GFPKI mice were sacrificed at steady state and cells were isolated from the colonic LP and caecum. Single live TCRβ$^+$CD4$^+$GFP$^+$ST2$^-$ and ST2$^+$ cells were FACS-sorted and RNA was extracted with no further manipulation. 5-10 colons and caeca were pooled for one sample of 2k-20k cells.
Graphs show the RT-qPCR analysis of the two sorted populations: ST2$^-$ and ST2$^+$ Tregs:

(A) Positive control *Il1rl1*.
(B) Genes found to be significantly upregulated in the ST2$^+$ Tregs in the microarray analysis.
(C) Genes that were significantly downregulated in the ST2$^+$ Tregs in the microarray analysis.

Relative expression levels were calculated using 2^(-ΔΔCT) method normalising to the housekeeper *Hprt* and the average expression in the ST2$^-$ samples.

Bars represent mean ±SEM (n=6 pairs of ST2$^+$ and ST2$^-$ samples).

Statistical significance was determined by Wilcoxon matched-pairs signed rank test. * P≤0.05.

3.2.7 An analysis of IL-18R expression amongst colonic Tregs.

Our transcriptional analysis revealed higher amounts of *Areg* in the ST2^{+} colonic Tregs (Fig. 3.6). A recent study showed that amphiregulin production by Tregs in the lung was critical for restoration of tissue homeostasis during influenza virus infection (Arpaia et al., 2015). While amphiregulin production could be induced by both IL-33 and IL-18, two related IL-1 cytokine family members, more than 75% of amphiregulin-producing cells expressed IL-18R and only approximately 25% expressed ST2 (Arpaia et al., 2015).

We therefore performed flow cytometric analysis to evaluate whether ST2-expressing Tregs in the colon also respond to IL-18, and thus, express IL-18R. IL-18R$^{-/-}$ mice were used to gate the IL-18R^{+} population (Fig. 3.8 A). We found that 40% of colonic Tregs expressed ST2 (mean of 39.4%), approximately 20% expressed IL-18R (mean of 23.3%), and on average 13.6% of colonic Tregs co-expressed ST2 and IL-18R (Fig. 3.8 B). To further understand the differences between these populations we investigated Gata3 and Rorγt expression (Fig. 3.8 C, D), which we previously defined to mark distinct Treg populations in the colon. Consistent with our previous findings, ST2^{+} Tregs expressed high amounts of Gata3, which were lower in both IL-18R^{+} and IL-18R^{+}ST2^{+} Treg populations. By contrast, Rorγt was significantly increased in IL-18R-expressing Tregs compared to IL-18R^{+}ST2^{+} and ST2^{+} Treg populations (Fig. 3.8 C, D).

Together these data show that ST2-expressing Tregs in the colonic LP largely do not co-express the IL-18R. Tregs that do express the IL-18R are predominately Rorγt^{+} Tregs, with the Rorγt^{+} and Gata3^{+} populations being distinct in their IL-18R expression.

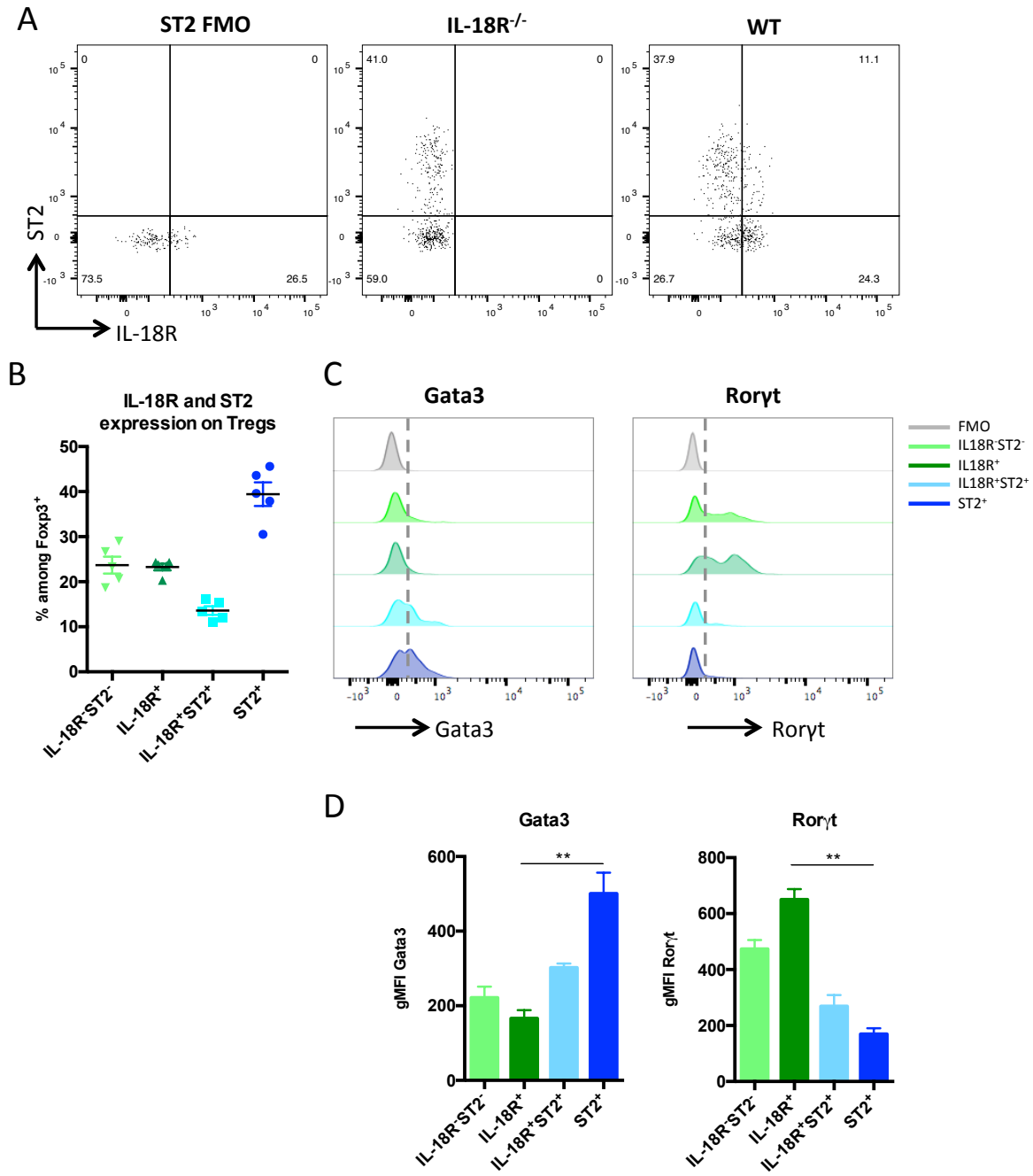


Figure 3.8. Analysis of IL-18R expression amongst colonic Tregs.

C57BL/6 and IL18R$^{-/-}$ mice were sacrificed at steady state and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing co-expression of ST2 and IL-18R on Tregs. Gated on single live TCRβ^{+}CD4^{+}Foxp3^{+} cells. ST2 FMO and IL-18R$^{-/-}$ mice were used to define gates.
(B) Frequency of double-negative IL-18R^{-}ST2^{-}, single positive IL-18R^{+}, double-positive IL-18R^{+}ST2^{+} and single positive ST2^{+} Treg populations among total Foxp3^{+} Tregs.
(C) Histograms showing expression of Gata3 and Rorγt^{+} on IL-18R^{-}ST2^{-}, IL-18R^{+}, IL-18R^{+}ST2^{+}, ST2^{+} colonic Foxp3^{+} cells. Gated on single live TCRβ^{+}CD4^{+}Foxp3^{+} cells. Gata3 and Rorγt FMOs are indicated in grey, IL-18R^{-}ST2^{-} in light green, IL-18R^{+} in dark green, IL-18R^{+}ST2^{+} in light blue, ST2^{+} in dark blue.
(D) A bar graph representing geometric mean fluorescent intensity (gMFI) of Gata3 and Rorγt^{+} in four colonic Treg populations indicated in (C).

Data shows representative results of one of two independent experiments (n=10 mice). Statistical significance was determined using a Friedman test followed by a Dunn's post-hoc test. ** $P \leq 0.01$.

3.2.8 Colonic Gata3^{+} Tregs have increased IL-10R expression.

IL-10 signalling into Tregs has been previously shown to be important for their control of immune responses (Chaudhry et al., 2011, Huber et al., 2011). In our comparative transcriptomic analysis expression of *Il10ra* was increased in the ST2^{+} colonic Treg population (Fig. 3.6). However, the results of the RT-qPCR performed on an independent data set failed to confirm this elevated expression (Fig. 3.7). Therefore, we set out to perform flow cytometric analysis of the IL-10R on colonic Tregs. A modified flow cytometry staining protocol was used with multiple rounds of amplification to enhance the signal to noise ratio (details can be found in Chapter 2).

Since staining with the anti-IL-10Rα antibody is technically challenging, CD45^{+}CD11b^{+} cells were firstly evaluated as a positive control. Multiple myeloid cell types are found in this gate, including macrophages, known to express IL-10R in the gut (Fig. 3.9 A). Secondly, the total population of colonic LP Tregs was studied and we observed that approximately 20% of CD4^{+}Foxp3^{+} cells expressed the IL-10R (Fig. 3.9 B). Thirdly, IL-10R expression was specifically examined in the Gata3^{+}, Rorγt^{+} and Gata3^{-}Rorγt^{-} Treg populations (Fig. 3.9 C, D). In this experimental set-up we were not able to assess ST2 expression in Tregs since the ST2 antibody is biotinylated and used in conjunction with streptavidin. This renders the ST2 antibody incompatible for use with the IL-10R amplification protocol, which requires streptavidin for the signal amplification. Analysis of the gMFI among the aforementioned populations revealed that Gata3^{+} Tregs expressed the highest amounts of IL-10R among the colonic LP Tregs (Fig. 3.9 E). This finding is consistent with the elevated *Il10ra* expression in ST2^{+} Tregs observed in the microarray given that ST2 and Gata3 are co-expressed in most of the colonic Tregs.

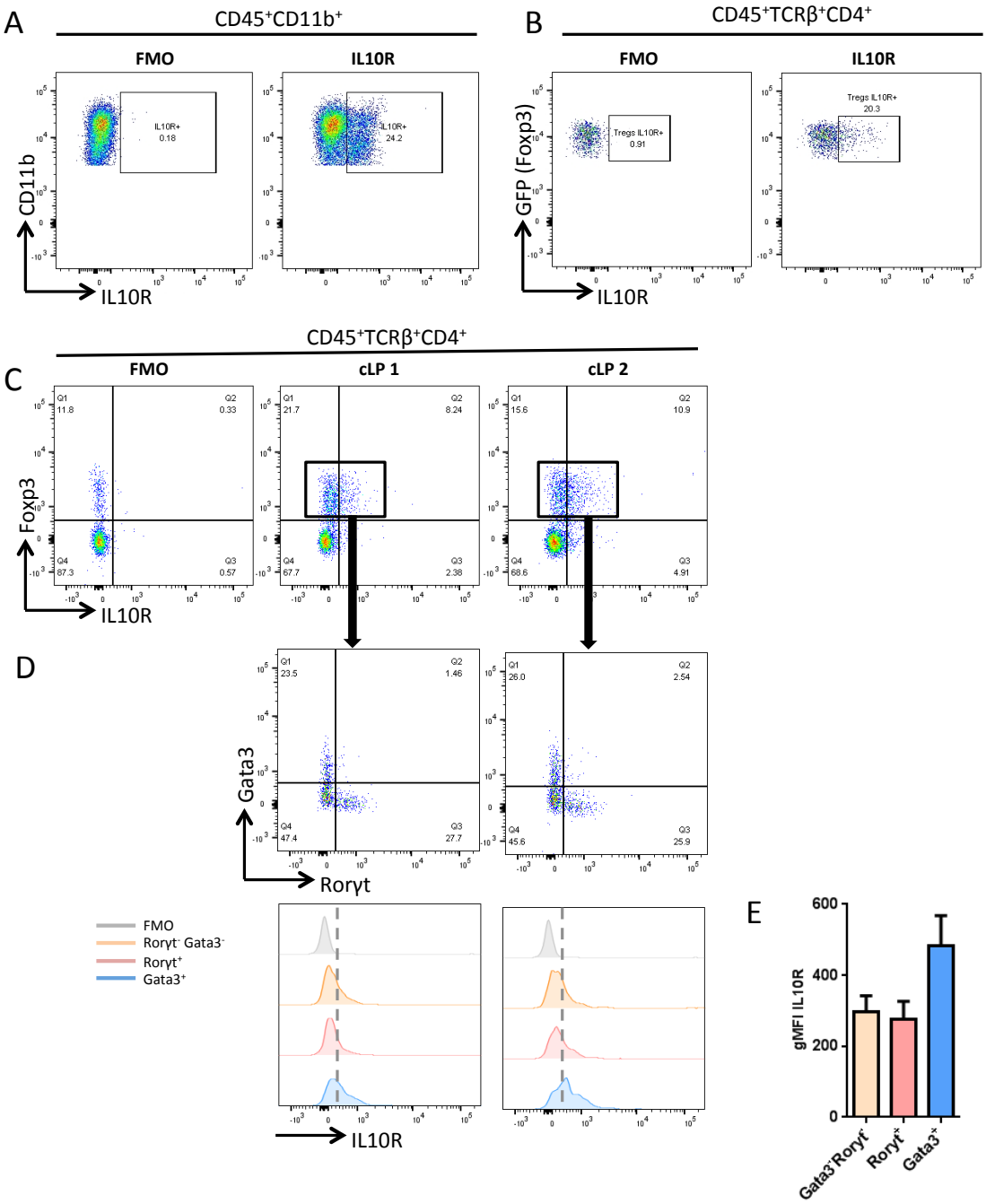


Figure 3.9. Colonic Gata3$^+$ Tregs have increased IL-10R expression.

Foxp3GFPKI (in A and B) or C57BL/6 (in C, D and E) mice were sacrificed at steady state and single cell suspensions from the colonic LP (cLP) were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing expression of IL-10R on CD11b$^+$ cells. Gated on single live CD45$^+$TCRβ$^-$CD11b$^+$ cells.
(B) Representative flow cytometry plots showing expression of IL-10R on CD4$^+$GFP$^+$ cells. Gated on single live CD45$^+$TCRβ$^+$CD4$^+$GFP$^+$ cells.
(C) Representative flow cytometry plots showing expression of IL-10R on CD4$^+$Foxp3$^+$ cells in two separate examples. Gated on single live CD45$^+$TCRβ$^+$CD4$^+$ cells.
(D) Flow cytometry plots showing gating strategy for the two respective examples of Gata3$^+$ and Rorγt$^+$ Treg populations, and the histograms depicting IL-10R expression levels in the three indicated populations: Rorγt$^-$ Gata3$^-$ (orange), Rorγt$^+$ (red), Gata3$^+$ (blue). Grey line indicates IL-10R FMO. Gated on single live CD45$^+$TCRβ$^+$CD4$^+$Foxp3$^+$cells.
(E) A bar graph representing geometric mean fluorescent intensity (gMFI) of IL-10R in three colonic Treg populations indicated in (D).

The above staining was performed in 3 individual experiments with n≥3 mice.

3.2.9 ST2^{+}/Gata3^{+} Tregs produce IL-10 in the colon under homeostatic conditions.

Tregs employ a variety of mechanisms to exert their regulatory function, including the production of immunosuppressive cytokines to downregulate effector responses. There are many studies reporting that IL-10 production in the colonic Tregs plays a non-redundant role in the control of inflammation in the gut (Asseman et al., 1999, Kamanaka et al., 2006, Rubtsov et al., 2008). It has been proposed that Rorγt^{+} Tregs are the major source of Treg-derived IL-10 in the gut (Ohnmacht et al., 2015, Yang et al., 2016a). However, our microarray analysis of ST2^{-} and ST2^{+} Tregs in the colon did not show a significant differential expression of *Il10*.

To investigate further, we used an IL-10^{GFP} reporter mouse (Kamanaka et al., 2006) crossed to a Foxp3^{hCD2} reporter mouse (Kendal et al., 2011) to interrogate production of IL-10 by different colonic Treg populations. On average 11.8% colonic Tregs expressed IL-10 under steady state conditions (Fig. 3.10 A, B). Among these 64.1%, 45.2%, and 21.0% co-expressed ST2, Gata3 and Rorγt, respectively (Fig. 3.10 C, D). The number of Foxp3^{+} Tregs in the colonic LP is largely influenced by the microbiota (Atarashi et al., 2011, Geuking et al., 2011) and certain microbial species specifically promote IL-10 production by colonic Tregs (Kullberg et al., 2002, Round and Mazmanian, 2010). We therefore investigated production of IL-10 in mice infected with *H. hepaticus.*

In line with published data (Kullberg et al., 2002), *H. hepaticus* infection resulted in an increase in the frequency of IL-10-producing Tregs (11.8% in steady state versus 28.8%

in the *Hh*-treated group) (Fig. 3.10 B). $ST2^+$ and $Gata3^+$ Tregs significantly decreased in frequency (64.1% vs 35.2% and 45.2% vs 11.1%, respectively) upon infection with *H. hepaticus* (Fig. 3.10 C, E), while an increase in the frequency and number of IL-10-producing $Ror\gamma t^+$ Tregs was observed (21.0% steady state vs 36.6% *Hh*) (Fig. 3.10 C, E).

Together these results demonstrate that under homeostatic conditions in our animal facility $ST2^+$ and $Gata3^+$ colonic Tregs are the major source of IL-10 among all colonic Tregs. However, upon infection with *H. hepaticus* the balance is reversed and $Ror\gamma t^+$ cells become the dominant population of IL-10-expressing Tregs.

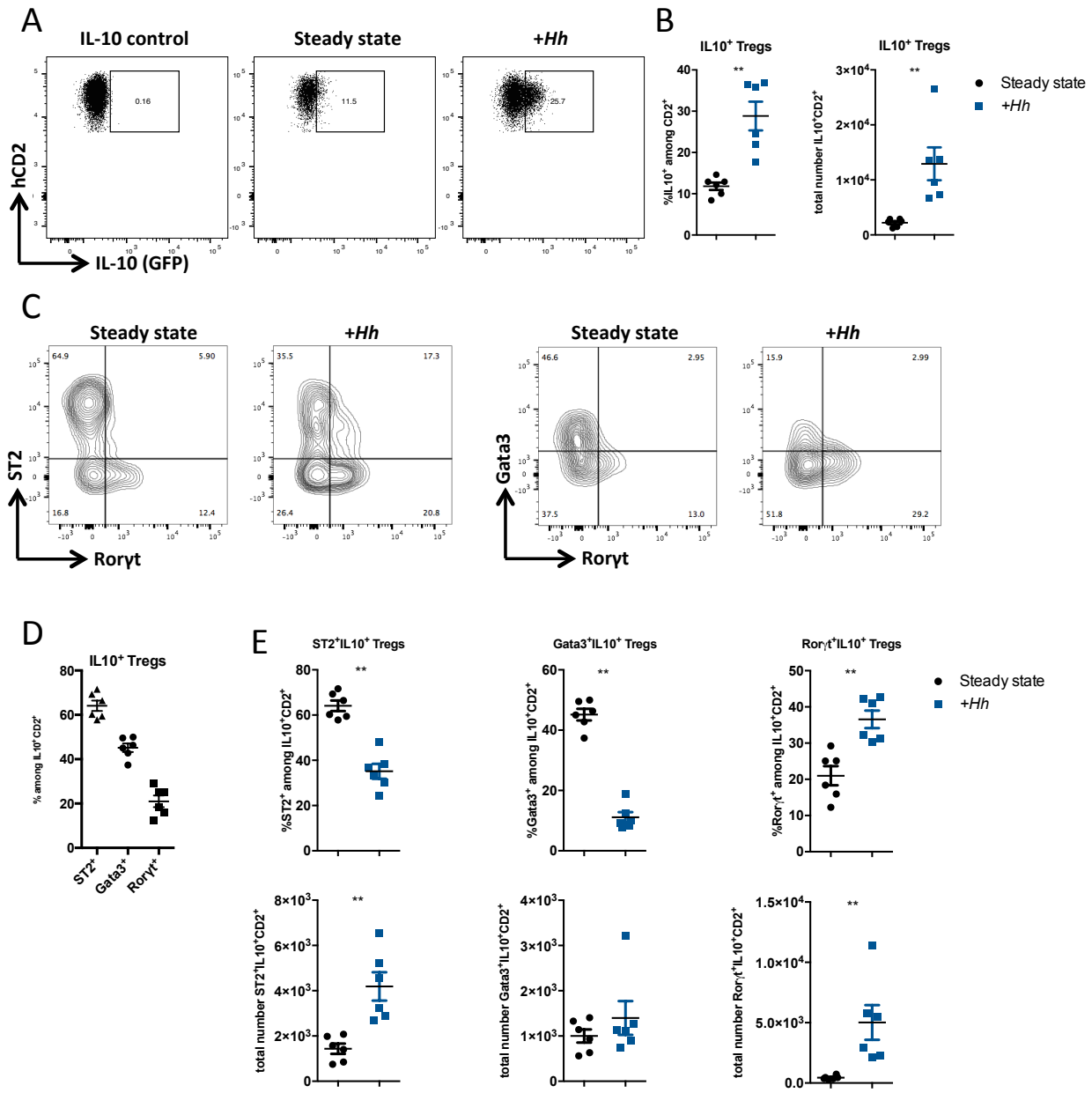


Figure 3.10. ST2$^+$/Gata3$^+$ Tregs produce IL-10 in the colon under homeostatic conditions.

Foxp3^{hCD2}xIL-10^{GFP} mice were sacrificed without treatment (steady state) or infected by oral gavage with *H. hepaticus* for two consecutive days (+*Hh*). Mice were sacrificed on day 14 and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing expression of IL-10 (GFP) by colonic Tregs. Gated on single live TCRβ$^+$CD4$^+$hCD2$^+$. Foxp3^{hCD2} mice were used for IL-10 control.
(B) Frequency (left) and total number (right) of colonic TCRβ$^+$CD4$^+$hCD2$^+$IL-10$^+$ Tregs.
(C) Representative flow cytometry plots showing expression of ST2, Gata3 and Rorγt by IL10(GFP)$^+$ colonic Tregs. Gated on single live TCRβ$^+$CD4$^+$hCD2$^+$IL-10$^+$ cells.
(D) Frequency of ST2$^+$, Gata3$^+$ and Rorγt$^+$ among TCRβ$^+$CD4$^+$hCD2$^+$IL-10$^+$ colonic Tregs at steady state.
(E) Frequency (top) and total number (bottom) ST2$^+$, Gata3$^+$ and Rorγt$^+$ colonic IL-10$^+$ Tregs.

Each point represents a single mouse and bars represent mean ±SEM.

Data are a representative of one experiment of two independent experiments (n=6 for each experimental group). Statistical significance was determined using a Mann Whitney U Test.
* P ≤ 0.05, ** P ≤ 0.01.

3.2.10 ST2^{+}/Gata3^{+} Tregs are reduced in the colon of IL-10-deficient mice.

It has been previously reported that IL-10 is not only important for Treg-mediated immunosuppression, but is also required for the stability and function of Tregs under inflammatory conditions (Murai et al., 2009, Chaudhry et al., 2011). Here, we set out to investigate colonic Treg populations in the absence of IL-10 using IL-10-deficient (IL-10$^{-/-}$) mice. IL-10$^{-/-}$ mice develop chronic enterocolitis when kept under conventional conditions ('dirty'), which becomes limited to the proximal colon under SPF conditions (Kuhn et al., 1993).

We used young, 6-7 week old, mice to minimise the confounding effects of possible intestinal inflammation development; however, histological analysis revealed that in our animal facility IL-10$^{-/-}$ mice did not develop macroscopic intestinal inflammation (Fig. 3.11 A, B). Nevertheless, there were more cells isolated from the colonic LP of the IL-10$^{-/-}$ mice when compared to age-matched wild type mice (data not shown).

Concordant with previous reports (Chaudhry et al., 2011), the frequency and number of total colonic Tregs was increased in the absence of IL-10 (22.8% in WT vs 27.8% in IL-10$^{-/-}$) (Fig. 3.11 C), and Foxp3 levels did not differ based on the gMFI of Foxp3 (data not shown).

On one hand, a significant decrease in the frequency of ST2^{+} Tregs (44.8% in WT vs 28.8% in IL-10$^{-/-}$) and Gata3^{+} Tregs (33.5% in WT and 17.9% in IL-10$^{-/-}$) was observed, which was consistent with a decrease in Helios^{+} Tregs (40.1% in WT and 25.1% in IL-10$^{-/-}$) (Fig. 3.12 A, B, C). On the other hand, Rorγt^{+} Tregs were significantly increased in the

absence of IL-10 (37.5% in WT vs 56.1% in IL-$10^{-/-}$) (Fig. 3.12 B, C). Due to an increase in total cell number in the IL-$10^{-/-}$ animals, Treg numbers of specific populations either remained unchanged ($ST2^+$ Tregs) or increased ($Gata3^+$, $Ror\gamma t^+$ and $Helios^+$) (Fig. 3.12 D).

Additionally, we investigated Th2 and Th17 cells defined as $TCR\beta^+CD4^+Foxp3^-Gata3^+$ and $TCR\beta^+CD4^+Foxp3^-Ror\gamma t^+$, respectively, and detected a decrease in the frequency of Th2 cells and an increase in Th17 cells (data not shown). Of note, no T-bet expression could be detected.

Finally, using IL-$10R^{-/-}$ mice in the same experimental setting described above, we obtained data that mirrored our findings described above from the IL-$10^{-/-}$ mice (data not shown).

Together the results demonstrate that in absence of IL-10 the balance of colonic LP Tregs changes and there is a significant decrease in thymic-derived $ST2^+$/$Gata3^+$ Tregs, and an associated expansion of induced $Ror\gamma t^+$ Tregs.

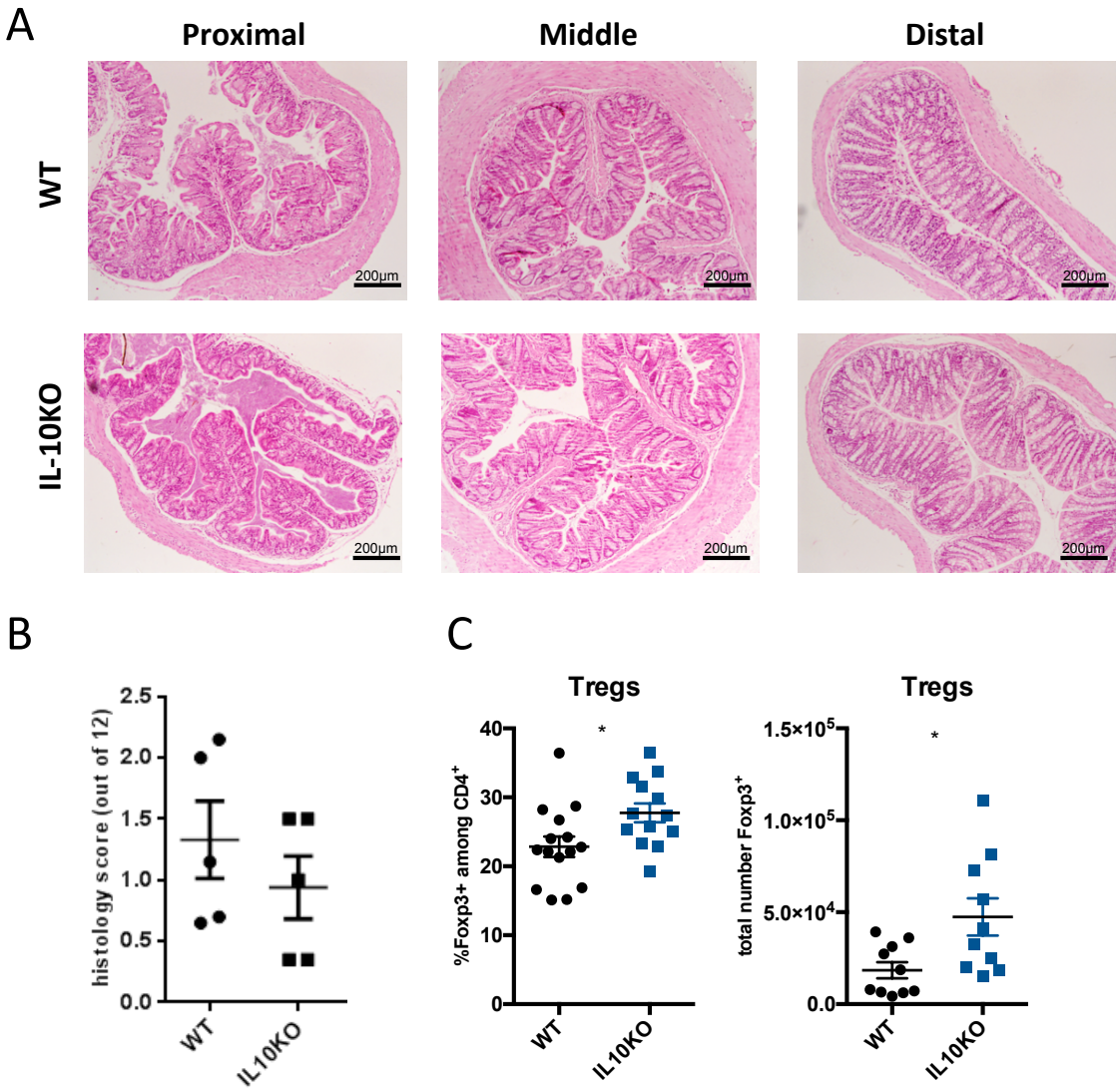


Figure 3.11. IL-10-deficient mice do not have obvious signs of inflammation in the colonic LP but harbour increased numbers of Tregs.

6-7 week old C57BL/6 (WT) and IL-10KO mice were sacrificed at steady state, and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative microphotographs of the colonic sections. Bar size - 200µm.
(B) Histological analysis of colonic inflammation.
(C) Frequency (left) and total number (right) colonic $TCR\beta^{+}CD4^{+}Foxp3^{+}$ Tregs.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents pooled results from three independent experiments (n=15 WT and n=13 IL-10KO). Statistical significance was determined using a Mann Whitney U Test. * $P \leq 0.05$.

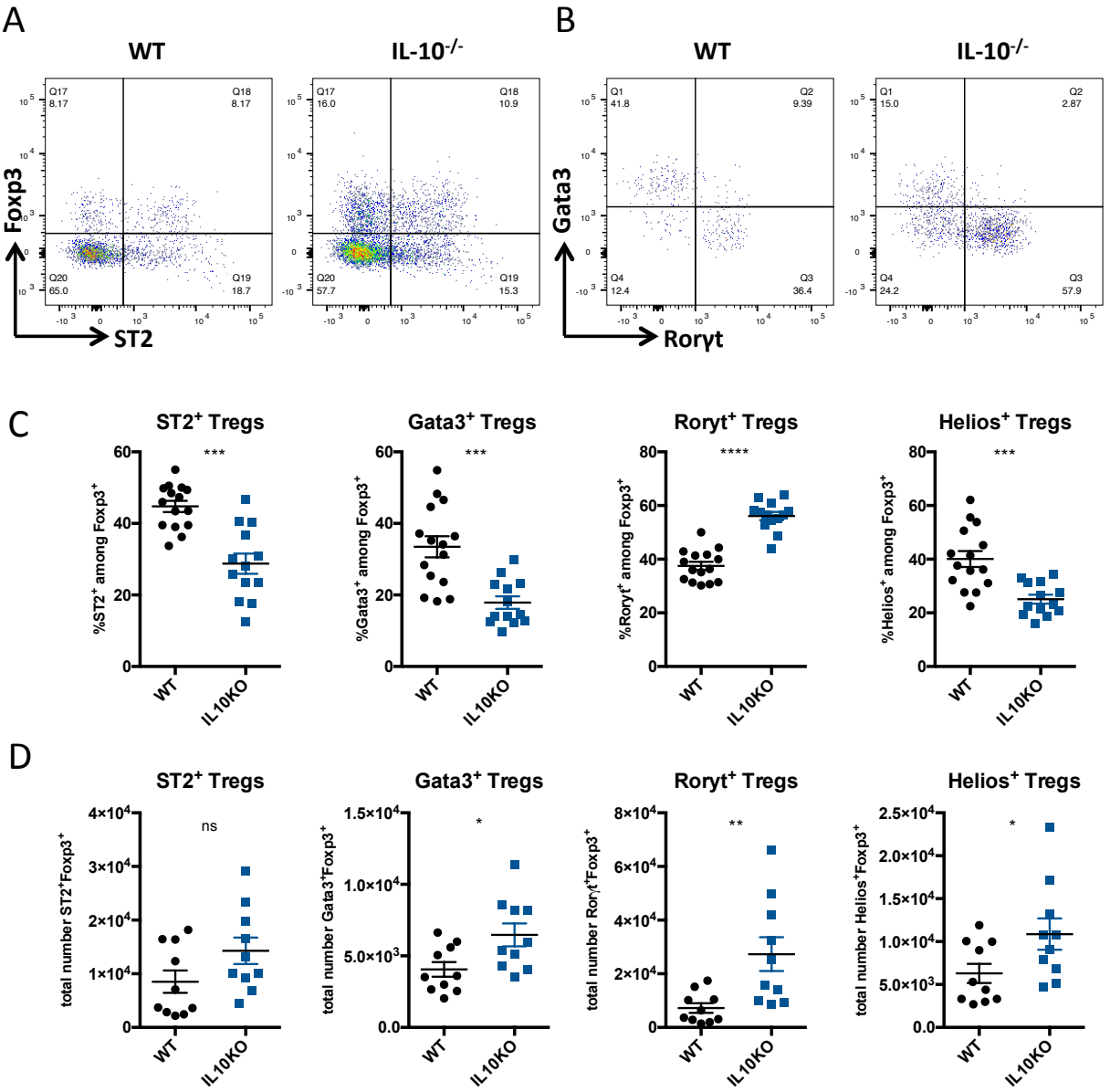


Figure 3.12. ST2$^+$ and Gata3$^+$ Tregs are reduced in the colon of IL-10-deficient mice.

6-7 week old C57BL/6 and IL-10KO mice were sacrificed at steady state, and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing expression of Foxp3 and ST2 in WT controls and IL-10KO. Gated on single live TCRβ$^+$CD4$^+$.
(B) Representative flow cytometry plots showing expression of Gata3 and Rorγt$^+$ in WT controls and IL-10KO. Gated on single live TCRβ$^+$CD4$^+$Foxp3$^+$.
(C) Frequency of ST2$^+$, Gata3$^+$, Rorγt$^+$ and Helios$^+$ among TCRβ$^+$CD4$^+$Foxp3$^+$ colonic Tregs.
(D) Total number of ST2$^+$, Gata3$^+$, Rorγt$^+$ and Helios$^+$ colonic TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents pooled results from three independent experiments (n=15 WT and n=13 IL-10KO). Statistical significance was determined using a Mann Whitney U Test. ns not significant, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

3.2.11 ST2$^+$/Gata3$^+$ Tregs are reduced in the colon upon colitis.

Tregs are key to maintaining intestinal homeostasis and their non-redundant role in the control of intestinal inflammation has been demonstrated multiple times, including the capacity of Tregs to prevent the onset and to even cure established colitis (Powrie et al., 1993, Powrie et al., 1994a, Maloy et al., 2003, Mottet et al., 2003).

To test whether the balance of distinct colonic Tregs populations described in detail in previous sections is altered upon the induction of colitis, we investigated colonic Tregs in the context of bacteria-driven intestinal inflammation in wild type mice. While the *H. hepaticus*/anti-IL-10R-driven model of colitis provides a tool to study Treg populations in a lymphocyte-replete setting, the blockade of IL-10 signalling in this model might influence the phenotype of these cells. Since bacterial colonisation alone can modulate Treg phenotype, we also assessed mice treated with *H. hepaticus* alone to understand the effects that can be attributed solely to bacterial colonisation.

As previously highlighted, Treg frequency was increased upon infection with *H. hepaticus* (30.3% steady state vs 42.7% *Hh*) (Fig. 3.13 A, B). This was associated with a significant decrease in the frequency of ST2$^+$ Tregs and a trend towards a decrease in Gata3$^+$ Tregs (57.9% steady state vs 43.0% *Hh* and 25.6% steady state vs 13.6% *Hh* for ST2 and Gata3, respectively) (Fig. 3.13 A, C). *H. hepaticus* infection led to an increase in the frequency of Rorγt$^+$ Tregs (14.7% steady state vs 29.9% *Hh*) (Fig. 3.13 A, C). This is consistent with Rorγt$^+$ Tregs representing pTregs and previous studies suggesting that Rorγt$^+$ Tregs require microbial signals for their development (Sefik et al., 2015, Ohnmacht et al., 2015, Yang et al., 2016a).

Under the inflammatory conditions induced by *H. hepaticus* infection with concomitant blockade of IL-10 signalling the decrease in frequency of both $ST2^+$ Tregs and $Gata3^+$ Tregs was even more pronounced (57.9% steady state vs 38.5% *Hh*/anti-IL-10R and 25.6% steady state vs 7.4% *Hh*/anti-IL-10R for ST2 and Gata3, respectively) (Fig. 3.13 A, C). By contrast, the increased frequency of $Ror\gamma t^+$ Tregs induced by bacterial colonisation was no longer significant in mice with colitis (14.7% steady state vs 20.2% *Hh*/anti-IL-10R) (Fig. 3.13 A, C). Nevertheless, $Ror\gamma t^+$ Treg numbers were increased during intestinal inflammation (Fig. 3.13 C). Helios expression was not investigated in this experimental set-up since it has been reported as a stable marker of tTregs only under homeostatic conditions (Thornton et al., 2010).

Together the results show that inflammatory environment, such as in mice treated with *H. hepaticus* and anti-IL-10R blocking antibody, negatively influences $ST2^+/Gata3^+$ colonic Tregs. $Ror\gamma t^+$ Tregs while increased upon bacterial infection do not change significantly in frequency in colitic animals.

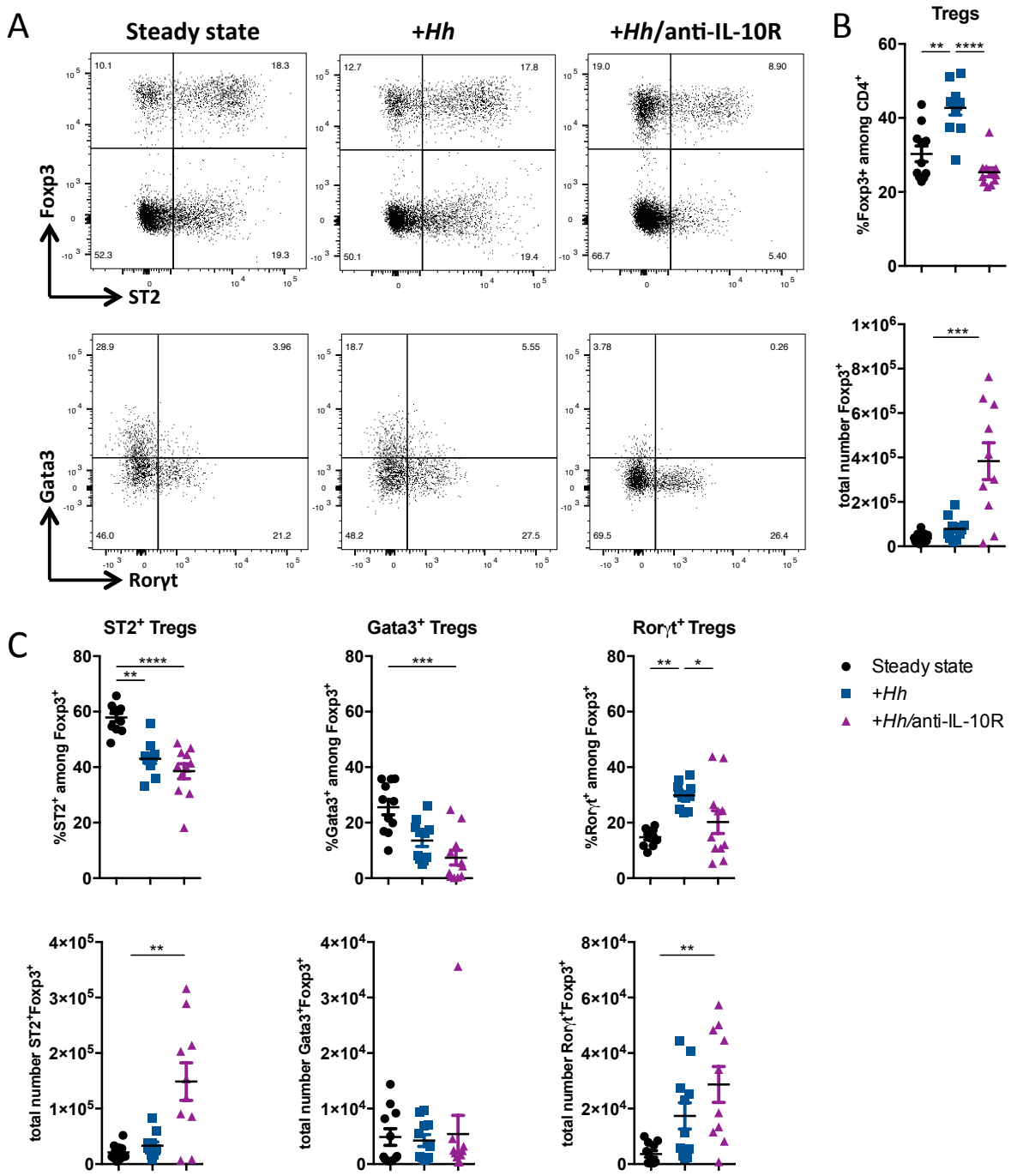


Figure 3.13. ST2$^+$ and Gata3$^+$ Tregs are reduced in the colon upon bacteria-driven colitis.

C57BL/6 mice were sacrificed without treatment (steady state) or infected with *Helicobacter hepaticus* either alone (+*Hh*) or with concomitant i.p. injections of a blocking anti-IL-10R mAb (+*Hh*/anti-IL-10R). Mice were sacrificed on day 14 and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing expression of Foxp3 and ST2 by colonic CD4$^+$ T cells (top) and Gata3 and Rorγt by colonic Tregs (bottom). Gated on single live TCRβ$^+$CD4$^+$ (top) and single live TCRβ$^+$CD4$^+$Foxp3$^+$ (bottom) cells isolated from the colon.
(B) Frequency (top) and total number (bottom) of colonic TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs.
(C) Frequency (top) and total number (bottom) of ST2$^+$, Gata3$^+$ and Rorγt$^+$ colonic TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents results of one experiment representative of five individual experiments (n=11 for each experimental group). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

3.3 Discussion

Tregs are a key immune cell type necessary for maintaining tolerance. They can be found throughout the body, both in the lymphoid and non-lymphoid tissues. Recently their unique role in non-lymphoid tissues has been highlighted. Heterogeneity amongst the tissue-resident Tregs exists, and here for the first time we have comprehensively evaluated distinct Treg populations found in the colonic LP.

Under steady state conditions primarily two distinct Treg populations can be identified in the colon, which are characterised by expression of master transcriptional regulators associated with Th2 and Th17 type immune responses, Gata3 and Rorγt, respectively. We have previously shown that ST2-expressing Tregs are enriched in the large intestine, and here, we identify ST2-expressing Tregs as predominantly Gata3^{+}. Gata3 has previously been shown to be an important transcription factor for Treg function and survival (Wang et al., 2011, Wohlfert et al., 2011, Rudra et al., 2012). Gata3-deficient Tregs have an intrinsic defect in homeostasis (Wang et al., 2011), exhibit reduced suppressive function and convert towards an effector T cell phenotype under inflammatory conditions (Wang et al., 2011, Wohlfert et al., 2011). Wohlfert *et al* (2011) also showed that there was a substantial population of Gata3-expressing Tregs in the small intestinal LP, and that the expression of Gata3 and Rorγt was mutually exclusive in these Tregs. This is consistent with our finding that ST2^{+} Tregs co-express Gata3 and are a distinct subset from the Rorγt-expressing colonic LP Tregs. Rorγt^{+} colonic Tregs have been the subject of three very recent studies (Ohnmacht et al., 2015, Sefik et al., 2015, Yang et al., 2016a) that identified these Tregs as the

microbiota-induced population, suggesting possible differences in antigen specificity between Rorγt^{+} and ST2^{+}/Gata3^{+} subsets.

We have found ST2^{+} Tregs to be most abundant in the lung, skin and colon, all of which are organs exposed to large numbers of bacteria. Notably ST2-expressing Tregs were also most enriched in the colon compared to the small intestine and while the gradient of dietary antigens decreases down the length of the GI tract, most of the commensal bacteria colonise the colon. While these observations would point to the induction of ST2^{+} Tregs by microbial signals, it is the Rorγt^{+} Treg population that was shown to be absent in germ-free mice or antibiotic-treated mice (Ohnmacht et al., 2015, Sefik et al., 2015). This data is in line with our finding that Rorγt^{+} Tregs are expanded upon weaning in mice, with the ST2^{+}/Gata3^{+} population found in the gut already present at 3 weeks of age. Concordant with this is the finding that ST2^{+} Tregs in the colonic LP are Helios^{+}, which under homeostatic conditions marks thymic-derived Tregs (Thornton et al., 2010). Moreover, a recent report described a population of Tregs that develops during the perinatal window of mouse development, persists into adulthood and is specialised in establishing self-tolerance and preventing autoimmunity (Yang et al., 2015). These Tregs had a distinct transcriptional profile, and notably, were enriched in *St2* expression. It could be speculated that the Treg population that develops early on in life in the thymus seeds the colonic niche first, and is subsequently complemented by Rorγt^{+} Tregs, which are induced by microbial signals upon exposure to the microbiota.

Further study of the ST2-expressing Tregs in the colon showed that they exhibit a highly suppressive phenotype as assessed by the expression of a number of surface markers previously associated with Treg regulatory function including CD25, CD103, KLRG-1, PD-1, TIGIT, GITR, ICOS and CTLA-4. They have also been shown to express high amounts of OX40 (Schiering et al., 2014), known to promote Treg accumulation in the colon (Griseri et al., 2010). Similarly, VAT Tregs, which also comprise a large population of ST2-expressing cells, expressed high amounts of activation markers associated with Treg function (Feuerer et al., 2009). Rorγt^{+} Tregs also displayed an activated phenotype characterised by CD44^{high}CD62L^{low} expression and had increased amounts of ICOS and CTLA4 (Ohnmacht et al., 2015), which reflects our own findings.

A comparative transcriptomic analysis of colonic ST2^{+} Tregs and their negative counterparts provided a global perspective on the profile of ST2-expressing Tregs in the colon. We saw enrichment of *Dgat2* in the ST2^{+} fraction. DGATs encoded by *Dgat1* and *Dgat2* are isoenzymes involved in lipid metabolism; they catalyse the final step in triglyceride biosynthesis. DGAT enzymes expressed in enterocytes were also reported to be important for intestinal fat absorption (Cheng et al., 2008). Interestingly, RNAseq analysis of Tregs residing in the VAT identified genes encoding DGAT enzymes as one of the most upregulated transcripts when compared to lymphoid Tregs (Feuerer et al., 2009). In addition, high levels of *Dgat* transcripts were also found in injured muscle Tregs (according to the RNAseq data found in Supplementary Table 1 in Panduro et al. (2016)). These results point to DGAT's potentially important role in tissue-specific metabolic adaptation. In addition, a high frequency of Tregs in both VAT and muscle express ST2, therefore, it would be interesting to investigate whether DGAT enzymes

are induced in response to IL-33. Furthermore, since there are no published reports on DGAT function in T cells, its functional relevance in Tregs is worth following up.

Since Tregs have been shown to promote tissue repair in a number of experimental settings, increased expression of *Areg*, which encodes amphiregulin – a member of the epidermal growth factor family, in colonic $ST2^+$ Tregs is worth highlighting. Both VAT- and skeletal muscle-resident Tregs express high levels of *Areg* (according to the RNAseq data found in Supplementary Table 1 in Panduro et al. (2016)). Burzyn *et al* (2013) demonstrated that depletion of Tregs in a muscle injury model inhibits muscle repair processes, and this could be rescued by administration of exogenous amphiregulin, suggesting that Tregs can mediate repair in the tissue at least partially by amphiregulin production. In further support of the amphiregulin-associated repair module of the tissue Treg population, lung damage upon influenza virus infection was significantly more pronounced in $Areg^{Fl/Fl}Foxp3^{YFP\text{-}cre}$ mice, in which amphiregulin is selectively depleted in $Foxp3^+$ Tregs, than in wild type controls (Arpaia et al., 2015). Amphiregulin has also been suggested to promote the suppressive capacity of Tregs by promoting Foxp3 expression and thus, inhibiting the GSK-3β/β-TrCP pathway (Wang et al., 2016, Zaiss et al., 2013). In our mice, however, we were neither able to detect the expression of the EGFR protein on Tregs nor any *Egfr* transcripts in any of the microarray analyses performed by our group. It is possible that under steady state conditions and in the colitis models we tested in our animal facility EGFR either does not become induced in Tregs or its levels are very low. Therefore, it could be speculated that amphiregulin binding to the EGFR on Tregs is not critical for the Treg function.

CXCR3 is a chemokine receptor that binds three different ligands induced upon IFN-γ signalling: CXCL9, CXLC10 and CXCL11 (Luster et al., 1985, Farber, 1990, Cole et al., 1998). It is primarily expressed on Th1 $CD4^+$ T cells and $CD8^+$ T cells and allows for efficient immune cell trafficking to sites of Th1-mediated inflammation (Groom and Luster, 2011). Tregs have been reported to upregulate T-bet and as a consequence CXCR3 expression in a Th-1 inflammatory milieu and were important for the regulation of effector responses in this setting (Koch et al., 2009). We found *Cxcr3* to be among the most significantly downregulated transcripts in the colonic $ST2^+$ Treg population, which is concordant with lack of T-bet expression in the colonic LP Tregs in our mice. Additionally, *Cxcr3* expression was reduced in VAT Tregs (according to the RNAseq data found in Supplementary Table 1 in Panduro et al. (2016)).

Data from our microarray analysis further supports the existence of multiple Treg populations, which represent some obvious functional specialisations. However, despite showing that there are differentially expressed genes between $ST2^+$ and $ST2^-$ Tregs in the colonic LP, the number of genes with substantial fold changes (>2) as well as the total number of significantly up- and downregulated genes was not high. This can be due to both technical issues related to the low number of colonic cells recovered from the colon and the isolation of Tregs from steady state mice. It can be speculated that upon challenge, such as inflammation, differences between the distinct populations would be more pronounced due to enhanced gene transcription as well as upregulation of genes in response to external stimuli.

The microbiota composition can have a profound effect on the colonic Tregs, and GF and antibiotic-treated mice have been reported to display decreased Treg numbers in the colonic LP (Atarashi et al., 2011, Geuking et al., 2011). In recent years, multiple reports were published describing the ability of specific microbial species or mixtures of bacteria to induce Tregs or to promote their accumulation in the colon (reviewed in Tanoue et al. (2016)). Among others, *Clostridia* (Atarashi et al., 2011), *Lactobacillus* (Tang et al., 2015) and *Bacteroides* (Round et al., 2011) could promote Tregs in the gut. The results from our laboratory demonstrated that infection with a mouse pathogen, *H. hepaticus*, also induces Tregs in the colonic LP (Powrie group, unpublished data). Here, we phenotyped these cells and showed that *H. hepaticus* infection resulted in an expansion of the Rorγt$^+$ Treg population. This was also associated with a decreased frequency of ST2$^+$/Gata3$^+$ Tregs. Rorγt$^+$ Tregs lacked Helios expression, suggesting that they were peripherally induced. Interestingly, Sefik et al (2015) showed that Rorγt$^+$ Tregs could be induced in the murine colon by colonisation with a broad range of bacterial species including but not limited to Firmicutes, Proteobacteria, or Bacteroidetes phyla. The mechanism of induction of Rorγt$^+$ Tregs remains unknown to date despite reports suggesting that DCs, MHCII and the vitamin A metabolite retinoic acid are required (Ohnmacht et al., 2015). Future experiments employing either tetramer staining or high-throughput TCR sequencing could allow to illuminate whether Rorγt$^+$ Treg are enriched for TCRs specific to microbial antigens.

Interestingly, infection with *H. hepaticus* also specifically leads to induction of IL-10-producing cells in the colon (Kullberg et al. (2002), (Powrie group, unpublished data), including Foxp3$^+$ Tregs. Colonic Tregs are known to produce unusually large amounts

of IL-10 (Kamanaka et al., 2006, Maynard et al., 2007, Atarashi et al., 2011, Schiering et al., 2014), and this is also a characteristic of other tissue-resident Tregs, such as those found in the VAT or the muscle (Feuerer et al., 2009, Burzyn et al., 2013). Here, we attributed most of the steady state IL-10 production to $ST2^+$/$Gata3^+$ Tregs. By contrast, IL-10^+ Tregs, which expanded upon *H. hepaticus* infection, were predominantly Rorγt$^+$. Other microbial species can also promote IL-10 production by colonic Tregs (Round et al., 2011), with some reports specifically highlighting IL-10-producing Rorγt$^+$ Tregs (Ohnmacht et al., 2015). Of note, Ohnmacht et al. (2015) and Yang et al. (2016a) observed higher IL-10 production by Rorγt$^+$ Tregs compared to their negative counterparts under homeostatic conditions, which contrasts our findings. This discrepancy could reflect differences of the endogenous flora or diet in the animal facilities where the experiments were performed, especially since microbial-derived and oral antigens are major drivers modulating the development of Rorγt$^+$ Tregs according to the outlined studies (Ohnmacht et al., 2015, Sefik et al., 2015).

Finally, the data from our group and others strongly implicate the microbiota as a major driver of the balance amongst distinct colonic Treg populations. Specifically the induction of IL-10-producing cells by the microbiota could represent a protective mechanism that prevents unnecessary inflammatory reactions in response to healthy gut flora. Furthermore, since IL-10 expression by $Foxp3^+$ T cells was demonstrated to be important in the cure model of colitis (Uhlig et al., 2006a), in which $CD4^+CD25^+$ T cells are administered to the mice only after inflammation has been already established, it would be of particular interest to establish which population of Tregs

produced IL-10 in this setting in order to enhance the capacity of Tregs to cure the intestinal pathology.

It has also been recently acknowledged that Tregs can adapt to specific inflammatory environments by upregulating the expression of transcription factors typically associated with the differentiation programme of effector Th cells, such as T-bet, Gata3, IRF4 or Stat3 (Liston and Gray, 2014). Deletion of the 'master regulators' of Th cell differentiation in Tregs leads to subset-specific deregulation of effector responses, which suggests that the ability of Treg cells to adapt to their surrounding inflammatory milieu is critical for preventing the development of immunopathology (Chaudhry et al., 2009, Koch et al., 2009). Here, we have investigated colonic Tregs in a bacteria-driven model of colitis, where intestinal inflammation is induced by *H. hepaticus* infection and concomitant blockade of IL-10 signalling. This model is IL-23-dependent and has a Th1/Th17 signature with IL-17A, IL17F and IFN-γ being highly elevated (Kullberg et al., 2006). The $ST2^+$/$Gata3^+$ Treg population was significantly reduced under inflammatory conditions, with the frequency of $Ror\gamma t^+$ Tregs not changing and absolute numbers increasing. While the investigated model allows studies of the colitic environment in a lymphocyte-replete setting in genetically unaltered mice, the blockade of IL-10 signalling might limit its utility in the investigation of Tregs. This is particularly worth highlighting because we observed increased expression of IL-10R on $Gata3^+$ Tregs compared to $Ror\gamma t^+$ Tregs, which could be of importance for $ST2^+$/$Gata3^+$ Treg homeostasis or function.

Previously, a specific ablation of IL-10Rα in Foxp3^{+} T cells has been shown to result in severe spontaneous colitis due to an impaired capacity of IL-10Rα-deficient Tregs to control Th17 responses (Chaudhry et al., 2011). IL-17-producing cells were also substantially expanded in the small intestinal LP of transgenic mice, which had IL-10Rα depleted selectively in CD4^{+} T cells through the expression of a dominant-negative IL-10Rα on all CD4^{+} T cells (Huber et al., 2011). Additionally, an earlier study demonstrated that mice with a specific deletion of STAT3 in Tregs (*Foxp3*$^{Cre/Cre}$*Stat3*$^{fl/fl}$) suffered from fatal intestinal inflammation (Chaudhry et al., 2009). This further supports the critical role of IL-10R expression on Tregs since IL-10 signals via STAT3. Here, we also reported an increase in Th17 cells in the intestine in absence of IL-10 (full IL-10KO). This was associated with an increase in Rorγt^{+} Tregs and corresponding decrease in ST2^{+}/Gata3^{+} Tregs. Exploring Tregs in a bone marrow chimaera setting with both IL-10R-deficient and –sufficient Tregs present would allow us to specifically investigate a potential role of IL-10R expression in distinct colonic Treg subtypes.

Previous work for our group also showed that ST2$^{-/-}$ Tregs had an impaired suppressive ability compared to WT Tregs in a Th1/Th17-driven T cell transfer model of colitis (Schiering et al., 2014). While further work on the functional relevance of ST2^{+}/Gata3^{+} Tregs is required, recent reports investigated the role of Rorγt^{+} Tregs in the control of the intestinal inflammation. Ohnmacht et al. (2015) for example, proposed that Rorγt^{+} Tregs contribute to the control of type 2 immunity. They demonstrated that Foxp3^{Cre}x Rorγt$^{fl/fl}$ mice, in which Rorγt is specifically deleted in Tregs, had elevated amounts of Th2-associated cytokines and suffered from exacerbated pathology, and even lethal colitis, in Oxazolone-induced colitis, which is typically considered a type 2 cytokine-

driven model of colitis. By contrast, Rorγt-deficiency in Tregs did not influence the outcome of DSS-induced colitis (a type 1/type 17 cytokine-driven model of colitis). Sefik et al. (2015), on the other hand, observed worsened pathology in Foxp3^{Cre}x Rorγt$^{fl/fl}$ mice upon Trinitrobenzene Sulfonic Acid (TNBS)-induced colitis (a type 1/type 17 cytokine-driven model of colitis) arguing that Rorγt^{+} Tregs play a role in the regulation of Th1/Th17 inflammatory responses. Both the animal model used in the outlined studies as well as the endogenous microflora could explain the differences in the results.

In summary, the data presented in this chapter demonstrated that ST2^{+}Gata3^{+} Tregs are likely a thymic-derived highly immunosuppressive population of Tregs enriched at barrier sites, including the colon. We also showed that the ST2^{+}Gata3^{+} Tregs are distinct from the Rorγt^{+} Tregs and that there is a clear balance between these two populations in the colonic LP, which might be due to functional differences between the two populations. Furthermore, we highlighted IL-10 as a major factor controlling the balance of ST2^{+}Gata3^{+} Tregs and Rorγt^{+} Tregs in the colonic LP.

Chapter 4. The role of IL-33 in the control of immune responses

4.1 Introduction

IL-33 is a member of the IL-1 family of cytokines, which are potent amplifiers of both innate and adaptive immune responses. IL-33 is a pleiotropic cytokine originally described as an inducer of type 2 immune responses (Xu et al., 1998); however, today it is well established that its receptor, ST2, is expressed on a wide range of immune cells. The mechanism of IL-33 signalling is similar to that of other TIR-domain-containing family members: IL-1RAcP is recruited upon IL-33 binding to ST2, leading to the formation of a signalling complex including MyD88, IRAK1 and 2, TRAF6, and subsequent activation of MAPK and NF-κB (reviewed in Liew et al (2010)).

As described in Chapter 1, IL-33 has been implicated in a number of disease conditions, including infections, inflammatory and autoimmune diseases, cancer, and graft rejections. Since its discovery in 2005 multiple studies have explored the role of IL-33 in the context of human IBD and various murine models of colitis. Genetic polymorphisms in both *IL33* and *IL1RL1* have been found to be significantly associated with both of the most common forms of IBD – CD and UC – in large patient cohorts (Latiano et al., 2013). Levels of IL-33 and ST2 have also been repeatedly reported to be increased in mucosal samples from both UC (Beltran et al., 2010, Kobori et al., 2010,

Pastorelli et al., 2010, Seidelin et al., 2010, Sedhom et al., 2013, Waddell et al., 2015) and CD (Pastorelli et al., 2010, Sedhom et al., 2013) patients, preferentially in the patients with active disease (Pastorelli et al., 2010, Sedhom et al., 2013).

Similar observations have been made in murine studies with increased amounts of IL-33 reported in mice with colitis induced by DSS (Pushparaj et al., 2013, Sedhom et al., 2013, Monticelli et al., 2015), TNBS (Duan et al., 2012, Sedhom et al., 2013) and Oxazolone (Waddell et al., 2015), as well as in SAMP1/YitFc mice, a spontaneous mixed Th1/Th2-driven model of IBD (Pastorelli et al., 2010). Studies employing genetically modified mouse strains, however, have not provided a clear answer on the role of IL-33 in colitis. Oboki et al (2010) generated the first IL-33KO mouse, and using an acute model of DSS-induced colitis showed that mice deficient in IL-33 displayed less severe pathology and experienced reduced mortality compared to wild type controls. This is in line with experiments conducted using ST2KO animals, in which mice unable to respond to IL-33 had reduced body weight loss, less diarrhoea and less pronounced colon shortening upon DSS-induced colitis (Pushparaj et al., 2013, Sedhom et al., 2013). Furthermore, this was replicated using an anti-ST2 antibody (Sedhom et al., 2013). Sedhom et al (2013) also demonstrated reduced disease severity in a TNBS-induced model of colitis in ST2-deficient mice. The pathological role of IL-33 in intestinal inflammation was also highlighted in a number of studies, in which exogenous IL-33 was administered, resulting in aggravated disease in an acute model of DSS-induced colitis (Groβ et al., 2012, Pushparaj et al., 2013, Sedhom et al., 2013). Sedhom et al (2013) observed increased translocation of fluorescently labelled dextran across the epithelial barrier upon IL-33 administration suggesting that IL-33 impacted

on barrier integrity. Conversely, Pushparaj et al (2013) attributed the pathological role of IL-33 to its ability to promote pro-inflammatory cytokines and chemokines. Surprisingly, a different study using the TNBS-induced model of colitis reported an amelioration of colonic tissue injury following injections of IL-33, attributing it to a change in the cytokine profile from Th1 to Th2 type cytokines (Duan et al., 2012). Finally, in Oxazolone-induced colitis, which is thought to recapitulate most symptoms of UC, due to its Th2-dominant profile, animals deficient in either IL-33 or its receptor ST2 suffered from worse disease pathology than the wild type mice (Waddell et al., 2015).

While IL-33 has been shown to have a predominantly pathological effect in acute models of colitis, its role in a chronic setting is less studied. In the first publication using IL-33-deficient mice, Oboki et al (2010) observed that IL-33-deficient mice had a longer recovery phase in DSS-induced colitis compared to the wild type animals. Subsequently, it has been shown that administration of recombinant IL-33 in a chronic model of DSS-induced colitis (during which animals receive recurrent rounds of DSS dissolved in water interspersed with rounds of pure water for an extended period of time) improved disease symptoms by promoting wound healing (Groβ et al., 2012, Zhu et al., 2015). This was due to a reduction in IFN-γ and IL-17A-producing $CD4^+$ T cells and a shift towards an increase in Th2 cells and corresponding Th2-associated cytokines (IL-4, IL-5 and IL-13). Since IL-33 induces enhanced epithelial cell hyperplasia, mucus production as well as goblet cell proliferation, all of which promote tissue barrier integrity, it is not surprising that it could play an important role in the recovery of damaged tissue.

In line with this promotion of barrier integrity is the fact that IL-33 functions as an alarmin, which is a molecule that is released upon cell injury to attract cells of the immune system to the site of tissue damage. Indeed, the role of IL-33 in the maintenance of tissue homeostasis and in the response to environmental changes has been emphasised recently. Tissue homeostasis is also tightly regulated by Tregs, which can limit tissue damage during infection (reviewed in Veiga-Parga et al. (2013)) and promote tissue repair (Burzyn et al., 2013, Arpaia et al., 2015). As discussed in Chapter 3, Tregs expressing the IL-33 receptor, ST2, are found primarily in non-lymphoid tissues, with Tregs found in the colon, skin, lung, VAT and muscle expressing the highest amounts of ST2. The exact role of IL-33 in Treg development, survival and function has yet to be defined, however, a few recent publications have addressed this question. Turnquist et al (2011) were the first to describe the capacity of IL-33 to promote ST2-expressing Tregs in a cardiac allograft model. IL-33 treatment preferentially expanded the $ST2^+$ population of Tregs improving allograft survival (Turnquist et al., 2011). In the VAT, IL-33 deficiency has been reported to result in a significant reduction in Treg numbers (Kolodin et al., 2015, Molofsky et al., 2015, Vasanthakumar et al., 2015), and exogenous administration of IL-33 significantly expanded the VAT Treg population with a preferential increase in $ST2^+KLRG\text{-}1^+$ Tregs (Han et al., 2015, Kolodin et al., 2015, Molofsky et al., 2015, Vasanthakumar et al., 2015). Miler et al (2010) also showed that recombinant IL-33 administration improved metabolic parameters in genetically obese *Ob/Ob* mice; however, in this study the authors did not investigate Foxp3 expression, and the improvement in disease was attributed to the expansion of Th2 cells and polarisation of adipose tissue macrophages towards the alternative M2 phenotype (Miller et al., 2010). Similarly to

VAT-resident Tregs, injection of IL-33 increased numbers of Tregs, specifically ST2-expressing Tregs, in the skeletal muscle, and improved muscle regeneration following a cryo-injury inflicted to the muscle (Kuswanto et al., 2016). In addition, this report also investigated the nature of the upregulated pathways in Tregs following exogenous IL-33 administration. It was demonstrated that IL-33 promoted cell proliferation, replication and cycling. This was in line with an experiment involving the transgenic Kaede mice (in these mice cells become photo-converted from green to red upon exposure to ultraviolet light), which showed that Tregs in response to IL-33 were not recruited from the circulation but rather proliferated *in situ* (Kuswanto et al., 2016). While most of the aforementioned studies have shown that IL-33 can directly influence Tregs by using *in vitro* stimulations (Turnquist et al., 2011, Vasanthakumar et al., 2015, Kuswanto et al., 2016), others have highlighted different mechanisms of action. For instance, Molofsky et al (2015) argued that ICOS/ICOSL interactions dependent on ILC2 were required for IL-33-mediated induction of Tregs in the VAT. By contrast, Matta et al (2014) suggested that ST2-expressing $CD11c^+$ DCs were critical for the expansion of $ST2^+$ Tregs through the production of IL-2. Mast cells were also reported as another source of innate-cell-derived IL-2 promoting Tregs (Morita et al., 2015). Since the described studies have used different experimental settings, it is likely that all of the investigated mechanisms might have relevance for the IL-33-mediated induction of Tregs; however, the inflammatory conditions, external environment and the immune cell network involved in the immune response are all likely to be determining factors of the precise mechanism of action of IL-33.

In this chapter we interrogate the IL-33 signalling pathway in Tregs, and how it is modulated by other factors using multiple *in vitro* and *in vivo* approaches. The main objectives of the chapter are:

- To determine the effects of absence of IL-33 signalling on the colonic Tregs
- To describe the effects of ST2 deficiency in a lymphocyte-dependent chronic model of colitis
- To define downstream events of IL-33 signalling in Tregs
- To identify negative regulators of IL-33 signalling.

4.2 Results

4.2.1 ST2 deficiency does not impair colonic Treg development.

Since we and others identified ST2 as an important marker of tissue-resident Tregs (Schiering et al., 2014, Vasanthakumar et al., 2015, Kuswanto et al., 2016) we set out to investigate whether IL-33 signalling is required for the development of colonic Tregs under homeostatic conditions.

Flow cytometric analysis of wild type and $ST2^{-/-}$ mice revealed that the colonic LP of ST2-deficient animals harboured a higher frequency of Tregs than the controls (23.5% in WT and 31.3% in $ST2^{-/-}$) (Fig. 4.1 A, B). Frequency of Foxp3-expressing T cells in the spleen was similar between the two groups (data not shown). To better understand the increase in Tregs in the absence of IL-33 signalling we also investigated whether the amount of Foxp3 on a per cell basis was different. However, colonic Tregs from both the wild type and $ST2^{-/-}$ mice expressed comparable amounts of the Foxp3 protein (Fig. 4.1 C). Intracellular transcription factor staining demonstrated that there were no differences in the distribution of $Gata3^{+}$ Tregs in the colonic LP (32.1% in WT and 30.5% in $ST2^{-/-}$) while $Ror\gamma t^{+}$ Tregs were minimally reduced in the ST2-deficient setting (37.5% in WT and 30.6% in $ST2^{-/-}$) (Fig. 4.1 D, E). Of note, there were no differences in other $CD4^{+}$ Th cell subsets (data not shown).

We also generated bone marrow chimaeras where bone marrow of wild type (WT), $ST2^{-/-}$ or a mix of wild type and $ST2^{-/-}$ (MIX) in 1:1 ratio was transferred into ST2-sufficient lethally irradiated $Rag1^{-/-}$ hosts. In this competitive mixed bone marrow

chimaera setting ST2-deficient Tregs were still significantly increased compared to wild type Tregs (19.1% of WT and 34.6% of $ST2^{-/-}$ in MIX) (Fig. 4.2 A, B). gMFI of the colonic Tregs in all the experimental groups was comparable (Fig. 4.2 C), and also Gata3 (14.8% of WT and 17.5% of $ST2^{-/-}$ in MIX) and Rorγt frequencies (38.0% of WT and 36.0% of $ST2^{-/-}$ in MIX) among Tregs were similar among the groups (Fig.4.2 D). These results indicate that expression of ST2 by the colonic Tregs is not required for their ability to accumulate in the colon.

Together, the data suggest that IL-33 signalling is neither required for the development of Tregs in the gut nor for their survival, and furthermore, in its absence the colonic LP Treg populations are significantly expanded with no corresponding difference in the effector $CD4^{+}$ Th cell subsets.

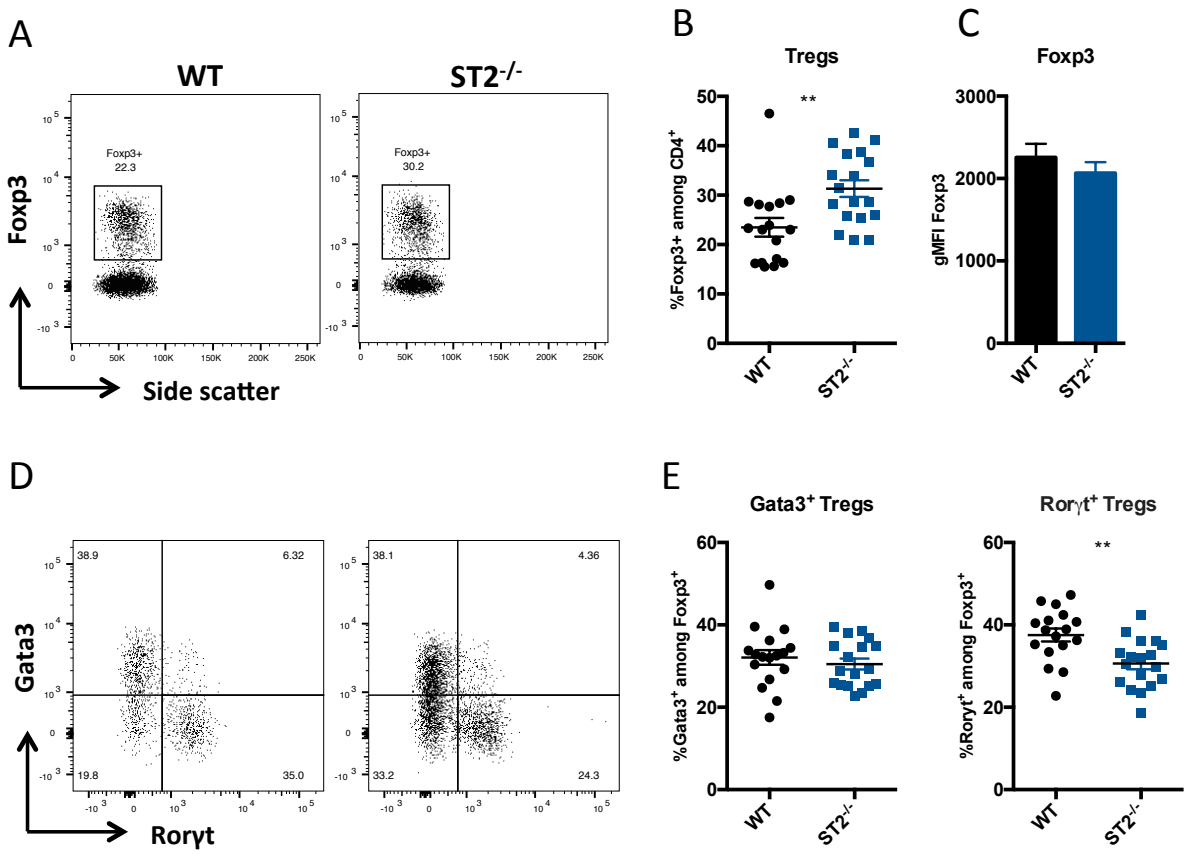


Figure 4.1. ST2 deficiency does not impair colonic Treg development.

C57BL/6 (WT) and ST2$^{-/-}$ mice were sacrificed at steady state and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Representative flow cytometry plots showing expression of Foxp3 in WT and ST2$^{-/-}$ steady state mice. Gated on single live TCRβ^{+}CD4^{+} cells.
(B) Frequency of Foxp3^{+} among TCRβ^{+}CD4^{+} T cells in the colon in WT and ST2$^{-/-}$ mice.
(C) Geometric mean fluorescence intensity (gMFI) of Foxp3 among single live TCRβ^{+}CD4^{+}Foxp3^{+} cells.
(D) Representative flow cytometry plots showing expression of Gata3 and Rorγt among colonic TCRβ^{+}CD4^{+}Foxp3^{+} Treg cells.
(E) Frequency of Gata3^{+} (left) and Rorγt^{+} (right) among the colonic TCRβ^{+}CD4^{+}Foxp3^{+} Tregs in the colon in WT and ST2$^{-/-}$ mice.

Each point represents an individual mouse and bars represent mean ±SEM.

Data represents pooled results of three independent experiments (n=17 WT and n=18 ST2$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. ** P ≤ 0.01.

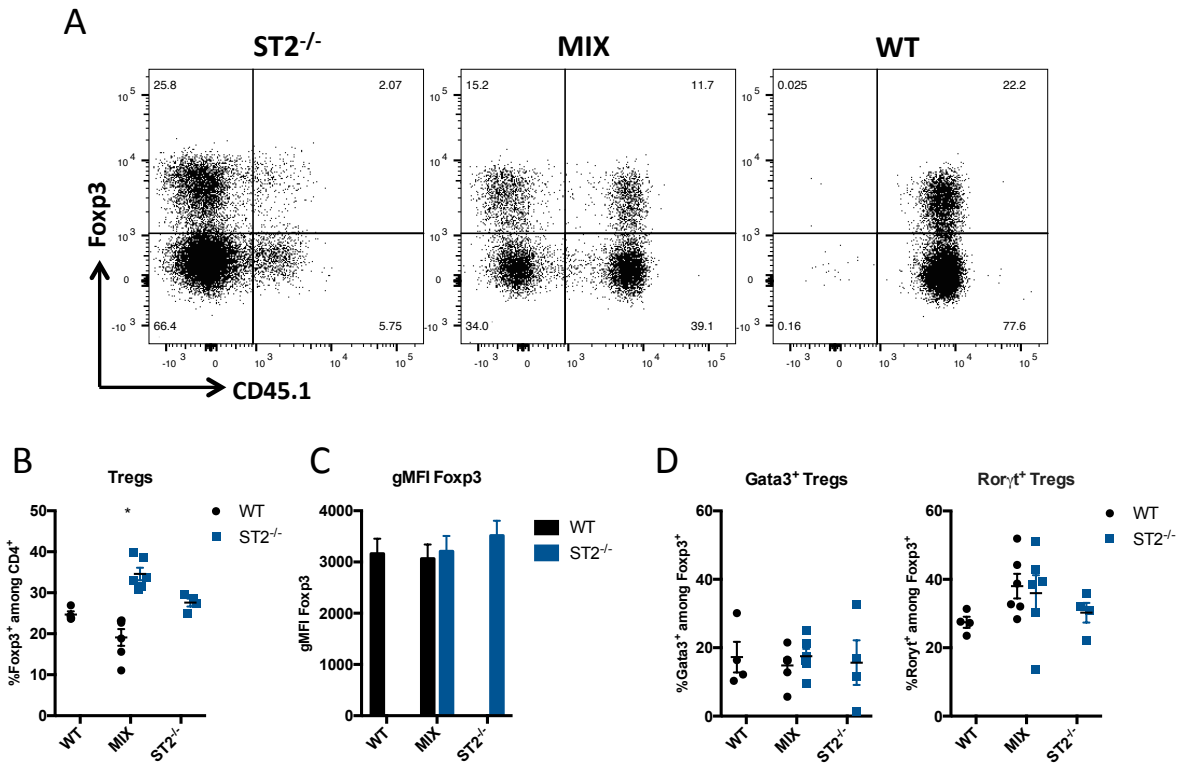


Figure 4.2. ST2$^{-/-}$ Tregs outcompete wild type Tregs in the colon in a MIX bone marrow chimaera setting.

Bone marrow chimaeras were generated by lethal irradiation of C57BL/6 Rag1$^{-/-}$ mice followed by i.v. injection of 10x10^6 WT (CD45.1$^+$), 10x10^6 ST2$^{-/-}$ (CD45.1$^-$) or a mix of 5x10^6 WT (CD45.1$^+$) and 5x10^6 ST2$^{-/-}$ (CD45.1$^-$) (MIX) bone marrow cells. Mice were allowed to rest for 10 weeks to allow full re-constitution of the haematopoietic system. Re-constituted mice were sacrificed at steady state and cells were isolated from the colonic LP for flow cytometric analysis.

(A) Representative flow cytometry plots showing expression of Foxp3 and CD45.1 among the colonic CD4$^+$ T cells. Gated on single live TCRβ$^+$CD4$^+$ cells.
(B) Frequency of Foxp3$^+$ among CD4$^+$ T cells in the colon of mice transferred with either WT, mixed (MIX) or ST2$^{-/-}$ bone marrow cells. Frequency is shown among CD4$^+$CD45.1$^+$ for full WT and WT population in the MIX setting and among CD4$^+$CD45.1$^-$ for full ST2$^{-/-}$ and ST2$^{-/-}$ population in the MIX setting.
(C) Geometric mean fluorescence intensity (gMFI) of Foxp3 among single live TCRβ$^+$CD4$^+$Foxp3$^+$ cells.
(D) Frequency of Gata3$^+$ (left) and Rorγt$^+$ (right) among the colonic Foxp3$^+$ Tregs in the colon in WT, MIX and ST2$^{-/-}$ mice. Gated on single live TCRβ$^+$CD4$^+$Foxp3$^+$ cells.

Each point represents an individual mouse and bars represent mean ±SEM.

Data represents results of a single experiment (n=4 WT, n=6 MIX and n=4 ST2$^{-/-}$). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test. * P ≤ 0.05.

4.2.2 ST2 deficiency in the *H. hepaticus*/anti-IL-10R-driven colitis.

The role of IL-33 in the development and pathology of IBD is not clear. Since studies to date investigating the role of IL-33 using IL-33- or ST2-deficient animals employed mainly chemical-induced colitis models (Oboki et al., 2010, Pushparaj et al., 2013, Sedhom et al., 2013, Waddell et al., 2015), we set out to identify the phenotype of mice deficient in ST2 in a chronic model of colitis. Wild type and $ST2^{-/-}$ were infected with *H. hepaticus* with concomitant anti-IL-10R blocking antibody administration.

Histological analysis revealed that there was no difference between the colitis score in $ST2^{-/-}$ mice and the wild type controls on day 14, which represents the peak of inflammation (Fig. 4.3 A, B). In wild type immunocompetent animals infection with *H. hepaticus* alone is not sufficient to cause the disease, and the blockade of the IL-10 pathway triggers disease only in the presence of disease-triggering microbiota, in this case *H. hepaticus*. We tested whether infection with *H. hepaticus* or blockade of IL-10R alone could promote disease in ST2-deficient mice. However, we did not observe any pathological changes in the absence of IL-33 signalling when mice were either infected with *H. hepaticus* or administered the anti-IL-10R blocking antibody alone (Fig. 4.3 C).

We also profiled immune cell populations recruited to the colon in this chronic model of colitis in the $ST2^{-/-}$ mice. There was no significant change in the frequency of total $TCR\beta^{+}CD4^{+}$ T cells (Fig. 4.4 A), while interestingly there was a significant increase in the frequency of $Foxp3^{+}$ Tregs (Fig. 4.4 B). Furthermore, intracellular staining of pro-inflammatory cytokines showed that IFN-γ^{+} $CD4^{+}$ T cells were significantly decreased in

the absence of IL-33 signalling (39.3% in WT vs 19.8% in $ST2^{-/-}$), while there was no difference in $IL\text{-}17A^{+}$, $IFN\gamma^{+}IL17A^{+}$ and $TNF\alpha^{+}$ $CD4^{+}$ T cells (Fig. 4.4 C).

Since multiple innate cell populations have been shown to express ST2 we also investigated whether ST2 deficiency would modulate them. However, there were no obvious differences in the frequency of myeloid cells in the colonic LP, including $Ly6C^{+}$ monocytes, $Ly6G^{+}$ neutrophils and Sig-$F^{int\ or\ high}$ eosinophils (that correspond to resting and activated eosinophils, respectively (Voehringer et al., 2007)) (Fig. 4.4 D).

Together, the data show that the pathology of intestinal inflammation in *H.hepaticus*/anti-IL-10R-driven colitis is not affected by the absence of IL-33 signalling in ST2-deficient mice, suggesting that this cytokine may not play a key role in the pathogenesis of colitis in the outlined model. Interestingly, we observed an increase in colonic LP Tregs upon intestinal inflammation and a corresponding decrease in $CD4^{+}IFN\gamma^{+}$ T cells in $ST2^{-/-}$ mice with no change amongst the innate immune cell populations.

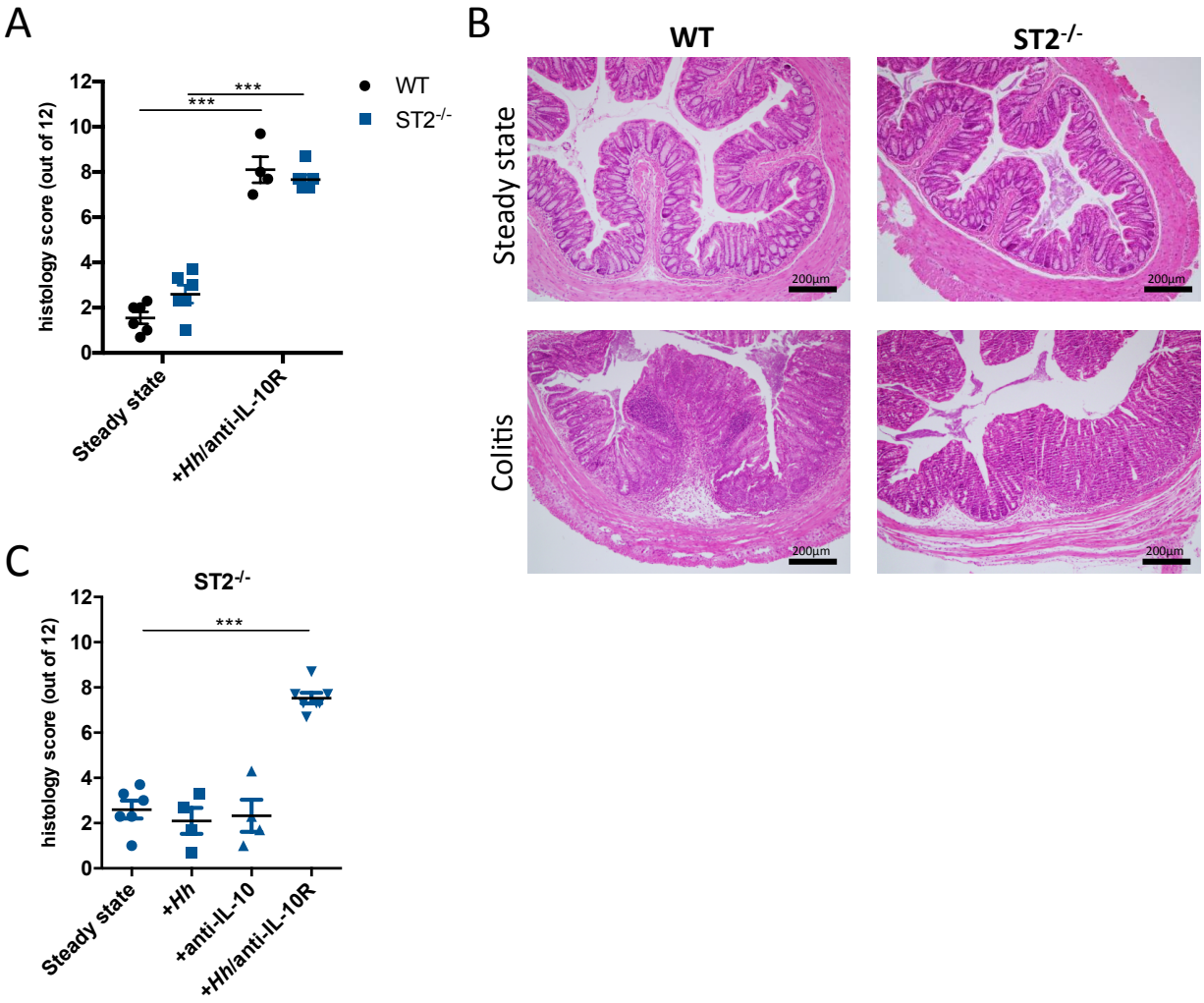


Figure 4.3. ST2 deficiency does not influence the severity of the *H.hepaticus*/anti-IL-10R-driven colitis.

C57BL/6 (WT) and ST2$^{-/-}$ mice were sacrificed without treatment (steady state) or infected with *Helicobacter hepaticus* either alone (+*Hh*) or with concomitant i.p. injections of a blocking anti-IL-10R mAb (+*Hh*/anti-IL-10R) or with i.p. injections of a blocking anti-IL-10R mAb alone (+anti-IL-10). Mice were sacrificed on day 14 and colons were harvested for histological analysis.

(A) Histological analysis of colonic inflammation in WT and ST2$^{-/-}$ mice at steady state versus at the peak of inflammation on day 14.
(B) Representative microphotographs of the proximal colon sections. Original magnification 10x, bars – 200 µm.
(C) Histological analysis of colonic inflammation in ST2$^{-/-}$ mice at steady state, treated only with *Hh*, treated only with anti-IL-10R or at the peak of inflammation (+*Hh*/anti-IL-10R).

Each point represents a single mouse and bars represent mean ±SEM.

Data represents representative results from one of two independent experiments (n=4 WT and n=7 ST2$^{-/-}$). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. *** $P \leq 0.001$.

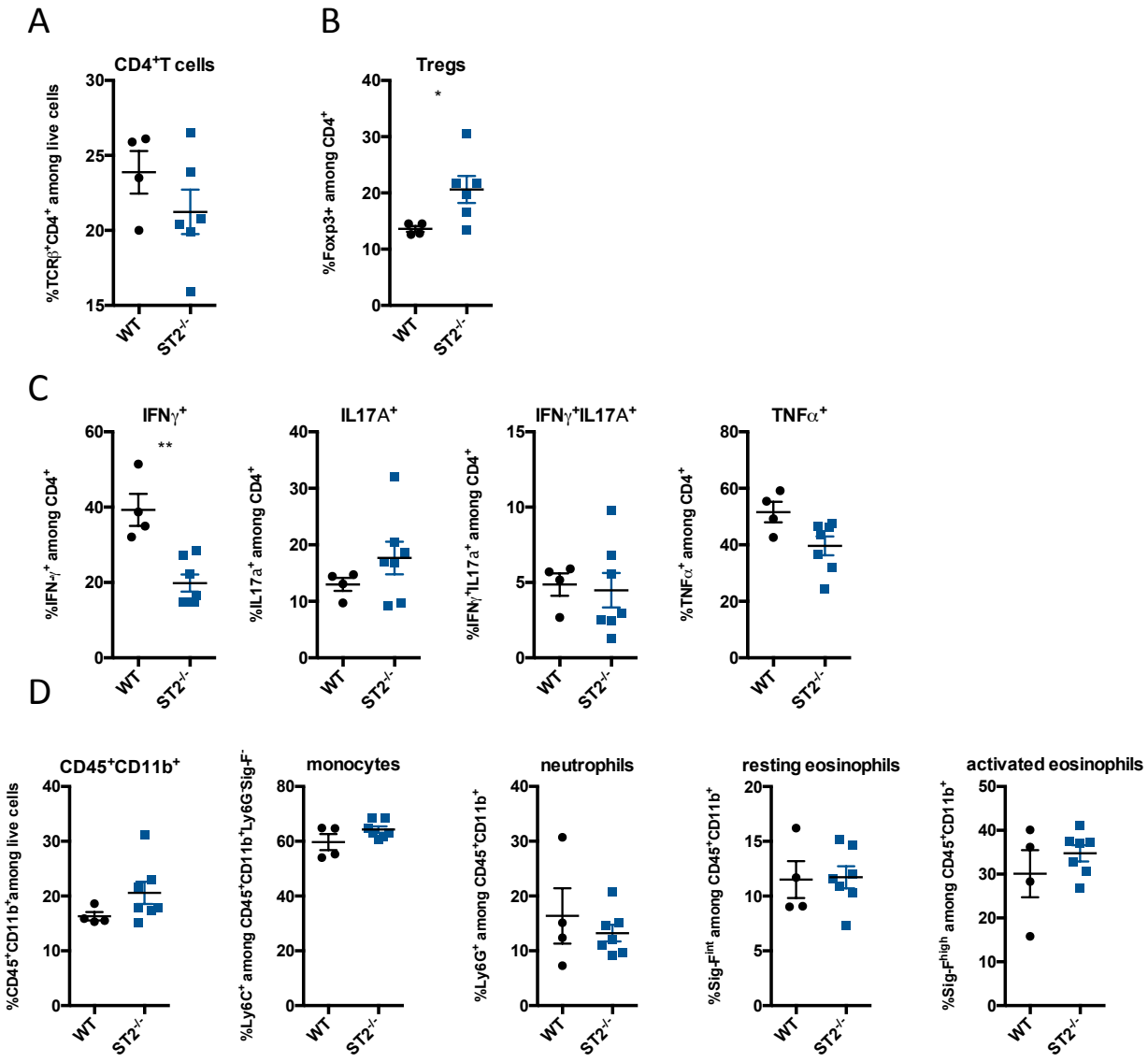


Figure 4.4. Immune cell populations in the colonic LP in wild type and ST2$^{-/-}$ mice during colitis.

C57BL/6 (WT) and ST2$^{-/-}$ mice were infected with *Helicobacter hepaticus* with concomitant i.p. injections of a blocking anti-IL-10R mAb (+*Hh*/anti-IL-10R). Mice were sacrificed on day 14 and cells were isolated from the colonic LP for flow cytometric analysis.

(A) Frequency of single live TCRβ^{+}CD4^{+} T cells in the colon.
(B) Frequency of single live TCRβ^{+}CD4^{+}Foxp3^{+} cells in the colon.
(C) Frequency of IFN-γ^{+}, IL-17a^{+}, IFN-γ^{+}IL-17a^{+} and TNFα^{+} among the colonic TCRβ^{+}CD4^{+} T cells following a 4-hour PMA/ionomycin restimulation.
(D) Frequency of myeloid cells (CD45^{+}CD11b^{+}), monocytes (Ly6C^{+}), neutrophils (Ly6G^{+}), resting (Sig-F^{intermediate}) and activated eosinophils (Sig-F^{high}).

Each point represents a single mouse and bars represent mean ±SEM.

Data represents representative results from one of two independent experiments (n=4 WT and n=7 ST2$^{-/-}$). Statistical significance was determined using a Mann Whitney U Test. * $P \leq 0.05$, ** $P \leq 0.01$.

4.2.3 MyD88 deficiency does not abrogate ST2$^+$ Tregs in the colon.

MyD88 is an essential adaptor protein in the IL-1R1 signalling pathway (Muzio et al., 1997, Wesche et al., 1997), which is recruited upon engagement of the IL-1 cytokine family primary receptor (IL-1R1, IL-18Rα, IL-1Rrp2 or ST2) with the IL-1RAcP co-receptor to further mediate signalling downstream of the IL-1 cytokine family members. MyD88 was also shown to be recruited to ST2 upon IL-33-stimulation (Schmitz et al., 2005). Surprisingly the role of MyD88 in Treg development has not been well studied. Since MyD88 is a component of the IL-33 signalling cascade, and we have previously shown enrichment of ST2 on colonic Tregs, we investigated whether MyD88-deficiency impacts on colonic Treg development.

The frequency and total number of Tregs was reduced in the colonic LP in MyD88$^{-/-}$ mice (34.8% in WT vs 22.6% in MyD88$^{-/-}$) (Fig. 4.5 A, B). Splenic Tregs were not affected by the absence of the MyD88 adaptor protein (data not shown). Similarly there was a lower abundance of ST2$^+$ and Gata3$^+$ Tregs in MyD88$^{-/-}$ animals compared to wild type controls, while Rorγt$^+$ Tregs did not show any significant difference (43.7% in WT vs 33.9% in MyD88$^{-/-}$, 47.0% in WT vs 37.3% in MyD88$^{-/-}$, 31.3% in WT vs 37.8% in MyD88$^{-/-}$ for ST2, Gata3, Rorγt, respectively) (Fig. 4.5 B, C). While the numbers of ST2$^+$ and Gata3$^+$ Tregs were reduced in the MyD88$^{-/-}$ mice compared to wild type controls, both subtypes of Tregs could be still detected in the colonic LP of mice deficient in MyD88.

Additionally, since MyD88 also regulates CD4$^+$ T cell effector responses we looked at the expression of ST2, Gata3 and Rorγt by CD4$^+$Foxp3$^-$ T cells. ST2 is preferentially

expressed on Th2 cells, therefore, both ST2 and Gata3 mark Th2 cells and Rorγt typically can be associated with Th17 cells. Consistent with a previously published report on MyD88$^{-/-}$ mice infected with *Chlamydia muridarum* that showed a Th2-dominant response (Chen et al., 2010), here, we observed an increase in Th2 cells: around 3-fold more CD4^{+}Foxp3^{-} T cells expressed ST2 or Gata3 in the colon of the MyD88$^{-/-}$ mice (8.0% in WT vs 25.8% in MyD88$^{-/-}$ and 10.0% in WT vs 33.6% in MyD88$^{-/-}$ for ST2 and Gata3, respectively) (Fig. 4.6). There was no difference in Th17 cells (5.1% in WT vs 6.7% in MyD88$^{-/-}$) (Fig 4.5 B and 4.6).

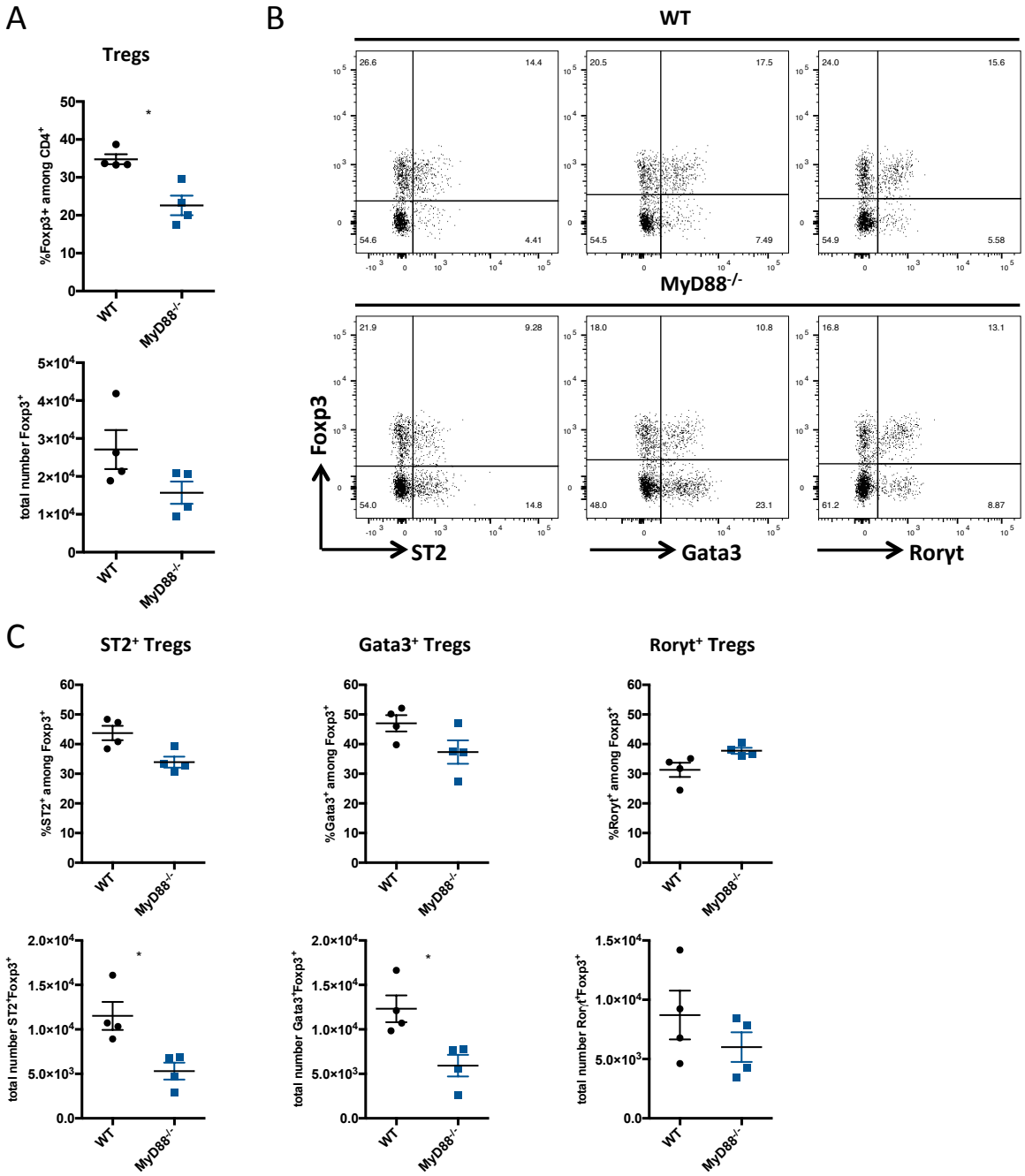


Figure 4.5. MyD88 signalling deficiency does not significantly impair ST2$^+$ Treg development.

C57BL/6 and MyD88$^{-/-}$ mice were sacrificed at steady state, and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

(A) Frequency (top) and total number (bottom) of colonic single live TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs.
(B) Representative flow cytometry plots showing expression of Foxp3 and ST2, Gata3 and Rorγt in WT controls and MyD88$^{-/-}$ mice. Gated on single live TCRβ$^+$CD4$^+$ cells.
(C) Frequency (top) and total number (bottom) of colonic ST2$^+$, Gata3$^+$ and Rorγt$^+$ Foxp3$^+$ Tregs.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents results from one experiment (n=4 for both groups). Statistical significance was determined using a Mann Whitney U Test. * P ≤ 0.05.

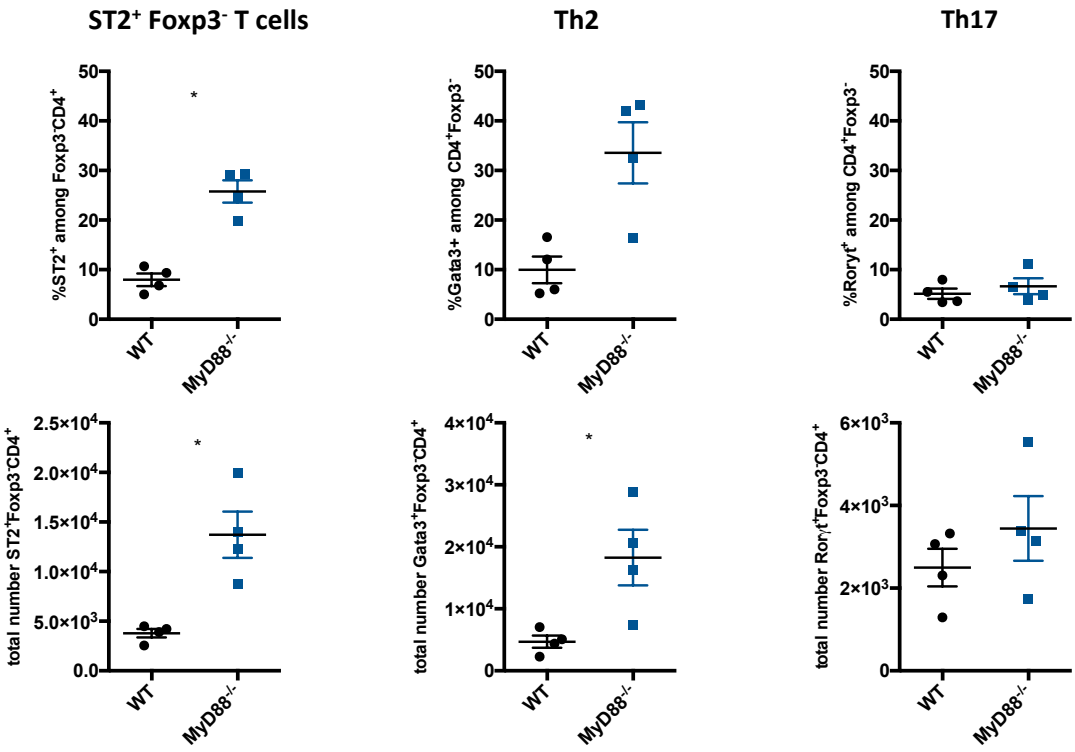


Figure 4.6. MyD88 signalling deficiency promotes Th2 cells in the colonic LP.

C57BL/6 and MyD88$^{-/-}$ mice were sacrificed at steady state, and single cell suspensions from the colonic LP were stained for flow cytometric analysis.

Frequency (top) and total number (bottom) of colonic ST2^{+}, Gata3^{+} (Th2) and Rorγt^{+} (Th17) single live TCRβ^{+}CD4^{+}Foxp3^{-} T cells.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents results from one experiment (n=4 for both groups). Statistical significance was determined using a Mann Whitney U Test. * $P \leq 0.05$.

4.2.4 Exogenous administration of IL-33 promotes splenic Tregs.

Exogenous administration of IL-33 has been previously investigated and, as mentioned before, implicated in both pathological and protective roles of the cytokine in the development of multiple conditions.

Here, we sought to determine whether IL-33 itself could modulate ST2 expression in Tregs. Daily injection of IL-33 i.p. over three days resulted in a robust increase in $CD4^+Foxp3^+$ Treg frequency and total number in the spleen (14.7% in PBS- vs 38.2% in IL-33-treated) (Fig. 4.7 A, B). In line with published reports (Turnquist et al., 2011), ST2-expressing Tregs were specifically increased (7.5% in PBS- vs 36.2% in IL-33-treated) (Fig. 4.7 A, B). To further validate that $ST2^+$ Tregs were preferentially targeted by IL-33, Ki67 staining was performed, which revealed that it was primarily $ST2^+$ Tregs that were proliferating (Fig. 4.7 C, D).

Interestingly, IL-33 administration led to increased mucus production, which in turn caused poor cell recovery from the colonic LP. This meant that it was not possible to analyse the colonic Tregs in detail within this experiment.

The described results demonstrate that IL-33 can influence Tregs *in vivo* and suggest a regulatory mechanism whereby IL-33 induces expression of its own receptor amplifying the signal.

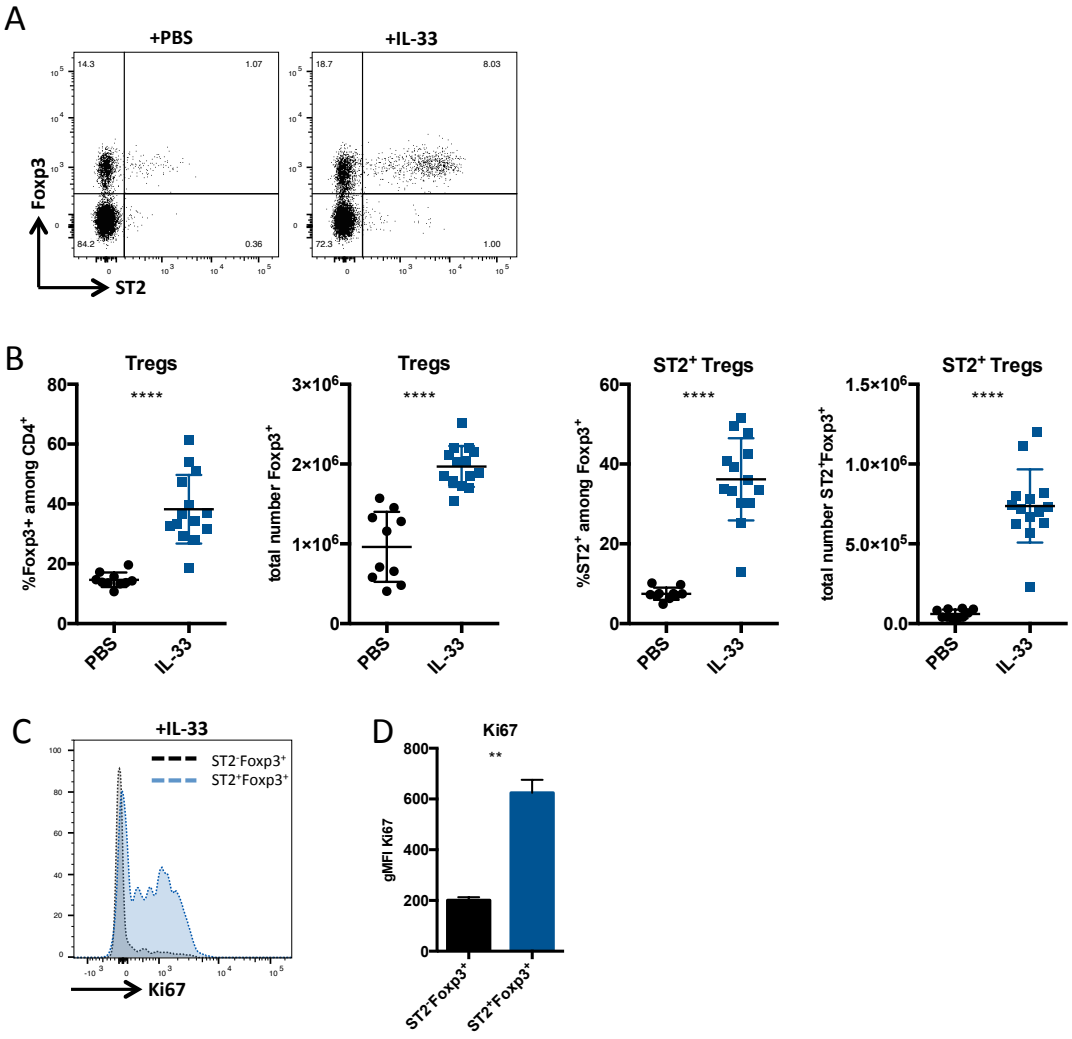


Figure 4.7. Exogenous administration of IL-33 promotes splenic Tregs and expression of ST2.

C57BL/6 mice were injected with 1µg recombinant IL-33 protein for three consecutive days and sacrificed one day after the last injection. Spleens were harvested and single cell suspensions were analysed by flow cytometry.

(A) Representative flow cytometry plots showing expression of ST2 on single live TCRβ$^+$CD4$^+$Foxp3$^+$ Tregs in the spleen in control mice (+PBS) and mice treated with IL-33 (+IL-33).
(B) Frequency (left) and total number (right) of Foxp3$^+$ cells and ST2$^+$ Foxp3$^+$ Tregs in the spleen.
(C) Representative histogram depicting Ki67 expression in ST2$^-$ (black) and ST2$^+$ (blue) TCRβ$^+$CD4$^+$Foxp3$^+$ Treg populations in IL-33-treated mice.
(D) Geometric mean fluorescence intensity (gMFI) of Ki67 in expression in ST2$^-$ and ST2$^+$ TCRβ$^+$CD4$^+$Foxp3$^+$ Treg populations in IL-33-treated mice.

Each point represents a single mouse and bars represent mean ±SEM.

Data represents pooled results from 3 independent experiments (n=10 for PBS and n=14 for +IL-33). Statistical significance was determined using a Mann Whitney U Test in (B) and a Wilcoxon matched-pairs signed rank test in (D). ** P ≤ 0.01, **** P ≤ 0.0001.

4.2.5 IL-33 activates the Gata3 pathway in Tregs.

To interrogate pathways regulated by IL-33 in Tregs we used an *in vitro* model, in which FACS-purified natural Tregs (nTregs) from Foxp3GFPKI mice are cultured in the presence of TCR stimulation followed by an acute stimulation with IL-33. Due to the very low cell numbers of Tregs recovered from the colonic LP no assays could be performed using the colonic Tregs and we therefore used splenic Tregs for these experiments.

As described Gata3 was preferentially co-expressed in the colonic $ST2^+$ Tregs, and recent studies have suggested a substantial role for the Gata3 pathway in Treg function and homeostasis (Rudra et al., 2012, Wang et al., 2011, Wohlfert et al., 2011). Previously the Gata3 pathway has also been shown to be activated in response to IL-33 in Th2 cells (Maneechotesuwan et al., 2007, Guo et al., 2009).

RT-qPCR analysis demonstrated that in response to acute IL-33 stimulation nTregs upregulated expression of both *Foxp3* and *Gata3* (Fig. 4.8 A). We confirmed activation of the Gata3 pathway by performing western blot analysis on nTreg protein lysates. After 30-60 minutes of stimulation with IL-33, we observed increased phosphorylation of Gata3 at serine308 with total levels of Gata3 remaining unchanged (Fig. 4.8 B). Since the results of stimulation *in vivo* with exogenous IL-33 showed a marked upregulation of ST2 on the surface of splenic Tregs we also interrogated ST2 expression in nTregs in response to IL-33 *in vitro*. Western blot analysis showed that ST2 was increased upon stimulation with IL-33 at time points between 40-240 minutes (Fig. 4.8 C).

Since a large population of colonic Tregs are peripherally induced Tregs, we also investigated IL-33 responses in Tregs differentiated *in vitro* from $CD4^+Foxp3^-$

precursors. To this end, FACS-sorted naïve $CD4^+$ T cells were cultured for 48-hours under Treg-differentiating conditions. We subsequently stimulated the cells with IL-33 for 45 minutes, which resulted in a robust activation of the Gata3 pathway as depicted by increased phosphorylation of Gata3 (Fig. 4.9 A).

Gata3 has been previously shown to bind to regulatory elements of the *Foxp3* locus to control its expression (Wang et al., 2011). Therefore, we hypothesized that upon IL-33 treatment Gata3 binds to the *Foxp3* locus to directly mediate its effects. To test this hypothesis we performed ChIP experiments on iTregs that were acutely stimulated with IL-33 for 45 minutes. qPCR was used to quantify binding of Gata3 to different gene loci. Gata3 was recruited to the *Foxp3* promoter but not to any of the well-defined CNS regions upon acute IL-33 stimulation in iTreg cultures (Fig. 4.9 B). This binding was accompanied by recruitment of RNA Polymerase II to the *Foxp3* promoter, demonstrating that expression of Foxp3 is activated by IL-33 and directly regulated by Gata3. Similarly, Gata3 binding to a regulatory region of the *Il1rl1* gene, *Il1rl1* enhancer, was observed upon IL-33 stimulation, suggesting that IL-33 also directly increases expression of its receptor in Tregs, allowing for the amplification of the IL-33 signalling in iTreg cultures (Fig. 4.9 C). RNA Polymerase II was also enriched at the *Il1rl1* promoter indicating active transcription of *Il1rl1* following IL-33 treatment (Fig. 4.9 C).

Altogether these results show that the Gata3 pathway is activated in Tregs in response to IL-33, and that this mechanism regulates Foxp3 and ST2 expression, thereby promoting Treg phenotype.

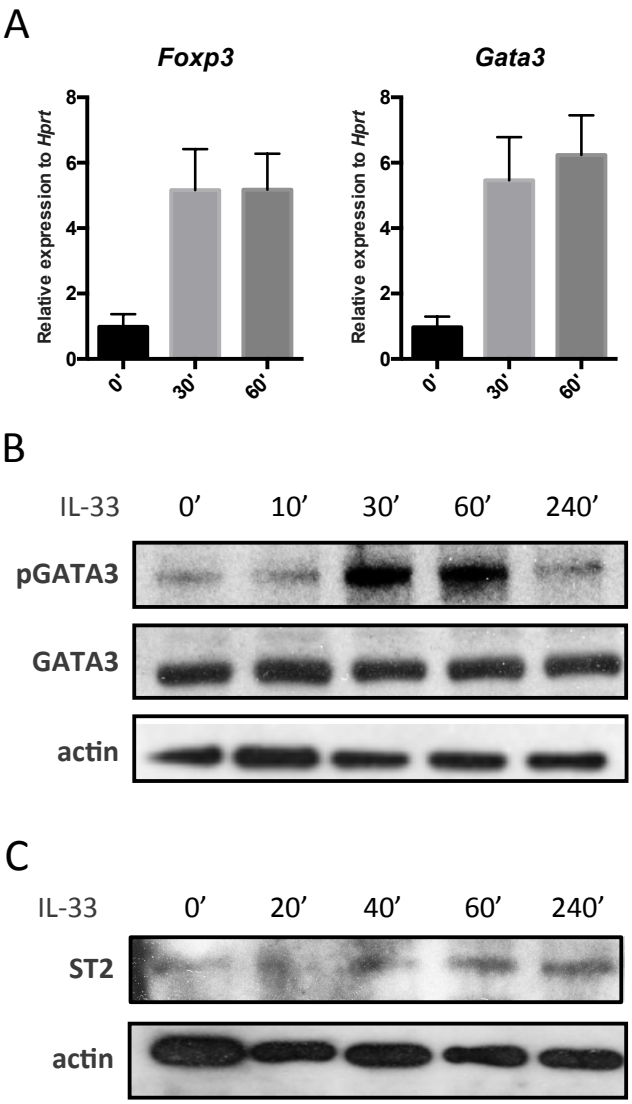


Figure 4.8. IL-33 promotes Foxp3 and ST2 expression and activates the Gata3 pathway in nTregs.

Foxp3GFPKI mice were sacrificed at steady state, $CD4^{+}GFP^{+}$ (nTregs) were FACS-sorted, plated with anti-CD3/anti-CD28, and after 24 hours stimulated with IL-33 for the indicated times. Cells were collected for RNA and protein analysis at the indicated time points.

(A) mRNA expression of *Foxp3* and *Gata3* at the indicated time points. ΔΔCt method was used for the analysis and results were normalised to 0' time point and *Hprt* levels. Assay was run in technical duplicates. Bars represent mean ±SD.
(B) Representative western blots showing expression of pGATA3, total GATA3 and actin of nTregs stimulated with IL-33 for 0'-240'.
(C) Representative western blots showing expression of ST2 and actin of nTregs stimulated with IL-33 for 0'-240'.

The results are representative of one of three independent experiments.

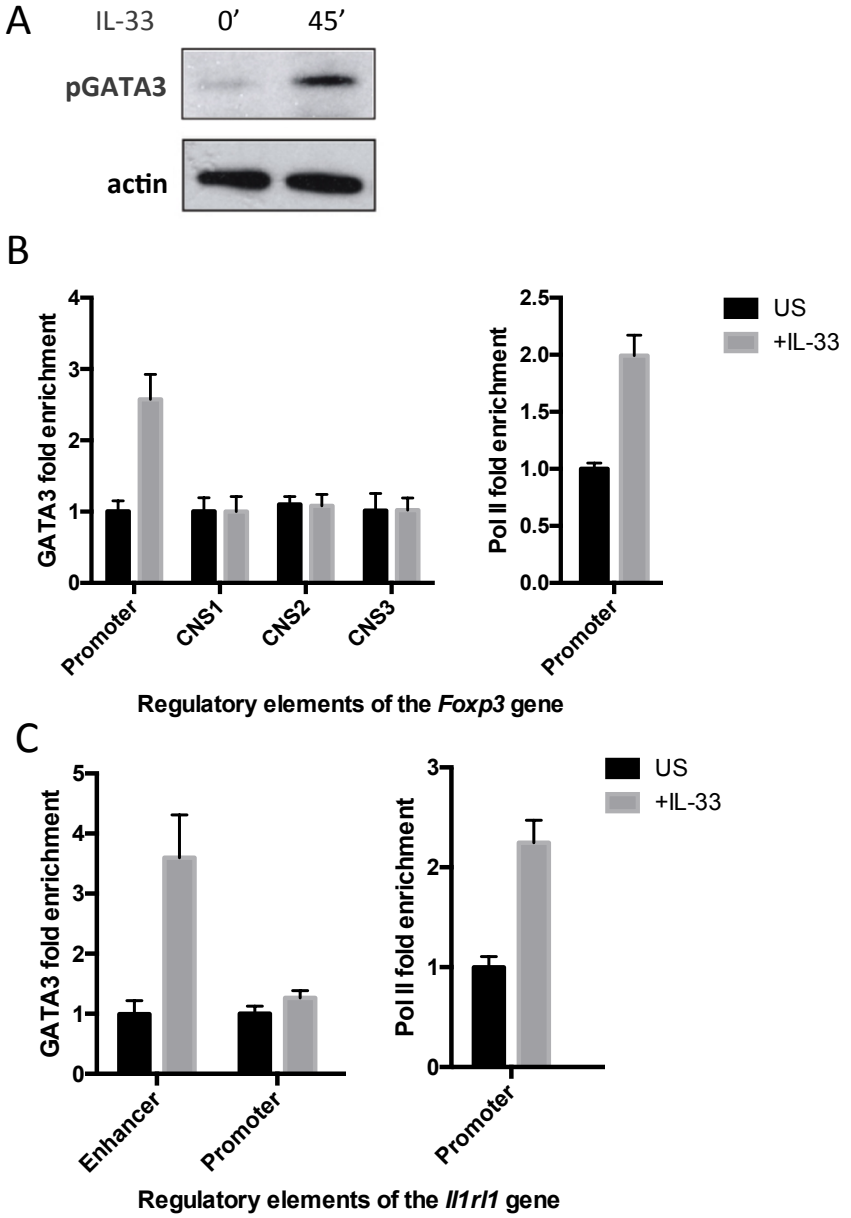


Figure 4.9. IL-33 activates the Gata3 pathway resulting in Gata3 recruitment to *Foxp3* and *Il1rl1* loci in iTregs.

Foxp3GFPKI mice were sacrificed at steady state, $CD4^{+}GFP^{-}CD44^{low}CD62L^{high}$ (naïve) T cells were FACS-sorted, plated with anti-CD3/anti-CD28 and cultured in presence of TGF-β1.

(A) Representative western blots showing expression of pGATA3 and actin of iTregs stimulated with IL-33 for 45' after 48 hours of differentiation with TGF-β1.
(B) Cells were cultured and stimulated as in (A), and processed for ChIP experiment. Recruitment of GATA3 and Polymerase II (Pol II) to specific regulatory regions of the *Foxp3* locus was assessed by qPCR. Results are normalized to genomic DNA (input) and presented as fold enrichment relative to unstimulated cells. CNS – conserved noncoding sequence, US – unstimulated cells, +IL-33 – cells stimulated with IL-33.
(C) Recruitment of GATA3 and Polymerase II (Pol II) to specific regulatory regions of the *Il1rl1* locus was assessed by qPCR.

Bars represent mean ±SD.

Blots in (A) are representative of one of three independent experiments. ChIP experiment results in (B) and (C) are representative of one of two independent experiments.

4.2.6 IL-33 promotes *Areg* expression in Tregs.

The results of the microarray analysis outlined in Chapter 3 highlighted amphiregulin-encoding *Areg* as a gene enriched in colonic ST2-expressing Tregs. Amphiregulin has also been shown to be enriched in Tregs found in multiple other tissues (Burzyn et al., 2013, Arpaia et al., 2015). Many immune cell populations, of both the innate and adaptive arms of the immune system, can produce amphiregulin. Interestingly, ILC2s, which are enriched at the barrier surfaces, have been recently found to produce large amounts of amphiregulin in response to IL-33 (Monticelli et al., 2015). Here, we investigate the link between the colon, IL-33, Tregs and amphiregulin.

RT-qPCR comparison of *Areg* expression between the lymphoid tissues, spleen and mLN, and the colon showed that *Areg* was highest in the colon in steady state mice (Fig. 4.10 A). Since amphiregulin plays a key role in the inflammatory response by promoting repair mechanisms and restoration of tissue homeostasis, we analysed its expression upon inflammation in a model of colitis driven by infection with *H. hepaticus* and concomitant injection of anti-IL-10R blocking antibody. *Areg* mRNA was upregulated about 150-fold in the colon of colitic mice (Fig. 4.10 B) suggesting it may play an important role in intestinal inflammation or its resolution.

To test whether amphiregulin is produced by Tregs in response to IL-33, we employed two models of stimulation with IL-33: an *in vivo* and *in vitro* one. As described in detail before (see section 4.2.4) administration of exogenous IL-33 resulted in a robust increase in ST2-expressing Tregs in the spleen of wild type mice. Here, we FACS-sorted four distinct populations of $CD4^+$ T cells (Fig 4.11 A): $Foxp3^-ST2^-$, $Foxp3^+ST2^-$, $Foxp3^-$

$ST2^+$, $Foxp3^+ST2^+$ and performed RT-qPCR on the extracted RNA. In both $Foxp3^-$ populations expression of *Areg* mRNA was almost not detectable. $Foxp3^+ST2^-$ expressed high levels of *Areg*, which was further 3-fold increased in the $Foxp3^+ST2^+$ Tregs suggesting that indeed IL-33 results in increased amphiregulin production by Tregs (Fig. 4.11 B).

Finally, FACS-sorted nTregs were stimulated for 30-60 minutes with IL-33 *in vitro,* and a 3-fold increase in *Areg* expression could be observed demonstrating that Tregs responded directly to IL-33 with an increase in *Areg* (Fig. 4.11 C).

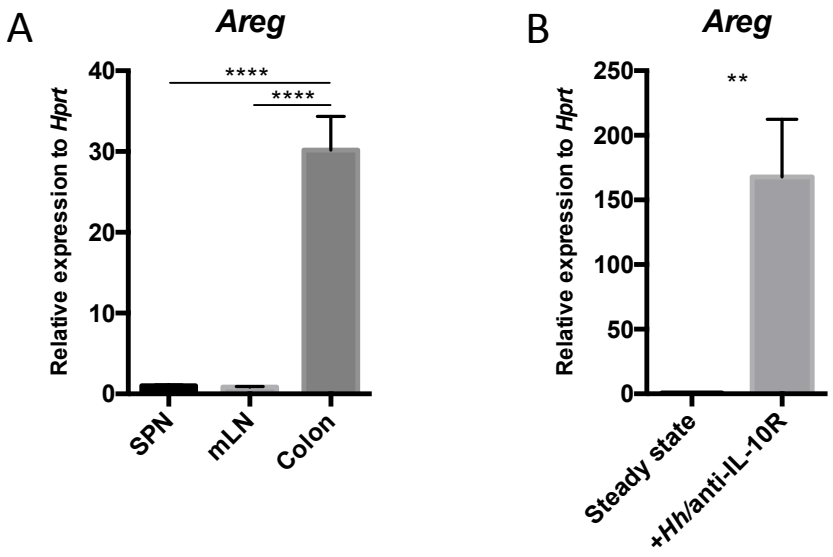


Figure 4.10. Areg is expressed in the colon and increased upon colitis.

Areg mRNA expression was quantified by RT-qPCR in the following experiments:

(A) Steady state C57BL/6 mice were sacrificed, spleens (SPN), mesenteric lymph nodes (mLN) and colons were harvested, and RNA from the tissues was extracted.
(B) C57BL/6 mice were sacrificed without treatment (steady state) or infected with *Helicobacter hepaticus* with concomitant i.p. injections of a blocking anti-IL-10R mAb (+*Hh*/anti-IL-10R). Mice were sacrificed on day 14 and colonic tissue was processed for RNA.

Bars represent mean ±SEM.

Data represents results from one experiment (n=5 for steady state and n=4 for *Hh*+anti-IL-10R). Statistical significance was determined using a Kruskal Wallis one- way analysis of variance followed by a Dunn's post-hoc test in (A) and a Mann Whitney U Test in (B). ** $P \leq 0.01$, **** $P \leq 0.0001$.

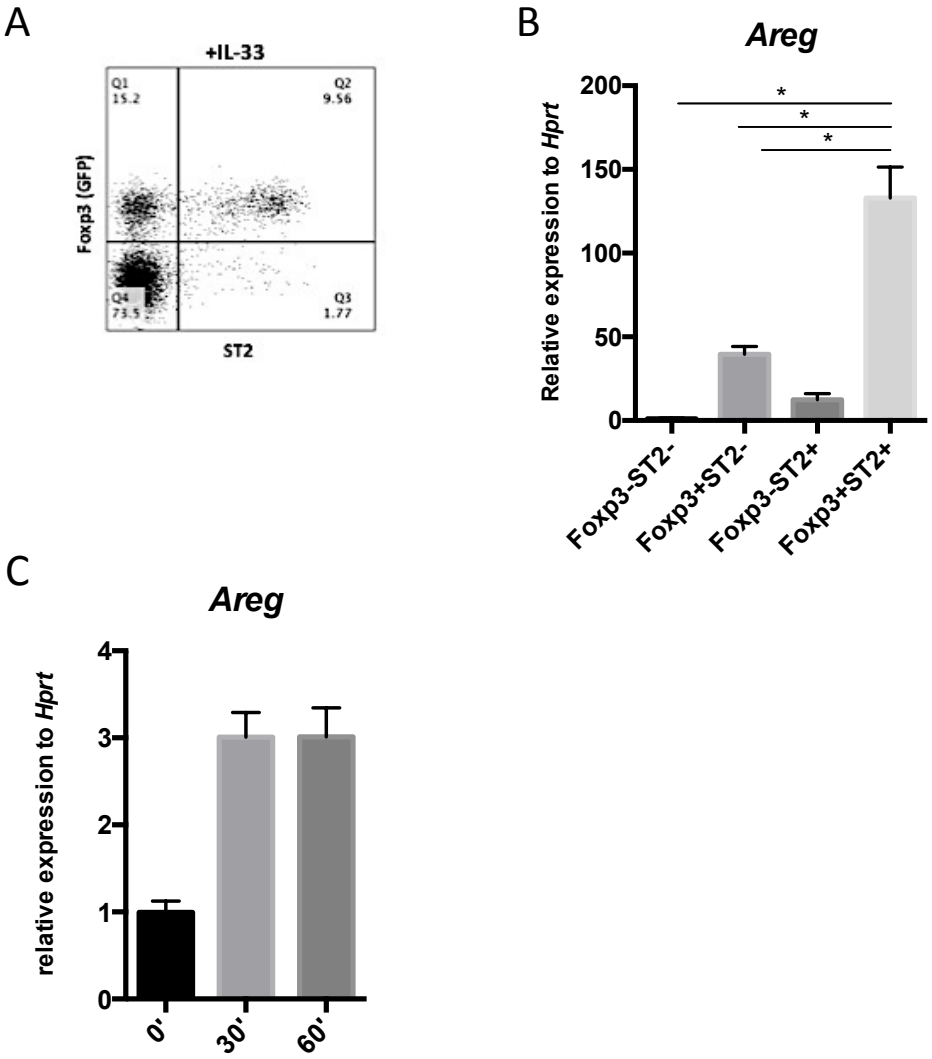


Figure 4.11. Areg is expressed upon IL-33 stimulation.

Areg mRNA expression was quantified by RT-qPCR in the following experiments:

(A) Foxp3GFPKI mice were injected with IL-33 1µg/day for three consecutive days and sacrificed one day after the last injection. A flow cytometry plot depicting gating strategy for the FACS sort of the populations analysed in (B).
(B) $Foxp3^{-}ST2^{-}$, $Foxp3^{+}ST2^{-}$, $Foxp3^{-}ST2^{+}$, $Foxp3^{+}ST2^{+}$ were FACS-sorted as described in (A) (n=4).
(C) Foxp3GFPKI mice were sacrificed and $CD4^{+}GFP^{+}$ cells were sorted and cultured with anti-CD3/CD28 for 24 hours followed by stimulation with IL-33.

Bars represent mean ±SEM.

Data represents results from a single experiment in (A, B) and results are a representative of one of three independent experiments in (C). Statistical significance was determined using a Wilcoxon matched-pairs signed rank test in (B). * $P \leq 0.05$.

4.2.7 Recovery is not impaired in Amphiregulin- and in ST2-deficient mice.

Areg is an EGF-like growth factor, which like EGF binds to the EGFR, and plays a role in tissue remodelling and repair processes (Enomoto et al., 2009, Monticelli et al., 2011, Burzyn et al., 2013). Recently, a novel function of Tregs implicated in repair has also been highlighted (Burzyn et al., 2013, Villalta et al., 2014, Arpaia et al., 2015). Since Tregs with reparative function have been described previously and we showed that IL-33 can directly induce *Areg* expression in Tregs, we hypothesise that mice deficient in Areg or ST2 display slower recovery following intestinal inflammation. To this end we used the modified protocol for the *H. hepaticus*/anti-IL-10R-driven colitis model: wild type, $ST2^{-/-}$ and $Areg^{-/-}$ mice were infected with *H. hepaticus* and administered the blocking anti-IL-10R mAb i.p. on day 0 and day 7, after which they were allowed to recover. In this model, on day 14, mice display highest inflammation, which is consistent with the worst pathology. It is followed by the recovery and resolution of inflammation phases that may take up to week 8-12, depending on the kinetics of the disease (Powrie group, unpublished data).

The histological assessment of the harvested colons on week 2 showed that wild type mice displayed epithelial cell hyperplasia, goblet cell depletion as well as dense cellular infiltrate following *H. hepaticus* infection and concomitant anti-IL-10R blocking mAb administration. In line with our previous results (section 4.2.2 and unpublished data, Powrie lab), both $ST2^{-/-}$ and $Areg^{-/-}$ mice developed similar disease to wild type controls (Fig 4.12 A, B). The colitis score was high reflecting severe inflammation in all experimental groups on week 4. After that mice started recovering as indicated by a lower histological score of colitis on week 6 and 8. By week 12 all mice recovered and

did not display any obvious signs of inflammation. There were no significant differences between $ST2^{-/-}$ and $Areg^{-/-}$ mice compared to wild type controls on any of the investigated time points (Fig. 4.12 A, B).

To complement the histological analysis an endoscopic analysis was performed, which provided a tool to track disease progression in the same individual animals over the course of the experiment. The assessment of the colitis severity was performed using the murine endoscopic index of colitis severity (MEICS), which includes parameters such as thickening of the colon, changes of the vascular pattern and the stool consistency (Becker et al., 2006). The severity of disease was significantly higher in ST2-deficient animals compared to wild type mice on week 3 when mice in all experimental groups displayed severe inflammation (Fig. 4.12 A, B). On week 10, wild type mice were healthy whereas $ST2^{-/-}$ animals did not fully recover and displayed mild symptoms of colitis. $Areg^{-/-}$ mice showed a similar trend to $ST2^{-/-}$ mice; however, the difference between $Areg^{-/-}$ and wild type mice was not significant. By week 14, mice in all experimental groups have recovered (Fig. 4.12 A, B).

It is important to note that only the distal parts of the colon can be evaluated using endoscopy. Therefore, we also tested whether histological assessment of the distal colon section alone was concordant with the endoscopy results. However, the analysis of the histology samples of the distal colon alone was consistent with the combined results of the proximal, middle and distal sections (data not shown). Therefore, further analysis is required to understand the discrepancy between the results of the histological and endoscopic investigation.

Altogether, the results of the histological analysis of the intestinal inflammation severity suggest that deficiency in ST2 and in Amphiregulin did not markedly alter the recovery from the intestinal inflammation induced by *H.hepaticus* and concomitant blockade of the IL-10R pathway.

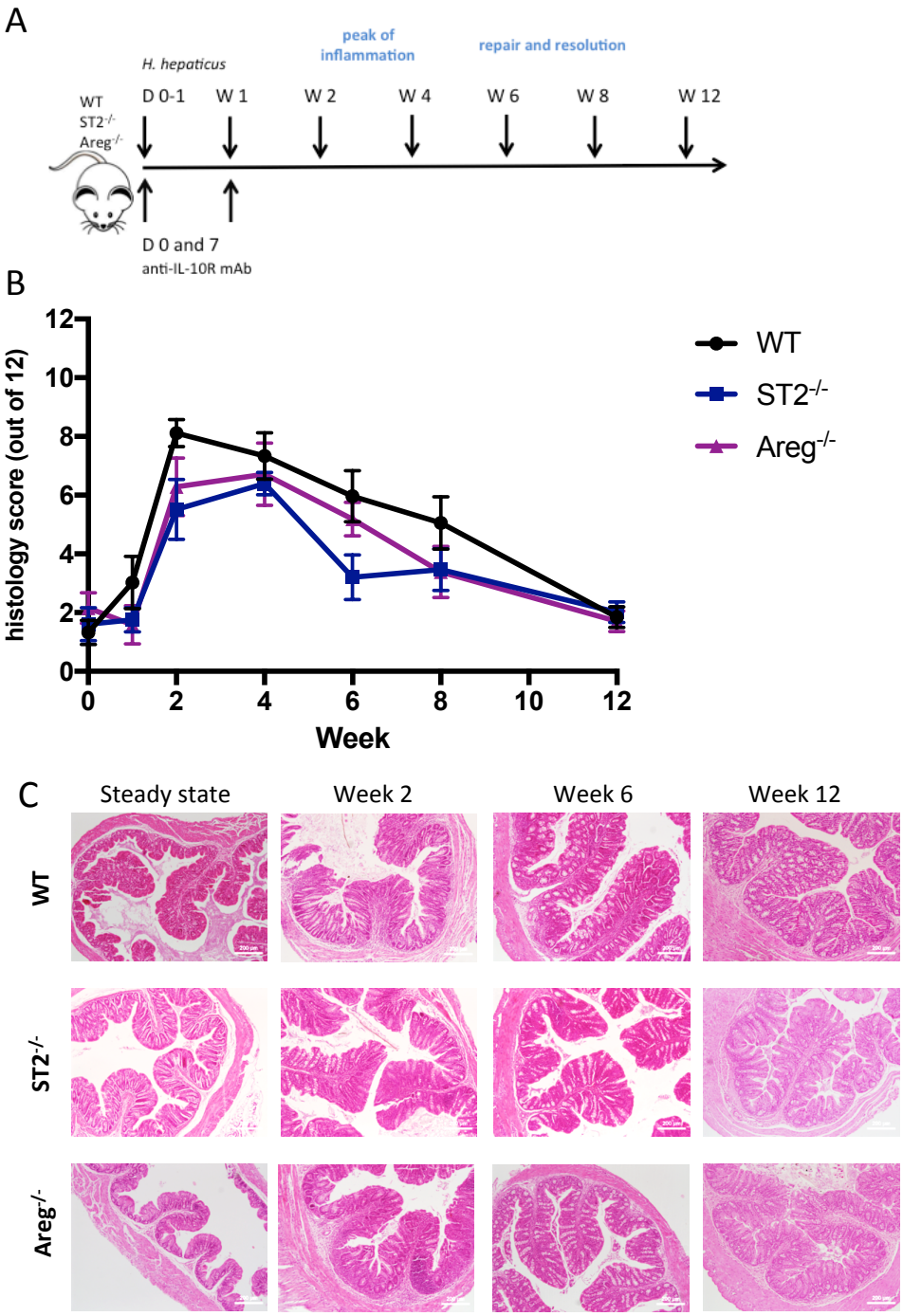


Figure 4.12. Recovery is not impaired in ST2- and Amphiregulin-deficient mice.

C57BL/6 (WT), ST2$^{-/-}$ and Areg$^{-/-}$ mice were infected with *H. hepaticus* and i.p. injected with an anti-IL-10R mAb on day 0 and 7. Mice were sacrificed at the indicated time points, and colons were harvested for histological analysis.

(A) A schematic diagram outlining the design of the experiment.
(B) Histological analysis of colonic inflammation in WT, ST2$^{-/-}$ and Areg$^{-/-}$ mice over the course of the experiment, from steady state to week 12.
(C) Representative microphotographs of the proximal colon sections. Original magnification 10x, bars – 200 µm.

Each point represents an average result of all mice of a given genotype sacrificed on one day and bars represent ±SEM.
Data represents results of one experiment (n=6 mice for each experimental group). Statistical significance was determined using an ordinary one way analysis of variance followed by a Bonferroni's multiple comparisons test. Differences between the experimental groups were not significant.

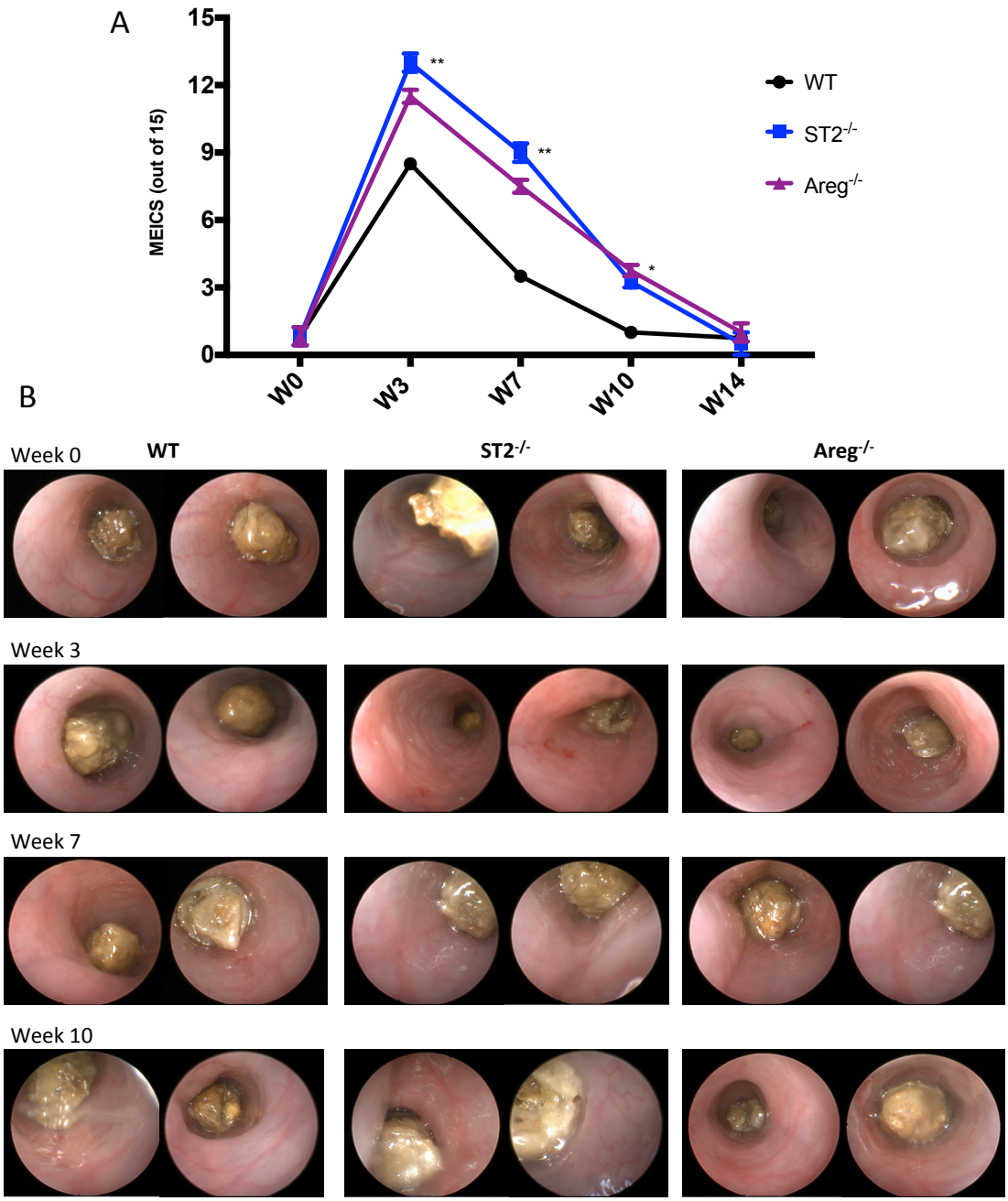


Figure 4.13. An endoscopic analysis of the colon in ST2- and Amphiregulin-deficient mice during *H. hepaticus*/anti-IL-10R driven colitis.

C57BL/6 (WT), ST2$^{-/-}$ and Areg$^{-/-}$ mice were infected with *H. hepaticus* and i.p. injected with an anti-IL-10R mAb on day 0 and 7. Mice were analysed by endoscopy at the indicated time points.

(A) Endoscopic analysis of the severity of colitis in WT, ST2$^{-/-}$ and Areg$^{-/-}$ mice over the course of the experiment, from steady state (week 0) to week 14.
(B) Representative endoscopic pictures showing the colon, with two examples for each group.

Each point represents an average result of all mice of a given genotype sacrificed on one day and bars represent ±SEM.
Data represents results of one experiment (n=4 mice for each experimental group). Statistical significance was determined using an ordinary one way analysis of variance followed by a Bonferroni's multiple comparisons test. * $P \leq 0.05$, ** $P \leq 0.01$.

4.2.8 IL-23 inhibits the effects of IL-33 on Tregs.

IL-23 is a pro-inflammatory cytokine that plays a crucial role in the pathogenesis of many inflammatory diseases, including IBD. Polymorphisms in the *IL23R* gene have been shown to be associated with increased susceptibility to both the CD and UC (Duerr et al., 2006, Wellcome Trust Case Control, 2007). Previous studies from our group showed that IL-23 can suppress immunosuppressive pathways during intestinal inflammation at least partially via inhibition of colonic Treg differentiation (Izcue et al., 2008, Ahern et al., 2010). As a follow-on from these observations, a microarray was performed on wild type and IL-23R$^{-/-}$ CD4^{+} T cells that were co-transferred into Rag1$^{-/-}$ mice and isolated from the colonic LP once the intestinal inflammation was established. The analysis of the microarray showed that IL-23R$^{-/-}$ CD4^{+} T cells had higher expression of *Foxp3* confirming a cell intrinsic role for IL-23 in Treg inhibition. Notably, *Il1rl1* was also among the upregulated transcripts in IL-23R-deficient T cells (Schiering et al., 2014), which led us to investigate whether IL-23 may inhibit colonic Tregs via downregulation of IL-33 signalling into these cells.

As described in the previous section nTregs were stimulated with IL-33 for 20-240 minutes, which resulted in the phosphorylation of Gata3, and thus, activation of the Gata3 signalling pathway. In the presence of IL-23 in nTreg cultures this effect was abrogated (Fig. 4.13 A). Next we wanted to understand how IL-23 inhibits activation of IL-33 signalling, and to this end, we used a STAT3 inhibitor in nTreg cultures, since IL-23 acts via STAT3. In the presence of both IL-23 and a STAT3 inhibitor, IL-33 promoted GATA3 activation, thus, reversing the inhibitory effect of IL-23 and indicating that it is STAT3-dependent (Fig. 4.13 B).

We have already demonstrated that upon IL-33 stimulation both *Foxp3* and *Gata3* are induced. Previously it has been shown that co-expression of both Foxp3 and Gata3 in Tregs is essential for their regulatory function (Rudra et al., 2012). Here, we analysed mRNA levels of representative genes, which are regulated either by both Foxp3 and Gata3 (*Pde3b*, *Sytl2*, *Ets2*, *Klf3*), or by Foxp3 alone (*Pdk1*, *Stx11*, *Ikzf2*, *Prdm1*). In line with the robust activation of the Gata3 pathway in response to IL-33 only the genes co-regulated by the two transcription factors were upregulated upon stimulation with IL-33. Furthermore, in the presence of IL-23 this was abolished (Fig. 4.13 C).

Previous work from our group has also implicated IL-33 as a co-factor enhancing TGF-β1-mediated iTreg differentiation *in vitro* (Schiering et al., 2014). Here, we tested whether the addition of IL-23 to the iTreg cultures would inhibit the effects of IL-33 on Tregs. Differentiation of naïve $CD4^+$ T cells with TGF-β1 and IL-33 alone upregulated expression of ST2, as shown in a western blot. By contrast, in presence of IL-23 this effect was largely diminished (4.14 A). We also performed ChIP experiments on naïve $CD4^+$ T cells differentiated for 48 hours in the presence of TGF-β1 followed by an acute 45-minute stimulation with IL-33 in the presence of IL-23. This showed that Gata3 was no longer recruited to the enhancer of the *Il1rl1* locus further confirming the inhibitory role of IL-23 in the IL-33-mediated Gata3 pathway activation (4.14 B).

In summary, our results demonstrate that the pro-inflammatory cytokine IL-23, which has been previously implicated in the inhibition of Tregs in the colon, is a negative regulator of IL-33 signalling in Tregs.

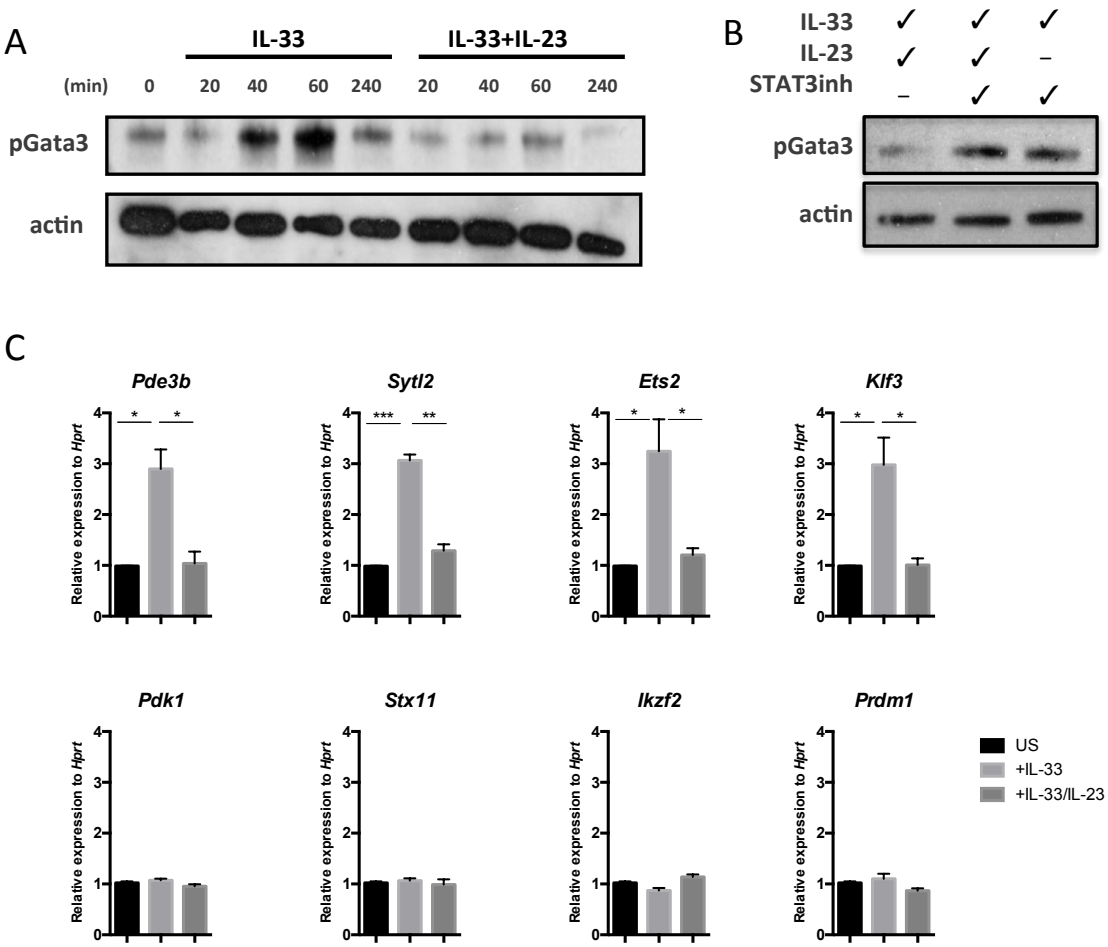


Figure 4.13. IL-23 inhibits the effects of IL-33 on nTregs.

Foxp3GFPKI mice were sacrificed at steady state, $CD4^{+}GFP^{+}$ (nTregs) were FACS-sorted, plated with anti-CD3/anti-CD28, and after 24 hours stimulated with IL-33 in the presence or absence of IL-23. Cells were collected for RNA and protein analysis at the indicated time points.

(A) Representative western blots showing expression of pGata3 and actin of nTregs stimulated with IL-33 only or with IL-23 for 0'-240'.
(B) Representative western blots showing expression of pGata3 and actin of nTregs stimulated with IL-33 and IL-23 or IL-33 and IL-23 and STAT3 inhibitor (STAT3inh) or IL-33 and STAT3inh for 45'. ✓- present, - - absent
(C) mRNA expression of Foxp3 and Gata3-target genes (top panel) or Foxp3-only target genes (bottom panel). ΔΔCt method was used for the analysis and results were normalised to US (unstimulated condition) and *Hprt* levels.

Bars represent mean ±SD.

The results are representative of one of three independent experiments.
Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

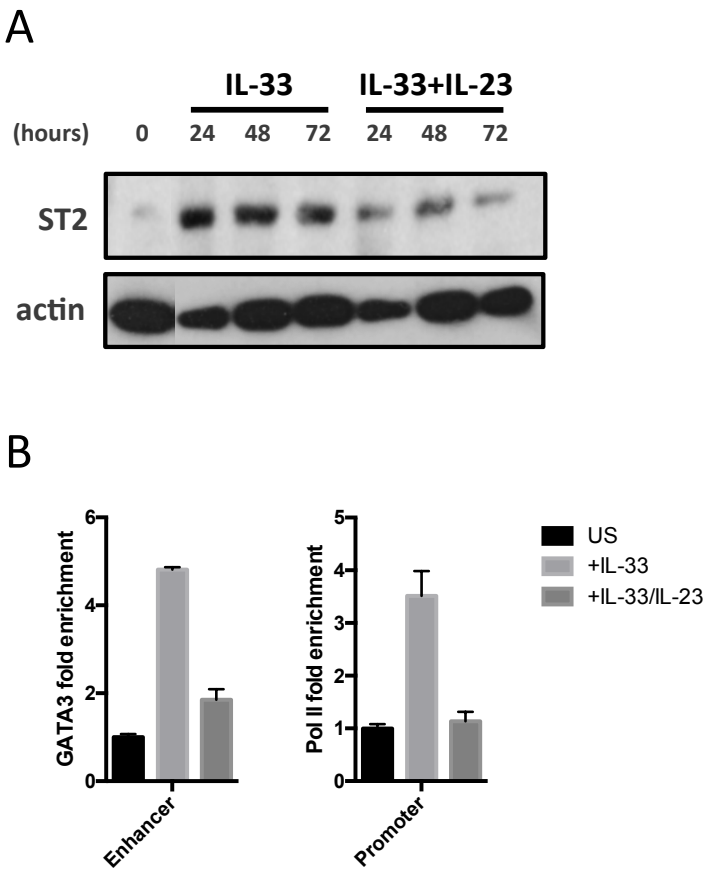


Figure 4.14. IL-23 inhibits IL-33-mediated recruitment of Gata3 to the *Il1rl1* enhancer.

Foxp3GFPKI mice were sacrificed at steady state, $CD4^{+}GFP^{-}CD44^{low}CD62L^{high}$ (naïve) T cells were FACS-sorted, plated with anti-CD3/anti-CD28 and cultured in the presence of TGF-β1.

(A) Representative western blots showing expression of ST2 and actin of iTregs differentiated with TGF-β1 with IL-33 in the presence or absence of IL-23. Time point '0' indicates cells lysed directly after the sort.
(B) Cells were cultured for 48 hours in the presence of TGF-β1 and stimulated with IL-33 only or IL-33 and IL-23, and subsequently processed for ChIP experiment. Recruitment of GATA3 and Polymerase II (Pol II) to specific regulatory regions of the *Il1rl1* locus was assessed by qPCR. Results are normalized to genomic DNA (input) and presented as fold enrichment relative to unstimulated cells. US – unstimulated cells, +IL-33 – cells stimulated with IL-33, +IL-33/IL-23 – cells stimulated with IL-33 and IL-23.

Bars represent mean ±SD.

Blots in (A) are representative of one of three independent experiments. ChIP experiment results (B) are a representative of one of two independent experiments.

4.2.9 IL-23 and IL-33 are expressed in the colonic LP during colitis.

We have already established by employing the *in vitro* stimulation experiments that a balance between IL-33 and IL-23 might be the key control point for Tregs. Here, we investigated the amounts of IL-23 and IL-33 in colon explant culture supernatants obtained from multiple different time points from the *H. hepaticus*/anti-IL-10R-driven model of colitis.

While IL-23 levels in the intestine increased over time in mice with chronic colitis, IL-33 levels increased initially and then declined during the chronic phase (Fig. 4.15). These results suggest the balance between IL-23 and IL-33 may be a major determinant of intestinal homeostasis.

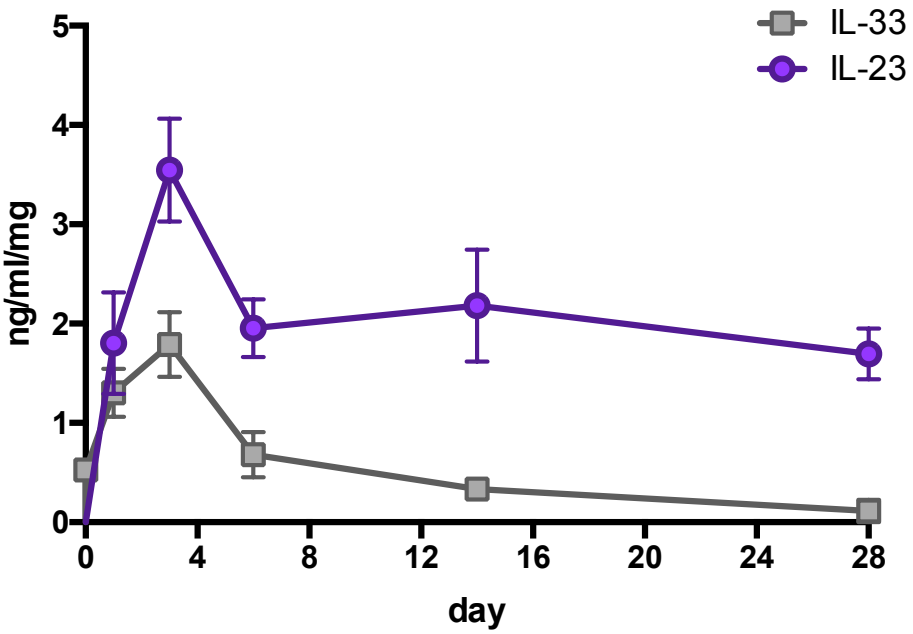


Figure 4.15. Expression of IL-23 and IL-33 *in vivo*.

C57BL/6 mice were sacrificed without treatment (steady state) or infected with *Helicobacter hepaticus* with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg weekly) (+*Hh*/anti-IL-10R).

Protein quantification of IL-33 and IL-23 by enzyme-linked immunosorbent assay in colon explant cultures incubated *ex vivo* for 24 hours at the indicated time points of the *Hh*/anti-IL-10R model of colitis. Graph shows amount of protein in ng/ml per mg of incubated tissue.

Bars represent mean ±SEM.

Data represent results from one experiment (n=5 for steady state and n=4 for *Hh*+anti-IL-10R).

4.2.10 Soluble ST2 negatively regulates IL-33 bioavailability during colitis.

Decoy receptors are important negative regulators of pro-inflammatory cytokines and are common in the IL-1 receptor family (reviewed in Garlanda et al (2013b)). The soluble form of ST2 (sST2) has been shown to act as a decoy receptor for IL-33 (Hayakawa et al., 2007) and increased sST2 levels have been recorded in IBD patients (Pastorelli et al., 2010).

Initial RT-qPCR analysis revealed that indeed levels of *St2* mRNA were significantly increased upon intestinal inflammation in the context of *H. hepaticus*/anti-IL-10R-driven colitis; specifically the soluble form of ST2 was substantially increased (Fig. 4.16 A). Investigating the kinetics of sST2 production during chronic colitis showed that the levels of sST2 drastically increased around day 10 (Fig. 4.16 B), which is also the time of the onset of clinical pathology in the described model (data not shown). This suggests that during intestinal inflammation sST2 may be released to reduce the amount of bioactive IL-33.

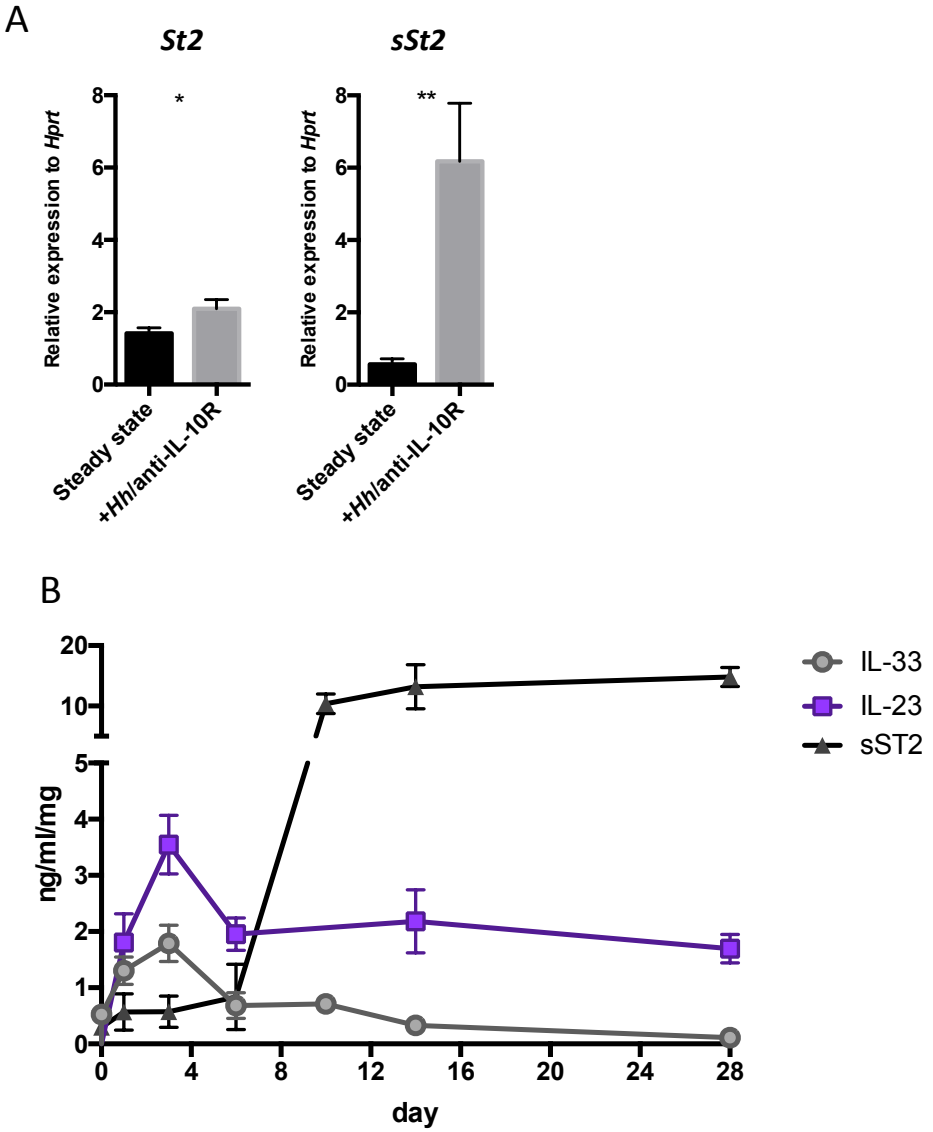


Figure 4.16. Soluble ST2 negatively regulates IL-33 bioavailability during colitis.

C57BL/6 mice were sacrificed without treatment (steady state) or infected with *Helicobacter hepaticus* with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg weekly) (+*Hh*/anti-IL-10R).

(A) mRNA expression of membrane-bound form of ST2 (*St2*) and soluble form of ST2 *(sSt2)* in the RNA extracted from the colon of steady state mice and of mice at the peak of colitis, day 14. ΔΔCt method was used for the analysis and results were normalised to steady state colon and *Hprt* levels.

(B) Protein quantification of IL-33, IL-23 and soluble ST2 (sST2) by enzyme-linked immunosorbent assay in colon explant cultures incubated *ex vivo* for 24 hours at the indicated time points of the *Hh*/anti-IL-10R model of colitis. Graph shows amount of protein in ng/ml per mg of incubated tissue.

Bars represent mean ±SEM.

Data represent results from one experiment (n=5 for steady state and n=4 for Hh/anti-IL-10R). Statistical significance was determined using the Mann Whitney U test. * $P \leq 0.05$, ** $P \leq 0.01$.

4.2.11 Soluble ST2 is produced by stromal cells.

We showed that sST2 is induced upon intestinal inflammation in the *H. hepaticus*/anti-IL-10R-driven model of colitis, which may reduce the bioavailability of IL-33 in the intestine. The cellular source of sST2 is, however, not clear (Palomo et al., 2015), and here, we set out to investigate which cells contribute to the production of sST2 in colitis.

Firstly, *sST2* mRNA levels were analysed in three different populations of cells present in the colon: epithelial cells, leukocytes and stromal cells. *sST2* was most highly expressed in the stroma fraction, which was further increased almost 10-fold upon colitis (Fig. 4.17 A). *sST2* was barely detectable in the epithelial cells, and its levels were low in the leukocyte population.

Next, we tested the capacity of stromal cells to produce sST2 in isolation by culturing stromal cells *ex vivo*, which were harvested from different time points of the *H. hepaticus*/anti-IL-10R model of colitis. This experiment showed that high amounts of sST2 could be detected in the supernatants, which likely contributes to the increase in sST2 protein levels during intestinal inflammation (Fig. 4.17 B).

To further delineate the factors that induce sST2 production by stromal cells, stromal cells cultured *in vitro* were stimulated with bacterial strains used in two common models of colitis, *H. hepaticus* and *Citrobacter rodentium*, as well as LPS. Both bacterial strains but not LPS induced robust sST2 production by the colonic stromal cells (Fig. 4.17 C).

We also tested a range of cytokines known to be abundant in the colon, and IL-1β, IL-33 and TNFα were able to significantly induce sST2 production in *in-vitro*-stimulated stromal cells (Fig. 4.17 D). These results suggest that both bacterial and immune factors could directly contribute to the induction of sST2 production by stromal cells in the context of intestinal inflammation.

In summary, we identified stromal cells in the colonic LP as a major cellular source of sST2 that limits the bioavailability of IL-33 during intestinal inflammation. We also showed that the production of sST2 by stromal cells could be induced by both bacterial stimuli and pro-inflammatory cytokines.

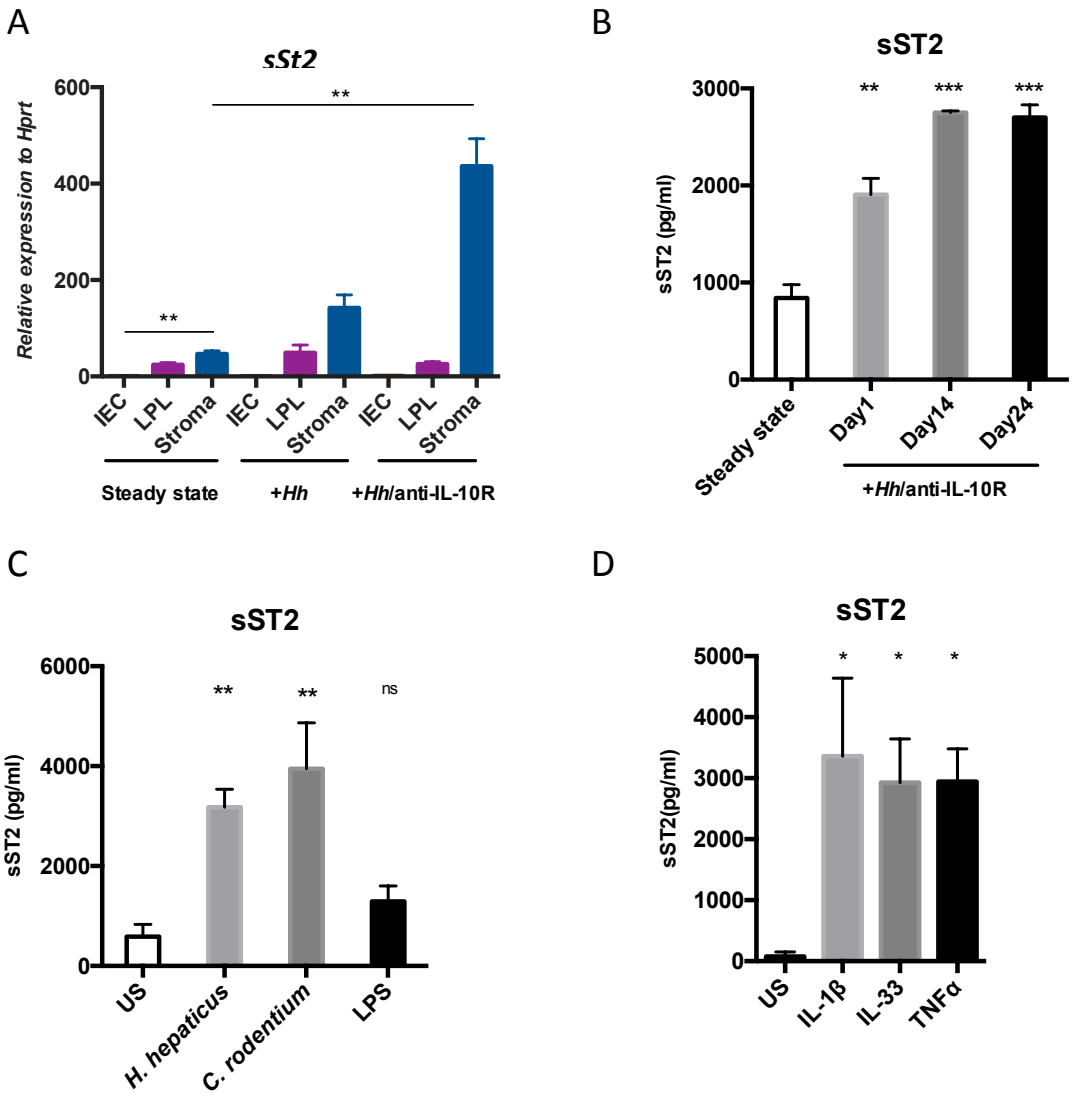


Figure 4.17. Stromal cells produce sST2.

(A) C57BL/6 mice were sacrificed without treatment (steady state) or infected with *Helicobacter hepaticus* either alone (+*Hh*) or with concomitant i.p. injections of a blocking anti-IL-10R mAb (1mg weekly) (+*Hh*/anti-IL-10R). Mice were sacrificed on day 10 and three different colonic cell populations were harvested (IEC following a 15 min EDTA wash, LPL from the 40/70% gradient interphase and stroma from the 30/40% gradient interphase) and RNA was extracted for subsequent RT-qPCR (n=5 for steady state and n=4 for +*Hh* and +*Hh*/anti-IL-10R). Relative expression levels were calculated using 2^(-ΔΔCT) method normalising to the housekeeper *Hprt* and the steady state IEC.

(B) Protein quantification of sST2 by ELISA in supernatants of stromal cultures of cells isolated from the colon at indicated time points of the *Hh*/anti-IL-10R model of colitis cultured *ex vivo* (n=3).

(C) Protein quantification of sST2 by ELISA in supernatants of stromal cells isolated from the colon of C57BL/6 steady state mice, and stimulated *in vitro* with *Helicobacter hepaticus*, *Citrobacter rodentium* or LPS (n=5).

(D) Protein quantification of sST2 by ELISA in supernatants of stromal cells isolated from the colon of C57BL/6 steady state mice and stimulated *in vitro* with indicated cytokines (n=3).

Bars represent mean ±SEM.

Data represents results of one experiment. Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test or ordinary one-way analysis of variance followed by Bonferroni's multiple comparisons test. *P ≤ 0.05, ** P ≤ 0.01, *** P≤ 0.001, **** P ≤ 0.0001, ns – not significant.

4.3 Discussion

In this chapter, we have investigated whether IL-33 modulates Treg responses, and while *in vitro* experiments showed that IL-33 directly promotes Treg phenotype, *in vivo*, the role of IL-33 signalling for Treg development was dispensable. We also identified IL-23 and sST2 as negative regulators of IL-33-mediated responses. Finally, for the first time, we showed that in a non-chemically-induced chronic model of colitis ST2-deficiency did not impact the intestinal pathology.

Recent studies from our and other laboratories have identified a sizable population of ST2-expressing Tregs residing in the non-lymphoid tissues suggesting that IL-33 may be an important factor for the survival or function of these cells (Schiering et al., 2014, Vasanthakumar et al., 2015, Kuswanto et al., 2016). We used *in vitro* cell culture systems to study the direct effects of IL-33 on Tregs.

We demonstrated that IL-33 could directly modulate nTregs and iTregs in culture without additional interactions with other immune or non-immune cells. IL-33 amplified a positive feedback loop, whereby the activation of the Gata3 pathway resulted in enhanced ST2 expression in Tregs. Gata3 has previously been shown to promote Foxp3 expression by binding to *Foxp3* regulatory regions (Wang et al., 2011, Rudra et al., 2012). Here, we showed that upon IL-33 stimulation Gata3 was recruited to the *Foxp3* promoter leading to increased Foxp3 expression; consistent with this, the genes co-regulated by Gata3 and Foxp3 were increased in response to IL-33. Therefore, we identified IL-33 as a direct upstream mediator of the Gata3 pathway activation in Tregs.

Similarly, expression of ST2 is also dependent on Gata3 in Th2 cells (Guo et al., 2009) and ILC2s (Yagi et al., 2014). In Th2 cells, Gata3 together with STAT5 co-regulate ST2 expression in response to IL-33 and a STAT5-activator, such as IL-2, IL-7 and TSLP. Both Gata3 and ST2 are expressed at low levels in resting Th2 cells. IL-33 together with a STAT5 activator increase Gata3 levels, in turn, enhancing ST2 expression and inducing IL-5 and IL-13 production by Th2 cells. Gata3 has also been shown to bind to the *Il1rl1* enhancer in Th2 cells upon IL-33 stimulation (Guo et al., 2009), mirroring our observations in Tregs. Of note, we did not observe any changes in STAT5 phosphorylation in response to IL-33 in nTreg and iTreg cultures (data not shown). Additionally, Gata3 is required for ILC2 development (Hoyler et al., 2012, Yagi et al., 2014), and shown to regulate transcription of the *Il1rl1* gene in these cells (Yagi et al., 2014). Other mechanisms, which regulate ST2 expression, have also been described. For instance, another study identified the Gfi1 transcription factor as a critical regulator of ST2 expression in ILC2 (Spooner et al., 2013). In contrast, in Th1 cells, in which ST2 is only induced under special conditions, for instance in highly activated effector cells during LCMV infection, the ST2 expression was controlled by the transcription factors T-bet and STAT4 (Baumann et al., 2015). These results suggest that, depending on the context and T cell type, IL-33 promotes 'master transcriptional regulators' characteristic of a given Th cell lineage, which in turn promote ST2 expression and amplify the positive feedback loop.

To test the requirement for IL-33 in Treg development and function *in vivo*, we investigated Tregs in ST2-deficient mice. Our results demonstrated that Treg development in the colonic LP was not affected in the absence of IL-33 signalling under

homeostatic conditions. In fact, the frequency of Foxp3^{+} T cells was higher in ST2$^{-/-}$ mice than in wild type controls. Since ST2 marks specifically Gata3-expressing Tregs we hypothesised that this Treg population could be preferentially affected; however, transcription factor expression analysis demonstrated a comparable frequency of Gata3^{+} Tregs in ST2-deficient and -sufficient mice. Furthermore, a mixed bone marrow chimaera generated from wild type and ST2$^{-/-}$ hematopoietic cells showed a similar increase in the proportion of ST2$^{-/-}$ Treg cells compared to the wild type counterparts. This indicated that ST2-deficient Treg cells can home, accumulate and survive in the colonic LP efficiently under homeostatic conditions. This contrasts studies investigating Tregs with a specific deletion of Gata3. Wang et al. (2011) argued for the critical role of Gata3 in the maintenance of Treg homeostasis since the genetic deletion of Gata3 specifically in Foxp3^{+} T cells resulted in a multiorgan inflammation. This was associated with reduced amounts of Foxp3 and other Treg effector genes (Wang et al., 2011). In contrast, in our study, ST2-deficiency did not alter Foxp3 expression in Tregs. Furthermore, while Wohlfert et al. (2011) did not observe any enhanced inflammatory responses upon Gata3 deletion in Tregs under steady state conditions, they demonstrated using murine mixed bone marrow chimaeras that Gata3-deficient Tregs failed to accumulate at inflamed sites (Wang et al., 2011, Wohlfert et al., 2011). These results suggested that Gata3 expression might be crucial for the control of inflammation. Again, here, we found that ST2-deficient Tregs performed to the same extent as wild type Tregs in the context of a competitive bone marrow chimaera setting.

Our data collected using *in vitro* systems suggested that IL-33 is a factor promoting Treg phenotype, particularly important for Gata3-expressing Tregs. By contrast, the *in vivo* studies showed that IL-33 is dispensable for both Treg development and the differentiation of the Gata3$^+$ Treg subset. However, while the *in vitro* experiments facilitate the understanding of the biological effects, they cannot recapitulate the complexity of the immune cell and mediator networks that orchestrate the immune responses *in vivo.* It is likely that other factors contribute to Gata3$^+$ Treg development and maintenance in such a complex environment such as the intestinal LP.

Similarly, while IL-33 is commonly associated with Th2 cells, it is not required for their differentiation. Th2 cells are found in ST2-deficient mice; however, they exhibit decreased Th2-associated cytokine production and reduced immune responses (Townsend et al., 2000). IL-33 is required in Th2 cells specifically for amplification of immune responses. For instance, in response to IL-33 stimulation Th2 cells produced IL-13 independent of TCR stimulation (Gao et al., 2015). It is likely that a similar mechanism underpins the role of IL-33 in promoting Treg function. In line with this, Wohlfert et al. (2011) demonstrated that TCR stimulation was sufficient to induce Gata3 expression in Tregs *in vitro*. Furthermore, this was enhanced upon IL-2 treatment, and consistently IL-2-deficient mice expressed lower amounts of Gata3 in the small intestinal LP Tregs (Wohlfert et al., 2011). Additionally, Gata3-deficient Tregs have been reported to express almost undetectable amounts of ST2 both *in vivo* and *in vitro* (Schiering et al., 2014, Gao et al., 2015). Overall, studies to date suggest that multiple signals, which may exhibit functional redundancy, exist that control development and maintenance of ST2$^+$/Gata3$^+$ Tregs. Drawing similarities between

Tregs and Th2 cells, it may be speculated that the initial expression of Gata3 in Tregs is induced by TCR stimulation, whereas the Treg phenotype is maintained and stabilised upon exposure to additional signals, such as IL-2 and IL-33.

Consistent with our results, a recent study reported that IL-33 deficiency did not alter Treg frequencies in the lymphoid tissues nor the lung (Siede et al., 2016). By contrast, multiple studies of VAT-resident Tregs demonstrated that IL-33 signalling was required for VAT-resident Treg development and maintenance. $ST2^{-/-}$ mice harboured a markedly lower number of Tregs in the VAT compared to wild type animals (Kolodin et al., 2015, Molofsky et al., 2015, Vasanthakumar et al., 2015). This was also recapitulated in mice that lack the MyD88 adaptor protein, via which IL-33, among others, signals (Vasanthakumar et al., 2015). Surprisingly, a targeted deletion of MyD88 in $Foxp3^{+}$ Treg cells (*Foxp3*$^{\text{YFP-cre}}$ × *Myd88*^{flox}) did not affect Treg responses to IL-33 in the VAT nor the lung (Molofsky et al., 2015). Here, while a decrease in Foxp3-expressing T cells in the colon of $MyD88^{-/-}$ mice was apparent, the frequencies of $ST2^{+}$, $Gata3^{+}$ and $Ror\gamma t^{+}$ Tregs were not significantly changed. However, the overall reduction in colonic Tregs in this context cannot be attributed to the absence of IL-33 signalling alone, since MyD88 is a shared adaptor protein for TLR and IL-1 family signalling. Therefore, the observed decrease in colonic Treg numbers could be due to the absence of different signals than IL-33. For instance, mice lacking TLR2, which also requires MyD88 to signal, displayed reduced Treg numbers in the spleen and blood (Sutmuller et al., 2006). Finally, differences between the colonic and VAT environment may drive the differential requirement for IL-33 signalling in colonic and VAT Tregs. Factors compensating for the lack of IL-33 may be absent from the VAT, while they

contribute to the maintenance of the ST2/Gata3-expressing population of Tregs in the colon. However, these organ-specific differences will have to be further elucidated.

To interrogate the role of IL-33 in the intestine in an inflammatory setting *H. hepaticus*/anti-IL-10R-driven model of colitis was performed in $ST2^{-/-}$ mice. To our knowledge, this is the first report of a chronic colitis model tested in an ST2-deficient setting, which is not chemically induced and is lymphocyte-dependent. The lack of IL-33 signalling did not impact on the severity of intestinal inflammation despite a reduction in IFN-γ-producing cells. Interestingly, as observed under steady state conditions, there was an increased frequency of Tregs in the colon strongly suggesting that colonic LP Tregs do not require IL-33 signalling under an inflammatory setting either. While no other studies have investigated IL-33 signalling in the context of *H.hepaticus*-induced intestinal inflammation, ST2-deficient Tregs exhibited a moderate but significant reduction in their capacity to prevent development of the intestinal pathology in a T cell transfer model of colitis (Schiering et al., 2014). This phenotype was further enhanced when Tregs deficient in Gata3 were investigated. Gata3-deficient Tregs failed to prevent wasting disease and the development of intestinal inflammation (Wang et al., 2011, Wohlfert et al., 2011). These results suggest that Gata3 governs a regulatory module in Tregs that is specifically required to inhibit potent effector responses, and IL-33 signalling may contribute to its regulation. However, while the T cell transfer model allows studies of the role of Tregs in preventing or limiting intestinal inflammation, it represents a particularly challenging environment represented by the robust proliferation of effector T cells producing high amounts of pro-inflammatory cytokines upon transfer into a lymphocyte-deficient

host. Therefore, studies of ST2- and Gata3-deficient Tregs in a more physiologically relevant setting could provide more insight into Treg biology.

Our studies in the *H. hepaticus*/anti-IL-10R-driven model of colitis have not provided an apparent pathological or protective role of IL-33 in chronic intestinal inflammation. It could be speculated that deficiency of IL-33, which can robustly promote type 2 immune responses, would only become functionally relevant in a Th2-driven model of intestinal inflammation. This is supported by the findings that IL-33 was significantly increased in human mucosal biopsies in UC patients (Beltran et al., 2010, Kobori et al., 2010, Pastorelli et al., 2010, Seidelin et al., 2010, Sedhom et al., 2013, Waddell et al., 2015).

In our studies, we identified *Areg* as a highly enriched transcript among colonic $ST2^{+}$ Tregs. *Areg* expression was also induced by stimulation with IL-33 in nTregs *in vitro*. Amphiregulin as an EGF-like growth factor has been repeatedly associated with the role in repair (Berasain et al., 2005, Enomoto et al., 2009, Shao and Sheng, 2010, Burzyn et al., 2013, Salimi et al., 2013, Arpaia et al., 2015, Meulenbroeks et al., 2015, Monticelli et al., 2011, 2015). Therefore, we hypothesised that IL-33 could be of relevance for Treg-mediated repair processes in the gut. To further interrogate the putative role of IL-33 in the context of intestinal immune responses and repair, we investigated whether ST2 or Areg deficiency would impair the repair of the colonic LP following *H. hepaticus*/anti-IL-10R-induced colitis. We found that $ST2^{-/-}$ and $Areg^{-/-}$ mice did not develop worse pathology over time and there was no visible delay in the recovery of the colonic LP in these animals compared to wild type controls. ST2

deficiency in all cells of the organism could eliminate IL-33 associated pro-inflammatory responses and thus, represent a potential confounding factor which influences the results; however, since ST2-deficient mice develop similar pathology to wild type animals, this is an unlikely explanation. Amphiregulin, on the other hand, is primarily involved in the restoration of tissue homeostasis by promoting both repair mechanisms and immunosuppression (Zaiss et al., 2015). It has previously been shown to be critical for the healing of the gut mucosa in a DSS-induced model of colitis, a process that was mediated by ILC2s (Monticelli et al., 2015). Amphiregulin produced by subepithelial myofibroblasts has also been shown to drive the renewal of the intestinal epithelium following a total body irradiation (Shao and Sheng, 2010). Additionally, both Tregs and ILC2s have been described to produce amphiregulin in an IL-33-dependent manner (Monticelli et al., 2011, Arpaia et al., 2015). This was required for restoration of the lung function and tissue repair after influenza virus infection. In contrast to the previous reports that demonstrated a central role for IL-33-induced amphiregulin production in tissue repair, our results suggest that the IL-33-amphiregulin axis is not necessary for the recovery of the colonic LP in the tested experimental setting. Further characterisation of the repair modules in the outlined model could potentially clarify our results. Additionally, transgenic mice with a cell-type-specific deletion in ST2 or Areg would be better suited to validate the results in the described set-up.

Our results have also highlighted a negative regulator of the IL-33-mediated effects on Tregs, i.e. the pro-inflammatory cytokine IL-23. Polymorphisms in *IL23R* gene have been shown to be associated with increased susceptibility to IBD (Duerr et al., 2006,

Wellcome Trust Case Control, 2007). A key role as a driver of intestinal inflammation for IL-23 has also been demonstrated in both T-cell-dependent and innate models of colitis (Hue et al., 2006, Kullberg et al., 2006, Uhlig et al., 2006b, Yen et al., 2006, Elson et al., 2007, Arnold et al., 2016). In addition to the well-established capacity of IL-23 to induce pro-inflammatory cytokines, it has also been recently appreciated that IL-23 could promote colitis by inhibiting Treg differentiation (Izcue et al., 2008, Ahern et al., 2010). Initially, an increased frequency of Tregs was found in the colonic LP following administration of naïve $CD4^+$ T cells into a lymphocyte-deficient host in the absence of IL-23 (*Il23a*$^{-/-}$*Rag1*$^{-/-}$) (Izcue et al., 2008). The cell-intrinsic role of IL-23 in limiting $Foxp3^+$ T cell differentiation was subsequently confirmed using a co-transfer system and a mixed bone marrow chimaera in the T-cell transfer and *H. hepaticus*/anti-IL10R-driven models of colitis, respectively. In both settings, IL-23R$^{-/-}$ T cells exhibited increased differentiation into Foxp3-expressing Tregs compared to the IL-23R-sufficient counterparts (Ahern et al., 2010). However, the IL-23-mediated mechanism that inhibits $Foxp3^+$ Treg-differentiation has not been defined. Here, using *in vitro* culture systems, we demonstrated that IL-23 could abrogate IL-33-mediated effects on Tregs. Specifically IL-23 inhibited the enhanced expression of ST2 and the activation of the Gata3 pathway and its downstream targets. We have also described the role of IL-33 as a co-factor in TGF-β1-mediated iTreg differentiation, and in this setting, IL-23 similarly inhibited the promoting effect of IL-33 (Schiering et al., 2014). In further support of the proposed mechanism, IL-23 and IL-33 were both expressed *in vivo* during intestinal inflammation in the *H. hepaticus*/anti-IL10R-driven models of colitis. This raises a possibility that the two can interact to regulate the pro-inflammatory and regulatory mechanisms in the colonic LP. It will be of interest to investigate whether IL-

23 can also negate IL-33-mediated effects in inflammatory settings in different tissues, for instance, in obesity-induced inflammation, in which $ST2^+$ Tregs have been shown to play a protective role (Han et al., 2015).

Furthermore, we observed high levels of the soluble form of ST2 upon intestinal inflammation. With IL-1 family cytokines abundantly produced during inflammatory responses a tight regulation of IL1R-dependent signalling is necessary in order to prevent damaging local and systemic inflammatory reactions. sST2 is a decoy receptor that lacks the intracellular signalling domain of the membrane-bound form of the ST2 and therefore, can bind to IL-33 scavenging it and reducing its bioavailability (Hayakawa et al., 2007). Elevated levels of sST2 have been noted in multiple health conditions (Mildner et al., 2010). However, its relevance has not been studied excessively. Asthmatic patients had increased sST2 levels detected in the serum (Oshikawa et al., 2001), and consistently pre-treatment with sST2 in a murine model of asthma reduced the disease-promoting type 2 immune response (Hayakawa et al., 2007). sST2 is also an important biomarker in cardiac diseases with high levels of sST2 associated with higher mortality (reviewed in Pascual-Figal et al. (2016)). Very recently levels of sST2 have also been linked to severity and activity of disease in IBD patients (Diaz-Jimenez et al., 2011, Boga et al., 2016). However, it remains to be elucidated whether increased levels of sST2 are just a consequence of an on-going inflammation or its activity contributes to the regulation of the disease condition in the context of IBD. Also, the cellular sources of sST2 have not been well studied. Murine fibroblast- and endothelial-derived cell lines were able to produce sST2 as well as human cardiac myocytes and lung alveolar epithelial cells (Kumar et al., 1997, Mildner et al., 2010).

Here, we identified stromal cells as the dominant sST2-producing cell population in the colon. Characterisation of the intestinal stromal cells performed by our group demonstrated that they are non-hematopoietic, non-epithelial and non-endothelial cells expressing glycoprotein gp38 ($CD45^{-}Epcam^{-}CD31^{-}gp38^{+}$, unpublished data, Powrie group). They have also been shown to mediate certain immune functions to a limited extent (e.g. antigen presentation) equivalent to the stromal cells previously described in the human colonic mucosa (Owens et al., 2013). Using *in vitro* culture system of the *ex vivo* cultured colonic stromal cells we showed that these cells substantially upregulated production of sST2 upon both bacterial and immune stimuli. Consistent with our findings IL-1β and TNFα have been previously shown to promote sST2 production (Kumar et al., 1997, Mildner et al., 2010). Of note, an early study by Pastorelli and colleagues (2010) suggested that expression of sST2 in the serum of IBD patients was modulated upon anti-TNF therapy. Additionally, our experiments also showed that IL-33 appears to self-regulate its bioavailability by inducing sST2 production by stromal cells. This is particularly interesting since IL-33 has been suggested to act as a transcriptional repressor, including downregulation of the *sST2* gene transcription (Shao et al., 2014). Finally, these results suggest that the colonic stromal cells have not only a capacity to respond to the surrounding inflammatory milieu but also to regulate it, and the elevated levels of sST2 could be a more general signature of inflammatory conditions, such as the intestinal inflammation.

In summary, we demonstrated here that IL-33 promotes Treg phenotype by activating the Gata3 pathway and repair module highlighted by amphiregulin expression. However, the requirement for IL-33 signalling in Treg development was not confirmed

in vivo, and furthermore, ST2 deficiency did not modulate the severity of colitis nor the subsequent recovery phase. Additionally, IL-23 was identified as a negative regulator of the IL-33 effects on Tregs *in vitro*. While the collected data strongly suggest that the balance of IL-33, IL-23 and sST2, produced by the intestinal stromal cells, could be a major determinant of the intestinal homeostasis, this remains to be validated in future experiments.

Chapter 5. Production of IL-33 in the colon

5.1 Introduction

IL-33 is regarded as a dual-function cytokine, similar to IL-1α and HMGB1. It can function at both an intra- and intercellular level: as a nuclear factor, which modulates the chromatin structure and gene expression, and as a conventional cytokine. The intracellular role of IL-33 has been understudied with most studies focusing on its pro-inflammatory capacity once it is secreted. IL-33 is not critical for the homeostasis but its role is essential upon stimulation. This was demonstrated in IL-33- and ST2-deficient animals, which do not develop any obvious abnormalities under steady state conditions. However, when challenged their immune response is substantially different from those of wild type animals (Hoshino et al., 1999, Senn et al., 2000, Townsend et al., 2000, Oboki et al., 2010, Louten et al., 2011, Pichery et al., 2012). To understand the role IL-33 plays in immune regulation it is critical to describe its cellular source.

IL-33 can be found associated with the chromatin in the nuclei (Carriere et al., 2007) of many different tissues across the body, including the lymph nodes, spleen, stomach, lung, skin and the brain (Schmitz et al., 2005). Its widespread expression in human organs is not surprising since it is expressed in most endothelial cells across the

vascular tree (Kuchler et al., 2008, Moussion et al., 2008). It was first described in humans as a nuclear factor preferentially expressed in high endothelial venules (HEVs) (Baekkevold et al., 2003), and consequently it has been extensively studied in these cells. HEVs are specialised blood vessels primarily found in organised secondary lymphoid tissues important for lymphocyte recruitment into lymphoid organs (Baekkevold et al., 2003, Carriere et al., 2007). High expression of IL-33 in these cells may suggest a role for IL-33 in lymphocyte trafficking. The constitutive expression of IL-33, however, appears to be species-specific, since IL-33 does not seem to be constitutively expressed throughout the blood vessels in mice (Pichery et al., 2012, Talabot-Ayer et al., 2012, Sedhom et al., 2013, Chen et al., 2015, Molofsky et al., 2015). A novel Il-33-LacZ gene trap reporter mouse strain allowed for evaluation of IL-33 expression in murine tissues (Pichery et al., 2012). IL-33 was detected in the adult tissues, including lymphoid organs, at barrier sites as well as in the brain, and during embryonic development (Pichery et al., 2012). Thus, there appears to be a consensus in both human and murine studies that the expression of IL-33 is enriched in structural cells, especially at barrier sites. These include: epithelial cells at mucosal sites (Schmitz et al., 2005, Moussion et al., 2008, Pichery et al., 2012, Hardman et al., 2013), keratinocytes in the skin (Moussion et al., 2008, Savinko et al., 2012, Sundnes et al., 2015), fibroblasts (Moussion et al., 2008, Bonilla et al., 2012, Pichery et al., 2012), smooth muscle cells (Miller et al., 2008, Prefontaine et al., 2009) and glial cells in nervous tissues (Pichery et al., 2012, Gadani et al., 2015). In general, IL-33 is upregulated upon stimulation; however, in some cells it is expressed constitutively, which suggests differential regulation of IL-33 expression in different cell types. For instance, keratinocytes in mice display a constitutive expression of IL-33, which is lost

upon inflammation (Sundnes et al., 2015). On the contrary, healthy human epidermis exhibits very low IL-33, which is rapidly induced upon IFN-γ exposure (Sundnes et al., 2015). Elevated levels of IL-33 can be found in many inflammatory diseases as well as during infections. A limited number of studies have employed murine bone marrow chimaera models or cell-specific gene deletion to identify relevant functional sources of IL-33. These studies showed that IL-33 production by non-hematopoietic cells was critical for the development of an adequate immune response. For instance, Nakanishi et al. (2013) showed that IL-33 produced by the epithelial cells of the nose was required in the house dust mite antigen-induced allergic rhinitis while Chen et al. (2015) demonstrated that IL-33-expressing endothelial cells drove systemic inflammation following myocardial pressure overload.

While there is substantial evidence that cells of the non-hematopoietic origin provide a primary source of IL-33, recent studies have also shed light on the expression of IL-33 in both human and murine immune cells. In unstimulated cells, hematopoietic-cells express no or low levels of IL-33; however, *Il33* mRNA can be induced upon stimulation (Polumuri et al., 2012, Talabot-Ayer et al., 2012), albeit to a lower level compared to *Il33* expression detected in non-haematopoietic cells (Schmitz et al., 2005, Byers et al., 2013). Most reports have focused on IL-33 expression in macrophages and DCs, in which IL-33 can be upregulated upon stimulation with LPS, TLR ligands, prostaglandin E2, and noradrenaline and adrenaline (Luthi et al., 2009, Ohno et al., 2009, Yanagawa et al., 2011a, 2011b, Polumuri et al., 2012, Talabot-Ayer et al., 2012, Su et al., 2013). Mast cells (Ohno et al., 2009, Hsu et al., 2010, Nile et al., 2010) and monocytes (Nile et al., 2010) can also produce IL-33. Importantly, most of these studies involved cells

cultured and stimulated *in vitro*. Therefore, the importance of IL-33 production by immune cells remains to be confirmed *in vivo*. Studies of expression of IL-33 are further complicated by the existence of two alternative promoters that in mice produce *Il33a* and *Il33b* transcripts. They both produce full-length 8-exon *Il33* and are expressed in a cell-type-specific manner with to date no functional differences between the two transcripts reported (Polumuri et al., 2012, Talabot-Ayer et al., 2012).

In the nucleus, IL-33 associates with chromatin by protein-protein interactions (Carriere et al., 2007, Roussel et al., 2008). Its N-terminal domain is required and sufficient for its nuclear localisation (Carriere et al., 2007) and therefore, not surprisingly the majority of studies reported nuclear staining in both human and mouse cells. However, there are a few reports describing cytoplasmic localisation of IL-33 in human cells, including monocytes (Nile et al., 2010), placenta-derived macrophages (Fock et al., 2013) or malignant tissues (Liu et al., 2014, Yang et al., 2016c). Additionally, IL-33 was found to localise to membrane-bound cytoplasmic vesicles in murine fibroblasts, which was suggested as a potential mechanism of release (Kakkar et al., 2012). However, since the specificity of the antibody staining was not verified using knock-out tissue in the above studies, these results remain to be verified.

There are many reports on the role of IL-33 in the context of IBD and in murine models of colitis, and the localisation of IL-33 expression was investigated in some of them. In the healthy human colon IL-33 was expressed in endothelial and epithelial cells (Moussion et al., 2008, Beltran et al., 2010, Sedhom et al., 2013). Epithelial cell

expression was increased upon inflammation in IBD patients compared to non-inflamed controls (Beltran et al., 2010, Pastorelli et al., 2010, Seidelin et al., 2010, Sedhom et al., 2013). Myofibroblasts defined by expression of α-SMA were consistently identified as a cellular source of IL-33 in inflamed UC patients (Kobori et al., 2010, Sponheim et al., 2010). Pastorelli et al. (2010) also described infiltration of IL-33-expressing $CD11b^{+}$ DCs and $CD19^{+}$ plasma cells into the lesions of IBD patients. Epithelial and myofibroblast expression in the inflammatory setting of a DSS-induced colitis was also observed (Sedhom et al., 2013); however, reports of IL-33 expression in the mouse gut are limited.

Recent studies showed expression of IL-33 in the intestinal epithelium in patients suffering from graft-versus-host disease following bone marrow transplantation (Reichenbach et al., 2015). Additionally, both epithelial cells and submucosal myofibroblasts produced IL-33 in the tumour tissue of human colorectal cancer patients and in the $Apc^{(Min/+)}$ mouse model of intestinal tumourigenesis (Maywald et al., 2015).

Despite abundant analysis of IL-33 expression, the relevant cellular sources in the gut are not known. The studies are hindered by lack of reliable tools required for the detection of IL-33. In addition, some conclusions were drawn based on the RNA expression, which particularly for IL-33, may not translate into protein expression. Due to recent advances in the availability of antibodies and generation of IL-33-deficient mice, here, we are able to reliably investigate the cellular source of IL-33 in the gut.

In this chapter we use a wide range of techniques to systematically describe expression and cellular sources of IL-33 in the murine colon. The two main objectives of this chapter are:

- To define which cells produce IL-33 in the colon under homeostatic conditions
- To determine spatial expression of IL-33 during intestinal inflammation.

5.2 Results

5.2.1 *Il33* mRNA is enriched in the stromal fraction of the colonic LP.

Many reports of cells expressing IL-33 in the GI tract exist in both human and mouse; however, the current results are not conclusive. Here, we set out to investigate which cells in the large intestine of wild type mice express IL-33. To this end we collected three main cell fractions representing intestinal epithelial cells (IEC), lamina propria leukocytes (LPL) and stromal cells, which correspond to large structural cells of the gut. It is important to note that the selection of the LPL and stroma fractions is obtained with a gradient-based method and the fractions do not represent pure but rather enriched populations.

Under steady state conditions there was a significant enrichment of *Il33* mRNA in the stromal population, some *Il33* was expressed in the LPL, and almost no *Il33* was detectable in epithelial cells (Fig. 5.1). To test whether epithelial cells upregulate *Il33* in response to pro-inflammatory stimuli we used the H. hepaticus/anti-IL-10R-driven model of colitis and isolated IEC, LPL and stromal cell populations from the colon on day 5 and 10 of the model. On day 5, pro-inflammatory cytokine genes are increased in the colon despite no clinical symptoms. By day 10, mice display clinical signs of intestinal inflammation (data not shown). Again no *Il33* could be found in the epithelial cells, while *Il33* was most highly enriched in the stromal fraction on both day 5 and 10. *Il33* mRNA levels were significantly decreased on day 10 compared to day 5 (Fig. 5.1).

Additionally, *H. hepaticus* infection alone did not induce any changes in *Il33* expression (data not shown).

Together the results indicate that large cells, which likely represent structural cells such as the stromal population, are a major source of IL-33 in the colon.

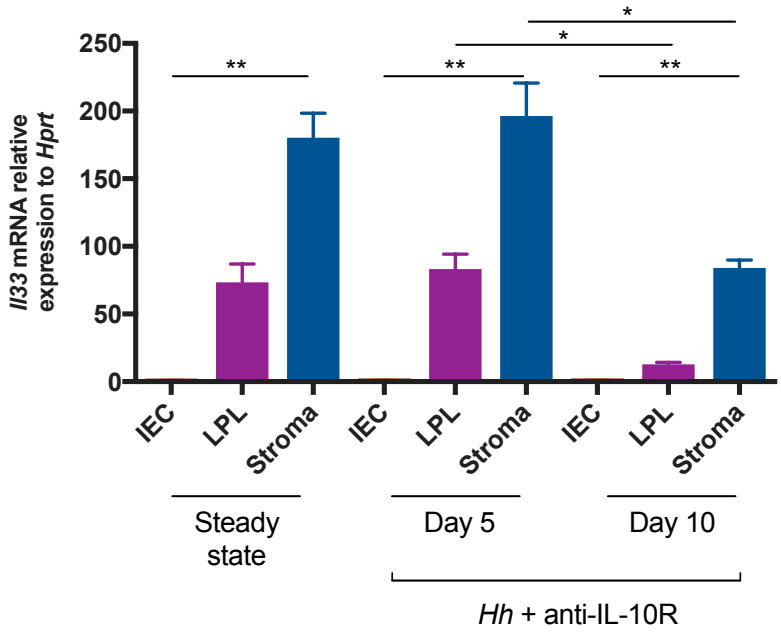


Figure 5.1. *Il33* mRNA is enriched in the colonic stromal fraction.

C57BL/6 mice were sacrificed without treatment (steady state) or infected with *H. hepaticus* with concomitant i.p. injections of a blocking anti-IL-10R mAb administered (1mg weekly) (*Hh* + anti-IL-10R). Mice were sacrificed at indicated time points and cells from three different fractions of the colon were harvested (IEC following a 15 min EDTA wash, LPL from the 40/70% gradient interphase and stroma from the 30/40% gradient interphase) and RNA was extracted for subsequent RT-qPCR.

Il33 expression in IEC, LPL and Stroma.

Relative expression levels were calculated using 2^(-ΔΔCT) method normalising to the housekeeper *Hprt* and the steady state IEC. Bars represent mean ±SEM.

Data represent results from a single experiment (n=5 for steady state and n=4 for *Hh*+anti-IL-10R). Statistical significance was determined using a Kruskal Wallis one-way analysis of variance followed by a Dunn's post-hoc test. * P≤0.05, **P≤0.01.

5.2.2 Validation of the IL-33$^{+/Citrine}$ reporter mouse strain in the lung.

The inability to pinpoint the precise cellular source of IL-33 has arisen, in part, from poor availability of research tools. Recent generation of two novel reporter mouse strains have advanced our understanding of IL-33 expression. The Il-33-LacZ gene trap reporter mouse strain provided substantial information on the expression of IL-33 in various tissues (Pichery et al., 2012); however, due to the nature of the LacZ system, IL-33 expression can only be evaluated on cryo-sections. Hardman et al. (2013) generated a fluorescent reporter mouse strain that allows for tracking of IL-33-expressing cells *in vivo* (IL-33$^{+/Citrine}$). In these mice the GFP-derived *Citrine* gene has been inserted directly downstream of the *Il33* start codon with Citrine fluorescence marking cells expressing *Il33* mRNA (Fig. 5.2 A). This knock-in results in a functional knock-out when mice are bred as homozygotes (IL-33$^{Cit/Cit}$) but allows tracking of IL-33-expressing cells in heterozygous mice (IL-33$^{+/Cit}$) (Hardman et al., 2013).

To make the IL-33$^{+/Cit}$ strain compatible with our in-house strains and mouse models of colitis we backcrossed the IL-33$^{+/Cit}$ mice from the BALB/c to C57BL/6 background for 8 generations until mice were 99.9% C57BL/6. This was assessed by SNP-based genotyping by Charles River Laboratories, UK. In order to validate the IL-33$^{+/Cit}$ strain on the C57BL/6 background single cell suspensions from the lung of IL-33$^{+/Cit}$ and wild type mice were analysed by flow cytometry. In line with previous results (Hardman et al., 2013), a small population of Citrine^{+} cells could be found in the lung of IL-33$^{+/Cit}$ mice (Fig. 5.2 B). In line with the report by Hardman et al. (2013), most cells expressing Citrine were non-haematopoietic CD45^{-} and up to 75% of Citrine^{+} cells were positive for the epithelial cell marker EpCam (Fig. 5.2 C).

Additionally, *ex vivo* imaging was used to evaluate Citrine expression in the lung. Citrine^{+} cells (in green) could be clearly seen in the lungs of IL-33$^{+/Cit}$ mice (Fig. 5.2 D), indicating that IL-33 expression could be reliably assessed using this mouse tool.

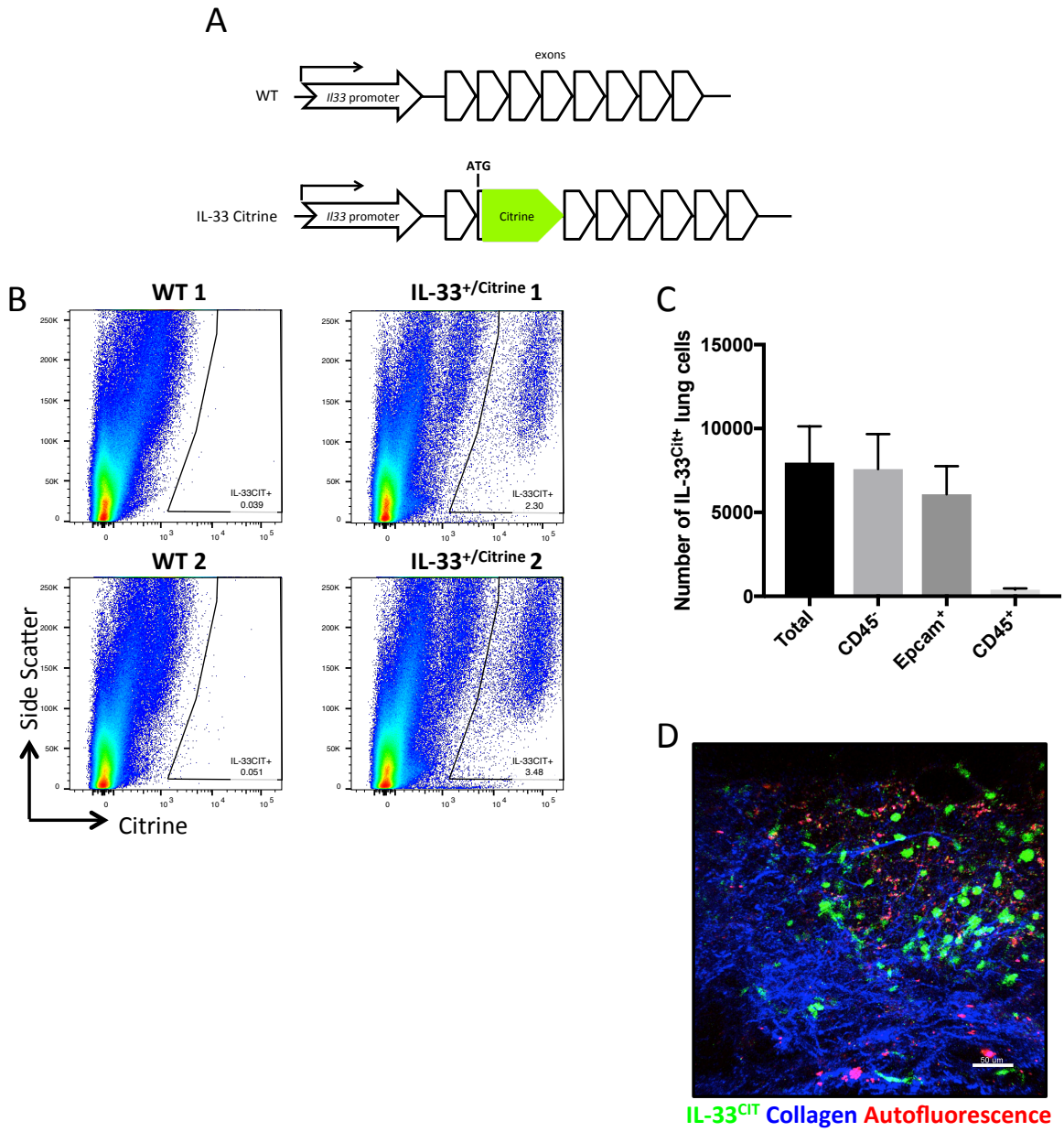


Figure 5.2. Validation of the IL-33$^{+/Cit}$ reporter mouse strain in the lung.

Lungs of IL-33$^{+/Cit}$ mice were isolated and processed for flow cytometric analysis or *ex vivo* imaging.

(A) IL-33$^{+/Cit}$ reporter mice have a citrine gene inserted directly downstream of the start codon ATG of the endogenous *Il33* gene.

(B) Two representative flow cytometry plots showing citrine expression in the single cell suspensions isolated from the lung of wild type (WT) (left panel) and IL-33$^{+/Cit}$ reporter mice (right panel).

(C) Characterisation of IL-33^{Cit+} cells in the lung assessed by expression of CD45 and EpCam using flow cytometric analysis. Gated on IL-33^{Cit+} cells as in (B). Bars represent mean ±SEM. Results represent data of a single experiment (n = 4 mice).

(D) An *ex vivo* image of lung tissue from an IL-33$^{+/Cit}$ reporter mouse. Original magnification 20X. Scale bar – 50 µm. IL-33^{citrine} in green, collagen in blue, autofluorescence in red.

5.2.3 IL-33 expression pattern varies across the length of the murine colon.

The GI tract is highly specialised with particular anatomical adaptations associated with function. There are obvious differences between the small and large intestine determined by their distinct functions; however, the colon itself also varies in its anatomy, cellular composition and bacterial colonisation along its length (reviewed in Mowat and Agace (2014)).

To obtain a comprehensive picture of spatial expression of IL-33 in the colon, two-photon microscopy was applied to image different colonic regions in IL-33$^{+/Cit}$ reporter mice. Proximal, middle and distal colon pieces were imaged directly *ex vivo* from both the lumen side and through the muscularis mucosae side into the LP (Fig. 5.3 A). Imaging from the luminal side shows the colonic crypts primarily composed of epithelial cells. While no obvious Citrine fluorescence could be observed in the proximal and middle colon, epithelial cells in the distal colon expressed low amounts of IL-33 (Fig. 5.3 B). Imaging through the muscularis mucosae revealed large 'clusters' of Citrine^{bright} cells expressing IL-33 in the proximal colon and smaller 'clusters' of Citrine^{bright} cells expressing IL-33 in the middle colon (Fig. 5.3 B, C). These 'clusters' of Citrine^{bright} cells were adjacent to the layer of collagen, which is present at the base of the LP as depicted in the diagram in Figure 5.3 A. The 9-µm-slice images confirmed the proximity of IL-33-expressing cells to the collagen layer without co-expression of IL-33 by these cells (Fig. 5.3 C). Additionally, while a few small 'clusters' of Citrine^{bright} cells could be spotted in the distal colon, most of the cells fluorescent with Citrine based on their morphology represented epithelial cells (Fig. 5.3 B, C). This pattern of IL-33^{Cit} expression was consistently replicated in six mice imaged on two separate occasions.

Since the colonic contents are highly fluorescent, colonic sections from wild type mice were imaged as controls for the fluorescence of microbial and food residues, which confirmed that the detected Citrine signal was real (Fig. 5.4 A).

Additionally, our image analysis also showed that many vascular cells present in both large and small vessels throughout the colon expressed IL-33 (Fig. 5.4 B).

In summary, our results indicate that the expression of IL-33 across the length of the colon is not uniform: clusters of undefined cells represented the primary IL-33 source in the proximal and middle colon while epithelial cells expressed IL-33 in the distal colon. Some vessels expressed IL-33 throughout the colon independent of their location.

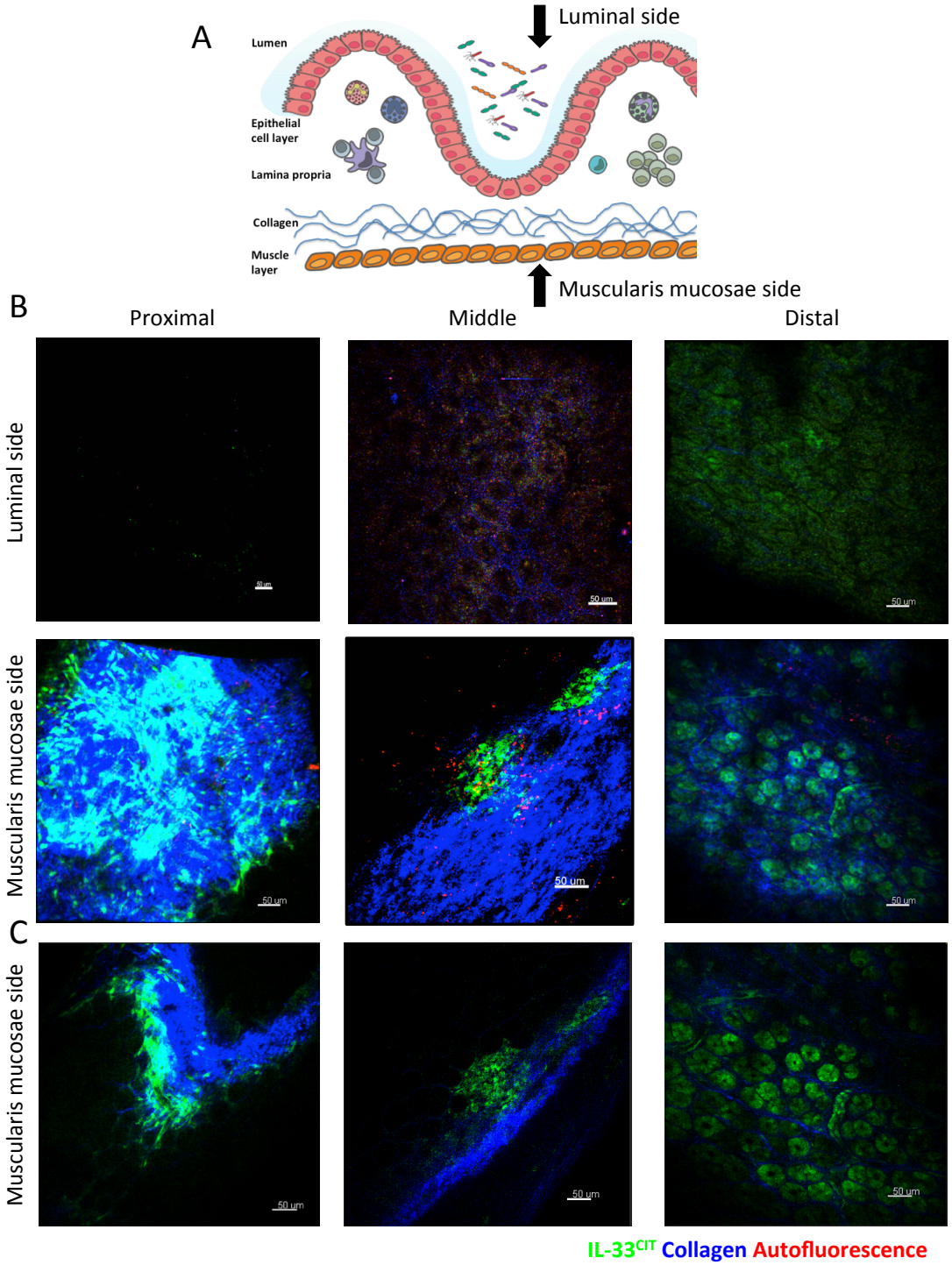


Figure 5.3. IL-33 expression varies along the length of the colon.

Colons were isolated from IL-33$^{+/Cit}$ reporter mice and directly imaged *ex vivo* with a spectral detector microscope. Sections representative of proximal, middle and distal colon are presented from both the luminal and muscularis mucosae sides.

(A) A schematic showing two sides from which the colon pieces were imaged.
(B) Representative e*x vivo* images of the proximal, middle and distal colon imaged from the luminal (top panel) and muscularis mucosae (bottom panel) sides. Images represent maximum intensity projection of 50-120 µm depending on the tissue.
(C) Representative 9 µm slice images corresponding to the images from (B) muscularis mucosae side.

Original magnification 20X. Scale bar – 50 µm.
IL-33^{citrine} in green, collagen in blue, autofluorescence in red.
Images are representative of two independent experiments (each n=3 mice).

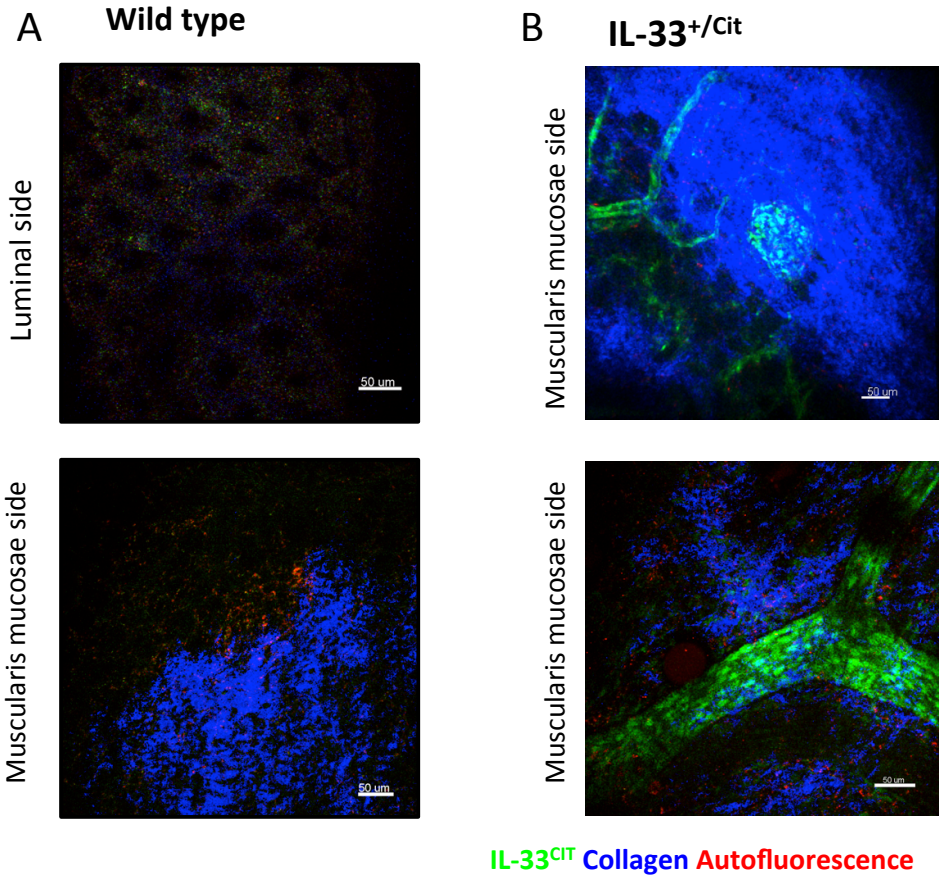


Figure 5.4. IL-33 expression in vessel structures.

Colons were isolated from C57BL/6 and IL-33$^{+/Cit}$ reporter mice and directly imaged *ex vivo* with a spectral detector microscope.

(A) Control images from a C57BL/6 mouse from the middle colon.
(B) Images depicting vessel-like structures expressing IL-33 in the colon of the IL-33$^{+/Cit}$ reporter mouse.

Top panel shows the luminal side of the colon, bottom panel shows the muscularis mucosae side.

Original magnification 20X. Scale bar – 50 µm.
IL-33^{citrine} in green, collagen in blue, autofluorescence in red.
Images are representative of two independent experiments (each n=3 mice).

5.2.4 IL-33 is highly enriched in the secondary lymphoid organs.

IL-33-expressing cells identified in the proximal and middle colon (section 5.2.3) were contained in discrete structures, like those found in lymph nodes or lymphoid follicles. These locations are primary sites where cells of the adaptive arm of the immune system are primed. Interestingly, in the original description of IL-33, it was found to be abundant in the T cell zone of the Peyer's patches (Baekkevold et al., 2003).

Next, we tested whether IL-33 could be detected in the Peyer's patches, which are found in the small intestine, as well as the caecal patch, a single macroscopic lymphoid follicle found in the appendix part of the caecum (Masahata et al., 2014, Mizoguchi et al., 1996). Peyer's patches and the caecal patch of IL-33$^{+/Cit}$ reporter mice were isolated from the small intestine and caecum, respectively, and imaged directly *ex vivo*. We found large areas of IL-33-expressing cells in both tested types of secondary lymphoid structures (Fig. 5.5 A, B). Citrine fluorescence was very intense and widespread throughout these structures. A common pattern could be observed with IL-33-expressing cells forming a ring around an area negative for Citrine fluorescence. Structurally, Peyer's and caecal patches consist of B cell areas in the middle surrounded by T cell zones (Mowat and Agace, 2014). Taken together, the data raised the possibility that IL-33 could be restricted to the T cell areas, consistent with the report by Baekkevold et al. (2003).

Together the data indicate that both Peyer's and caecal patches were highly enriched in IL-33-expressing cells.

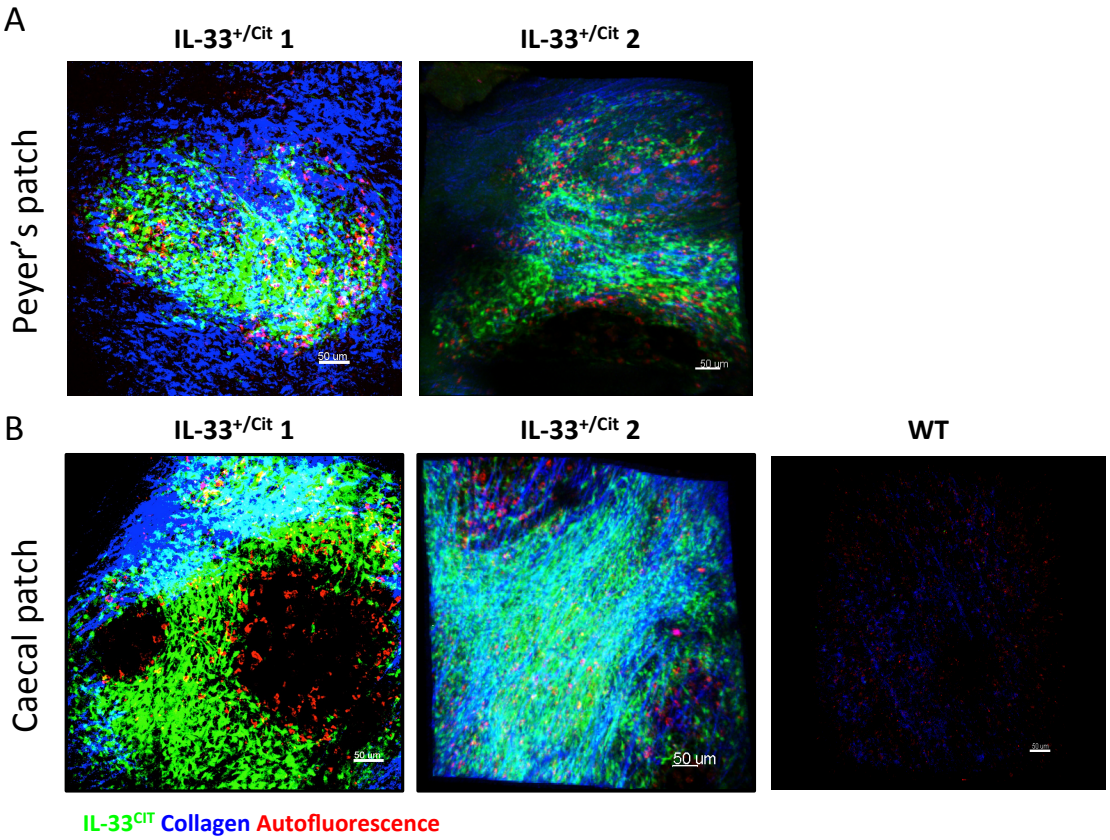


Figure 5.5. IL-33 is highly enriched in the secondary lymphoid structures.

Peyer's patches of the small intestine and the caecal patch of the caecum were isolated from IL-33$^{+/Cit}$ reporter and C57BL/6 mice and directly imaged *ex vivo* with a spectral detector microscope.

(A) Representative images of the Peyer's patches from IL-33$^{+/Cit}$ reporter mice.
(B) Representative images of the caecal patches from IL-33$^{+/Cit}$ reporter mice and C57BL/6 wild type (WT) control.

Original magnification 20X. Scale bar – 50 µm.
IL-33^{citrine} in green, collagen in blue, autofluorescence in red.
Images are representative of two independent experiments (each n=3 mice).

5.2.5 Validation of the anti-IL-33 antibody.

Two-photon imaging of *ex vivo* isolated tissue is a powerful tool, with which the expression of IL-33 across a whole tissue can be evaluated. However, it is difficult to precisely phenotype cells expressing IL-33 without crossing the IL-33$^{+/Cit}$ reporter mouse to a different cell-specific reporter strain or using an excessive amount of antibody to identify cells of interest *in vivo*. Unfortunately isolation of single cell suspensions from the IL-33$^{+/Cit}$ reporter mice for flow cytometric analysis was not feasible as the Citrine signal was markedly upregulated upon collagenase digestion (data not shown). This is likely due to the extensive cell death taking place during tissue digestion, which can result in the induction of *Il33* expression. Recent advances and increase in the availability of specific antibodies allowed us to identify a polyclonal antibody against murine IL-33, which we subsequently used in immunohistochemistry experiments.

Firstly, the antibody was validated in two organs where IL-33 expression has been already clearly defined: type-2 pneumocytes in the lung (Hardman et al., 2013) (Fig. 5.6 A) and endothelial cells lining the blood vessels in the VAT (Molofsky et al., 2015) (Fig. 5.6 B). No IL-33 signal could be detected in IL-33-deficient lung and VAT tissue, validating the specificity of the antibody (Fig. 5.6 A, B).

Secondly, to confirm that the expression of IL-33 determined by the IL-33$^{+/Cit}$ reporter mouse strain was consistent with protein expression, an anti-GFP antibody (which also detects Citrine) was combined with an anti-IL-33 staining in the lung and colon (Fig. 5.7). It is worth noting that while IL-33 is usually found in the nuclei, the Citrine protein

localises in the cytoplasm. IL-33-expressing cells in the lung as well as both in the clusters and in the vessels in the colon co-expressed Citrine and IL-33 (Fig. 5.7 A, B, C); thus, demonstrating that the antibody against IL-33 is specific. Additionally, this result confirmed that the protein expression correlates well with the transcriptional activity of the IL-33 promoter in the IL-33$^{+/Cit}$ reporter mouse strain.

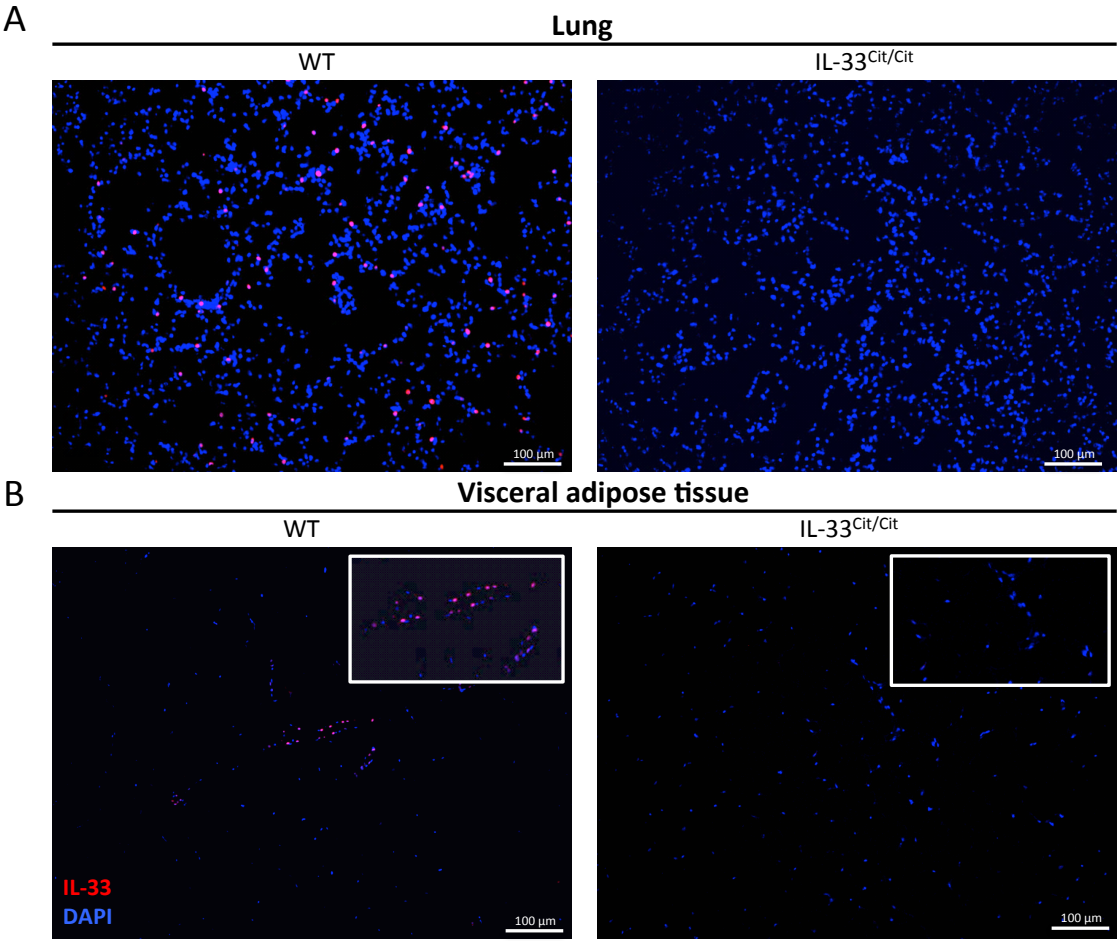


Figure 5.6. IL-33 expression in the lung and VAT.

Lungs and visceral adipose tissue of C57BL/6 (WT) and IL-33$^{Cit/Cit}$ mice were isolated and processed for IHC staining.

(A) Representative images of immunofluorescence staining of lung tissue in wild type C57BL/6 (WT) and IL33KO (IL-33$^{Cit/Cit}$) mice.
(B) Representative images of immunofluorescence staining of visceral adipose tissue in wild type C57BL/6 (WT) and IL33KO (IL-33$^{Cit/Cit}$) mice. Framed images in the right-hand corner represent zoomed-in regions.

Original magnification 20X. Scale bar – 100 µm.
IL-33 in red and nuclei in blue (DAPI).
The staining was performed in n=3 mice for lung and n=2 mice for VAT.

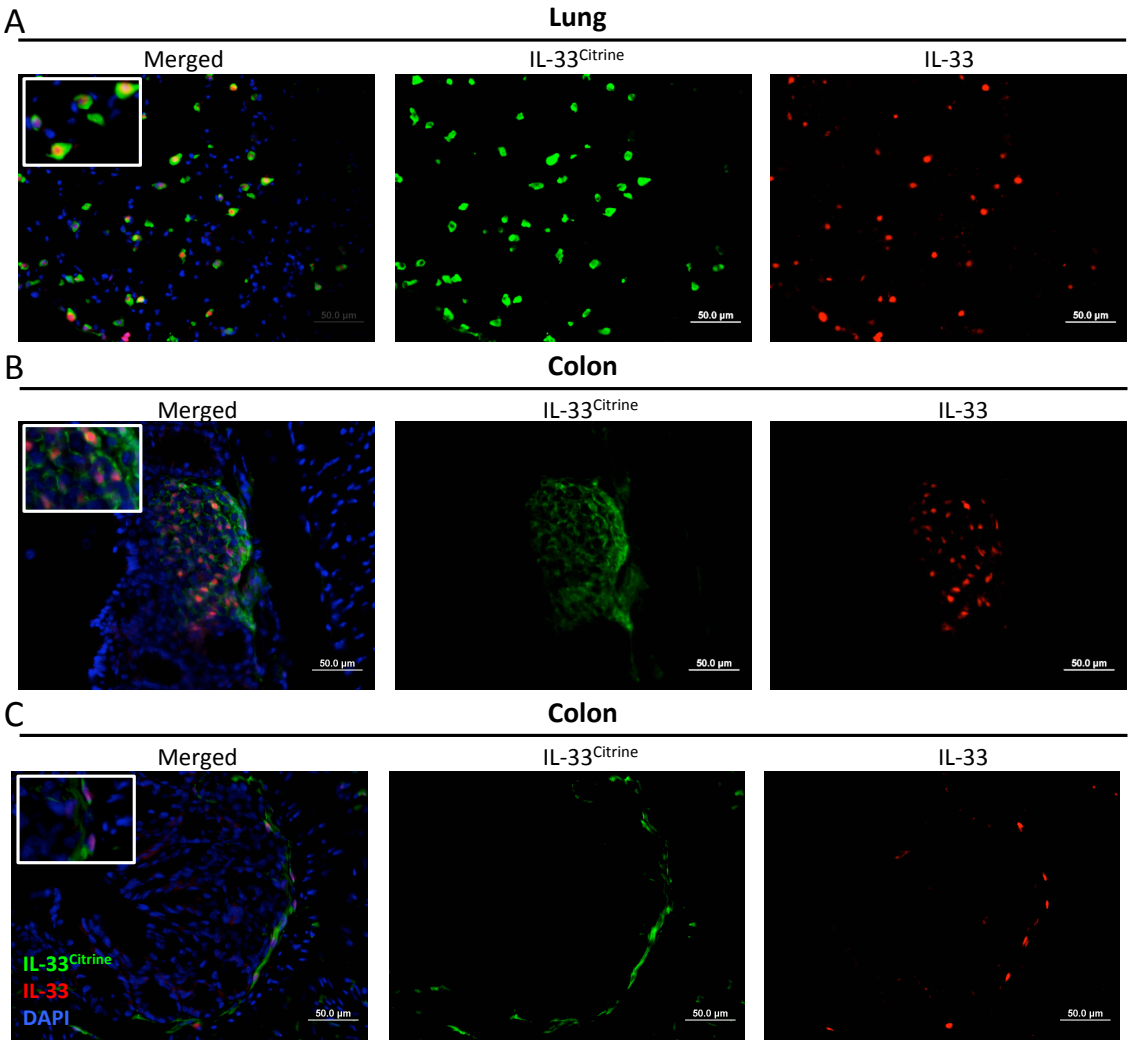


Figure 5.7. Co-expression of IL-33^{Citrine} and IL-33 protein.

Lungs and colons were isolated from IL-33$^{+/Cit}$ reporter mice and processed for IHC staining.

(A) Representative images of immunofluorescence staining of lung tissue
(B) Representative images of immunofluorescence staining of a 'cluster' of cells in the middle colon.
(C) Representative images of immunofluorescence staining of a proximal colon.

Left panels show merged images, middle panels show anti-GFP staining detecting Citrine protein, and right panels show the anti-IL33 antibody staining marking the IL-33 protein. Framed images in the left-hand corner represent zoomed-in areas.

Original magnification 40X. Scale bar – 50 µm. Citrine in green, IL-33 in red and nuclei in blue (DAPI). The staining was performed in n=2 mice.

5.2.6 IL-33 is expressed in SILTs in the colonic LP.

Ex vivo imaging of the colonic tissue provided a better insight into the expression of IL-33 throughout the colon and indicated that the cellular source of IL-33 may not be the same across the length of the intestine. To appreciate IL-33 expression across a range of cell types and locations, staining of fixed colonic sections was performed to simplify visualisation of IL-33 expression in the context of the intestinal architecture.

Due to the known technical issues associated with IL-33 staining experiments, IL-33KO ($IL\text{-}33^{Cit/Cit}$) tissue was always used as a control in all IL-33 staining experiments. No IL-33-expressing cells were detected throughout the IL-33-deficient colon (Fig. 5.8. A, B).

Firstly, $IL\text{-}33^{+}$ cells could be found throughout the colon underlying the colonic LP, specifically located within muscularis mucosae or the submucosa area (Fig. 5.9 A, B), likely representing structural cells producing IL-33. A similar pattern of expression could be observed in the proximal colon across the villus-like structures, resembling blood or lymphatic vessels (Fig. 5.9 A).

Secondly, some IL-33 expression could be detected in epithelial cells at the base of the crypts exclusively in the distal colon (Fig. 5.9 B).

Thirdly, consistent with the observations made using the *ex vivo* imaging technique, 'clusters' of IL-33-expressing cells could also be identified throughout the colon. Both smaller and larger 'cluster-like' structures containing $IL\text{-}33^{+}$ cells were recorded in the colonic LP (Fig. 5.9. C, D). These aggregates of cells found in the intestine are solitary

isolated lymphoid tissues (SILTs), which depending on the size, can be classified as cryptopatches or more mature isolated lymphoid follicles (ILFs) (smaller and larger, respectively) (Mowat and Agace, 2014).

Finally, IL-33 protein localisation was also assessed by the co-expression of IL-33 and nuclear 4',6-diamidino-2-phenylindole (DAPI) staining. IL-33 was consistently expressed in the nuclei of the cells (Fig. 5.10. A, B). This is in line with published reports describing IL-33 association with chromatin (Carriere et al., 2007).

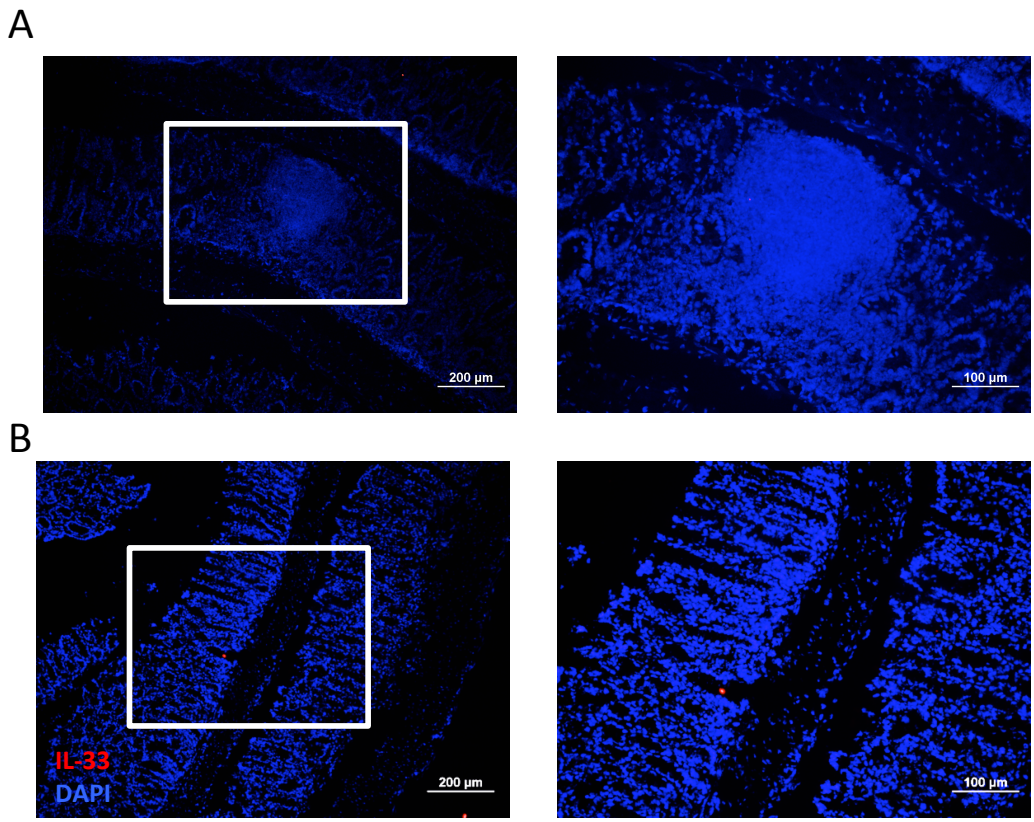


Figure 5.8. Absence of IL-33 staining in IL-33$^{Cit/Cit}$ mice.

IL-33$^{Cit/Cit}$ mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of a colonic SILT.
(B) Representative images of immunofluorescence staining of the colon.

Panels on the right show the zoomed-in areas indicated with the white squares.
Original magnification 10X (left panel) and 20X (right panel). Scale bar – 200µm (left) and 100µm (right). IL-33 in red and nuclei in blue (DAPI).

This staining was performed as a control to any other staining performed using the anti-IL-33 antibody in a total of n=3 IL-33$^{Cit/Cit}$ mice.

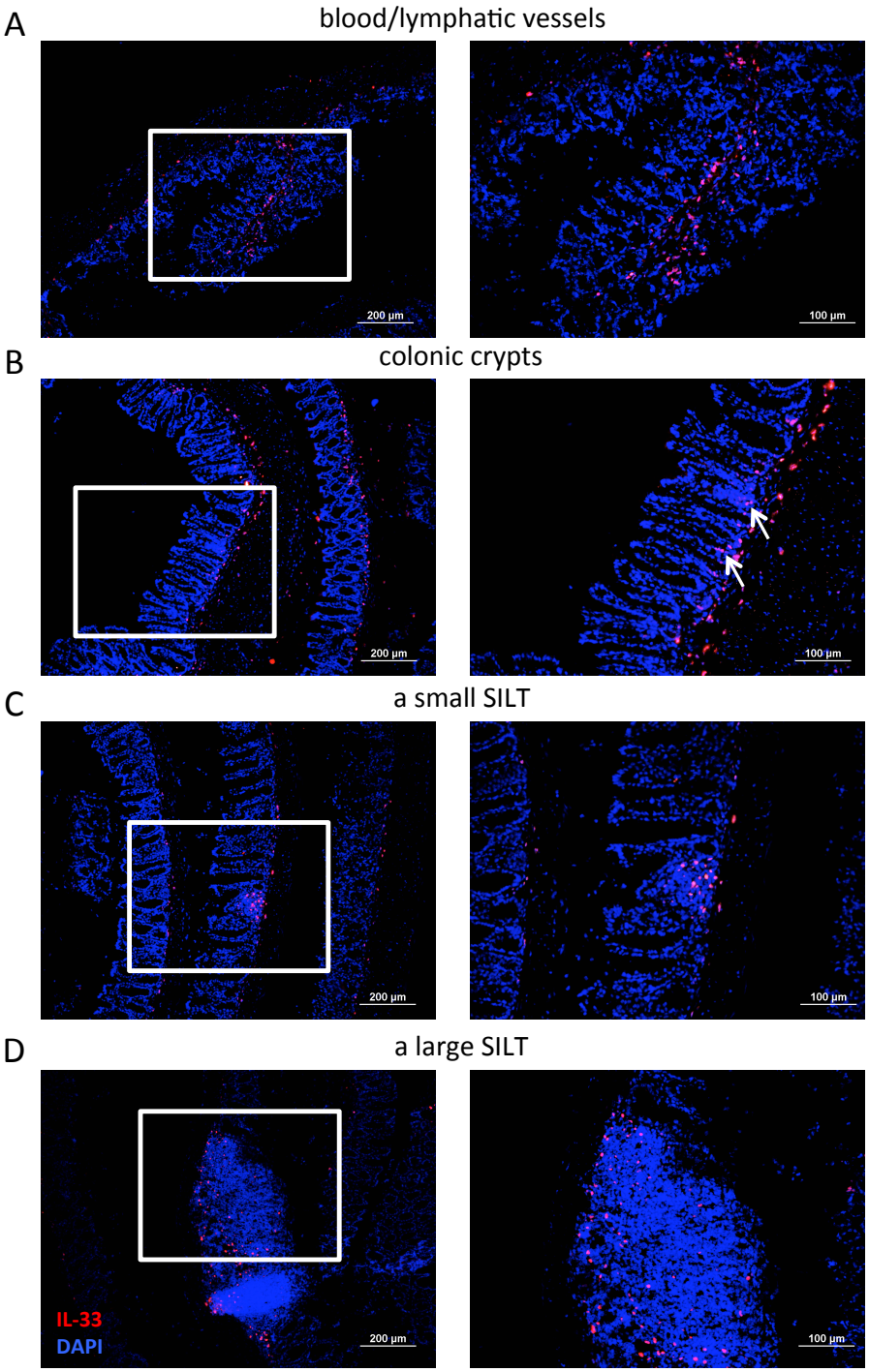


Figure 5.9. IL-33 expression in the colon under homeostatic conditions.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of a villus-like structure in the proximal colon.
(B) Representative images of immunofluorescence staining of a section from the distal colon. White arrows in the right image indicate epithelial cells at the base of the crypt expressing IL-33.
(C) Representative images of immunofluorescence staining of a small SILT.
(D) Representative images of immunofluorescence staining of a large SILT.

Panels on the right show zoomed-in area indicated with a white square.
Original magnification 10X (left panel) and 20X (right panel). Scale bar – 200µm (left) and 100µm (right).
IL-33 in red and nuclei in blue (DAPI).
Each staining was performed in n=5 mice.

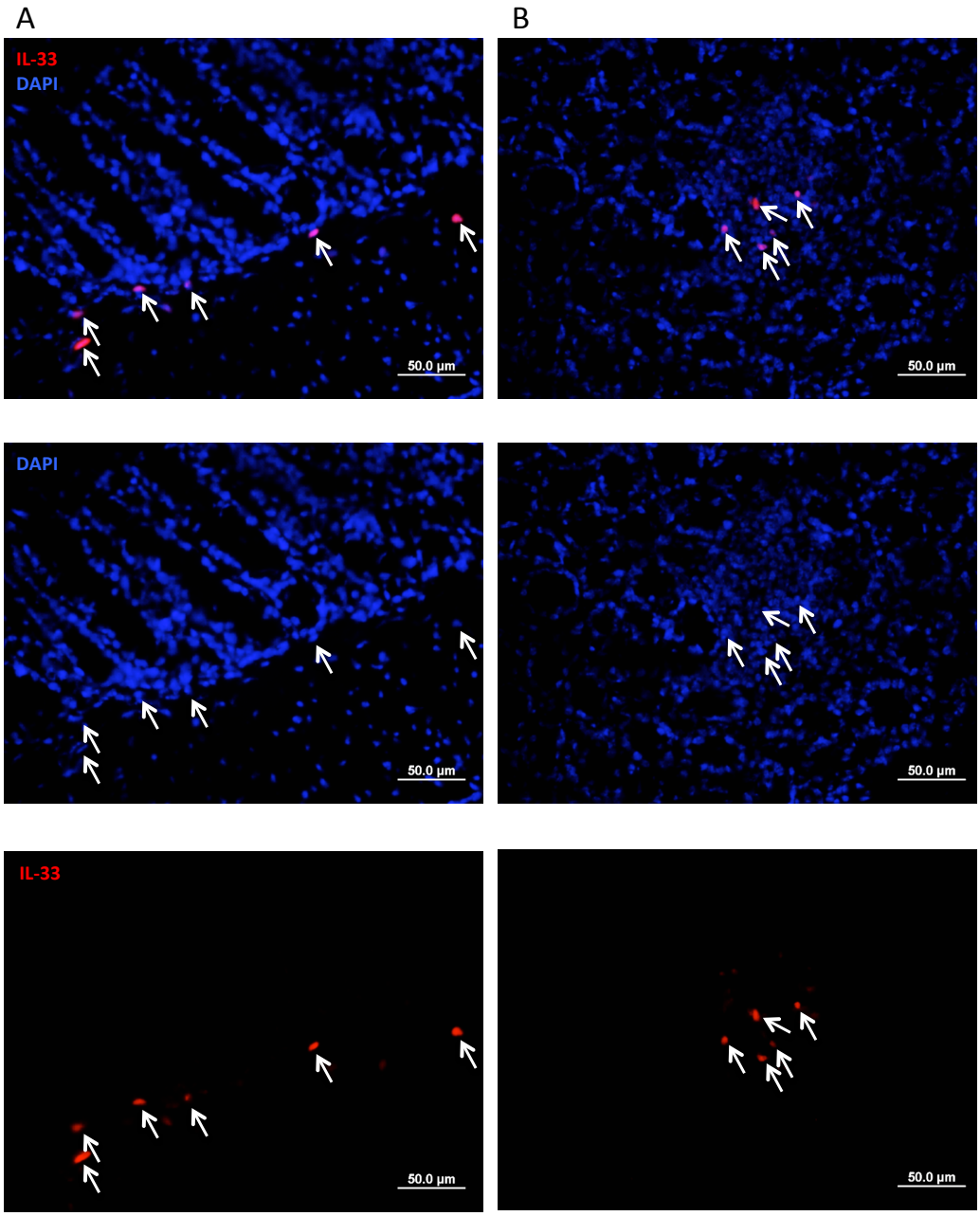


Figure 5.10. IL-33 is localised in the nuclei of cells in the colon.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of IL-33-expressing cells in the submucosa.
(B) Representative images of immunofluorescence staining of IL-33-expressing cells in a small SILT. White arrows indicate cells expressing IL-33.

Top panel represents merged images, middle – nuclear staining with DAPI, bottom – IL-33 staining. Original magnification 40X. Scale bar – 50µm. IL-33 in red and nuclei in blue (DAPI).

Nuclear localisation was investigated for every performed staining whenever possible (for large SILTs with many nuclei overlapping it was not possible to discriminate IL-33 and DAPI staining well enough with the available microscope).

5.2.7 Expression of IL-33 by non-hematopoietic cells.

Most studies have identified non-hematopoietic cells as major producers of IL-33 throughout the body organs, including epithelial cells, endothelial cells, fibroblast-like cells and others (reviewed in Liew et al. (2016)). To establish which cells express IL-33 in the colon, we performed a number of co-immunolabelling studies on cryo-frozen swiss-roll colonic sections with an anti-IL-33 antibody with either the anti-E-cadherin, anti-CD31 or anti-α-SMA antibodies.

Firstly, an anti-E-cadherin antibody was used to establish whether epithelial cells in the colon express IL-33. The vast majority of E-cadherin^{+} cells did not express IL-33 (Fig. 5.11). Similar to the expression pattern identified using *ex vivo* imaging of the IL-33$^{+/Cit}$ reporter mouse line, IL-33 could be found in a few E-cadherin^{+} cells in the distal colon. These cells were localised close to the base of the crypts suggesting they could be more 'stem-like' (Fig. 5.11 A). Lgr5 co-staining could be used to establish whether E-cadherin^{+}IL-33^{+} cells are stem cells. It could also clearly be seen that SILTs were clear of any E-cadherin^{+} epithelial cells (Fig. 5.11 B, C).

Secondly, we investigated expression of IL-33 in endothelial cells using an anti-CD31 antibody, which marks both blood and lymphatic vessels. A low magnification image of the colon showed that IL-33^{+} cells underlie the colonic crypts, while the anti-CD31 staining marked vessels running through the submucosal regions mirroring this pattern (Fig. 5.12. A). Figure 5.12 shows that most IL-33-expressing cells underlying colonic crypts (Fig. 5.12 A, C) as well as cells in the middle of villus-like structures in the proximal colon (Fig. 5.12 B) co-expressed CD31 suggesting that some IL-33^{+} cells in the

colon could be lining either blood or lymphatic vessels. Performing a more complex staining with additional anti-gp38 (podoplanin) antibody would help to determine whether these cells belong to the blood ($gp38^-$) or lymphatic ($gp38^+$) vessel system.

Next, an anti-α-SMA antibody was used to assess IL-33 expression in myofibroblasts that are found primarily in the submucosal and muscularis mucosae regions. The majority of α-SMA^+ cells were IL-33-negative (Fig. 5.13 A). In addition, no positive α-SMA staining could be identified in the SILTs excluding conventional myofibroblasts as a possible cellular source of IL-33 in the colonic SILTs (Fig. 5.13 B).

Finally, we assessed the contribution of an immune-competent subset of stromal cells, the platelet-derived growth factor receptor (PDGFR)α^+ population of subepithelial fibroblasts, that can be found both in the mouse and human colonic LP (Kurahashi et al., 2013). To this end, we used PDGFRα^{eGFP} reporter mouse strain, in which the H2B-eGFP fusion gene is expressed from the endogenous *Pdgfra* locus (Driskell et al., 2013). GFP localises to the nuclei facilitating a clear assessment of the co-expression with the nuclear IL-33 staining. This is particularly important in the context of the SILTs since they are composed of many cells overlying each other and identifying individual cells is complicated using conventional fluorescence microscopy. A preliminary experiment showed that PDGFRα^+ cells could be indeed found in the colonic SILTs, and what is more, all IL-33-expressing cells were PDGFRα^+ but only a subset of PDGFRα^+ cells expressed IL-33 (Fig. 5.14).

Additionally, it can be seen that PDGFRα^{+} cells in the colonic LP can be divided into PDGFRα^{intermediate} and PDGFRα^{high} based on the fluorescent signal intensity with PDGFRα^{+} cells localised to the SILTs representing the PDGFRα^{intermediate} subset. A range in PDGFRα intensity among PDGFRα^{+} cells in the colon has been documented previously (Kurahashi et al., 2013). Interestingly, *Il33* mRNA was enriched in FACS-sorted PDGFRα^{intermediate} stromal cells isolated from the colonic LP (M. Friedrich, Powrie group, unpublished data), further confirming this finding.

Furthermore, since the PDGFRα^{eGFP} reporter mouse strain allows clear evaluation of co-expression of IL-33 due to its nuclear expression, we further used this mouse line to investigate cells expressing IL-33 that underlay the colonic crypts and ran through the middle of the villus-like structures of the proximal colon (Fig. 5.9).
A preliminary staining showed that IL-33-expressing cells found in the middle of the villus-like structures in the proximal colon were all PDGFRα^{+} (Fig. 5.15 A). Since PDGFRα and CD31 are not co-expressed by the same cells, the data suggested that these IL-33^{+} cells are a subset of stromal cells adjacent to the vessels rather than the CD31^{+} endothelial cells themselves. By contrast, IL-33-expressing cells underlying the colonic crypts were not all PDGFRα^{+}, suggesting that different mesenchymal cell subsets may produce IL-33 (Fig. 5.15 B). However, further experiments employing the PDGFRα^{eGFP} reporter mouse line, the anti-IL-33 antibody and other markers will be required to establish this. A clear subset division of PDGFRα^{+} and α-SMA^{+} cells has been described for both the murine and human colonic mucosa (Kurahashi et al., 2013), therefore, careful evaluation of these stromal populations is necessary.

In summary, PDGFRα^{+} stromal cells appear to be the major producers of IL-33 in the colonic SILTs. E-cadherin^{+} epithelial cells at the base of the colonic crypts in the distal colon can also express IL-33. Additionally, either cells of endothelial (CD31^{+}) or other mesenchymal origin (α-SMA^{+}) could be contributing to the production of IL-33 under homeostatic conditions in the mouse colon.

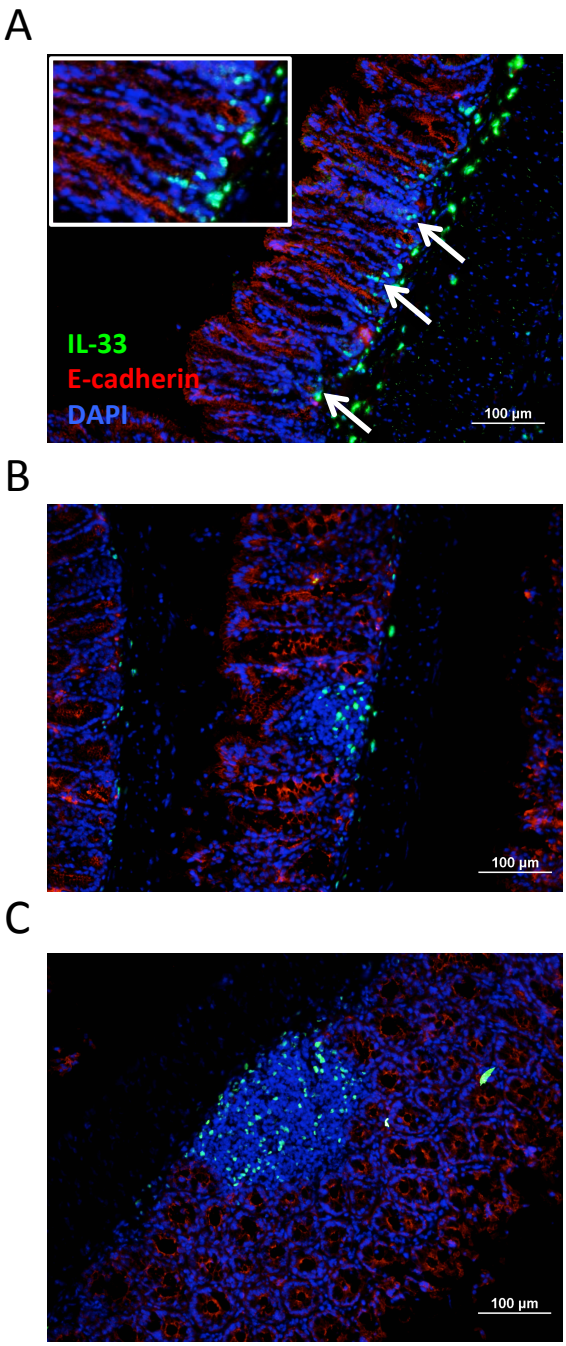


Figure 5.11. E-cadherin and IL-33 expression in the colon under homeostatic conditions.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of the distal colon. White arrows point to the IL-33-expressing cells at the base of the crypts. The framed image in the left-hand corner represents a zoomed-in area.
(B) Representative images of immunofluorescence staining of the colon with a small SILT.
(C) Representative images of immunofluorescence staining of the colon with a large SILT.

Original magnification 20X. Scale bar – 100µm. IL-33 in green, E-cadherin in red and nuclei in blue (DAPI). Each staining was performed in n=4 mice.

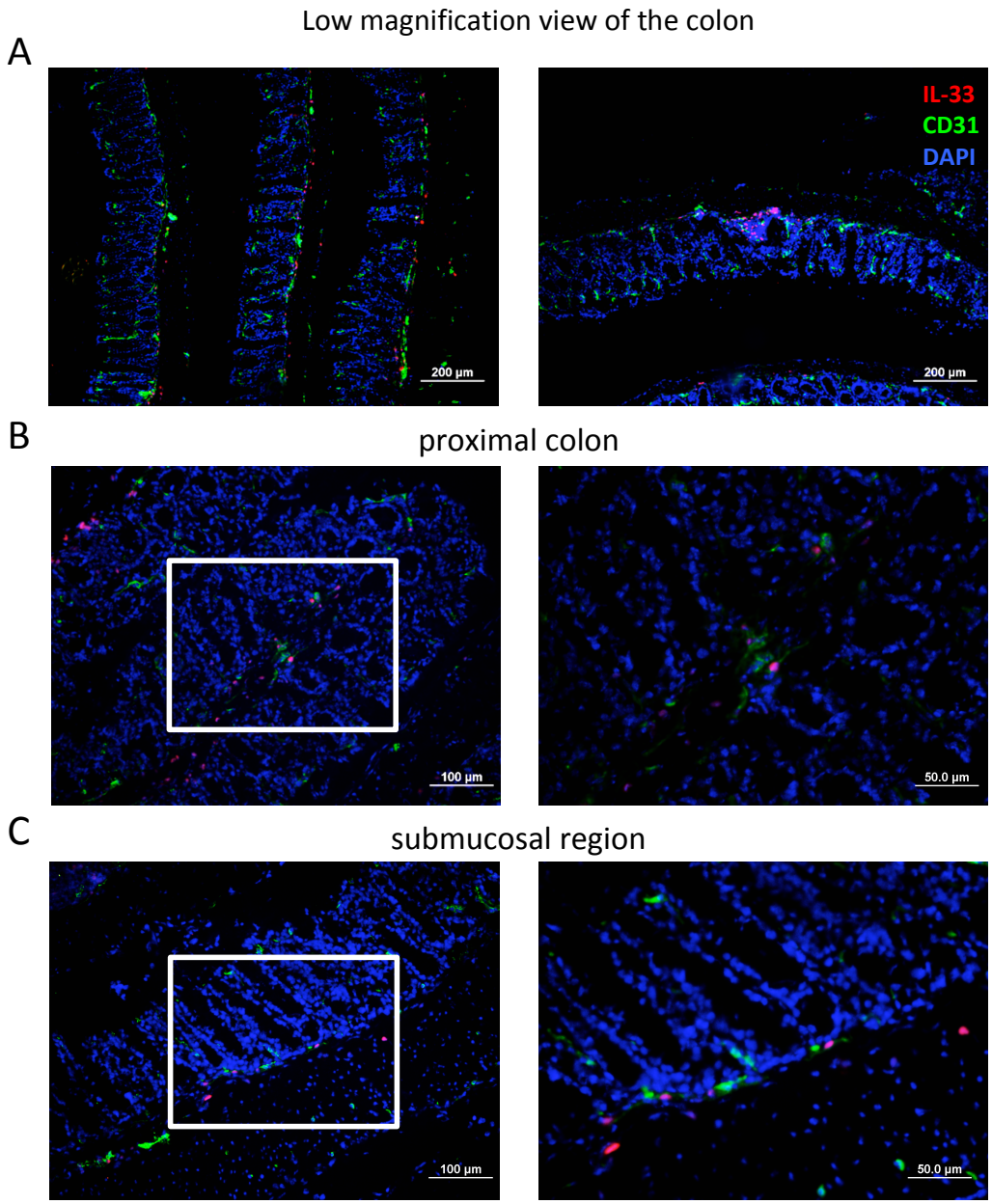


Figure 5.12. CD31 and IL-33 expression in the colon under homeostatic conditions.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of the colon.
(B) Representative images of immunofluorescence staining of the proximal colon.
(C) Representative images of immunofluorescence staining of IL-33-expressing cells underlying the lamina propria in the submucosal region.

Panels on the right in (B) and (C) show zoomed-in areas indicated with the white squares. Original magnification 10X in (A), 20X (left panel) and 40X (right panel) in (B) and (C). Scale bar – 200µm in (A), 100µm (left panel) and 50µm (right panel) in (B) and (C). IL-33 in red, CD31 in green and nuclei in blue (DAPI).
Each staining was performed in n=3 mice.

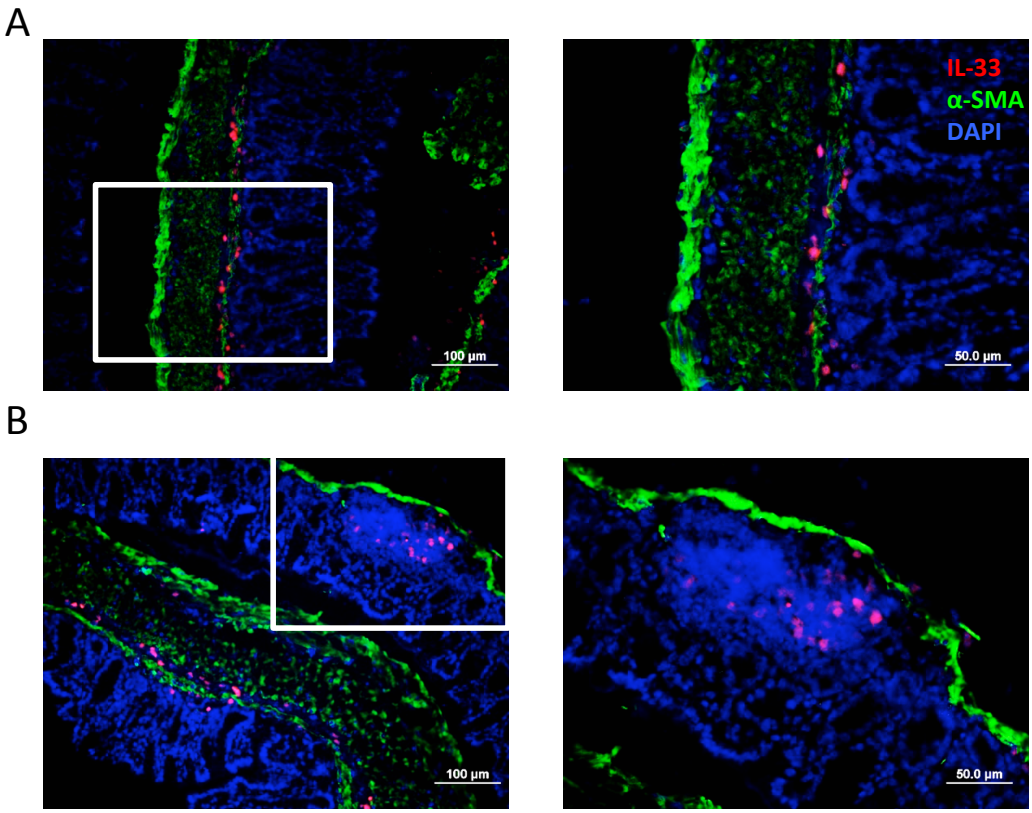


Figure 5.13. α-SMA and IL-33 expression in the colon under homeostatic conditions.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of the colon highlighting the submucosa and muscle layers.
(B) Representative images of immunofluorescence staining of the colon including a colonic SILT.

Panels on the right show zoomed-in areas indicated with the white squares.
Original magnification 20X (left panel) and 40X (right panel). Scale bar – 100µm (left panel) and 50µm (right panel). IL-33 in red, α-SMA in green and nuclei in blue (DAPI).
Each staining was performed in n=2 mice.

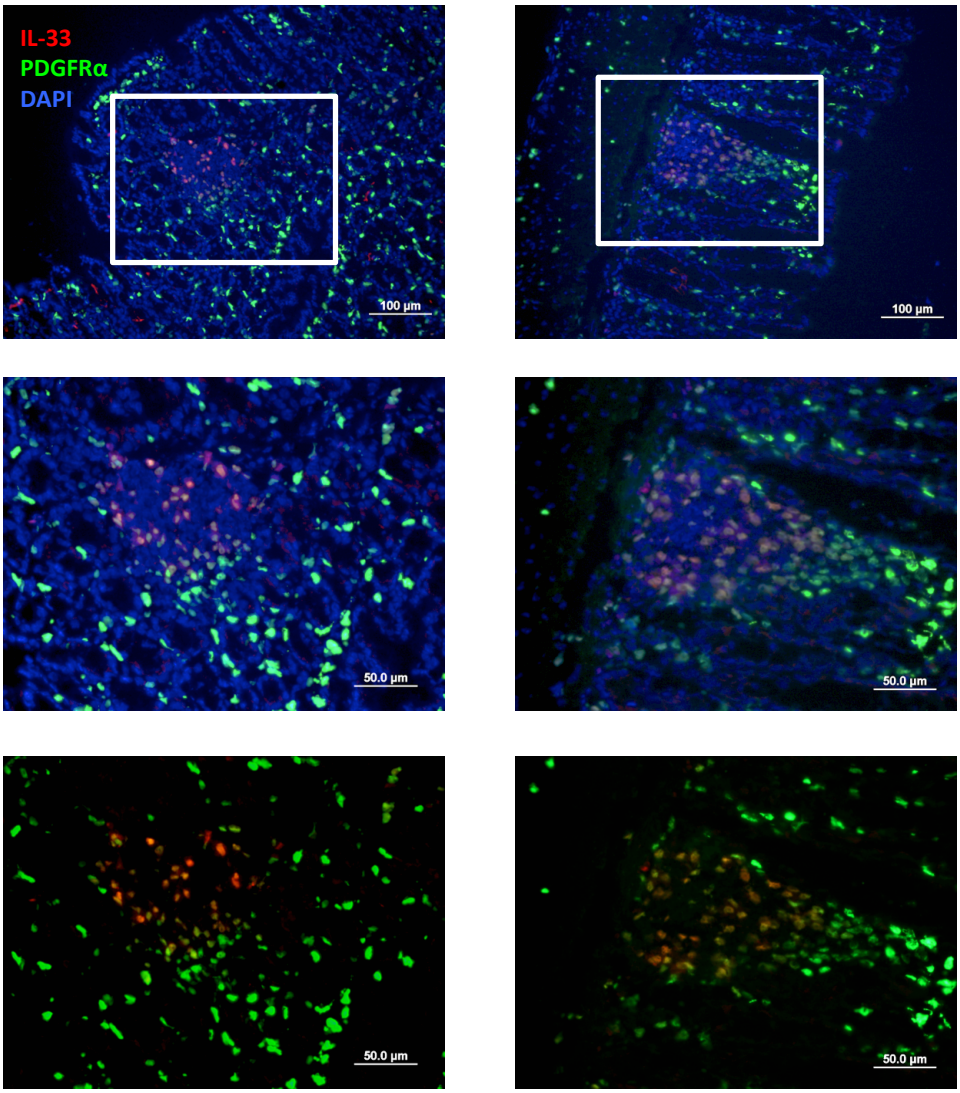


Figure 5.14. PDGFRα and IL-33 expression in the colonic SILTs under homeostatic conditions.

PDGFRα^{eGFP} reporter mice were sacrificed and their colons processed for IHC staining.

Representative images of immunofluorescence staining of the colon including two examples of SILTs.

Panels in the middle and bottom show zoomed-in areas indicated with the white squares. Bottom panel shows IL-33 and PDGFRα only.

Original magnification 20X (top panel) and 40X (middle and bottom panel). Scale bar – 100µm (top panel) and 50µm (middle and bottom panel). IL-33 in red, PDGFRα in green and nuclei in blue (DAPI). The staining was performed in n=1 mouse.

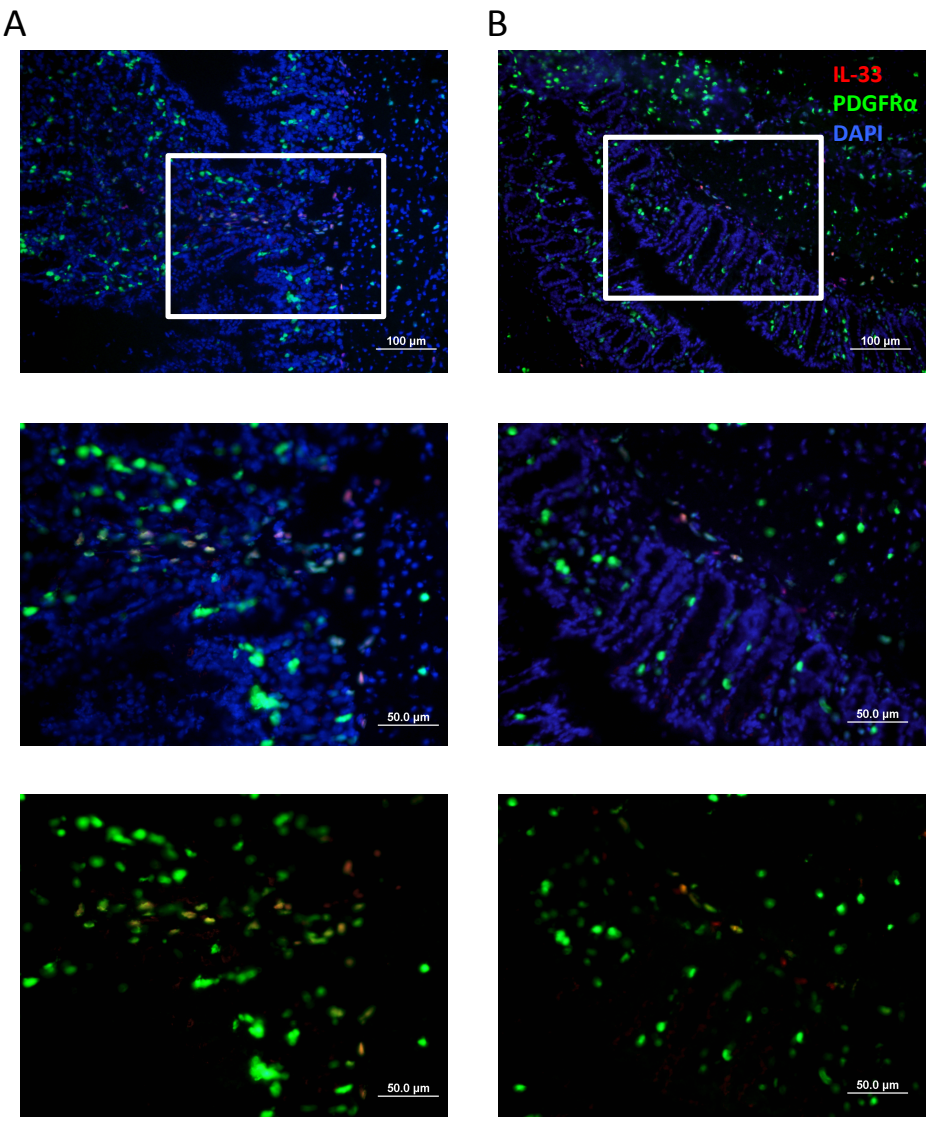


Figure 5.15. PDGFRα and IL-33 expression in the colon under homeostatic conditions.

PDGFRα^{eGFP} reporter mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of the proximal colon.
(B) Representative images of immunofluorescence staining of the colon.

Panels in the middle and bottom show zoomed-in areas indicated with the white squares. Bottom panel shows IL-33 and PDGFRα only.

Original magnification 20X (top panel) and 40X (middle and bottom panel). Scale bar – 100µm (top panel) and 50µm (middle and bottom panel). IL-33 in red, PDGFRα in green and nuclei in blue (DAPI). The staining was performed in n=1 mouse.

5.2.8 IL-33-expressing cells are found in the T cell zone in the colonic SILTs.

Stromal cells in lymphoid tissues can coordinate the immune system by forming a dense network and by secretion of chemokines, which attract both T and B cells (reviewed in Fletcher et al. (2015)). Here, we used anti-CD4 and anti-B220 antibodies together with the anti-IL-33 antibody to assess the location of IL-33-expressing cells in the context of the colonic SILTs.

Firstly, CD4 staining was performed in order to mark T cell-rich areas of the SILTs. In both smaller and larger SILTs IL-33-expressing cells co-localised with the $CD4^+$ cells suggesting that IL-33 is produced in the T cell zones (Fig. 5.16 A, B).

Secondly, B-cells were marked with an anti-B220 antibody. $B220^+$ cells were found in the middle of the SILT, marking the B-cell zone. IL-33-expressing cells did not preferentially co-localise to this area (Fig. 5.17 A, B).

As described in Chapter 3, we identified a population of Tregs preferentially located to the colon that expresses high amounts of ST2, the IL-33 receptor. We also demonstrated that IL-33 promotes Treg phenotype upon IL-33 stimulation. Here, we also investigated whether IL-33-producing cells could be found in the proximity of Tregs in the colonic LP. Foxp3-expressing cells were found in the SILTs. As depicted in Figure 5.18, cells producing IL-33 were identified in the same SILTs as $Foxp3^+$ cells, raising the possibility that indeed IL-33 produced in the SILTs could be influencing Tregs in the proximity.

Altogether our results suggest that IL-33 production in the SILTs is enriched in the T cell zones and Foxp3-expressing cells can be found close to the source of IL-33, posing a possibility that IL-33 can influence their phenotype and function *in vivo*.

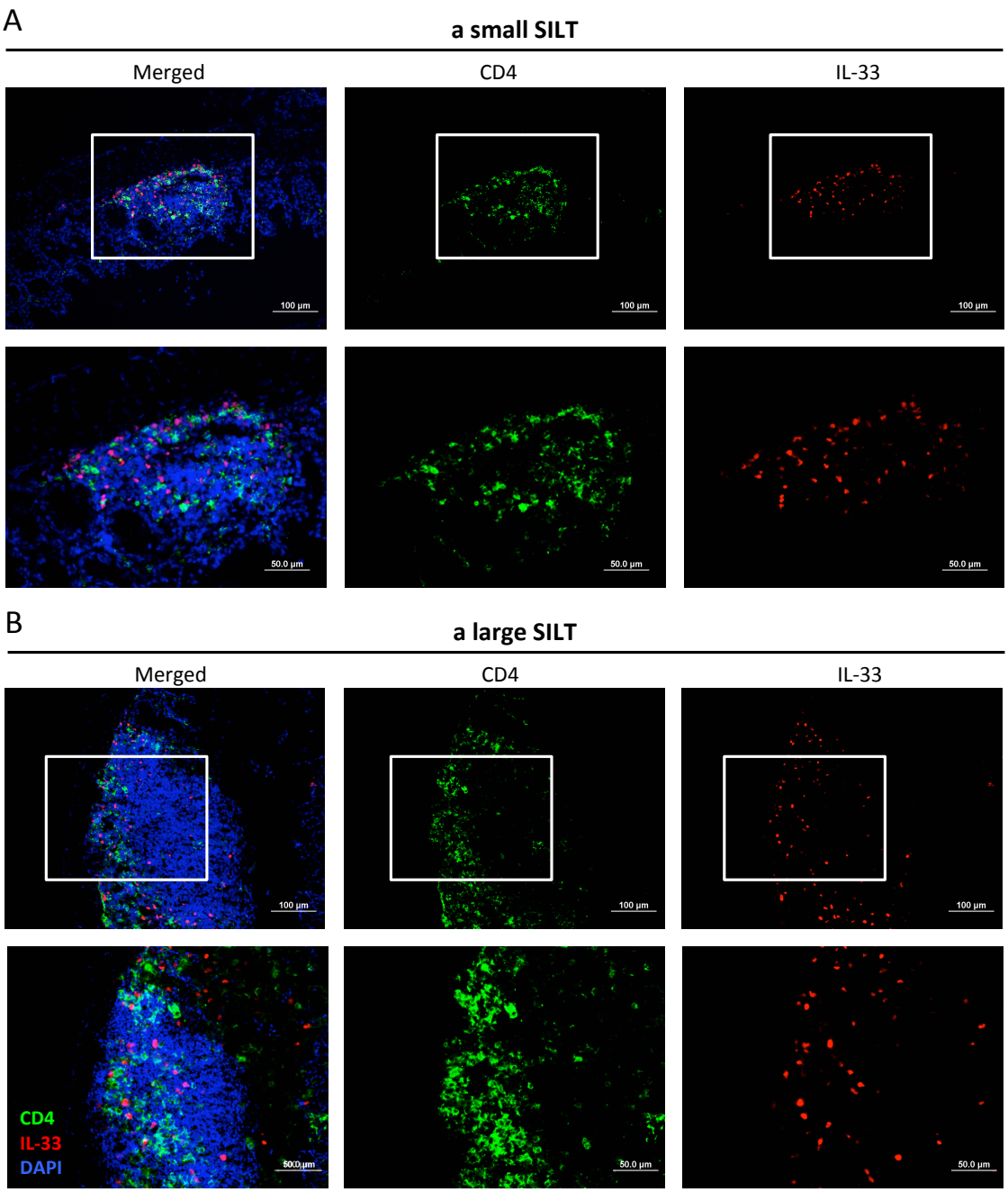


Figure 5.16. IL-33-expressing cells can be found within the T cell zone in the colonic SILTs.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of a small SILT found in the colon showing merged image (left), CD4 (middle), and IL-33 staining (right). Lower panel shows zoomed-in area indicated with a white square.
(B) Representative images of immunofluorescence staining of a large SILT found in the colon showing merged image (left), CD4 (middle), and IL-33 staining (right). Lower panel shows zoomed-in area indicated with a white square.

Original magnification 20X (top panel) and 40X (bottom panel). Scale bar – 100µm (top) and 50µm (bottom). CD4 in green, IL-33 in red and nuclei in blue (DAPI).
Each staining was performed in n=2 mice.

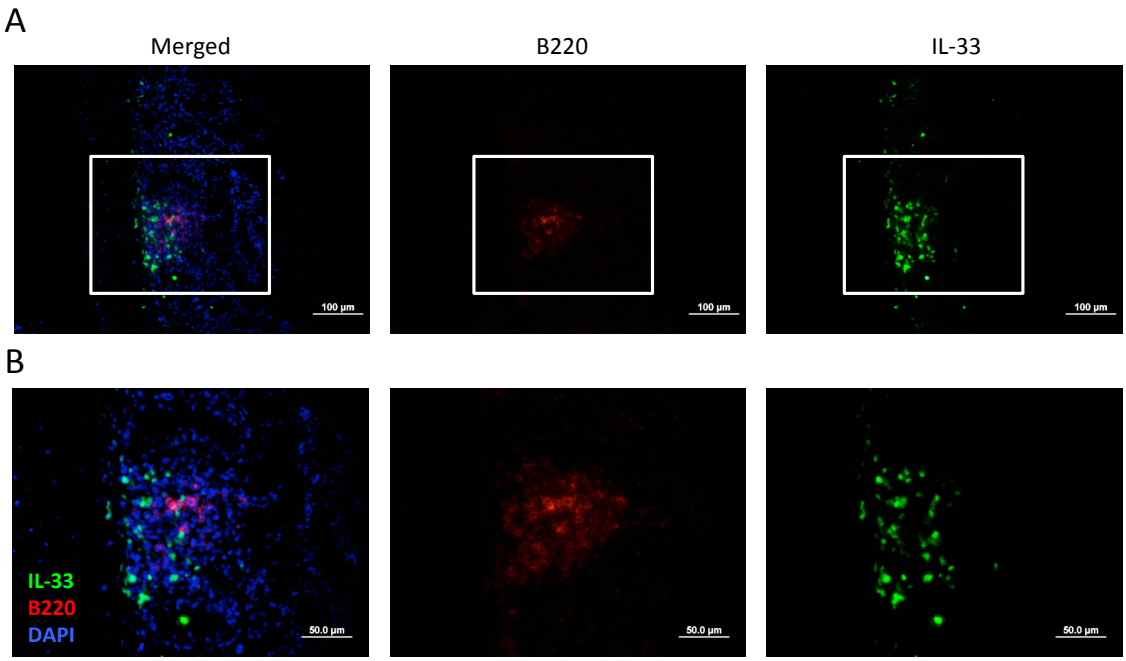


Figure 5.17. Visualisation of the B-cell zone and IL-33-expressing cells in a colonic SILT.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining of a SILT found in the colon showing merged image (left), B220 (middle), and IL-33 staining (right).
(B) Zoomed-in areas indicated with a white square in (A).

Original magnification 20X (A) and 40X (B). Scale bar – 100µm (A) and 50µm (B). IL-33 in green, B220 in red and nuclei in blue (DAPI).
Each staining was performed in n=2 mice.

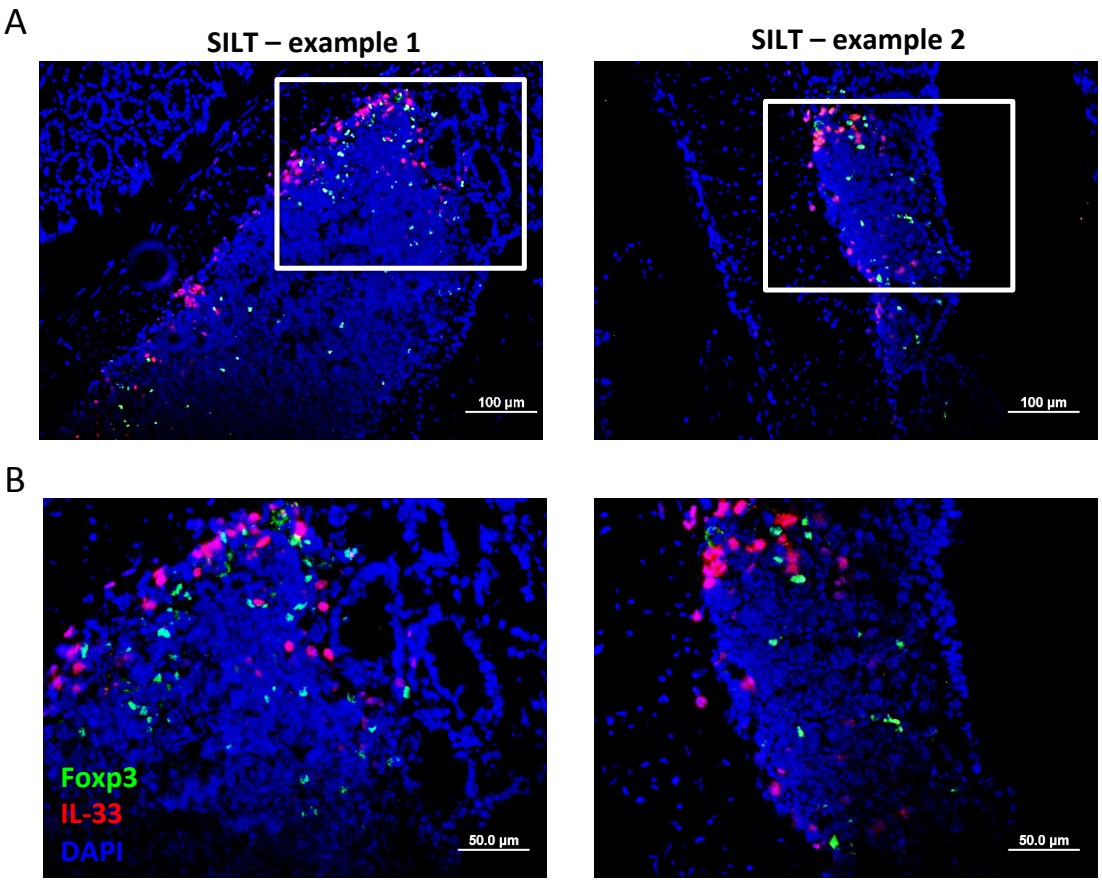


Figure 5.18. Foxp3^{+} cells are located close to IL-33-expressing cells in the colonic SILTs.

C57BL/6 mice were sacrificed and their colons processed for IHC staining.

(A) Representative images of immunofluorescence staining showing two examples of SILTs found in the colon.
(B) Zoomed-in areas indicated with a white square in (A).

Original magnification 20X (A) and 40X (B). Scale bar – 100µm (A) and 50µm (B). Foxp3 in green, IL-33 in red and nuclei in blue (DAPI).
Each staining was performed in n=2 mice.

5.2.9 IL-33 expression in the colon in colitis.

IL-33 can be expressed constitutively under homeostatic conditions but can also be induced upon cell activation and is involved in the modulation of immune response in many distinct diseases (reviewed in Liew et al. (2016)). IL-33 has been shown to be upregulated in IBD and mouse models of colitis (Beltran et al., 2010, Pastorelli et al., 2010, Seidelin et al., 2010, Sedhom et al., 2013); therefore, we next assessed IL-33 expression in the *H. hepaticus*/anti-IL-10R-driven model of colitis.

Wild type mice were infected with *H. hepaticus* with concomitant administration of an anti-IL-10R blocking antibody, and IL-33 expression was investigated in the colonic tissue on day 14 – the peak of inflammation – by immunofluorescence staining. Similarly to steady state tissue (see Fig. 5.9), IL-33-expressing cells could be found in the villus-like structures in the proximal colon and underlying the colonic crypts throughout the colon (Fig. 5.19 A). Clear IL-33 expression could be also identified in the colonic SILTs (Fig. 5.19 B). However, while in a healthy colon every single cluster of cells contained some IL-33^{+} cells, here, we could identify some SILTs with no IL-33-expressing cells (Fig. 5.19 C). Additionally, in contrast to steady state animals, the enlarged inflamed submucosal region contained multiple IL-33^{+} cells (Fig. 5.19 D). Finally, no epithelial expression could be detected in the inflamed tissue.

Overall, the immunofluorescence staining results suggest that there are minor differences in IL-33 expression upon intestinal inflammation in the *H. hepaticus*/anti-IL-10R-driven model of colitis when tissue is investigated at the peak of intestinal inflammation.

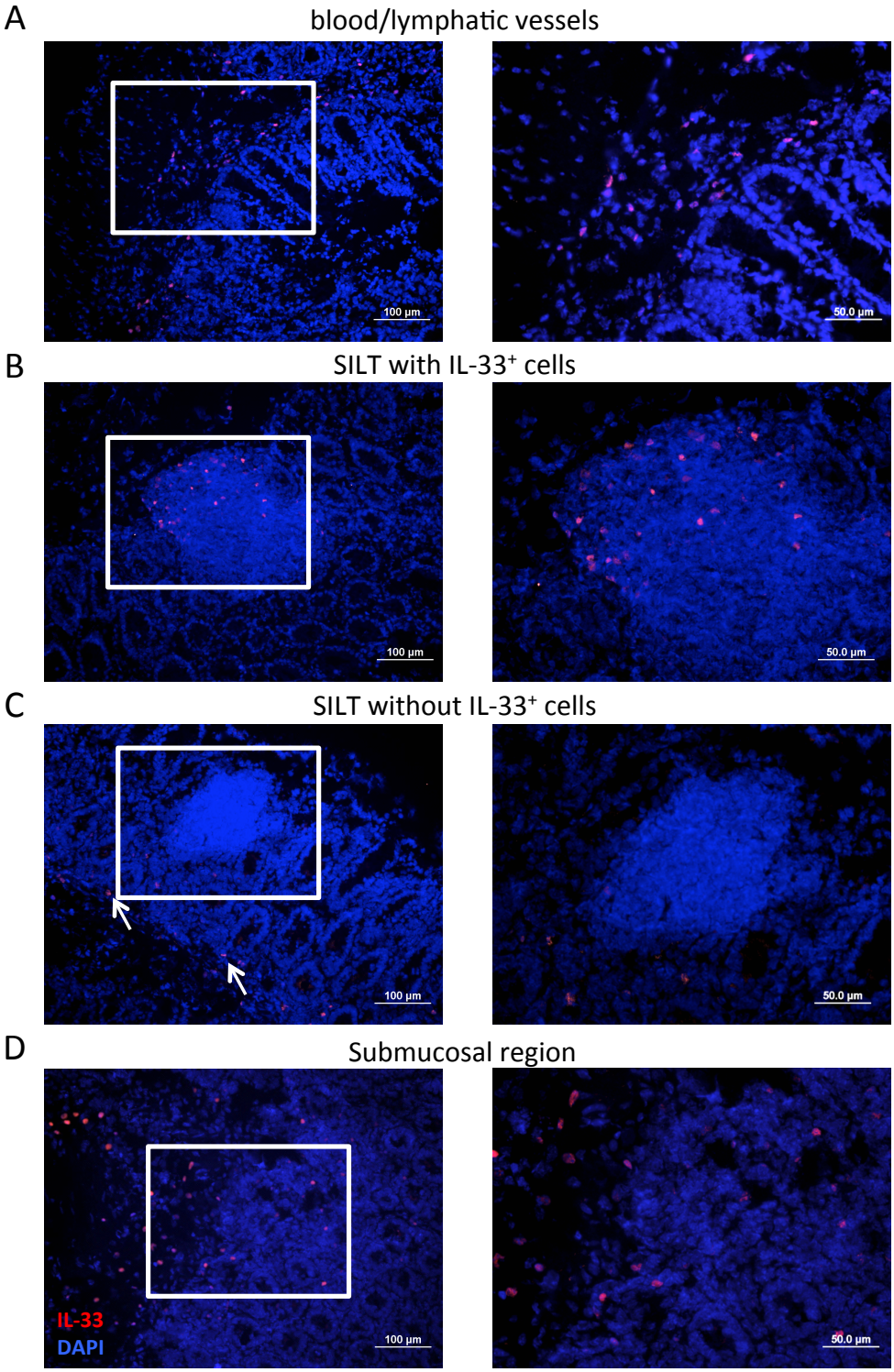


Figure 5.19. IL-33 expression in the colon during *H.hepaticus*/anti-IL-10R-driven colitis.

C57BL/6 mice were sacrificed on day 14 following oral infection with *H. Hepaticus* and weekly i.p. injections of a blocking anti-IL-10R antibody. Colonic tissue was processed for IHC staining.

(A) Representative images of immunofluorescence staining of the proximal colon.
(B) Representative images of immunofluorescence staining of a SILT with IL-33-expressing cells.
(C) Representative images of immunofluorescence staining of a SILT without IL-33-expressing cells. White arrows in the left image indicate cells in the submucosal region expressing IL-33.
(D) Representative images of immunofluorescence staining of IL-33-expressing cells in the submucosal region.

Panels on the right show zoomed-in area indicated with a white square.
Original magnification 20X (left panel) and 40X (right panel). Scale bar – 100µm (left) and 50µm (right). IL-33 in red and nuclei in blue (DAPI).
Each staining was performed in n=2 mice.

5.3 Discussion

Unfortunately, to date studies of IL-33 have not provided adequate information to define the cellular source of IL-33 in the intestine. Therefore, the principal objective of the experiments conducted in this chapter was to identify which cells produce IL-33 in the gut. We found IL-33-producing cells primarily localised within colonic SILTs. These cells expressed PDFGRα, suggesting they are of stromal origin.

Recently a valuable tool, the IL-33$^{+/Cit}$ reporter mouse strain, was generated, enabling studies of IL-33 *in vivo*. Hardman et al. (2013) employed it to identify type-2 pneumocytes as a major cellular source of IL-33 in the lung during experimental allergic-driven lung inflammation. Here, we used the IL-33$^{+/Cit}$ reporter strain to describe IL-33 expression in the intestine. A few previous reports suggested a lack of correlation between RNA and protein data (Schmitz et al., 2005, Moussion et al., 2008); however, importantly, we showed that the transcriptional activity of the IL-33 reporter corresponded well with the protein expression, and therefore, could be used to reliably evaluate IL-33 expression in the gut.

The original report describing IL-33 (then called NF-HEV) highlighted its abundant expression in the Peyer's patches (Baekkevold et al., 2003). Using the IL-33$^{+/Cit}$ reporter mouse strain we also found that IL-33 was highly expressed in both Peyer's patches and the caecal patch. Similarly, an increased density of IL-33-expressing cells was identified in SILTs, lymphoid tissue aggregates distributed throughout the colon and the rectum (Owen et al., 1991). The described structures are all part of the GALT and

constitute the sites of T cell priming and IgA production (Lee et al., 2008, Masahata et al., 2014). Peyer's patches have clearly defined B cell follicles flanked by T cell areas and are lined with a follicle-associated epithelium, which contains M cells (Neutra et al., 2001). While Peyer's patches have been well characterised, a very limited number of studies have investigated the relevance of the caecal patch, which is located around the ileocaecal valve of the caecum, and the SILTs, which can be found across the length of the colon. Both T cell priming and IgA production have been described in the context of the caecal patch (Owen et al., 1991). In addition, the caecal patch might be of relevance for the development of the pathological response driving colitis since removal of the structure in TCRα-deficient mice leads to suppression of the disease development (Mizoguchi et al., 1996). Both Peyer's patches and the caecal patch can be identified macroscopically, whereas SILTs are small microscopic aggregates of lymphoid tissue with a rather less defined structure. SILTs can be divided into smaller cryptopatches and larger ILFs, with the latter representing a more mature state of an organised lymphoid structure (Herbrand et al., 2008, Pabst et al., 2005). Pabst et al. (2006) also suggested that, in contrast to Peyer's patches and the caecal patch, which start developing during embryonic life, the phenotype and development of SILTs are more adaptable to the environment and their response to the stimuli can be more rapid. Interestingly, we observed that all the SILTs found in the colonic LP contained IL-33^{+} cells, independent of their size, suggesting that IL-33 expression could be a signature of organised lymphoid tissue. It will be particularly interesting to understand whether IL-33 expression in the lymphoid tissues is important for their development or their function, or potentially both.

Interestingly we found that *Il33* expression was enriched within the stromal cell fraction. This was consistent with our experiments performed using a PDGFRα reporter mouse line, which suggested that PDGFRα^{+} intestinal stromal cells present in the SILTs produced IL-33. Stromal cells constitute a wide range of non-hematopoietic (CD45^{-}), non-epithelial (EpCAM^{-}) mesenchymal cells throughout the body. Depending on the function and origin they can express gp38, PDGFRα, α-SMA, CD31, desmin, vimentin and other markers. Of particular relevance here are stromal cells that are involved in the development of lymphoid tissues, such as the follicular reticular cells (FRCs) that constitute a large proportion of non-hematopoietic cells in the lymphoid organs. FRCs are CD45^{-}EpCAM^{-}CD31^{-}gp38^{+}PDGFRα^{+}, and create a scaffolding for leukocytes to migrate on (reviewed in Fletcher et al. (2015)). They also produce a range of chemokines, which regulate immune cell trafficking. For instance, chemokines released by FRCs influence the compartmentalisation of T and B cells into their respective zones (reviewed in Malhotra et al. (2013)). Tertiary tissue development in the gut, such as that of the colonic SILTs, also requires stromal interactions. Initial clustering of lymphoid tissue inducer cells (CD45^{+}IL-7Rα^{+}Rorγt^{+}) together with stromal lymphoid tissue organiser cells (CD45^{-}ICAM-1^{+}VCAM-1^{+}gp38^{+} PDGFRα^{+}) is critical for the formation of colonic SILTs. However, in contrast to well-established stromal contributions in the peripheral lymphoid tissues, these populations have not been well studied in the context of lymphoid tissue development in the gut. Peduto et al. (2009) described a gp38^{+} stromal cell population present in the small intestinal SILTs during early ontogeny. They found that differentiation of these cells was also promoted upon tertiary structure formation in response to immune cell infiltration during chronic inflammation (Peduto et al., 2009). However, it remains to be determined whether the

PDGFRα$^+$ cells identified here as IL-33-producing cells in the colonic SILTs represent the same stromal population. In a different experimental setting, IL-33 was produced by PDGFRβ$^+$NG2$^+$ pericytes, fibroblast-like cells surrounding the vessels, in response to PDFF-BB stimulation, which led to tumour-associated macrophage recruitment and promoted metastasis (Yang et al., 2016b). Pericytes, however, do not co-express PDGFRα$^+$; therefore, they are unlikely to represent the IL-33-expressing population we identified in the colon. Finally, it would be of particular interest to determine the functional relevance of IL-33 produced by PDGFRα$^+$ cells: whether it is involved in tissue development or whether its role is restricted to modulation of the immune responses.

Production of IL-33 by stromal cells is indeed not surprising since, in addition to a key role of stromal cells in lymphoid tissue development, recently it has been appreciated that stromal cells are also capable of modulating immune responses (reviewed in Powell et al. (2011), Owens and Simmons (2013)). A key role in orchestrating intestinal immunity was attributed to stromal cells in the TNFΔARE mouse model of IBD, in which TNFα is continuously overexpressed (Kontoyiannis et al., 2002). The ability to respond to TNFα by mesenchymal cells was sufficient to drive intestinal pathology in this model (Armaka et al., 2008). Multiple characteristics make stromal cells relevant in the context of immune responses. For instance, intestinal stromal cells, both in human and mouse, have been shown to express TLRs, allowing them to mount a response to a microbial challenge (Otte et al., 2003, Walton et al., 2009). There is also some evidence that stromal cells can express MHCII and present antigens, acting as non-professional APCs (Saada et al., 2006, Owens et al., 2013). Production of various cytokines and

chemokines as well as upregulation of ICAM1 have also been attributed to stromal cells, thus demonstrating that they can actively contribute to the coordination of immune responses in the gut (Pang et al., 1994, Roulis et al., 2011). Human intestinal stromal cells have also been suggested to promote $CD25^{+}CD127^{-}Foxp3^{+}$ T cells via secretion of prostaglandin E2 in *in vitro* co-culture systems (Pinchuk et al., 2011). Here, we found IL-33-expressing cells and Tregs located in the T cell areas in the colonic SILTs; however, the functional importance of this finding, and whether stromal-derived IL-33 can modulate T cell function remain to be established.

A few reports of stromal cells modulating immune responses via IL-33 production exist. For instance, IL-33 produced by synovial fibroblasts could directly bind to mast cells and promote their activation and tryptase/protease granule accumulation, which led to an exacerbation of collagen-induced arthritis (Kaieda et al., 2010, Xu et al., 2008). Also, myofibroblasts expressed IL-33 in the murine small intestine and colonic mucosa samples from UC patients (Kobori et al., 2010, Sponheim et al., 2010). In our studies we did not confirm expression of IL-33 by α-SMA^{+} myofibroblasts, and the functional relevance of IL-33 expression by $PDGFR\alpha^{+}$ stromal cells in the colonic SILTs will be investigated further.

In addition to IL-33-expressing stromal cells in the colonic SILTs, we could also detect some epithelial cell expression exclusively under steady state conditions at the base of the crypts primarily in the distal colon. The consensus in the literature is that epithelial cells are the major source of IL-33 in the gut; this is primarily due to high IL-33 expression commonly found in epithelial cells in other barrier organs such as the skin,

or stomach. We did not investigate factors that could account for the restricted production of IL-33 by epithelial cells in the distal colon. However, it is widely recognised that anatomy, morphology, bacterial load as well as the cellular composition are heterogeneous along the GI tract (reviewed in Mowat and Agace (2014)). For instance, microbiota composition changes along the proximal-distal axis in the colon, and it has been previously suggested to modulate expression of TLRs as well as heat shock proteins, both of which are important for the maintenance of intestinal homeostasis (Hu et al., 2010, Wang et al., 2010). Studying the modulatory effects of the microbiota on the expression of IL-33 could provide further insight into the pattern of IL-33 expression we observed along the GI tract.

Interestingly, we did not observe any major differences in the expression of IL-33 in colitic mice in the *H. hepaticus*/anti-IL-10R-driven model of colitis. Changes in IL-33 expression upon inflammatory stimuli have been reported previously. For instance, mouse keratinocytes lose IL-33 expression under inflammatory settings, while in humans, IFN-γ exposure leads to induction of IL-33 by keratinocytes (Sundnes et al., 2015). It is well established that synovial fibroblasts are a rich source of IL-33 during RA and in murine models of arthritis (Xu et al., 2008, Palmer et al., 2009). While an increase in IL-33 has been reported in IBD patients, very limited information exists on cells producing IL-33 in murine models of colitis. Additionally, we investigated only a snapshot of IL-33 expression in the context of *H. hepaticus*/anti-IL-10R-driven model of colitis focusing on tissue expression only at the peak of inflammation. Our previous results (see Chapter 4 for details) showed an increase in IL-33 production in the colonic explant cultures on day 3 following the induction of colitis in the described model of

colitis; therefore, further studies looking at the temporal response that could be related to repair processes and tissue healing could prove more informative.

Studies of cells expressing IL-33 in the SILTs as well as the secondary lymphoid structures may be hindered by the difficulty associated with the isolation of cells. Stromal cells form a structural component of the tissue, and their isolation requires both mechanical and enzymatic treatment. In addition, it is worth highlighting that IL-33 becomes upregulated by cells upon cell death and mechanical stress (Cayrol and Girard, 2009, Kakkar et al., 2012). Therefore, harsh isolation protocols may lead to false-positive results when studying these cells by flow cytometry or recovering them for *ex vivo* manipulation. Recent developments in immunofluorescence staining protocols, such as histo-cytometry that allows for the use of multiple antibodies simultaneously (Gerner et al., 2012), could be employed to better characterise the stromal cell subset expressing IL-33 in the colonic SILTs.

In summary, we employed multiple imaging techniques and identified IL-33 expression to be enriched in colonic stromal cells. We described a non-uniform pattern of IL-33 expression along the GI tract and substantial enrichment of IL-33-expressing cells in secondary and tertiary lymphoid tissues. Identification of IL-33 expression in the colonic SILTs is novel, and its functional relevance warrants further study.

Chapter 6. General discussion

IL-33 is a pleiotropic immune mediator that orchestrates both innate and adaptive immunity. It is a potent inducer of type 2 immune responses, but can also promote anti-viral responses and repair processes. A role for IL-33 in the pathogenesis of IBD has also been speculated since increased IL-33 levels were found in inflamed mucosal samples from IBD patients. However, little is known about the cellular source of IL-33 in the gut and the function that IL-33 may perform in intestinal homeostasis. ST2 is abundantly expressed on Tregs found in non-lymphoid tissues, including the colon. Nevertheless, its functional relevance has not been investigated in the context of intestinal tolerance. Therefore, the experiments described in this thesis were performed in order to understand the contribution of IL-33 to the maintenance of intestinal homeostasis, and in particular to understand how IL-33 may modulate Treg phenotype and function. We identified stromal cells found in the organised lymphoid structures, SILTs, in the colon as the dominant cellular source of IL-33 in the gut. We also showed that IL-33 induces Gata3-expressing Tregs enriched in amphiregulin, which likely promote a repair module in Tregs. These $ST2^{+}$/$Gata3^{+}$ Tregs were shown to be distinct from $Ror\gamma t^{+}$ Tregs, which represent another abundant Treg population found in the colon.

Solitary intestinal lymphoid tissues are organised lymphoid tissue structures where immune responses are primed. They are abundant in the intestine under homeostatic conditions but have also been shown to form during colitis (Buettner and Lochner,

2016). Intestinal stromal cells, which are one of the cellular components of SILTs, have previously been described to represent an immunocompetent cell type capable of cytokine production and antigen presentation. Stromal cells producing IL-33 were highly concentrated in the SILTs both under steady state and inflammatory conditions. Both IL-33 mRNA and protein were highly enriched in the stromal compartment. However, the ability of stromal cells to release bioactive IL-33 was not tested and remains to be investigated. Nonetheless, intestinal stromal cells were shown to produce sST2 in response to both bacterial and pro-inflammatory stimuli. Interestingly, intestinal stromal cells secreted sST2 in response to IL-33 stimulation, suggesting that these cells might self-regulate the activity of IL-33 in an autocrine fashion. Although, exploration of the role of IL-33 as a nuclear factor was not within the scope of this thesis, investigating this function in stromal cells could provide valuable insight into the biology of these cells. Previously, FRCs present in the spleen have been shown to produce IL-33 in response to a viral infection, which promoted robust anti-viral CTL responses (Bonilla et al., 2012). Intestinal stromal cells found in the SILTs are strategically positioned to influence a broad range of immune responses, and therefore, could represent sentinel cells producing IL-33 early in an immune response to cell death triggered by pathogens or mechanical stress.

Stromal cells producing IL-33 and found in the SILTs were located in the T cell zone. Tregs were also present in the same SILTs with IL-33^{+} stromal cells, raising the possibility that stromal cell-derived IL-33 could directly modulate Tregs. Consistent with this, we identified a population of ST2-expressing Tregs, which was especially abundant in the colon. It will be particularly informative to assess the co-localisation of

the ST2-expressing Tregs with the IL-33-producing stromal cells in the SILTs in order to test whether Tregs may respond to stromal-derived IL-33. Additionally, our *in vitro* experiments revealed that IL-33 induced amphiregulin, a repair mediator, in Tregs. However in an *in vivo* colitis model, deficiency in IL-33 signalling or amphiregulin did not reveal a requirement for either of these factors for repair and resolution to occur. This absence of a repair phenotype could be due to functional redundancy with other immune mediators. Nonetheless, an analogous mechanism, whereby IL-33 induces amphiregulin production in Tregs has also been proposed in the context of other tissues: in the skeletal muscle upon injury and the lung during influenza infection (Burzyn et al., 2013, Arpaia et al., 2015). Non-lymphoid tissue-resident Tregs could therefore have a unique role in mediating repair.

Further underscoring a possible unique role for non-lymphoid tissue-resident Tregs is the observation that $ST2^+$ Tregs are abundant in the colon, muscle, VAT, lung and skin. Others have demonstrated that IL-33 signalling was critical for the accumulation and maintenance of VAT Tregs and promoted both immune and non-immune processes *in situ*. IL-33-producing FRC-like cells in the VAT were critical in these processes, highlighting another example of possible Treg-stroma interactions (Kolodin et al., 2015). However, in the colon, IL-33 was dispensable for the development of Tregs and their function during a bacterially driven model of experimental colitis. This suggests that while IL-33 may promote a specific Treg phenotype and function *in vivo*, the colonic environment is rich in other factors sufficient for their development and maintenance. Of note, another IL-1 cytokine family member, IL-18, which is also abundant in the colon, was found to be essential for Treg-mediated control of colitis

(Harrison et al., 2015). Interestingly, while IL-33 expression was predominantly restricted to the stromal compartment, the epithelial cells produced IL-18. This raises the possibility that spatial compartmentalisation may exist to provide multiple mechanisms that support Treg-mediated intestinal homeostasis.

Importantly, the murine colon harbours two distinct Treg populations $ST2^+/Gata3^+$ and $Ror\gamma t^+$ Tregs. These Treg cell populations displayed a differential marker expression profile. These include markers of activation, such as KLRG1 and CD103, cytokine receptors, including ST2, IL-10R and IL-18R, as well as IL-10. Defining the TCR repertoire of $ST2^+/Gata3^+$ and $Ror\gamma t^+$ Tregs would provide further insight into the biology of these cells. Furthermore, the $ST2^+/Gata3^+$ Treg population was found in the intestine earlier in life than $Ror\gamma t^+$ Tregs. Data from our lab and others suggest that while $ST2^+/Gata3^+$ Tregs are thymic-derived, $Ror\gamma t^+$ Tregs require microbial exposure for their development (Schiering et al., 2014, Ohnmacht et al., 2015, Sefik et al., 2015, Yang et al., 2016a). Altogether these results suggest that the two Treg populations require different signals for their development. Consistent with this, recently a perinatally generated Treg population, which is essential to prevent autoimmunity development, has been described (Yang et al., 2015). These Tregs were enriched in *St2*, raising the possibility that these cells are the $ST2^+/Gata3^+$ Treg cells, which can be found throughout the non-lymphoid tissues. Whether indeed these cells can be found early on in the SILTs in the colon, and whether IL-33 produced by the intestinal stromal cells is one of the signals supporting this population warrants further investigation.

Studies to date, which investigated the function of specialised Treg populations by targeted deletion of either Gata3 or Rorγt reported inconsistent results. This was likely due to differences in the experimental models used or changes in the microbiota across the different animal facilities, in which the experiments were performed (Ohnmacht et al., 2015, Sefik et al., 2015, Yang et al., 2016a). Future studies of human samples will be key to determining whether ST2/Gata3- and Rorγt-expressing Tregs exist in the human gut and whether this balance is disrupted in IBD patients. Rorγt^{+} Tregs in the colon of both healthy and IBD patients have been described (Sefik et al., 2015). In addition, ST2^{+} Tregs have been reported in the human VAT (Vasanthakumar et al., 2015) but their presence in other tissues, including the colon, warrants further investigation. The role of IL-33 in the human setting both in healthy and in the inflamed gut remains to be elucidated. Importantly, tertiary lymphoid structures are also abundant in the human colon, and their formation can be observed in IBD patients (Buettner and Lochner, 2016). Therefore, studies of IL-33 expression in these structures in the human setting would be particularly interesting to understand the functional relevance of this cytokine.

Taken together, we have identified intestinal stromal cells localised to the SILTs to be the dominant source of IL-33 in the colon under both homeostatic and inflammatory conditions. ST2^{+}/Gata3^{+} Tregs were also abundant in the murine colon, and we showed that these cells are distinct from Rorγt^{+} Tregs. IL-33 upregulated amphiregulin expression in Tregs, suggesting that it may promote a population of Tregs with reparative function. However, this hypothesis remains to be confirmed *in vivo*. These findings open an exciting avenue to explore heterocellular signalling between stromal

cells and Tregs. We hypothesize that signals received by the stromal compartment upon cell injury may promote a specific phenotype of Tregs with a repair capacity, and thus, promote intestinal homeostasis.

References

ABRAHAM, C. & CHO, J. H. 2009. Inflammatory bowel disease. *N Engl J Med,* 361**,** 2066-78.

ABREU, M. T. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol,* 10**,** 131-44.

AFKARIAN, M., SEDY, J. R., YANG, J., JACOBSON, N. G., CEREB, N., YANG, S. Y., MURPHY, T. L. & MURPHY, K. M. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol,* 3**,** 549-57.

AHERN, P. P., IZCUE, A., MALOY, K. J. & POWRIE, F. 2008. The interleukin-23 axis in intestinal inflammation. *Immunol Rev,* 226**,** 147-59.

AHERN, P. P., SCHIERING, C., BUONOCORE, S., MCGEACHY, M. J., CUA, D. J., MALOY, K. J. & POWRIE, F. 2010. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity,* 33**,** 279-88.

AL-HADDAD, S. & RIDDELL, R. H. 2005. The role of eosinophils in inflammatory bowel disease. *Gut,* 54**,** 1674-5.

ALI, S., MOHS, A., THOMAS, M., KLARE, J., ROSS, R., SCHMITZ, M. L. & MARTIN, M. U. 2011. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *J Immunol,* 187**,** 1609-16.

ALLAKHVERDI, Z., SMITH, D. E., COMEAU, M. R. & DELESPESSE, G. 2007. Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J Immunol,* 179**,** 2051-4.

ALVES-FILHO, J. C., SONEGO, F., SOUTO, F. O., FREITAS, A., VERRI, W. A., JR., AUXILIADORA-MARTINS, M., BASILE-FILHO, A., MCKENZIE, A. N., XU, D., CUNHA, F. Q. & LIEW, F. Y. 2010. Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nat Med,* 16**,** 708-12.

ANDERSON, M. S., VENANZI, E. S., KLEIN, L., CHEN, Z., BERZINS, S. P., TURLEY, S. J., VON BOEHMER, H., BRONSON, R., DIERICH, A., BENOIST, C. & MATHIS, D. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science,* 298**,** 1395-401.

ANDRONICOS, N. M., MCNALLY, J., KOTZE, A. C., HUNT, P. W. & INGHAM, A. 2012. Trichostrongylus colubriformis larvae induce necrosis and release of IL33 from intestinal epithelial cells in vitro: implications for gastrointestinal nematode vaccine design. *Int J Parasitol,* 42**,** 295-304.

ANSEL, K. M., DJURETIC, I., TANASA, B. & RAO, A. 2006. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol,* 24**,** 607-56.

AOKI, R., KAWAMURA, T., GOSHIMA, F., OGAWA, Y., NAKAE, S., NAKAO, A., MORIISHI, K., NISHIYAMA, Y. & SHIMADA, S. 2013. Mast cells play a key role in host defense against herpes simplex virus infection through TNF-alpha and IL-6 production. *J Invest Dermatol,* 133**,** 2170-9.

ARMAKA, M., APOSTOLAKI, M., JACQUES, P., KONTOYIANNIS, D. L., ELEWAUT, D. & KOLLIAS, G. 2008. Mesenchymal cell targeting by TNF as a common pathogenic principle in chronic inflammatory joint and intestinal diseases. *J Exp Med,* 205**,** 331-7.

ARNOLD, I. C., MATHISEN, S., SCHULTHESS, J., DANNE, C., HEGAZY, A. N. & POWRIE, F. 2016. CD11c(+) monocyte/macrophages promote chronic Helicobacter hepaticus-induced intestinal inflammation through the production of IL-23. *Mucosal Immunol,* 9**,** 352-63.

ARPAIA, N., CAMPBELL, C., FAN, X., DIKIY, S., VAN DER VEEKEN, J., DEROOS, P., LIU, H., CROSS, J. R., PFEFFER, K., COFFER, P. J. & RUDENSKY, A. Y. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature,* 504**,** 451-5.

ARPAIA, N., GREEN, J. A., MOLTEDO, B., ARVEY, A., HEMMERS, S., YUAN, S., TREUTING, P. M. & RUDENSKY, A. Y. 2015. A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell,* 162**,** 1078-89.

ARTIS, D., KANE, C. M., FIORE, J., ZAPH, C., SHAPIRA, S., JOYCE, K., MACDONALD, A., HUNTER, C., SCOTT, P. & PEARCE, E. J. 2005. Dendritic cell-intrinsic expression of NF-kappa B1 is required to promote optimal Th2 cell differentiation. *J Immunol,* 174**,** 7154-9.

ASANO, M., TODA, M., SAKAGUCHI, N. & SAKAGUCHI, S. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med,* 184**,** 387-96.

ASSEMAN, C., MAUZE, S., LEACH, M. W., COFFMAN, R. L. & POWRIE, F. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med,* 190**,** 995-1004.

ATARASHI, K., TANOUE, T., ANDO, M., KAMADA, N., NAGANO, Y., NARUSHIMA, S., SUDA, W., IMAOKA, A., SETOYAMA, H., NAGAMORI, T., ISHIKAWA, E., SHIMA, T., HARA, T., KADO, S., JINNOHARA, T., OHNO, H., KONDO, T., TOYOOKA, K., WATANABE, E., YOKOYAMA, S., TOKORO, S., MORI, H., NOGUCHI, Y., MORITA, H., IVANOV, II, SUGIYAMA, T., NUNEZ, G., CAMP, J. G., HATTORI, M., UMESAKI, Y. & HONDA, K. 2015. Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell,* 163**,** 367-80.

ATARASHI, K., TANOUE, T., OSHIMA, K., SUDA, W., NAGANO, Y., NISHIKAWA, H., FUKUDA, S., SAITO, T., NARUSHIMA, S., HASE, K., KIM, S., FRITZ, J. V., WILMES, P., UEHA, S., MATSUSHIMA, K., OHNO, H., OLLE, B., SAKAGUCHI, S., TANIGUCHI, T., MORITA, H., HATTORI, M. & HONDA, K. 2013. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature,* 500**,** 232-6.

ATARASHI, K., TANOUE, T., SHIMA, T., IMAOKA, A., KUWAHARA, T., MOMOSE, Y., CHENG, G., YAMASAKI, S., SAITO, T., OHBA, Y., TANIGUCHI, T., TAKEDA, K., HORI, S., IVANOV, II, UMESAKI, Y., ITOH, K. & HONDA, K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science,* 331**,** 337-41.

AWASTHI, A., RIOL-BLANCO, L., JAGER, A., KORN, T., POT, C., GALILEOS, G., BETTELLI, E., KUCHROO, V. K. & OUKKA, M. 2009. Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. *J Immunol,* 182**,** 5904-8.

BAEKKEVOLD, E. S., ROUSSIGNE, M., YAMANAKA, T., JOHANSEN, F. E., JAHNSEN, F. L., AMALRIC, F., BRANDTZAEG, P., ERARD, M., HARALDSEN, G. & GIRARD, J. P. 2003. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol,* 163**,** 69-79.

BAILEY-BUCKTROUT, S. L., MARTINEZ-LLORDELLA, M., ZHOU, X., ANTHONY, B., ROSENTHAL, W., LUCHE, H., FEHLING, H. J. & BLUESTONE, J. A. 2013. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity,* 39**,** 949-62.

BARLOW, J. L., PEEL, S., FOX, J., PANOVA, V., HARDMAN, C. S., CAMELO, A., BUCKS, C., WU, X., KANE, C. M., NEILL, D. R., FLYNN, R. J., SAYERS, I., HALL, I. P. & MCKENZIE, A. N. 2013. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J Allergy Clin Immunol,* 132**,** 933-41.

BARNES, M. J., KREBS, P., HARRIS, N., EIDENSCHENK, C., GONZALEZ-QUINTIAL, R., ARNOLD, C. N., CROZAT, K., SOVATH, S., MORESCO, E. M., THEOFILOPOULOS, A. N., BEUTLER, B. & HOEBE, K. 2009. Commitment to the regulatory T cell lineage requires CARMA1 in the thymus but not in the periphery. *PLoS Biol,* 7**,** e51.

BAUMANN, C., BONILLA, W. V., FROHLICH, A., HELMSTETTER, C., PEINE, M., HEGAZY, A. N., PINSCHEWER, D. D. & LOHNING, M. 2015. T-bet- and STAT4-dependent IL-33 receptor expression directly promotes antiviral Th1 cell responses. *Proc Natl Acad Sci U S A,* 112**,** 4056-61.

BECKER, C., FANTINI, M. C. & NEURATH, M. F. 2006. High resolution colonoscopy in live mice. *Nat Protoc,* 1**,** 2900-4.

BELTRAN, C. J., NUNEZ, L. E., DIAZ-JIMENEZ, D., FARFAN, N., CANDIA, E., HEINE, C., LOPEZ, F., GONZALEZ, M. J., QUERA, R. & HERMOSO, M. A. 2010. Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflamm Bowel Dis,* 16**,** 1097-107.

BENNETT, C. L., CHRISTIE, J., RAMSDELL, F., BRUNKOW, M. E., FERGUSON, P. J., WHITESELL, L., KELLY, T. E., SAULSBURY, F. T., CHANCE, P. F. & OCHS, H. D. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet,* 27**,** 20-1.

BENSON, M. J., PINO-LAGOS, K., ROSEMBLATT, M. & NOELLE, R. J. 2007. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med,* 204**,** 1765-74.

BERASAIN, C., GARCIA-TREVIJANO, E. R., CASTILLO, J., ERROBA, E., LEE, D. C., PRIETO, J. & AVILA, M. A. 2005. Amphiregulin: an early trigger of liver regeneration in mice. *Gastroenterology,* 128**,** 424-32.

BERER, K., MUES, M., KOUTROLOS, M., RASBI, Z. A., BOZIKI, M., JOHNER, C., WEKERLE, H. & KRISHNAMOORTHY, G. 2011. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature,* 479**,** 538-41.

BERG, D. J., DAVIDSON, N., KUHN, R., MULLER, W., MENON, S., HOLLAND, G., THOMPSON-SNIPES, L., LEACH, M. W. & RENNICK, D. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest,* 98**,** 1010-20.

BERTHELOOT, D. & LATZ, E. 2016. HMGB1, IL-1alpha, IL-33 and S100 proteins: dual-function alarmins. *Cell Mol Immunol*.

BESSA, J., MEYER, C. A., DE VERA MUDRY, M. C., SCHLICHT, S., SMITH, S. H., IGLESIAS, A. & COTE-SIERRA, J. 2014. Altered subcellular localization of IL-33 leads to non-resolving lethal inflammation. *J Autoimmun,* 55**,** 33-41.

BETTELLI, E., CARRIER, Y., GAO, W., KORN, T., STROM, T. B., OUKKA, M., WEINER, H. L. & KUCHROO, V. K. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature,* 441**,** 235-8.

BJARNASON, I., O'MORAIN, C., LEVI, A. J. & PETERS, T. J. 1983. Absorption of 51chromium-labeled ethylenediaminetetraacetate in inflammatory bowel disease. *Gastroenterology,* 85**,** 318-22.

BOGA, S., ALKIM, H., KOKSAL, A. R., OZAGARI, A. A., BAYRAM, M., TEKIN NEIJMANN, S., SEN, I. & ALKIM, C. 2016. Serum ST2 in inflammatory bowel disease: a potential biomarker for disease activity. *J Investig Med,* 64**,** 1016-24.

BOGUNOVIC, M., GINHOUX, F., HELFT, J., SHANG, L., HASHIMOTO, D., GRETER, M., LIU, K., JAKUBZICK, C., INGERSOLL, M. A., LEBOEUF, M., STANLEY, E. R., NUSSENZWEIG, M., LIRA, S. A., RANDOLPH, G. J. & MERAD, M. 2009. Origin of the lamina propria dendritic cell network. *Immunity,* 31**,** 513-25.

BONILLA, W. V., FROHLICH, A., SENN, K., KALLERT, S., FERNANDEZ, M., JOHNSON, S., KREUTZFELDT, M., HEGAZY, A. N., SCHRICK, C., FALLON, P. G., KLEMENZ, R., NAKAE, S., ADLER, H., MERKLER, D., LOHNING, M. & PINSCHEWER, D. D. 2012. The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses. *Science,* 335**,** 984-9.

BONO, M. R., FERNANDEZ, D., FLORES-SANTIBANEZ, F., ROSEMBLATT, M. & SAUMA, D. 2015. CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. *FEBS Lett,* 589**,** 3454-60.

BOURGEOIS, E., VAN, L. P., SAMSON, M., DIEM, S., BARRA, A., ROGA, S., GOMBERT, J. M., SCHNEIDER, E., DY, M., GOURDY, P., GIRARD, J. P. & HERBELIN, A. 2009. The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *Eur J Immunol,* 39**,** 1046-55.

BOURGEOIS, E. A., LEVESCOT, A., DIEM, S., CHAUVINEAU, A., BERGES, H., MILPIED, P., LEHUEN, A., DAMOTTE, D., GOMBERT, J. M., SCHNEIDER, E., GIRARD, J. P., GOURDY, P. & HERBELIN, A. 2011. A natural protective function of invariant NKT cells in a mouse model of innate-cell-driven lung inflammation. *Eur J Immunol,* 41**,** 299-305.

BRADY, G., BOGGAN, L., BOWIE, A. & O'NEILL, L. A. 2005. Schlafen-1 causes a cell cycle arrest by inhibiting induction of cyclin D1. *J Biol Chem,* 280**,** 30723-34.

BRESTOFF, J. R., KIM, B. S., SAENZ, S. A., STINE, R. R., MONTICELLI, L. A., SONNENBERG, G. F., THOME, J. J., FARBER, D. L., LUTFY, K., SEALE, P. & ARTIS, D. 2015. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature,* 519**,** 242-6.

BROQUET, A. H., HIRATA, Y., MCALLISTER, C. S. & KAGNOFF, M. F. 2011. RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium. *J Immunol,* 186**,** 1618-26.

BRUNKOW, M. E., JEFFERY, E. W., HJERRILD, K. A., PAEPER, B., CLARK, L. B., YASAYKO, S. A., WILKINSON, J. E., GALAS, D., ZIEGLER, S. F. & RAMSDELL, F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet,* 27**,** 68-73.

BUETTNER, M. & LOCHNER, M. 2016. Development and Function of Secondary and Tertiary Lymphoid Organs in the Small Intestine and the Colon. *Front Immunol,* 7**,** 342.

BULEK, K., SWAIDANI, S., QIN, J., LU, Y., GULEN, M. F., HERJAN, T., MIN, B., KASTELEIN, R. A., ARONICA, M., KOSZ-VNENCHAK, M. & LI, X. 2009. The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response. *J Immunol,* 182**,** 2601-9.

BUONOCORE, S., AHERN, P. P., UHLIG, H. H., IVANOV, II, LITTMAN, D. R., MALOY, K. J. & POWRIE, F. 2010. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature,* 464**,** 1371-5.

BURCHILL, M. A., YANG, J., VANG, K. B., MOON, J. J., CHU, H. H., LIO, C. W., VEGOE, A. L., HSIEH, C. S., JENKINS, M. K. & FARRAR, M. A. 2008. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity,* 28**,** 112-21.

BURCHILL, M. A., YANG, J., VOGTENHUBER, C., BLAZAR, B. R. & FARRAR, M. A. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol,* 178**,** 280-90.

BURICH, A., HERSHBERG, R., WAGGIE, K., ZENG, W., BRABB, T., WESTRICH, G., VINEY, J. L. & MAGGIO-PRICE, L. 2001. Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol,* 281**,** G764-78.

BURISCH, J., JESS, T., MARTINATO, M., LAKATOS, P. L. & EPICOM, E. 2013. The burden of inflammatory bowel disease in Europe. *J Crohns Colitis,* 7**,** 322-37.

BURZYN, D., KUSWANTO, W., KOLODIN, D., SHADRACH, J. L., CERLETTI, M., JANG, Y., SEFIK, E., TAN, T. G., WAGERS, A. J., BENOIST, C. & MATHIS, D. 2013. A special population of regulatory T cells potentiates muscle repair. *Cell,* 155**,** 1282-95.

BYERS, D. E., ALEXANDER-BRETT, J., PATEL, A. C., AGAPOV, E., DANG-VU, G., JIN, X., WU, K., YOU, Y., ALEVY, Y., GIRARD, J. P., STAPPENBECK, T. S., PATTERSON, G. A., PIERCE, R. A., BRODY, S. L. & HOLTZMAN, M. J. 2013. Long-term IL-33-producing epithelial progenitor cells in chronic obstructive lung disease. *J Clin Invest,* 123**,** 3967-82.

CARRIERE, V., ROUSSEL, L., ORTEGA, N., LACORRE, D. A., AMERICH, L., AGUILAR, L., BOUCHE, G. & GIRARD, J. P. 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A,* 104**,** 282-7.

CASES, S., STONE, S. J., ZHOU, P., YEN, E., TOW, B., LARDIZABAL, K. D., VOELKER, T. & FARESE, R. V., JR. 2001. Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. *J Biol Chem,* 276**,** 38870-6.

CASH, H. L., WHITHAM, C. V., BEHRENDT, C. L. & HOOPER, L. V. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science,* 313**,** 1126-30.

CAYROL, C. & GIRARD, J. P. 2009. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc Natl Acad Sci U S A,* 106**,** 9021-6.

CAYROL, C. & GIRARD, J. P. 2014. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr Opin Immunol,* 31**,** 31-7.

CEBULA, A., SEWERYN, M., REMPALA, G. A., PABLA, S. S., MCINDOE, R. A., DENNING, T. L., BRY, L., KRAJ, P., KISIELOW, P. & IGNATOWICZ, L. 2013. Thymus-derived

regulatory T cells contribute to tolerance to commensal microbiota. *Nature,* 497**,** 258-62.
CERUTTI, A. 2008. The regulation of IgA class switching. *Nat Rev Immunol,* 8**,** 421-34.
CHACKERIAN, A. A., OLDHAM, E. R., MURPHY, E. E., SCHMITZ, J., PFLANZ, S. & KASTELEIN, R. A. 2007. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J Immunol,* 179**,** 2551-5.
CHAN, H. C. & NG, S. C. 2016. Emerging biologics in inflammatory bowel disease. *J Gastroenterol*.
CHANG, Y. J., KIM, H. Y., ALBACKER, L. A., BAUMGARTH, N., MCKENZIE, A. N., SMITH, D. E., DEKRUYFF, R. H. & UMETSU, D. T. 2011. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol,* 12**,** 631-8.
CHASSAING, B. & DARFEUILLE-MICHAUD, A. 2011. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. *Gastroenterology,* 140**,** 1720-28.
CHAUDHRY, A., RUDRA, D., TREUTING, P., SAMSTEIN, R. M., LIANG, Y., KAS, A. & RUDENSKY, A. Y. 2009. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science,* 326**,** 986-91.
CHAUDHRY, A., SAMSTEIN, R. M., TREUTING, P., LIANG, Y., PILS, M. C., HEINRICH, J. M., JACK, R. S., WUNDERLICH, F. T., BRUNING, J. C., MULLER, W. & RUDENSKY, A. Y. 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity,* 34**,** 566-78.
CHEN, L. & FLIES, D. B. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol,* 13**,** 227-42.
CHEN, L., LEI, L., CHANG, X., LI, Z., LU, C., ZHANG, X., WU, Y., YEH, I. T. & ZHONG, G. 2010. Mice deficient in MyD88 Develop a Th2-dominant response and severe pathology in the upper genital tract following Chlamydia muridarum infection. *J Immunol,* 184**,** 2602-10.
CHEN, W., JIN, W., HARDEGEN, N., LEI, K. J., LI, L., MARINOS, N., MCGRADY, G. & WAHL, S. M. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med,* 198**,** 1875-86.
CHEN, W. Y., HONG, J., GANNON, J., KAKKAR, R. & LEE, R. T. 2015. Myocardial pressure overload induces systemic inflammation through endothelial cell IL-33. *Proc Natl Acad Sci U S A,* 112**,** 7249-54.
CHENG, D., IQBAL, J., DEVENNY, J., CHU, C. H., CHEN, L., DONG, J., SEETHALA, R., KEIM, W. J., AZZARA, A. V., LAWRENCE, R. M., PELLEYMOUNTER, M. A. & HUSSAIN, M. M. 2008. Acylation of acylglycerols by acyl coenzyme A:diacylglycerol acyltransferase 1 (DGAT1). Functional importance of DGAT1 in the intestinal fat absorption. *J Biol Chem,* 283**,** 29802-11.
CHEROUTRE, H., LAMBOLEZ, F. & MUCIDA, D. 2011. The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol,* 11**,** 445-56.
CHERRY, W. B., YOON, J., BARTEMES, K. R., IIJIMA, K. & KITA, H. 2008. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *J Allergy Clin Immunol,* 121**,** 1484-90.

CHOI, Y. S., PARK, J. A., KIM, J., RHO, S. S., PARK, H., KIM, Y. M. & KWON, Y. G. 2012. Nuclear IL-33 is a transcriptional regulator of NF-kappaB p65 and induces endothelial cell activation. *Biochem Biophys Res Commun,* 421**,** 305-11.

CHU, V. T., BELLER, A., RAUSCH, S., STRANDMARK, J., ZANKER, M., ARBACH, O., KRUGLOV, A. & BEREK, C. 2014. Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and contribute to gut immune homeostasis. *Immunity,* 40**,** 582-93.

CHUNG, Y., CHANG, S. H., MARTINEZ, G. J., YANG, X. O., NURIEVA, R., KANG, H. S., MA, L., WATOWICH, S. S., JETTEN, A. M., TIAN, Q. & DONG, C. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity,* 30**,** 576-87.

CIOFANI, M., MADAR, A., GALAN, C., SELLARS, M., MACE, K., PAULI, F., AGARWAL, A., HUANG, W., PARKHURST, C. N., MURATET, M., NEWBERRY, K. M., MEADOWS, S., GREENFIELD, A., YANG, Y., JAIN, P., KIRIGIN, F. K., BIRCHMEIER, C., WAGNER, E. F., MURPHY, K. M., MYERS, R. M., BONNEAU, R. & LITTMAN, D. R. 2012. A validated regulatory network for Th17 cell specification. *Cell,* 151**,** 289-303.

CIPOLLETTA, D., COHEN, P., SPIEGELMAN, B. M., BENOIST, C. & MATHIS, D. 2015. Appearance and disappearance of the mRNA signature characteristic of Treg cells in visceral adipose tissue: age, diet, and PPARgamma effects. *Proc Natl Acad Sci U S A,* 112**,** 482-7.

CIPOLLETTA, D., FEUERER, M., LI, A., KAMEI, N., LEE, J., SHOELSON, S. E., BENOIST, C. & MATHIS, D. 2012. PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature,* 486**,** 549-53.

COHEN, E. S., SCOTT, I. C., MAJITHIYA, J. B., RAPLEY, L., KEMP, B. P., ENGLAND, E., REES, D. G., OVERED-SAYER, C. L., WOODS, J., BOND, N. J., VEYSSIER, C. S., EMBREY, K. J., SIMS, D. A., SNAITH, M. R., VOUSDEN, K. A., STRAIN, M. D., CHAN, D. T., CARMEN, S., HUNTINGTON, C. E., FLAVELL, L., XU, J., POPOVIC, B., BRIGHTLING, C. E., VAUGHAN, T. J., BUTLER, R., LOWE, D. C., HIGAZI, D. R., CORKILL, D. J., MAY, R. D., SLEEMAN, M. A. & MUSTELIN, T. 2015. Oxidation of the alarmin IL-33 regulates ST2-dependent inflammation. *Nat Commun,* 6**,** 8327.

COLE, K. E., STRICK, C. A., PARADIS, T. J., OGBORNE, K. T., LOETSCHER, M., GLADUE, R. P., LIN, W., BOYD, J. G., MOSER, B., WOOD, D. E., SAHAGAN, B. G. & NEOTE, K. 1998. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med,* 187**,** 2009-21.

COLLISON, L. W., WORKMAN, C. J., KUO, T. T., BOYD, K., WANG, Y., VIGNALI, K. M., CROSS, R., SEHY, D., BLUMBERG, R. S. & VIGNALI, D. A. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature,* 450**,** 566-9.

COOMBES, J. L., SIDDIQUI, K. R., ARANCIBIA-CARCAMO, C. V., HALL, J., SUN, C. M., BELKAID, Y. & POWRIE, F. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med,* 204**,** 1757-64.

COYLE, A. J., LLOYD, C., TIAN, J., NGUYEN, T., ERIKKSON, C., WANG, L., OTTOSON, P., PERSSON, P., DELANEY, T., LEHAR, S., LIN, S., POISSON, L., MEISEL, C., KAMRADT, T., BJERKE, T., LEVINSON, D. & GUTIERREZ-RAMOS, J. C. 1999. Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J Exp Med,* 190**,** 895-902.

CROTTY, S. 2014. T follicular helper cell differentiation, function, and roles in disease. *Immunity,* 41**,** 529-42.

CUA, D. J., SHERLOCK, J., CHEN, Y., MURPHY, C. A., JOYCE, B., SEYMOUR, B., LUCIAN, L., TO, W., KWAN, S., CHURAKOVA, T., ZURAWSKI, S., WIEKOWSKI, M., LIRA, S. A., GORMAN, D., KASTELEIN, R. A. & SEDGWICK, J. D. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature,* 421**,** 744-8.

D'ARGENIO, V. & SALVATORE, F. 2015. The role of the gut microbiome in the healthy adult status. *Clin Chim Acta,* 451**,** 97-102.

DE LANGE, K. M. & BARRETT, J. C. 2015. Understanding inflammatory bowel disease via immunogenetics. *J Autoimmun,* 64**,** 91-100.

DE SOUZA, H. S. & FIOCCHI, C. 2016. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol,* 13**,** 13-27.

DEAGLIO, S., DWYER, K. M., GAO, W., FRIEDMAN, D., USHEVA, A., ERAT, A., CHEN, J. F., ENJYOJI, K., LINDEN, J., OUKKA, M., KUCHROO, V. K., STROM, T. B. & ROBSON, S. C. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med,* 204**,** 1257-65.

DELGOFFE, G. M., KOLE, T. P., ZHENG, Y., ZAREK, P. E., MATTHEWS, K. L., XIAO, B., WORLEY, P. F., KOZMA, S. C. & POWELL, J. D. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity,* 30**,** 832-44.

DIAZ-JIMENEZ, D., NUNEZ, L. E., BELTRAN, C. J., CANDIA, E., SUAZO, C., ALVAREZ-LOBOS, M., GONZALEZ, M. J., HERMOSO, M. A. & QUERA, R. 2011. Soluble ST2: a new and promising activity marker in ulcerative colitis. *World J Gastroenterol,* 17**,** 2181-90.

DRISKELL, R. R., LICHTENBERGER, B. M., HOSTE, E., KRETZSCHMAR, K., SIMONS, B. D., CHARALAMBOUS, M., FERRON, S. R., HERAULT, Y., PAVLOVIC, G., FERGUSON-SMITH, A. C. & WATT, F. M. 2013. Distinct fibroblast lineages determine dermal architecture in skin development and repair. *Nature,* 504**,** 277-81.

DRUBE, S., HEINK, S., WALTER, S., LOHN, T., GRUSSER, M., GERBAULET, A., BEROD, L., SCHONS, J., DUDECK, A., FREITAG, J., GROTHA, S., REICH, D., RUDESCHKO, O., NORGAUER, J., HARTMANN, K., ROERS, A. & KAMRADT, T. 2010. The receptor tyrosine kinase c-Kit controls IL-33 receptor signaling in mast cells. *Blood,* 115**,** 3899-906.

DU, P., KIBBE, W. A. & LIN, S. M. 2008. lumi: a pipeline for processing Illumina microarray. *Bioinformatics,* 24**,** 1547-8.

DUAN, L., CHEN, J., ZHANG, H., YANG, H., ZHU, P., XIONG, A., XIA, Q., ZHENG, F., TAN, Z., GONG, F. & FANG, M. 2012. Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3(+) regulatory T-cell responses in mice. *Mol Med,* 18**,** 753-61.

DUARTE, J. H., ZELENAY, S., BERGMAN, M. L., MARTINS, A. C. & DEMENGEOT, J. 2009. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. *Eur J Immunol,* 39**,** 948-55.

DUERR, R. H., TAYLOR, K. D., BRANT, S. R., RIOUX, J. D., SILVERBERG, M. S., DALY, M. J., STEINHART, A. H., ABRAHAM, C., REGUEIRO, M., GRIFFITHS, A., DASSOPOULOS, T., BITTON, A., YANG, H., TARGAN, S., DATTA, L. W., KISTNER, E. O., SCHUMM, L. P., LEE, A. T., GREGERSEN, P. K., BARMADA, M. M., ROTTER, J. I., NICOLAE, D.

L. & CHO, J. H. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science,* 314**,** 1461-3.
DUPAGE, M. & BLUESTONE, J. A. 2016. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol,* 16**,** 149-63.
EBERL, G. & LITTMAN, D. R. 2003. The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. *Immunol Rev,* 195**,** 81-90.
ELINAV, E., HENAO-MEJIA, J. & FLAVELL, R. A. 2013. Integrative inflammasome activity in the regulation of intestinal mucosal immune responses. *Mucosal Immunol,* 6**,** 4-13.
ELINAV, E., STROWIG, T., KAU, A. L., HENAO-MEJIA, J., THAISS, C. A., BOOTH, C. J., PEAPER, D. R., BERTIN, J., EISENBARTH, S. C., GORDON, J. I. & FLAVELL, R. A. 2011. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell,* 145**,** 745-57.
ELSON, C. O., CONG, Y., WEAVER, C. T., SCHOEB, T. R., MCCLANAHAN, T. K., FICK, R. B. & KASTELEIN, R. A. 2007. Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology,* 132**,** 2359-70.
ENOMOTO, Y., ORIHARA, K., TAKAMASU, T., MATSUDA, A., GON, Y., SAITO, H., RA, C. & OKAYAMA, Y. 2009. Tissue remodeling induced by hypersecreted epidermal growth factor and amphiregulin in the airway after an acute asthma attack. *J Allergy Clin Immunol,* 124**,** 913-20 e1-7.
FAHLEN, L., READ, S., GORELIK, L., HURST, S. D., COFFMAN, R. L., FLAVELL, R. A. & POWRIE, F. 2005. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med,* 201**,** 737-46.
FARBER, J. M. 1990. A macrophage mRNA selectively induced by gamma-interferon encodes a member of the platelet factor 4 family of cytokines. *Proc Natl Acad Sci U S A,* 87**,** 5238-42.
FATHMAN, C. G. & LINEBERRY, N. B. 2007. Molecular mechanisms of CD4+ T-cell anergy. *Nat Rev Immunol,* 7**,** 599-609.
FENG, Y., ARVEY, A., CHINEN, T., VAN DER VEEKEN, J., GASTEIGER, G. & RUDENSKY, A. Y. 2014. Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. *Cell,* 158**,** 749-63.
FEUERER, M., HERRERO, L., CIPOLLETTA, D., NAAZ, A., WONG, J., NAYER, A., LEE, J., GOLDFINE, A. B., BENOIST, C., SHOELSON, S. & MATHIS, D. 2009. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med,* 15**,** 930-9.
FINNISH-GERMAN, A. C. 1997. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet,* 17**,** 399-403.
FINOTTO, S., NEURATH, M. F., GLICKMAN, J. N., QIN, S., LEHR, H. A., GREEN, F. H., ACKERMAN, K., HALEY, K., GALLE, P. R., SZABO, S. J., DRAZEN, J. M., DE SANCTIS, G. T. & GLIMCHER, L. H. 2002. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science,* 295**,** 336-8.
FLACZYK, A., DUERR, C. U., SHOURIAN, M., LAFFERTY, E. I., FRITZ, J. H. & QURESHI, S. T. 2013. IL-33 signaling regulates innate and adaptive immunity to Cryptococcus neoformans. *J Immunol,* 191**,** 2503-13.

FLETCHER, A. L., ACTON, S. E. & KNOBLICH, K. 2015. Lymph node fibroblastic reticular cells in health and disease. *Nat Rev Immunol,* 15**,** 350-61.

FOCK, V., MAIRHOFER, M., OTTI, G. R., HIDEN, U., SPITTLER, A., ZEISLER, H., FIALA, C., KNOFLER, M. & POLLHEIMER, J. 2013. Macrophage-derived IL-33 is a critical factor for placental growth. *J Immunol,* 191**,** 3734-43.

FOLTZ, C. J., FOX, J. G., CAHILL, R., MURPHY, J. C., YAN, L., SHAMES, B. & SCHAUER, D. B. 1998. Spontaneous inflammatory bowel disease in multiple mutant mouse lines: association with colonization by Helicobacter hepaticus. *Helicobacter,* 3**,** 69-78.

FONTENOT, J. D., GAVIN, M. A. & RUDENSKY, A. Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol,* 4**,** 330-6.

FOX, J. G., DEWHIRST, F. E., TULLY, J. G., PASTER, B. J., YAN, L., TAYLOR, N. S., COLLINS, M. J., JR., GORELICK, P. L. & WARD, J. M. 1994. Helicobacter hepaticus sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J Clin Microbiol,* 32**,** 1238-45.

FRANK, D. N., ST AMAND, A. L., FELDMAN, R. A., BOEDEKER, E. C., HARPAZ, N. & PACE, N. R. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A,* 104**,** 13780-5.

FU, W., ERGUN, A., LU, T., HILL, J. A., HAXHINASTO, S., FASSETT, M. S., GAZIT, R., ADORO, S., GLIMCHER, L., CHAN, S., KASTNER, P., ROSSI, D., COLLINS, J. J., MATHIS, D. & BENOIST, C. 2012. A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells. *Nat Immunol,* 13**,** 972-80.

FURUSAWA, Y., OBATA, Y., FUKUDA, S., ENDO, T. A., NAKATO, G., TAKAHASHI, D., NAKANISHI, Y., UETAKE, C., KATO, K., KATO, T., TAKAHASHI, M., FUKUDA, N. N., MURAKAMI, S., MIYAUCHI, E., HINO, S., ATARASHI, K., ONAWA, S., FUJIMURA, Y., LOCKETT, T., CLARKE, J. M., TOPPING, D. L., TOMITA, M., HORI, S., OHARA, O., MORITA, T., KOSEKI, H., KIKUCHI, J., HONDA, K., HASE, K. & OHNO, H. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature,* 504**,** 446-50.

FUSS, I. J., HELLER, F., BOIRIVANT, M., LEON, F., YOSHIDA, M., FICHTNER-FEIGL, S., YANG, Z., EXLEY, M., KITANI, A., BLUMBERG, R. S., MANNON, P. & STROBER, W. 2004. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest,* 113**,** 1490-7.

FUSS, I. J., NEURATH, M., BOIRIVANT, M., KLEIN, J. S., DE LA MOTTE, C., STRONG, S. A., FIOCCHI, C. & STROBER, W. 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol,* 157**,** 1261-70.

FYDEREK, K., STRUS, M., KOWALSKA-DUPLAGA, K., GOSIEWSKI, T., WEDRYCHOWICZ, A., JEDYNAK-WASOWICZ, U., SLADEK, M., PIECZARKOWSKI, S., ADAMSKI, P., KOCHAN, P. & HECZKO, P. B. 2009. Mucosal bacterial microflora and mucus layer thickness in adolescents with inflammatory bowel disease. *World J Gastroenterol,* 15**,** 5287-94.

GACHTER, T., WERENSKIOLD, A. K. & KLEMENZ, R. 1996. Transcription of the interleukin-1 receptor-related T1 gene is initiated at different promoters in mast cells and fibroblasts. *J Biol Chem,* 271**,** 124-9.

GADANI, S. P., WALSH, J. T., SMIRNOV, I., ZHENG, J. & KIPNIS, J. 2015. The glia-derived alarmin IL-33 orchestrates the immune response and promotes recovery following CNS injury. *Neuron,* 85**,** 703-9.

GAO, X., WANG, X., YANG, Q., ZHAO, X., WEN, W., LI, G., LU, J., QIN, W., QI, Y., XIE, F., JIANG, J., WU, C., ZHANG, X., CHEN, X., TURNQUIST, H., ZHU, Y. & LU, B. 2015. Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells. *J Immunol,* 194**,** 438-45.

GARIN, M. I., CHU, C. C., GOLSHAYAN, D., CERNUDA-MOROLLON, E., WAIT, R. & LECHLER, R. I. 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood,* 109**,** 2058-65.

GARLANDA, C., DINARELLO, C. A. & MANTOVANI, A. 2013a. The interleukin-1 family: back to the future. *Immunity,* 39**,** 1003-18.

GARLANDA, C., RIVA, F., BONAVITA, E., GENTILE, S. & MANTOVANI, A. 2013b. Decoys and Regulatory "Receptors" of the IL-1/Toll-Like Receptor Superfamily. *Front Immunol,* 4**,** 180.

GARRETT, W. S., GALLINI, C. A., YATSUNENKO, T., MICHAUD, M., DUBOIS, A., DELANEY, M. L., PUNIT, S., KARLSSON, M., BRY, L., GLICKMAN, J. N., GORDON, J. I., ONDERDONK, A. B. & GLIMCHER, L. H. 2010. Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. *Cell Host Microbe,* 8**,** 292-300.

GAVIN, M. A., RASMUSSEN, J. P., FONTENOT, J. D., VASTA, V., MANGANIELLO, V. C., BEAVO, J. A. & RUDENSKY, A. Y. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature,* 445**,** 771-5.

GEREMIA, A., ARANCIBIA-CARCAMO, C. V., FLEMING, M. P., RUST, N., SINGH, B., MORTENSEN, N. J., TRAVIS, S. P. & POWRIE, F. 2011. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J Exp Med,* 208**,** 1127-33.

GERMAIN, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol,* 2**,** 309-22.

GERNER, M. Y., KASTENMULLER, W., IFRIM, I., KABAT, J. & GERMAIN, R. N. 2012. Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity,* 37**,** 364-76.

GEUKING, M. B., CAHENZLI, J., LAWSON, M. A., NG, D. C., SLACK, E., HAPFELMEIER, S., MCCOY, K. D. & MACPHERSON, A. J. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity,* 34**,** 794-806.

GHORESCHI, K., LAURENCE, A., YANG, X. P., TATO, C. M., MCGEACHY, M. J., KONKEL, J. E., RAMOS, H. L., WEI, L., DAVIDSON, T. S., BOULADOUX, N., GRAINGER, J. R., CHEN, Q., KANNO, Y., WATFORD, W. T., SUN, H. W., EBERL, G., SHEVACH, E. M., BELKAID, Y., CUA, D. J., CHEN, W. & O'SHEA, J. J. 2010. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature,* 467**,** 967-71.

GLOCKER, E. O., KOTLARZ, D., BOZTUG, K., GERTZ, E. M., SCHAFFER, A. A., NOYAN, F., PERRO, M., DIESTELHORST, J., ALLROTH, A., MURUGAN, D., HATSCHER, N.,

PFEIFER, D., SYKORA, K. W., SAUER, M., KREIPE, H., LACHER, M., NUSTEDE, R., WOELLNER, C., BAUMANN, U., SALZER, U., KOLETZKO, S., SHAH, N., SEGAL, A. W., SAUERBREY, A., BUDERUS, S., SNAPPER, S. B., GRIMBACHER, B. & KLEIN, C. 2009. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med,* 361**,** 2033-45.

GONDEK, D. C., LU, L. F., QUEZADA, S. A., SAKAGUCHI, S. & NOELLE, R. J. 2005. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol,* 174**,** 1783-6.

GOSAVI, S., WHITFORD, P. C., JENNINGS, P. A. & ONUCHIC, J. N. 2008. Extracting function from a beta-trefoil folding motif. *Proc Natl Acad Sci U S A,* 105**,** 10384-9.

GOTTSCHALK, R. A., CORSE, E. & ALLISON, J. P. 2012. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J Immunol,* 188**,** 976-80.

GRISERI, T., ASQUITH, M., THOMPSON, C. & POWRIE, F. 2010. OX40 is required for regulatory T cell-mediated control of colitis. *J Exp Med,* 207**,** 699-709.

GROOM, J. R. & LUSTER, A. D. 2011. CXCR3 in T cell function. *Exp Cell Res,* 317**,** 620-31.

GROSCHWITZ, K. R., AHRENS, R., OSTERFELD, H., GURISH, M. F., HAN, X., ABRINK, M., FINKELMAN, F. D., PEJLER, G. & HOGAN, S. P. 2009. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. *Proc Natl Acad Sci U S A,* 106**,** 22381-6.

GROSSMAN, W. J., VERBSKY, J. W., BARCHET, W., COLONNA, M., ATKINSON, J. P. & LEY, T. J. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity,* 21**,** 589-601.

GROTENBOER, N. S., KETELAAR, M. E., KOPPELMAN, G. H. & NAWIJN, M. C. 2013. Decoding asthma: translating genetic variation in IL33 and IL1RL1 into disease pathophysiology. *J Allergy Clin Immunol,* 131**,** 856-65.

GROB, P., DOSER, K., FALK, W., OBERMEIER, F. & HOFMANN, C. 2012. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflamm Bowel Dis,* 18**,** 1900-9.

GUGLANI, L. & KHADER, S. A. 2010. Th17 cytokines in mucosal immunity and inflammation. *Curr Opin HIV AIDS,* 5**,** 120-7.

GUO, L., WEI, G., ZHU, J., LIAO, W., LEONARD, W. J., ZHAO, K. & PAUL, W. 2009. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc Natl Acad Sci U S A,* 106**,** 13463-8.

GUPTA, S., MANICASSAMY, S., VASU, C., KUMAR, A., SHANG, W. & SUN, Z. 2008. Differential requirement of PKC-theta in the development and function of natural regulatory T cells. *Mol Immunol,* 46**,** 213-24.

HADIS, U., WAHL, B., SCHULZ, O., HARDTKE-WOLENSKI, M., SCHIPPERS, A., WAGNER, N., MULLER, W., SPARWASSER, T., FORSTER, R. & PABST, O. 2011. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity,* 34**,** 237-46.

HAENUKI, Y., MATSUSHITA, K., FUTATSUGI-YUMIKURA, S., ISHII, K. J., KAWAGOE, T., IMOTO, Y., FUJIEDA, S., YASUDA, M., HISA, Y., AKIRA, S., NAKANISHI, K. & YOSHIMOTO, T. 2012. A critical role of IL-33 in experimental allergic rhinitis. *J Allergy Clin Immunol,* 130**,** 184-94 e11.

HAMADA, H., HIROI, T., NISHIYAMA, Y., TAKAHASHI, H., MASUNAGA, Y., HACHIMURA, S., KAMINOGAWA, S., TAKAHASHI-IWANAGA, H., IWANAGA, T., KIYONO, H., YAMAMOTO, H. & ISHIKAWA, H. 2002. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol,* 168**,** 57-64.

HAN, J. M., WU, D., DENROCHE, H. C., YAO, Y., VERCHERE, C. B. & LEVINGS, M. K. 2015. IL-33 Reverses an Obesity-Induced Deficit in Visceral Adipose Tissue ST2+ T Regulatory Cells and Ameliorates Adipose Tissue Inflammation and Insulin Resistance. *J Immunol,* 194**,** 4777-83.

HANSEN, J. J. 2015. Immune Responses to Intestinal Microbes in Inflammatory Bowel Diseases. *Curr Allergy Asthma Rep,* 15**,** 61.

HAPFELMEIER, S., LAWSON, M. A., SLACK, E., KIRUNDI, J. K., STOEL, M., HEIKENWALDER, M., CAHENZLI, J., VELYKOREDKO, Y., BALMER, M. L., ENDT, K., GEUKING, M. B., CURTISS, R., 3RD, MCCOY, K. D. & MACPHERSON, A. J. 2010. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science,* 328**,** 1705-9.

HARADA, Y., HARADA, Y., ELLY, C., YING, G., PAIK, J. H., DEPINHO, R. A. & LIU, Y. C. 2010. Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. *J Exp Med,* 207**,** 1381-91.

HARDMAN, C. S., PANOVA, V. & MCKENZIE, A. N. 2013. IL-33 citrine reporter mice reveal the temporal and spatial expression of IL-33 during allergic lung inflammation. *Eur J Immunol,* 43**,** 488-98.

HARIBHAI, D., WILLIAMS, J. B., JIA, S., NICKERSON, D., SCHMITT, E. G., EDWARDS, B., ZIEGELBAUER, J., YASSAI, M., LI, S. H., RELLAND, L. M., WISE, P. M., CHEN, A., ZHENG, Y. Q., SIMPSON, P. M., GORSKI, J., SALZMAN, N. H., HESSNER, M. J., CHATILA, T. A. & WILLIAMS, C. B. 2011. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity,* 35**,** 109-22.

HARRIS, C. A., HAAS, J. T., STREEPER, R. S., STONE, S. J., KUMARI, M., YANG, K., HAN, X., BROWNELL, N., GROSS, R. W., ZECHNER, R. & FARESE, R. V., JR. 2011. DGAT enzymes are required for triacylglycerol synthesis and lipid droplets in adipocytes. *J Lipid Res,* 52**,** 657-67.

HARRIS, T. J., GROSSO, J. F., YEN, H. R., XIN, H., KORTYLEWSKI, M., ALBESIANO, E., HIPKISS, E. L., GETNET, D., GOLDBERG, M. V., MARIS, C. H., HOUSSEAU, F., YU, H., PARDOLL, D. M. & DRAKE, C. G. 2007. Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. *J Immunol,* 179**,** 4313-7.

HARRISON, O. J., SRINIVASAN, N., POTT, J., SCHIERING, C., KRAUSGRUBER, T., ILOTT, N. E. & MALOY, K. J. 2015. Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3(+) Treg cell function in the intestine. *Mucosal Immunol,* 8**,** 1226-36.

HAXHINASTO, S., MATHIS, D. & BENOIST, C. 2008. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med,* 205**,** 565-74.

HAYAKAWA, H., HAYAKAWA, M., KUME, A. & TOMINAGA, S. 2007. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J Biol Chem,* 282**,** 26369-80.

HAYDAY, A. & GIBBONS, D. 2008. Brokering the peace: the origin of intestinal T cells. *Mucosal Immunol,* 1**,** 172-4.

HAZLETT, L. D., MCCLELLAN, S. A., BARRETT, R. P., HUANG, X., ZHANG, Y., WU, M., VAN ROOIJEN, N. & SZLITER, E. 2010. IL-33 shifts macrophage polarization, promoting resistance against Pseudomonas aeruginosa keratitis. *Invest Ophthalmol Vis Sci,* 51**,** 1524-32.

HENDRICKSON, B. A., GOKHALE, R. & CHO, J. H. 2002. Clinical aspects and pathophysiology of inflammatory bowel disease. *Clin Microbiol Rev,* 15**,** 79-94.

HERBRAND, H., BERNHARDT, G., FORSTER, R. & PABST, O. 2008. Dynamics and function of solitary intestinal lymphoid tissue. *Crit Rev Immunol,* 28**,** 1-13.

HERMAN, A. E., FREEMAN, G. J., MATHIS, D. & BENOIST, C. 2004. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J Exp Med,* 199**,** 1479-89.

HERMISTON, M. L. & GORDON, J. I. 1995. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science,* 270**,** 1203-7.

HORAI, R., ZARATE-BLADES, C. R., DILLENBURG-PILLA, P., CHEN, J., KIELCZEWSKI, J. L., SILVER, P. B., JITTAYASOTHORN, Y., CHAN, C. C., YAMANE, H., HONDA, K. & CASPI, R. R. 2015. Microbiota-Dependent Activation of an Autoreactive T Cell Receptor Provokes Autoimmunity in an Immunologically Privileged Site. *Immunity,* 43**,** 343-53.

HORI, S. 2012. The Foxp3 interactome: a network perspective of T(reg) cells. *Nat Immunol,* 13**,** 943-5.

HORI, S., NOMURA, T. & SAKAGUCHI, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science,* 299**,** 1057-61.

HOSHINO, K., KASHIWAMURA, S., KURIBAYASHI, K., KODAMA, T., TSUJIMURA, T., NAKANISHI, K., MATSUYAMA, T., TAKEDA, K. & AKIRA, S. 1999. The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *J Exp Med,* 190**,** 1541-8.

HOYLER, T., KLOSE, C. S., SOUABNI, A., TURQUETI-NEVES, A., PFEIFER, D., RAWLINS, E. L., VOEHRINGER, D., BUSSLINGER, M. & DIEFENBACH, A. 2012. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity,* 37**,** 634-48.

HSU, C. L., NEILSEN, C. V. & BRYCE, P. J. 2010. IL-33 is produced by mast cells and regulates IgE-dependent inflammation. *PLoS One,* 5**,** e11944.

HU, S., WANG, Y., LICHTENSTEIN, L., TAO, Y., MUSCH, M. W., JABRI, B., ANTONOPOULOS, D., CLAUD, E. C. & CHANG, E. B. 2010. Regional differences in colonic mucosa-associated microbiota determine the physiological expression of host heat shock proteins. *Am J Physiol Gastrointest Liver Physiol,* 299**,** G1266-75.

HUBER, S., GAGLIANI, N., ESPLUGUES, E., O'CONNOR, W., JR., HUBER, F. J., CHAUDHRY, A., KAMANAKA, M., KOBAYASHI, Y., BOOTH, C. J., RUDENSKY, A. Y., RONCAROLO, M. G., BATTAGLIA, M. & FLAVELL, R. A. 2011. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity,* 34**,** 554-65.

HUE, S., AHERN, P., BUONOCORE, S., KULLBERG, M. C., CUA, D. J., MCKENZIE, B. S., POWRIE, F. & MALOY, K. J. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med,* 203**,** 2473-83.

HUMAN MICROBIOME PROJECT, C. 2012. Structure, function and diversity of the healthy human microbiome. *Nature,* 486**,** 207-14.

HUMPHREYS, N. E., XU, D., HEPWORTH, M. R., LIEW, F. Y. & GRENCIS, R. K. 2008. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol,* 180**,** 2443-9.

HUNG, L. Y., LEWKOWICH, I. P., DAWSON, L. A., DOWNEY, J., YANG, Y., SMITH, D. E. & HERBERT, D. R. 2013. IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity against hookworms. *Proc Natl Acad Sci U S A,* 110**,** 282-7.

IMAI, Y., YASUDA, K., SAKAGUCHI, Y., HANEDA, T., MIZUTANI, H., YOSHIMOTO, T., NAKANISHI, K. & YAMANISHI, K. 2013. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci U S A,* 110**,** 13921-6.

ISOMURA, I., PALMER, S., GRUMONT, R. J., BUNTING, K., HOYNE, G., WILKINSON, N., BANERJEE, A., PROIETTO, A., GUGASYAN, R., WU, L., MCNALLY, A., STEPTOE, R. J., THOMAS, R., SHANNON, M. F. & GERONDAKIS, S. 2009. c-Rel is required for the development of thymic Foxp3+ CD4 regulatory T cells. *J Exp Med,* 206**,** 3001-14.

ITOH, M., TAKAHASHI, T., SAKAGUCHI, N., KUNIYASU, Y., SHIMIZU, J., OTSUKA, F. & SAKAGUCHI, S. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol,* 162**,** 5317-26.

IVANOV, II, ATARASHI, K., MANEL, N., BRODIE, E. L., SHIMA, T., KARAOZ, U., WEI, D., GOLDFARB, K. C., SANTEE, C. A., LYNCH, S. V., TANOUE, T., IMAOKA, A., ITOH, K., TAKEDA, K., UMESAKI, Y., HONDA, K. & LITTMAN, D. R. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell,* 139**,** 485-98.

IVANOV, II, FRUTOS RDE, L., MANEL, N., YOSHINAGA, K., RIFKIN, D. B., SARTOR, R. B., FINLAY, B. B. & LITTMAN, D. R. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe,* 4**,** 337-49.

IVANOV, II & HONDA, K. 2012. Intestinal commensal microbes as immune modulators. *Cell Host Microbe,* 12**,** 496-508.

IVANOV, II, MCKENZIE, B. S., ZHOU, L., TADOKORO, C. E., LEPELLEY, A., LAFAILLE, J. J., CUA, D. J. & LITTMAN, D. R. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell,* 126**,** 1121-33.

IVANOV, II, ZHOU, L. & LITTMAN, D. R. 2007. Transcriptional regulation of Th17 cell differentiation. *Semin Immunol,* 19**,** 409-17.

IWAHANA, H., YANAGISAWA, K., ITO-KOSAKA, A., KUROIWA, K., TAGO, K., KOMATSU, N., KATASHIMA, R., ITAKURA, M. & TOMINAGA, S. 1999. Different promoter usage and multiple transcription initiation sites of the interleukin-1 receptor-related human ST2 gene in UT-7 and TM12 cells. *Eur J Biochem,* 264**,** 397-406.

IZCUE, A., COOMBES, J. L. & POWRIE, F. 2009. Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol,* 27**,** 313-38.

IZCUE, A., HUE, S., BUONOCORE, S., ARANCIBIA-CARCAMO, C. V., AHERN, P. P., IWAKURA, Y., MALOY, K. J. & POWRIE, F. 2008. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity,* 28**,** 559-70.

JACKSON, D. J., MAKRINIOTI, H., RANA, B. M., SHAMJI, B. W., TRUJILLO-TORRALBO, M. B., FOOTITT, J., JERICO, D.-R., TELCIAN, A. G., NIKONOVA, A., ZHU, J., ANISCENKO, J., GOGSADZE, L., BAKHSOLIANI, E., TRAUB, S., DHARIWAL, J., PORTER, J., HUNT, D., HUNT, T., HUNT, T., STANCIU, L. A., KHAITOV, M., BARTLETT, N. W., EDWARDS, M. R., KON, O. M., MALLIA, P., PAPADOPOULOS, N. G., AKDIS, C. A., WESTWICK, J., EDWARDS, M. J., COUSINS, D. J., WALTON, R. P. & JOHNSTON, S. L. 2014. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. *Am J Respir Crit Care Med,* 190**,** 1373-82.

JANSSEN RESEARCH & DEVELOPMENT, L. 2014. *A Single Ascending Dose Study in Healthy Participants and Multiple Ascending Dose Study of CNTO 7160 in Participants With Asthma and Participants With Atopic Dermatitis* [Online]. ClinicalTrials.gov. Available: https://clinicaltrials.gov/ct2/show/NCT02345928 [Accessed 16 12 2016].

JOHANSSON, M. E., PHILLIPSON, M., PETERSSON, J., VELCICH, A., HOLM, L. & HANSSON, G. C. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A,* 105**,** 15064-9.

JOHANSSON-LINDBOM, B., SVENSSON, M., PABST, O., PALMQVIST, C., MARQUEZ, G., FORSTER, R. & AGACE, W. W. 2005. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med,* 202**,** 1063-73.

JOLLER, N., LOZANO, E., BURKETT, P. R., PATEL, B., XIAO, S., ZHU, C., XIA, J., TAN, T. G., SEFIK, E., YAJNIK, V., SHARPE, A. H., QUINTANA, F. J., MATHIS, D., BENOIST, C., HAFLER, D. A. & KUCHROO, V. K. 2014. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity,* 40**,** 569-81.

JORDAN, M. S., BOESTEANU, A., REED, A. J., PETRONE, A. L., HOLENBECK, A. E., LERMAN, M. A., NAJI, A. & CATON, A. J. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol,* 2**,** 301-6.

JOSTINS, L., RIPKE, S., WEERSMA, R. K., DUERR, R. H., MCGOVERN, D. P., HUI, K. Y., LEE, J. C., SCHUMM, L. P., SHARMA, Y., ANDERSON, C. A., ESSERS, J., MITROVIC, M., NING, K., CLEYNEN, I., THEATRE, E., SPAIN, S. L., RAYCHAUDHURI, S., GOYETTE, P., WEI, Z., ABRAHAM, C., ACHKAR, J. P., AHMAD, T., AMININEJAD, L., ANANTHAKRISHNAN, A. N., ANDERSEN, V., ANDREWS, J. M., BAIDOO, L., BALSCHUN, T., BAMPTON, P. A., BITTON, A., BOUCHER, G., BRAND, S., BUNING, C., COHAIN, A., CICHON, S., D'AMATO, M., DE JONG, D., DEVANEY, K. L., DUBINSKY, M., EDWARDS, C., ELLINGHAUS, D., FERGUSON, L. R., FRANCHIMONT, D., FRANSEN, K., GEARRY, R., GEORGES, M., GIEGER, C., GLAS, J., HARITUNIANS, T., HART, A., HAWKEY, C., HEDL, M., HU, X., KARLSEN, T. H., KUPCINSKAS, L., KUGATHASAN, S., LATIANO, A., LAUKENS, D., LAWRANCE, I. C., LEES, C. W., LOUIS, E., MAHY, G., MANSFIELD, J., MORGAN, A. R., MOWAT, C., NEWMAN, W., PALMIERI, O., PONSIOEN, C. Y., POTOCNIK, U., PRESCOTT, N. J., REGUEIRO, M., ROTTER, J. I., RUSSELL, R. K., SANDERSON, J. D., SANS, M., SATSANGI, J., SCHREIBER, S., SIMMS, L. A., SVENTORAITYTE, J., TARGAN, S. R., TAYLOR, K. D., TREMELLING, M., VERSPAGET, H. W., DE VOS, M., WIJMENGA, C., WILSON, D. C., WINKELMANN, J., XAVIER, R. J., ZEISSIG, S., ZHANG, B., ZHANG, C. K., ZHAO, H., INTERNATIONAL, I. B. D. G. C., SILVERBERG, M. S.,

ANNESE, V., HAKONARSON, H., BRANT, S. R., RADFORD-SMITH, G., MATHEW, C. G., RIOUX, J. D., et al. 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature,* 491**,** 119-24.

KAIEDA, S., SHIN, K., NIGROVIC, P. A., SEKI, K., LEE, R. T., STEVENS, R. L. & LEE, D. M. 2010. Synovial fibroblasts promote the expression and granule accumulation of tryptase via interleukin-33 and its receptor ST-2 (IL1RL1). *J Biol Chem,* 285**,** 21478-86.

KAKKAR, R., HEI, H., DOBNER, S. & LEE, R. T. 2012. Interleukin 33 as a mechanically responsive cytokine secreted by living cells. *J Biol Chem,* 287**,** 6941-8.

KAMANAKA, M., KIM, S. T., WAN, Y. Y., SUTTERWALA, F. S., LARA-TEJERO, M., GALAN, J. E., HARHAJ, E. & FLAVELL, R. A. 2006. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity,* 25**,** 941-52.

KAPLAN, M. H., HUFFORD, M. M. & OLSON, M. R. 2015. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol,* 15**,** 295-307.

KARIMI, K., INMAN, M. D., BIENENSTOCK, J. & FORSYTHE, P. 2009. Lactobacillus reuteri-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med,* 179**,** 186-93.

KASER, A., LEE, A. H., FRANKE, A., GLICKMAN, J. N., ZEISSIG, S., TILG, H., NIEUWENHUIS, E. E., HIGGINS, D. E., SCHREIBER, S., GLIMCHER, L. H. & BLUMBERG, R. S. 2008. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell,* 134**,** 743-56.

KASER, A., MARTINEZ-NAVES, E. & BLUMBERG, R. S. 2010a. Endoplasmic reticulum stress: implications for inflammatory bowel disease pathogenesis. *Curr Opin Gastroenterol,* 26**,** 318-26.

KASER, A., ZEISSIG, S. & BLUMBERG, R. S. 2010b. Inflammatory bowel disease. *Annu Rev Immunol,* 28**,** 573-621.

KAWAHATA, K., MISAKI, Y., YAMAUCHI, M., TSUNEKAWA, S., SETOGUCHI, K., MIYAZAKI, J. & YAMAMOTO, K. 2002. Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J Immunol,* 168**,** 4399-405.

KAYAMA, H., UEDA, Y., SAWA, Y., JEON, S. G., MA, J. S., OKUMURA, R., KUBO, A., ISHII, M., OKAZAKI, T., MURAKAMI, M., YAMAMOTO, M., YAGITA, H. & TAKEDA, K. 2012. Intestinal CX3C chemokine receptor 1(high) (CX3CR1(high)) myeloid cells prevent T-cell-dependent colitis. *Proc Natl Acad Sci U S A,* 109**,** 5010-5.

KEARLEY, J., BUCKLAND, K. F., MATHIE, S. A. & LLOYD, C. M. 2009. Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med,* 179**,** 772-81.

KENDAL, A. R., CHEN, Y., REGATEIRO, F. S., MA, J., ADAMS, E., COBBOLD, S. P., HORI, S. & WALDMANN, H. 2011. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. *J Exp Med,* 208**,** 2043-53.

KERDILES, Y. M., STONE, E. L., BEISNER, D. R., MCGARGILL, M. A., CH'EN, I. L., STOCKMANN, C., KATAYAMA, C. D. & HEDRICK, S. M. 2010. Foxo transcription factors control regulatory T cell development and function. *Immunity,* 33**,** 890-904.

KHATTRI, R., COX, T., YASAYKO, S. A. & RAMSDELL, F. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol,* 4**,** 337-42.

KIESLER, P., FUSS, I. J. & STROBER, W. 2015. Experimental Models of Inflammatory Bowel Diseases. *Cell Mol Gastroenterol Hepatol,* 1**,** 154-170.

KIM, H. P. & LEONARD, W. J. 2007. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med,* 204**,** 1543-51.

KIM, K. S., HONG, S. W., HAN, D., YI, J., JUNG, J., YANG, B. G., LEE, J. Y., LEE, M. & SURH, C. D. 2016. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science,* 351**,** 858-63.

KITOH, A., ONO, M., NAOE, Y., OHKURA, N., YAMAGUCHI, T., YAGUCHI, H., KITABAYASHI, I., TSUKADA, T., NOMURA, T., MIYACHI, Y., TANIUCHI, I. & SAKAGUCHI, S. 2009. Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3+ regulatory T cells. *Immunity,* 31**,** 609-20.

KLEIN, L., KYEWSKI, B., ALLEN, P. M. & HOGQUIST, K. A. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol,* 14**,** 377-91.

KOBAYASHI, T., OKAMOTO, S., HISAMATSU, T., KAMADA, N., CHINEN, H., SAITO, R., KITAZUME, M. T., NAKAZAWA, A., SUGITA, A., KOGANEI, K., ISOBE, K. & HIBI, T. 2008. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut,* 57**,** 1682-9.

KOBIE, J. J., SHAH, P. R., YANG, L., REBHAHN, J. A., FOWELL, D. J. & MOSMANN, T. R. 2006. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J Immunol,* 177**,** 6780-6.

KOBORI, A., YAGI, Y., IMAEDA, H., BAN, H., BAMBA, S., TSUJIKAWA, T., SAITO, Y., FUJIYAMA, Y. & ANDOH, A. 2010. Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. *J Gastroenterol,* 45**,** 999-1007.

KOCH, M. A., TUCKER-HEARD, G., PERDUE, N. R., KILLEBREW, J. R., URDAHL, K. B. & CAMPBELL, D. J. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol,* 10**,** 595-602.

KOLODIN, D., VAN PANHUYS, N., LI, C., MAGNUSON, A. M., CIPOLLETTA, D., MILLER, C. M., WAGERS, A., GERMAIN, R. N., BENOIST, C. & MATHIS, D. 2015. Antigen- and cytokine-driven accumulation of regulatory T cells in visceral adipose tissue of lean mice. *Cell Metab,* 21**,** 543-57.

KOMATSU, N., MARIOTTI-FERRANDIZ, M. E., WANG, Y., MALISSEN, B., WALDMANN, H. & HORI, S. 2009. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A,* 106**,** 1903-8.

KONDO, Y., YOSHIMOTO, T., YASUDA, K., FUTATSUGI-YUMIKURA, S., MORIMOTO, M., HAYASHI, N., HOSHINO, T., FUJIMOTO, J. & NAKANISHI, K. 2008. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int Immunol,* 20**,** 791-800.

KONTOYIANNIS, D., BOULOUGOURIS, G., MANOLOUKOS, M., ARMAKA, M., APOSTOLAKI, M., PIZARRO, T., KOTLYAROV, A., FORSTER, I., FLAVELL, R.,

GAESTEL, M., TSICHLIS, P., COMINELLI, F. & KOLLIAS, G. 2002. Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. *J Exp Med,* 196**,** 1563-74.

KRAUSGRUBER, T., SCHIERING, C., ADELMANN, K., HARRISON, O. J., CHOMKA, A., PEARSON, C., AHERN, P. P., SHALE, M., OUKKA, M. & POWRIE, F. 2016. T-bet is a key modulator of IL-23-driven pathogenic CD4(+) T cell responses in the intestine. *Nat Commun,* 7**,** 11627.

KRETSCHMER, K., APOSTOLOU, I., HAWIGER, D., KHAZAIE, K., NUSSENZWEIG, M. C. & VON BOEHMER, H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol,* 6**,** 1219-27.

KRIEGEL, M. A., SEFIK, E., HILL, J. A., WU, H. J., BENOIST, C. & MATHIS, D. 2011. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc Natl Acad Sci U S A,* 108**,** 11548-53.

KUCHLER, A. M., POLLHEIMER, J., BALOGH, J., SPONHEIM, J., MANLEY, L., SORENSEN, D. R., DE ANGELIS, P. M., SCOTT, H. & HARALDSEN, G. 2008. Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. *Am J Pathol,* 173**,** 1229-42.

KUHN, R., LOHLER, J., RENNICK, D., RAJEWSKY, K. & MULLER, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell,* 75**,** 263-74.

KULLBERG, M. C., JANKOVIC, D., FENG, C. G., HUE, S., GORELICK, P. L., MCKENZIE, B. S., CUA, D. J., POWRIE, F., CHEEVER, A. W., MALOY, K. J. & SHER, A. 2006. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. *J Exp Med,* 203**,** 2485-94.

KULLBERG, M. C., JANKOVIC, D., GORELICK, P. L., CASPAR, P., LETTERIO, J. J., CHEEVER, A. W. & SHER, A. 2002. Bacteria-triggered CD4(+) T regulatory cells suppress Helicobacter hepaticus-induced colitis. *J Exp Med,* 196**,** 505-15.

KUMAR, S., TZIMAS, M. N., GRISWOLD, D. E. & YOUNG, P. R. 1997. Expression of ST2, an interleukin-1 receptor homologue, is induced by proinflammatory stimuli. *Biochem Biophys Res Commun,* 235**,** 474-8.

KURAHASHI, M., NAKANO, Y., PERI, L. E., TOWNSEND, J. B., WARD, S. M. & SANDERS, K. M. 2013. A novel population of subepithelial platelet-derived growth factor receptor alpha-positive cells in the mouse and human colon. *Am J Physiol Gastrointest Liver Physiol,* 304**,** G823-34.

KUROWSKA-STOLARSKA, M., KEWIN, P., MURPHY, G., RUSSO, R. C., STOLARSKI, B., GARCIA, C. C., KOMAI-KOMA, M., PITMAN, N., LI, Y., NIEDBALA, W., MCKENZIE, A. N., TEIXEIRA, M. M., LIEW, F. Y. & XU, D. 2008. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol,* 181**,** 4780-90.

KUROWSKA-STOLARSKA, M., STOLARSKI, B., KEWIN, P., MURPHY, G., CORRIGAN, C. J., YING, S., PITMAN, N., MIRCHANDANI, A., RANA, B., VAN ROOIJEN, N., SHEPHERD, M., MCSHARRY, C., MCINNES, I. B., XU, D. & LIEW, F. Y. 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol,* 183**,** 6469-77.

KUSWANTO, W., BURZYN, D., PANDURO, M., WANG, K. K., JANG, Y. C., WAGERS, A. J., BENOIST, C. & MATHIS, D. 2016. Poor Repair of Skeletal Muscle in Aging Mice

Reflects a Defect in Local, Interleukin-33-Dependent Accumulation of Regulatory T Cells. *Immunity,* 44**,** 355-67.

LANGRISH, C. L., CHEN, Y., BLUMENSCHEIN, W. M., MATTSON, J., BASHAM, B., SEDGWICK, J. D., MCCLANAHAN, T., KASTELEIN, R. A. & CUA, D. J. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med,* 201**,** 233-40.

LATHROP, S. K., BLOOM, S. M., RAO, S. M., NUTSCH, K., LIO, C. W., SANTACRUZ, N., PETERSON, D. A., STAPPENBECK, T. S. & HSIEH, C. S. 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature,* 478**,** 250-4.

LATIANO, A., PALMIERI, O., PASTORELLI, L., VECCHI, M., PIZARRO, T. T., BOSSA, F., MERLA, G., AUGELLO, B., LATIANO, T., CORRITORE, G., SETTESOLDI, A., VALVANO, M. R., D'INCA, R., STRONATI, L., ANNESE, V. & ANDRIULLI, A. 2013. Associations between genetic polymorphisms in IL-33, IL1R1 and risk for inflammatory bowel disease. *PLoS One,* 8**,** e62144.

LEE, A. Y., CHANG, S. Y., KIM, J. I., CHA, H. R., JANG, M. H., YAMAMOTO, M. & KWEON, M. N. 2008. Dendritic cells in colonic patches and iliac lymph nodes are essential in mucosal IgA induction following intrarectal administration via CCR7 interaction. *Eur J Immunol,* 38**,** 1127-37.

LEE, J., MO, J. H., KATAKURA, K., ALKALAY, I., RUCKER, A. N., LIU, Y. T., LEE, H. K., SHEN, C., COJOCARU, G., SHENOUDA, S., KAGNOFF, M., ECKMANN, L., BEN-NERIAH, Y. & RAZ, E. 2006. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat Cell Biol,* 8**,** 1327-36.

LEE, Y., AWASTHI, A., YOSEF, N., QUINTANA, F. J., XIAO, S., PETERS, A., WU, C., KLEINEWIETFELD, M., KUNDER, S., HAFLER, D. A., SOBEL, R. A., REGEV, A. & KUCHROO, V. K. 2012. Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol,* 13**,** 991-9.

LEFRANCAIS, E., DUVAL, A., MIREY, E., ROGA, S., ESPINOSA, E., CAYROL, C. & GIRARD, J. P. 2014. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc Natl Acad Sci U S A,* 111**,** 15502-7.

LEFRANCAIS, E., ROGA, S., GAUTIER, V., GONZALEZ-DE-PEREDO, A., MONSARRAT, B., GIRARD, J. P. & CAYROL, C. 2012. IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc Natl Acad Sci U S A,* 109**,** 1673-8.

LEPPKES, M., BECKER, C., IVANOV, II, HIRTH, S., WIRTZ, S., NEUFERT, C., POULY, S., MURPHY, A. J., VALENZUELA, D. M., YANCOPOULOS, G. D., BECHER, B., LITTMAN, D. R. & NEURATH, M. F. 2009. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology,* 136**,** 257-67.

LEUNG, B. P., XU, D., CULSHAW, S., MCINNES, I. B. & LIEW, F. Y. 2004. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. *J Immunol,* 173**,** 145-50.

LI, D., GUABIRABA, R., BESNARD, A. G., KOMAI-KOMA, M., JABIR, M. S., ZHANG, L., GRAHAM, G. J., KUROWSKA-STOLARSKA, M., LIEW, F. Y., MCSHARRY, C. & XU, D. 2014a. IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. *J Allergy Clin Immunol,* 134**,** 1422-1432 e11.

LI, X., LIANG, Y., LEBLANC, M., BENNER, C. & ZHENG, Y. 2014b. Function of a Foxp3 cis-element in protecting regulatory T cell identity. *Cell,* 158**,** 734-48.

LIANG, B., WORKMAN, C., LEE, J., CHEW, C., DALE, B. M., COLONNA, L., FLORES, M., LI, N., SCHWEIGHOFFER, E., GREENBERG, S., TYBULEWICZ, V., VIGNALI, D. & CLYNES, R. 2008. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol,* 180**,** 5916-26.

LIEW, F. Y., GIRARD, J. P. & TURNQUIST, H. R. 2016. Interleukin-33 in health and disease. *Nat Rev Immunol,* 16**,** 676-689.

LIEW, F. Y., PITMAN, N. I. & MCINNES, I. B. 2010. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol,* 10**,** 103-10.

LIGHVANI, A. A., FRUCHT, D. M., JANKOVIC, D., YAMANE, H., ALIBERTI, J., HISSONG, B. D., NGUYEN, B. V., GADINA, M., SHER, A., PAUL, W. E. & O'SHEA, J. J. 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A,* 98**,** 15137-42.

LIN, W., HARIBHAI, D., RELLAND, L. M., TRUONG, N., CARLSON, M. R., WILLIAMS, C. B. & CHATILA, T. A. 2007. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol,* 8**,** 359-68.

LINGEL, A., WEISS, T. M., NIEBUHR, M., PAN, B., APPLETON, B. A., WIESMANN, C., BAZAN, J. F. & FAIRBROTHER, W. J. 2009. Structure of IL-33 and its interaction with the ST2 and IL-1RAcP receptors--insight into heterotrimeric IL-1 signaling complexes. *Structure,* 17**,** 1398-410.

LIO, C. W. & HSIEH, C. S. 2008. A two-step process for thymic regulatory T cell development. *Immunity,* 28**,** 100-11.

LISTON, A. & GRAY, D. H. 2014. Homeostatic control of regulatory T cell diversity. *Nat Rev Immunol,* 14**,** 154-65.

LIU, J., SHEN, J. X., HU, J. L., HUANG, W. H. & ZHANG, G. J. 2014. Significance of interleukin-33 and its related cytokines in patients with breast cancers. *Front Immunol,* 5**,** 141.

LIU, J. Z., VAN SOMMEREN, S., HUANG, H., NG, S. C., ALBERTS, R., TAKAHASHI, A., RIPKE, S., LEE, J. C., JOSTINS, L., SHAH, T., ABEDIAN, S., CHEON, J. H., CHO, J., DARYANI, N. E., FRANKE, L., FUYUNO, Y., HART, A., JUYAL, R. C., JUYAL, G., KIM, W. H., MORRIS, A. P., POUSTCHI, H., NEWMAN, W. G., MIDHA, V., ORCHARD, T. R., VAHEDI, H., SOOD, A., SUNG, J. J., MALEKZADEH, R., WESTRA, H. J., YAMAZAKI, K., YANG, S. K., INTERNATIONAL MULTIPLE SCLEROSIS GENETICS, C., INTERNATIONAL, I. B. D. G. C., BARRETT, J. C., FRANKE, A., ALIZADEH, B. Z., PARKES, M., B, K. T., DALY, M. J., KUBO, M., ANDERSON, C. A. & WEERSMA, R. K. 2015. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet,* 47**,** 979-86.

LIU, X., HAMMEL, M., HE, Y., TAINER, J. A., JENG, U. S., ZHANG, L., WANG, S. & WANG, X. 2013. Structural insights into the interaction of IL-33 with its receptors. *Proc Natl Acad Sci U S A,* 110**,** 14918-23.

LIU, X., LI, M., WU, Y., ZHOU, Y., ZENG, L. & HUANG, T. 2009. Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma. *Biochem Biophys Res Commun,* 386**,** 181-5.

LOHNING, M., STROEHMANN, A., COYLE, A. J., GROGAN, J. L., LIN, S., GUTIERREZ-RAMOS, J. C., LEVINSON, D., RADBRUCH, A. & KAMRADT, T. 1998. T1/ST2 is

preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc Natl Acad Sci U S A,* 95**,** 6930-5.

LONG, M., PARK, S. G., STRICKLAND, I., HAYDEN, M. S. & GHOSH, S. 2009. Nuclear factor-kappaB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. *Immunity,* 31**,** 921-31.

LOPETUSO, L. R., CHOWDHRY, S. & PIZARRO, T. T. 2013. Opposing Functions of Classic and Novel IL-1 Family Members in Gut Health and Disease. *Front Immunol,* 4**,** 181.

LORD, J. D. 2015. Promises and paradoxes of regulatory T cells in inflammatory bowel disease. *World J Gastroenterol,* 21**,** 11236-45.

LORENZ, R. G. & NEWBERRY, R. D. 2004. Isolated lymphoid follicles can function as sites for induction of mucosal immune responses. *Ann N Y Acad Sci,* 1029**,** 44-57.

LOUTEN, J., RANKIN, A. L., LI, Y., MURPHY, E. E., BEAUMONT, M., MOON, C., BOURNE, P., MCCLANAHAN, T. K., PFLANZ, S. & DE WAAL MALEFYT, R. 2011. Endogenous IL-33 enhances Th2 cytokine production and T-cell responses during allergic airway inflammation. *Int Immunol,* 23**,** 307-15.

LUCKHEERAM, R. V., ZHOU, R., VERMA, A. D. & XIA, B. 2012. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol,* 2012**,** 925135.

LUSTER, A. D., UNKELESS, J. C. & RAVETCH, J. V. 1985. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature,* 315**,** 672-6.

LUTHI, A. U., CULLEN, S. P., MCNEELA, E. A., DURIEZ, P. J., AFONINA, I. S., SHERIDAN, C., BRUMATTI, G., TAYLOR, R. C., KERSSE, K., VANDENABEELE, P., LAVELLE, E. C. & MARTIN, S. J. 2009. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity,* 31**,** 84-98.

MABBOTT, N. A., DONALDSON, D. S., OHNO, H., WILLIAMS, I. R. & MAHAJAN, A. 2013. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol,* 6**,** 666-77.

MACPHERSON, A. J. & HARRIS, N. L. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol,* 4**,** 478-85.

MACPHERSON, A. J. & UHR, T. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science,* 303**,** 1662-5.

MAGER, L. F., RIETHER, C., SCHURCH, C. M., BANZ, Y., WASMER, M. H., STUBER, R., THEOCHARIDES, A. P., LI, X., XIA, Y., SAITO, H., NAKAE, S., BAERLOCHER, G. M., MANZ, M. G., MCCOY, K. D., MACPHERSON, A. J., OCHSENBEIN, A. F., BEUTLER, B. & KREBS, P. 2015. IL-33 signaling contributes to the pathogenesis of myeloproliferative neoplasms. *J Clin Invest,* 125**,** 2579-91.

MAIZELS, R. M., PEARCE, E. J., ARTIS, D., YAZDANBAKHSH, M. & WYNN, T. A. 2009. Regulation of pathogenesis and immunity in helminth infections. *J Exp Med,* 206**,** 2059-66.

MALHOTRA, D., FLETCHER, A. L. & TURLEY, S. J. 2013. Stromal and hematopoietic cells in secondary lymphoid organs: partners in immunity. *Immunol Rev,* 251**,** 160-76.

MALOY, K. J. & KULLBERG, M. C. 2008. IL-23 and Th17 cytokines in intestinal homeostasis. *Mucosal Immunol,* 1**,** 339-49.

MALOY, K. J. & POWRIE, F. 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature,* 474**,** 298-306.

MALOY, K. J., SALAUN, L., CAHILL, R., DOUGAN, G., SAUNDERS, N. J. & POWRIE, F. 2003. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med,* 197**,** 111-9.

MANEECHOTESUWAN, K., XIN, Y., ITO, K., JAZRAWI, E., LEE, K. Y., USMANI, O. S., BARNES, P. J. & ADCOCK, I. M. 2007. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *J Immunol,* 178**,** 2491-8.

MANEL, N., UNUTMAZ, D. & LITTMAN, D. R. 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol,* 9**,** 641-9.

MANGAN, P. R., HARRINGTON, L. E., O'QUINN, D. B., HELMS, W. S., BULLARD, D. C., ELSON, C. O., HATTON, R. D., WAHL, S. M., SCHOEB, T. R. & WEAVER, C. T. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature,* 441**,** 231-4.

MANKERTZ, J. & SCHULZKE, J. D. 2007. Altered permeability in inflammatory bowel disease: pathophysiology and clinical implications. *Curr Opin Gastroenterol,* 23**,** 379-83.

MANNON, P. J., FUSS, I. J., MAYER, L., ELSON, C. O., SANDBORN, W. J., PRESENT, D., DOLIN, B., GOODMAN, N., GRODEN, C., HORNUNG, R. L., QUEZADO, M., YANG, Z., NEURATH, M. F., SALFELD, J., VELDMAN, G. M., SCHWERTSCHLAG, U., STROBER, W. & ANTI, I. L. C. S. D. S. G. 2004. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med,* 351**,** 2069-79.

MARSON, A., KRETSCHMER, K., FRAMPTON, G. M., JACOBSEN, E. S., POLANSKY, J. K., MACISAAC, K. D., LEVINE, S. S., FRAENKEL, E., VON BOEHMER, H. & YOUNG, R. A. 2007. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature,* 445**,** 931-5.

MARTIN, M. U. 2013. Special aspects of interleukin-33 and the IL-33 receptor complex. *Semin Immunol,* 25**,** 449-57.

MASAHATA, K., UMEMOTO, E., KAYAMA, H., KOTANI, M., NAKAMURA, S., KURAKAWA, T., KIKUTA, J., GOTOH, K., MOTOOKA, D., SATO, S., HIGUCHI, T., BABA, Y., KUROSAKI, T., KINOSHITA, M., SHIMADA, Y., KIMURA, T., OKUMURA, R., TAKEDA, A., TAJIMA, M., YOSHIE, O., FUKUZAWA, M., KIYONO, H., FAGARASAN, S., IIDA, T., ISHII, M. & TAKEDA, K. 2014. Generation of colonic IgA-secreting cells in the caecal patch. *Nat Commun,* 5**,** 3704.

MATTA, B. M., LOTT, J. M., MATHEWS, L. R., LIU, Q., ROSBOROUGH, B. R., BLAZAR, B. R. & TURNQUIST, H. R. 2014. IL-33 is an unconventional Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. *J Immunol,* 193**,** 4010-20.

MATTA, B. M., REICHENBACH, D. K., ZHANG, X., MATHEWS, L., KOEHN, B. H., DWYER, G. K., LOTT, J. M., UHL, F. M., PFEIFER, D., FESER, C. J., SMITH, M. J., LIU, Q., ZEISER, R., BLAZAR, B. R. & TURNQUIST, H. R. 2016. Peri-alloHCT IL-33 administration expands recipient T-regulatory cells that protect mice against acute GVHD. *Blood,* 128**,** 427-39.

MAVROMMATIS, E., FISH, E. N. & PLATANIAS, L. C. 2013. The schlafen family of proteins and their regulation by interferons. *J Interferon Cytokine Res,* 33**,** 206-10.

MAYNARD, C. L., HARRINGTON, L. E., JANOWSKI, K. M., OLIVER, J. R., ZINDL, C. L., RUDENSKY, A. Y. & WEAVER, C. T. 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol,* 8**,** 931-41.

MAYNARD, C. L. & WEAVER, C. T. 2009. Intestinal effector T cells in health and disease. *Immunity,* 31**,** 389-400.

MAYWALD, R. L., DOERNER, S. K., PASTORELLI, L., DE SALVO, C., BENTON, S. M., DAWSON, E. P., LANZA, D. G., BERGER, N. A., MARKOWITZ, S. D., LENZ, H. J., NADEAU, J. H., PIZARRO, T. T. & HEANEY, J. D. 2015. IL-33 activates tumor stroma to promote intestinal polyposis. *Proc Natl Acad Sci U S A,* 112**,** E2487-96.

MAZMANIAN, S. K., LIU, C. H., TZIANABOS, A. O. & KASPER, D. L. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell,* 122**,** 107-18.

MAZMANIAN, S. K., ROUND, J. L. & KASPER, D. L. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature,* 453**,** 620-5.

MCDOLE, J. R., WHEELER, L. W., MCDONALD, K. G., WANG, B., KONJUFCA, V., KNOOP, K. A., NEWBERRY, R. D. & MILLER, M. J. 2012. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature,* 483**,** 345-9.

MCGEACHY, M. J., BAK-JENSEN, K. S., CHEN, Y., TATO, C. M., BLUMENSCHEIN, W., MCCLANAHAN, T. & CUA, D. J. 2007. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol,* 8**,** 1390-7.

MCGEACHY, M. J., CHEN, Y., TATO, C. M., LAURENCE, A., JOYCE-SHAIKH, B., BLUMENSCHEIN, W. M., MCCLANAHAN, T. K., O'SHEA, J. J. & CUA, D. J. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol,* 10**,** 314-24.

MCGEACHY, M. J., STEPHENS, L. A. & ANDERTON, S. M. 2005. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol,* 175**,** 3025-32.

MCHEDLIDZE, T., WALDNER, M., ZOPF, S., WALKER, J., RANKIN, A. L., SCHUCHMANN, M., VOEHRINGER, D., MCKENZIE, A. N., NEURATH, M. F., PFLANZ, S. & WIRTZ, S. 2013. Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity,* 39**,** 357-71.

MCHUGH, R. S., WHITTERS, M. J., PICCIRILLO, C. A., YOUNG, D. A., SHEVACH, E. M., COLLINS, M. & BYRNE, M. C. 2002. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity,* 16**,** 311-23.

MCLAREN, J. E., MICHAEL, D. R., SALTER, R. C., ASHLIN, T. G., CALDER, C. J., MILLER, A. M., LIEW, F. Y. & RAMJI, D. P. 2010. IL-33 reduces macrophage foam cell formation. *J Immunol,* 185**,** 1222-9.

MEDOFF, B. D., SANDALL, B. P., LANDRY, A., NAGAHAMA, K., MIZOGUCHI, A., LUSTER, A. D. & XAVIER, R. J. 2009. Differential requirement for CARMA1 in agonist-selected T-cell development. *Eur J Immunol,* 39**,** 78-84.

MEDRIKOVA, D., SIJMONSMA, T. P., SOWODNIOK, K., RICHARDS, D. M., DELACHER, M., STICHT, C., GRETZ, N., SCHAFMEIER, T., FEUERER, M. & HERZIG, S. 2015. Brown

adipose tissue harbors a distinct sub-population of regulatory T cells. *PLoS One,* 10**,** e0118534.

MESTECKY, J. & RUSSELL, M. W. 2009. Specific antibody activity, glycan heterogeneity and polyreactivity contribute to the protective activity of S-IgA at mucosal surfaces. *Immunol Lett,* 124**,** 57-62.

MEULENBROEKS, C., VAN WEELDEN, H., SCHWARTZ, C., VOEHRINGER, D., REDEGELD, F. A., RUTTEN, V. P., WILLEMSE, T., SIJTS, A. J. & ZAISS, D. M. 2015. Basophil-derived amphiregulin is essential for UVB irradiation-induced immune suppression. *J Invest Dermatol,* 135**,** 222-8.

MICHELUCCI, A., CORDES, T., GHELFI, J., PAILOT, A., REILING, N., GOLDMANN, O., BINZ, T., WEGNER, A., TALLAM, A., RAUSELL, A., BUTTINI, M., LINSTER, C. L., MEDINA, E., BALLING, R. & HILLER, K. 2013. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proc Natl Acad Sci U S A,* 110**,** 7820-5.

MILDNER, M., STORKA, A., LICHTENAUER, M., MLITZ, V., GHANNADAN, M., HOETZENECKER, K., NICKL, S., DOME, B., TSCHACHLER, E. & ANKERSMIT, H. J. 2010. Primary sources and immunological prerequisites for sST2 secretion in humans. *Cardiovasc Res,* 87**,** 769-77.

MILLER, A. M., ASQUITH, D. L., HUEBER, A. J., ANDERSON, L. A., HOLMES, W. M., MCKENZIE, A. N., XU, D., SATTAR, N., MCINNES, I. B. & LIEW, F. Y. 2010. Interleukin-33 induces protective effects in adipose tissue inflammation during obesity in mice. *Circ Res,* 107**,** 650-8.

MILLER, A. M., XU, D., ASQUITH, D. L., DENBY, L., LI, Y., SATTAR, N., BAKER, A. H., MCINNES, I. B. & LIEW, F. Y. 2008. IL-33 reduces the development of atherosclerosis. *J Exp Med,* 205**,** 339-46.

MISHRA, A., HOGAN, S. P., LEE, J. J., FOSTER, P. S. & ROTHENBERG, M. E. 1999. Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. *J Clin Invest,* 103**,** 1719-27.

MIYAO, T., FLOESS, S., SETOGUCHI, R., LUCHE, H., FEHLING, H. J., WALDMANN, H., HUEHN, J. & HORI, S. 2012. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity,* 36**,** 262-75.

MIZOGUCHI, A., MIZOGUCHI, E., CHIBA, C. & BHAN, A. K. 1996. Role of appendix in the development of inflammatory bowel disease in TCR-alpha mutant mice. *J Exp Med,* 184**,** 707-15.

MOFFATT, M. F., GUT, I. G., DEMENAIS, F., STRACHAN, D. P., BOUZIGON, E., HEATH, S., VON MUTIUS, E., FARRALL, M., LATHROP, M., COOKSON, W. O. & CONSORTIUM, G. 2010. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med,* 363**,** 1211-21.

MOLINERO, L. L., YANG, J., GAJEWSKI, T., ABRAHAM, C., FARRAR, M. A. & ALEGRE, M. L. 2009. CARMA1 controls an early checkpoint in the thymic development of FoxP3+ regulatory T cells. *J Immunol,* 182**,** 6736-43.

MOLOFSKY, A. B., VAN GOOL, F., LIANG, H. E., VAN DYKEN, S. J., NUSSBAUM, J. C., LEE, J., BLUESTONE, J. A. & LOCKSLEY, R. M. 2015. Interleukin-33 and Interferon-gamma Counter-Regulate Group 2 Innate Lymphoid Cell Activation during Immune Perturbation. *Immunity,* 43**,** 161-74.

MOMBAERTS, P., IACOMINI, J., JOHNSON, R. S., HERRUP, K., TONEGAWA, S. & PAPAIOANNOU, V. E. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell,* 68**,** 869-77.

MONDOT, S., KANG, S., FURET, J. P., AGUIRRE DE CARCER, D., MCSWEENEY, C., MORRISON, M., MARTEAU, P., DORE, J. & LECLERC, M. 2011. Highlighting new phylogenetic specificities of Crohn's disease microbiota. *Inflamm Bowel Dis,* 17**,** 185-92.

MONTICELLI, L. A., OSBORNE, L. C., NOTI, M., TRAN, S. V., ZAISS, D. M. & ARTIS, D. 2015. IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin-EGFR interactions. *Proc Natl Acad Sci U S A,* 112**,** 10762-7.

MONTICELLI, L. A., SONNENBERG, G. F., ABT, M. C., ALENGHAT, T., ZIEGLER, C. G., DOERING, T. A., ANGELOSANTO, J. M., LAIDLAW, B. J., YANG, C. Y., SATHALIYAWALA, T., KUBOTA, M., TURNER, D., DIAMOND, J. M., GOLDRATH, A. W., FARBER, D. L., COLLMAN, R. G., WHERRY, E. J. & ARTIS, D. 2011. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol,* 12**,** 1045-54.

MOORE, K. W., DE WAAL MALEFYT, R., COFFMAN, R. L. & O'GARRA, A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol,* 19**,** 683-765.

MORA, J. R., BONO, M. R., MANJUNATH, N., WENINGER, W., CAVANAGH, L. L., ROSEMBLATT, M. & VON ANDRIAN, U. H. 2003. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature,* 424**,** 88-93.

MORITA, H., ARAE, K., UNNO, H., MIYAUCHI, K., TOYAMA, S., NAMBU, A., OBOKI, K., OHNO, T., MOTOMURA, K., MATSUDA, A., YAMAGUCHI, S., NARUSHIMA, S., KAJIWARA, N., IIKURA, M., SUTO, H., MCKENZIE, A. N., TAKAHASHI, T., KARASUYAMA, H., OKUMURA, K., AZUMA, M., MORO, K., AKDIS, C. A., GALLI, S. J., KOYASU, S., KUBO, M., SUDO, K., SAITO, H., MATSUMOTO, K. & NAKAE, S. 2015. An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity,* 43**,** 175-86.

MORO, K., YAMADA, T., TANABE, M., TAKEUCHI, T., IKAWA, T., KAWAMOTO, H., FURUSAWA, J., OHTANI, M., FUJII, H. & KOYASU, S. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature,* 463**,** 540-4.

MORRISSEY, P. J., CHARRIER, K., BRADDY, S., LIGGITT, D. & WATSON, J. D. 1993. CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. *J Exp Med,* 178**,** 237-44.

MOSMANN, T. R., CHERWINSKI, H., BOND, M. W., GIEDLIN, M. A. & COFFMAN, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol,* 136**,** 2348-57.

MOTTET, C., UHLIG, H. H. & POWRIE, F. 2003. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *J Immunol,* 170**,** 3939-43.

MOUSSION, C., ORTEGA, N. & GIRARD, J. P. 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One,* 3**,** e3331.

MOW, W. S., VASILIAUSKAS, E. A., LIN, Y. C., FLESHNER, P. R., PAPADAKIS, K. A., TAYLOR, K. D., LANDERS, C. J., ABREU-MARTIN, M. T., ROTTER, J. I., YANG, H. & TARGAN, S. R. 2004. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. *Gastroenterology,* 126**,** 414-24.

MOWAT, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol,* 3**,** 331-41.

MOWAT, A. M. & AGACE, W. W. 2014. Regional specialization within the intestinal immune system. *Nat Rev Immunol,* 14**,** 667-85.

MUNIZ, L. R., KNOSP, C. & YERETSSIAN, G. 2012. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Front Immunol,* 3**,** 310.

MURAI, M., TUROVSKAYA, O., KIM, G., MADAN, R., KARP, C. L., CHEROUTRE, H. & KRONENBERG, M. 2009. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol,* 10**,** 1178-84.

MURPHY, K. M., OUYANG, W., FARRAR, J. D., YANG, J., RANGANATH, S., ASNAGLI, H., AFKARIAN, M. & MURPHY, T. L. 2000. Signaling and transcription in T helper development. *Annu Rev Immunol,* 18**,** 451-94.

MUZIO, M., NI, J., FENG, P. & DIXIT, V. M. 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science,* 278**,** 1612-5.

NAKANISHI, W., YAMAGUCHI, S., MATSUDA, A., SUZUKAWA, M., SHIBUI, A., NAMBU, A., KONDO, K., SUTO, H., SAITO, H., MATSUMOTO, K., YAMASOBA, T. & NAKAE, S. 2013. IL-33, but not IL-25, is crucial for the development of house dust mite antigen-induced allergic rhinitis. *PLoS One,* 8**,** e78099.

NARUSHIMA, S., SUGIURA, Y., OSHIMA, K., ATARASHI, K., HATTORI, M., SUEMATSU, M. & HONDA, K. 2014. Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia. *Gut Microbes,* 5**,** 333-9.

NEILL, D. R., WONG, S. H., BELLOSI, A., FLYNN, R. J., DALY, M., LANGFORD, T. K., BUCKS, C., KANE, C. M., FALLON, P. G., PANNELL, R., JOLIN, H. E. & MCKENZIE, A. N. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature,* 464**,** 1367-70.

NELSON, M. P., CHRISTMANN, B. S., WERNER, J. L., METZ, A. E., TREVOR, J. L., LOWELL, C. A. & STEELE, C. 2011. IL-33 and M2a alveolar macrophages promote lung defense against the atypical fungal pathogen Pneumocystis murina. *J Immunol,* 186**,** 2372-81.

NEURATH, M. F., WEIGMANN, B., FINOTTO, S., GLICKMAN, J., NIEUWENHUIS, E., IIJIMA, H., MIZOGUCHI, A., MIZOGUCHI, E., MUDTER, J., GALLE, P. R., BHAN, A., AUTSCHBACH, F., SULLIVAN, B. M., SZABO, S. J., GLIMCHER, L. H. & BLUMBERG, R. S. 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med,* 195**,** 1129-43.

NEUTRA, M. R., MANTIS, N. J. & KRAEHENBUHL, J. P. 2001. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol,* 2**,** 1004-9.

NICKLIN, M. J., BARTON, J. L., NGUYEN, M., FITZGERALD, M. G., DUFF, G. W. & KORNMAN, K. 2002. A sequence-based map of the nine genes of the human interleukin-1 cluster. *Genomics,* 79**,** 718-25.

NIESS, J. H., BRAND, S., GU, X., LANDSMAN, L., JUNG, S., MCCORMICK, B. A., VYAS, J. M., BOES, M., PLOEGH, H. L., FOX, J. G., LITTMAN, D. R. & REINECKER, H. C. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science,* 307**,** 254-8.

NILE, C. J., BARKSBY, E., JITPRASERTWONG, P., PRESHAW, P. M. & TAYLOR, J. J. 2010. Expression and regulation of interleukin-33 in human monocytes. *Immunology,* 130**,** 172-80.

O'NEILL, L. A. & BOWIE, A. G. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol,* 7**,** 353-64.

OBOKI, K., OHNO, T., KAJIWARA, N., ARAE, K., MORITA, H., ISHII, A., NAMBU, A., ABE, T., KIYONARI, H., MATSUMOTO, K., SUDO, K., OKUMURA, K., SAITO, H. & NAKAE, S. 2010. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A,* 107**,** 18581-6.

OHNMACHT, C., PARK, J. H., CORDING, S., WING, J. B., ATARASHI, K., OBATA, Y., GABORIAU-ROUTHIAU, V., MARQUES, R., DULAUROY, S., FEDOSEEVA, M., BUSSLINGER, M., CERF-BENSUSSAN, N., BONECA, I. G., VOEHRINGER, D., HASE, K., HONDA, K., SAKAGUCHI, S. & EBERL, G. 2015. MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORgammat(+) T cells. *Science,* 349**,** 989-93.

OHNO, T., OBOKI, K., KAJIWARA, N., MORII, E., AOZASA, K., FLAVELL, R. A., OKUMURA, K., SAITO, H. & NAKAE, S. 2009. Caspase-1, caspase-8, and calpain are dispensable for IL-33 release by macrophages. *J Immunol,* 183**,** 7890-7.

OLDENHOVE, G., BOULADOUX, N., WOHLFERT, E. A., HALL, J. A., CHOU, D., DOS SANTOS, L., O'BRIEN, S., BLANK, R., LAMB, E., NATARAJAN, S., KASTENMAYER, R., HUNTER, C., GRIGG, M. E. & BELKAID, Y. 2009. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity,* 31**,** 772-86.

ONISHI, Y., FEHERVARI, Z., YAMAGUCHI, T. & SAKAGUCHI, S. 2008. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A,* 105**,** 10113-8.

OSHIKAWA, K., KUROIWA, K., TAGO, K., IWAHANA, H., YANAGISAWA, K., OHNO, S., TOMINAGA, S. I. & SUGIYAMA, Y. 2001. Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *Am J Respir Crit Care Med,* 164**,** 277-81.

OTTE, J. M., ROSENBERG, I. M. & PODOLSKY, D. K. 2003. Intestinal myofibroblasts in innate immune responses of the intestine. *Gastroenterology,* 124**,** 1866-78.

OUYANG, W., BECKETT, O., MA, Q., PAIK, J. H., DEPINHO, R. A. & LI, M. O. 2010. Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. *Nat Immunol,* 11**,** 618-27.

OWEN, R. L., PIAZZA, A. J. & ERMAK, T. H. 1991. Ultrastructural and cytoarchitectural features of lymphoreticular organs in the colon and rectum of adult BALB/c mice. *Am J Anat,* 190**,** 10-8.

OWENS, B. M. & SIMMONS, A. 2013. Intestinal stromal cells in mucosal immunity and homeostasis. *Mucosal Immunol,* 6**,** 224-34.

OWENS, B. M., STEEVELS, T. A., DUDEK, M., WALCOTT, D., SUN, M. Y., MAYER, A., ALLAN, P. & SIMMONS, A. 2013. CD90(+) Stromal Cells are Non-Professional Innate Immune Effectors of the Human Colonic Mucosa. *Front Immunol,* 4**,** 307.

PABST, O., HERBRAND, H., FRIEDRICHSEN, M., VELAGA, S., DORSCH, M., BERHARDT, G., WORBS, T., MACPHERSON, A. J. & FORSTER, R. 2006. Adaptation of solitary intestinal lymphoid tissue in response to microbiota and chemokine receptor CCR7 signaling. *J Immunol,* 177**,** 6824-32.

PABST, O., HERBRAND, H., WORBS, T., FRIEDRICHSEN, M., YAN, S., HOFFMANN, M. W., KORNER, H., BERNHARDT, G., PABST, R. & FORSTER, R. 2005. Cryptopatches and isolated lymphoid follicles: dynamic lymphoid tissues dispensable for the generation of intraepithelial lymphocytes. *Eur J Immunol,* 35**,** 98-107.

PALM, N. W., DE ZOETE, M. R., CULLEN, T. W., BARRY, N. A., STEFANOWSKI, J., HAO, L., DEGNAN, P. H., HU, J., PETER, I., ZHANG, W., RUGGIERO, E., CHO, J. H., GOODMAN, A. L. & FLAVELL, R. A. 2014. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell,* 158**,** 1000-10.

PALMER, G., TALABOT-AYER, D., LAMACCHIA, C., TOY, D., SEEMAYER, C. A., VIATTE, S., FINCKH, A., SMITH, D. E. & GABAY, C. 2009. Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis. *Arthritis Rheum,* 60**,** 738-49.

PALOMO, J., DIETRICH, D., MARTIN, P., PALMER, G. & GABAY, C. 2015. The interleukin (IL)-1 cytokine family--Balance between agonists and antagonists in inflammatory diseases. *Cytokine,* 76**,** 25-37.

PANDIYAN, P., ZHENG, L., ISHIHARA, S., REED, J. & LENARDO, M. J. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol,* 8**,** 1353-62.

PANDURO, M., BENOIST, C. & MATHIS, D. 2016. Tissue Tregs. *Annu Rev Immunol,* 34**,** 609-33.

PANG, G., COUCH, L., BATEY, R., CLANCY, R. & CRIPPS, A. 1994. GM-CSF, IL-1 alpha, IL-1 beta, IL-6, IL-8, IL-10, ICAM-1 and VCAM-1 gene expression and cytokine production in human duodenal fibroblasts stimulated with lipopolysaccharide, IL-1 alpha and TNF-alpha. *Clin Exp Immunol,* 96**,** 437-43.

PASCUAL-FIGAL, D. A., LAX, A., PEREZ-MARTINEZ, M. T., DEL CARMEN ASENSIO-LOPEZ, M., SANCHEZ-MAS, J. & NETWORK, G. 2016. Clinical relevance of sST2 in cardiac diseases. *Clin Chem Lab Med,* 54**,** 29-35.

PASTORELLI, L., GARG, R. R., HOANG, S. B., SPINA, L., MATTIOLI, B., SCARPA, M., FIOCCHI, C., VECCHI, M. & PIZARRO, T. T. 2010. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proc Natl Acad Sci U S A,* 107**,** 8017-22.

PAUL, W. E. & ZHU, J. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol,* 10**,** 225-35.

PEARSON, C., THORNTON, E. E., MCKENZIE, B., SCHAUPP, A. L., HUSKENS, N., GRISERI, T., WEST, N., TUNG, S., SEDDON, B. P., UHLIG, H. H. & POWRIE, F. 2016. ILC3 GM-CSF production and mobilisation orchestrate acute intestinal inflammation. *Elife,* 5**,** e10066.

PECARIC-PETKOVIC, T., DIDICHENKO, S. A., KAEMPFER, S., SPIEGL, N. & DAHINDEN, C. A. 2009. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood,* 113**,** 1526-34.

PEDUTO, L., DULAUROY, S., LOCHNER, M., SPATH, G. F., MORALES, M. A., CUMANO, A. & EBERL, G. 2009. Inflammation recapitulates the ontogeny of lymphoid stromal cells. *J Immunol,* 182**,** 5789-99.

PEYRIN-BIROULET, L. 2010. Anti-TNF therapy in inflammatory bowel diseases: a huge review. *Minerva Gastroenterol Dietol,* 56**,** 233-43.

PICHERY, M., MIREY, E., MERCIER, P., LEFRANCAIS, E., DUJARDIN, A., ORTEGA, N. & GIRARD, J. P. 2012. Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: in situ analysis using a novel Il-33-LacZ gene trap reporter strain. *J Immunol,* 188**,** 3488-95.

PIEHLER, D., GRAHNERT, A., ESCHKE, M., RICHTER, T., KOHLER, G., STENZEL, W. & ALBER, G. 2013. T1/ST2 promotes T helper 2 cell activation and polyfunctionality in bronchopulmonary mycosis. *Mucosal Immunol,* 6**,** 405-14.

PINCHUK, I. V., BESWICK, E. J., SAADA, J. I., BOYA, G., SCHMITT, D., RAJU, G. S., BRENMOEHL, J., ROGLER, G., REYES, V. E. & POWELL, D. W. 2011. Human colonic myofibroblasts promote expansion of CD4+ CD25high Foxp3+ regulatory T cells. *Gastroenterology,* 140**,** 2019-30.

POLUMURI, S. K., JAYAKAR, G. G., SHIREY, K. A., ROBERTS, Z. J., PERKINS, D. J., PITHA, P. M. & VOGEL, S. N. 2012. Transcriptional regulation of murine IL-33 by TLR and non-TLR agonists. *J Immunol,* 189**,** 50-60.

POWELL, D. W., PINCHUK, I. V., SAADA, J. I., CHEN, X. & MIFFLIN, R. C. 2011. Mesenchymal cells of the intestinal lamina propria. *Annu Rev Physiol,* 73**,** 213-37.

POWRIE, F., CORREA-OLIVEIRA, R., MAUZE, S. & COFFMAN, R. L. 1994a. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med,* 179**,** 589-600.

POWRIE, F., LEACH, M. W., MAUZE, S., CADDLE, L. B. & COFFMAN, R. L. 1993. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int Immunol,* 5**,** 1461-71.

POWRIE, F., LEACH, M. W., MAUZE, S., MENON, S., CADDLE, L. B. & COFFMAN, R. L. 1994b. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity,* 1**,** 553-62.

POWRIE, F. & MASON, D. 1990. OX-22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22low subset. *J Exp Med,* 172**,** 1701-8.

PREFONTAINE, D., LAJOIE-KADOCH, S., FOLEY, S., AUDUSSEAU, S., OLIVENSTEIN, R., HALAYKO, A. J., LEMIERE, C., MARTIN, J. G. & HAMID, Q. 2009. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol,* 183**,** 5094-103.

PRICE, A. E., LIANG, H. E., SULLIVAN, B. M., REINHARDT, R. L., EISLEY, C. J., ERLE, D. J. & LOCKSLEY, R. M. 2010. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A,* 107**,** 11489-94.

PUSHPARAJ, P. N., LI, D., KOMAI-KOMA, M., GUABIRABA, R., ALEXANDER, J., MCSHARRY, C. & XU, D. 2013. Interleukin-33 exacerbates acute colitis via interleukin-4 in mice. *Immunology,* 140**,** 70-7.

RAK, G. D., OSBORNE, L. C., SIRACUSA, M. C., KIM, B. S., WANG, K., BAYAT, A., ARTIS, D. & VOLK, S. W. 2016. IL-33-Dependent Group 2 Innate Lymphoid Cells Promote Cutaneous Wound Healing. *J Invest Dermatol,* 136**,** 487-96.

RAKOFF-NAHOUM, S., PAGLINO, J., ESLAMI-VARZANEH, F., EDBERG, S. & MEDZHITOV, R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell,* 118**,** 229-41.

RAMSAY, D. B., STEPHEN, S., BORUM, M., VOLTAGGIO, L. & DOMAN, D. B. 2010. Mast cells in gastrointestinal disease. *Gastroenterol Hepatol (N Y),* 6**,** 772-7.

RANK, M. A., KOBAYASHI, T., KOZAKI, H., BARTEMES, K. R., SQUILLACE, D. L. & KITA, H. 2009. IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin Immunol,* 123**,** 1047-54.

READ, S., GREENWALD, R., IZCUE, A., ROBINSON, N., MANDELBROT, D., FRANCISCO, L., SHARPE, A. H. & POWRIE, F. 2006. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. *J Immunol,* 177**,** 4376-83.

REICHENBACH, D. K., SCHWARZE, V., MATTA, B. M., TKACHEV, V., LIEBERKNECHT, E., LIU, Q., KOEHN, B. H., PFEIFER, D., TAYLOR, P. A., PRINZ, G., DIERBACH, H., STICKEL, N., BECK, Y., WARNCKE, M., JUNT, T., SCHMITT-GRAEFF, A., NAKAE, S., FOLLO, M., WERTHEIMER, T., SCHWAB, L., DEVLIN, J., WATKINS, S. C., DUYSTER, J., FERRARA, J. L., TURNQUIST, H. R., ZEISER, R. & BLAZAR, B. R. 2015. The IL-33/ST2 axis augments effector T-cell responses during acute GVHD. *Blood,* 125**,** 3183-92.

RESCIGNO, M., URBANO, M., VALZASINA, B., FRANCOLINI, M., ROTTA, G., BONASIO, R., GRANUCCI, F., KRAEHENBUHL, J. P. & RICCIARDI-CASTAGNOLI, P. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol,* 2**,** 361-7.

RIMOLDI, M., CHIEPPA, M., SALUCCI, V., AVOGADRI, F., SONZOGNI, A., SAMPIETRO, G. M., NESPOLI, A., VIALE, G., ALLAVENA, P. & RESCIGNO, M. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol,* 6**,** 507-14.

RITCHIE, M. E., PHIPSON, B., WU, D., HU, Y., LAW, C. W., SHI, W. & SMYTH, G. K. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res,* 43**,** e47.

ROOKS, M. G. & GARRETT, W. S. 2016. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol,* 16**,** 341-52.

ROULIS, M., ARMAKA, M., MANOLOUKOS, M., APOSTOLAKI, M. & KOLLIAS, G. 2011. Intestinal epithelial cells as producers but not targets of chronic TNF suffice to cause murine Crohn-like pathology. *Proc Natl Acad Sci U S A,* 108**,** 5396-401.

ROUND, J. L., LEE, S. M., LI, J., TRAN, G., JABRI, B., CHATILA, T. A. & MAZMANIAN, S. K. 2011. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science,* 332**,** 974-7.

ROUND, J. L. & MAZMANIAN, S. K. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A,* 107**,** 12204-9.

ROUSSEL, L., ERARD, M., CAYROL, C. & GIRARD, J. P. 2008. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. *EMBO Rep,* 9**,** 1006-12.

ROVEDATTI, L., KUDO, T., BIANCHERI, P., SARRA, M., KNOWLES, C. H., RAMPTON, D. S., CORAZZA, G. R., MONTELEONE, G., DI SABATINO, A. & MACDONALD, T. T. 2009. Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. *Gut,* 58**,** 1629-36.

ROY, A., GANESH, G., SIPPOLA, H., BOLIN, S., SAWESI, O., DAGALV, A., SCHLENNER, S. M., FEYERABEND, T., RODEWALD, H. R., KJELLEN, L., HELLMAN, L. & ABRINK, M. 2014. Mast cell chymase degrades the alarmins heat shock protein 70, biglycan, HMGB1, and interleukin-33 (IL-33) and limits danger-induced inflammation. *J Biol Chem,* 289**,** 237-50.

RUBTSOV, Y. P., RASMUSSEN, J. P., CHI, E. Y., FONTENOT, J., CASTELLI, L., YE, X., TREUTING, P., SIEWE, L., ROERS, A., HENDERSON, W. R., JR., MULLER, W. & RUDENSKY, A. Y. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity,* 28**,** 546-58.

RUDRA, D., DEROOS, P., CHAUDHRY, A., NIEC, R. E., ARVEY, A., SAMSTEIN, R. M., LESLIE, C., SHAFFER, S. A., GOODLETT, D. R. & RUDENSKY, A. Y. 2012. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol,* 13**,** 1010-9.

RUDRA, D., EGAWA, T., CHONG, M. M., TREUTING, P., LITTMAN, D. R. & RUDENSKY, A. Y. 2009. Runx-CBFbeta complexes control expression of the transcription factor Foxp3 in regulatory T cells. *Nat Immunol,* 10**,** 1170-7.

SAADA, J. I., PINCHUK, I. V., BARRERA, C. A., ADEGBOYEGA, P. A., SUAREZ, G., MIFFLIN, R. C., DI MARI, J. F., REYES, V. E. & POWELL, D. W. 2006. Subepithelial myofibroblasts are novel nonprofessional APCs in the human colonic mucosa. *J Immunol,* 177**,** 5968-79.

SAGLANI, S., LUI, S., ULLMANN, N., CAMPBELL, G. A., SHERBURN, R. T., MATHIE, S. A., DENNEY, L., BOSSLEY, C. J., OATES, T., WALKER, S. A., BUSH, A. & LLOYD, C. M. 2013. IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma. *J Allergy Clin Immunol,* 132**,** 676-685 e13.

SAKAGUCHI, S., SAKAGUCHI, N., ASANO, M., ITOH, M. & TODA, M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol,* 155**,** 1151-64.

SAKAGUCHI, S., TAKAHASHI, T. & NISHIZUKA, Y. 1982. Study on cellular events in postthymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. *J Exp Med,* 156**,** 1565-76.

SALAS, A. & PANES, J. 2015. IBD. Regulatory T cells for treatment of Crohn's disease. *Nat Rev Gastroenterol Hepatol,* 12**,** 315-6.

SALIMI, M., BARLOW, J. L., SAUNDERS, S. P., XUE, L., GUTOWSKA-OWSIAK, D., WANG, X., HUANG, L. C., JOHNSON, D., SCANLON, S. T., MCKENZIE, A. N., FALLON, P. G. & OGG, G. S. 2013. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med,* 210**,** 2939-50.

SANADA, S., HAKUNO, D., HIGGINS, L. J., SCHREITER, E. R., MCKENZIE, A. N. & LEE, R. T. 2007. IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. *J Clin Invest,* 117**,** 1538-49.

SANDBORN, W. J., GASINK, C., GAO, L. L., BLANK, M. A., JOHANNS, J., GUZZO, C., SANDS, B. E., HANAUER, S. B., TARGAN, S., RUTGEERTS, P., GHOSH, S., DE

VILLIERS, W. J., PANACCIONE, R., GREENBERG, G., SCHREIBER, S., LICHTIGER, S., FEAGAN, B. G. & GROUP, C. S. 2012. Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *N Engl J Med,* 367**,** 1519-28.

SATOH-TAKAYAMA, N., DUMOUTIER, L., LESJEAN-POTTIER, S., RIBEIRO, V. S., MANDELBOIM, O., RENAULD, J. C., VOSSHENRICH, C. A. & DI SANTO, J. P. 2009. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. *J Immunol,* 183**,** 6579-87.

SATOH-TAKAYAMA, N., VOSSHENRICH, C. A., LESJEAN-POTTIER, S., SAWA, S., LOCHNER, M., RATTIS, F., MENTION, J. J., THIAM, K., CERF-BENSUSSAN, N., MANDELBOIM, O., EBERL, G. & DI SANTO, J. P. 2008. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity,* 29**,** 958-70.

SAUER, S., BRUNO, L., HERTWECK, A., FINLAY, D., LELEU, M., SPIVAKOV, M., KNIGHT, Z. A., COBB, B. S., CANTRELL, D., O'CONNOR, E., SHOKAT, K. M., FISHER, A. G. & MERKENSCHLAGER, M. 2008. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A,* 105**,** 7797-802.

SAVINKO, T., MATIKAINEN, S., SAARIALHO-KERE, U., LEHTO, M., WANG, G., LEHTIMAKI, S., KARISOLA, P., REUNALA, T., WOLFF, H., LAUERMA, A. & ALENIUS, H. 2012. IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. *J Invest Dermatol,* 132**,** 1392-400.

SCHATZ, D. G. & JI, Y. 2011. Recombination centres and the orchestration of V(D)J recombination. *Nat Rev Immunol,* 11**,** 251-63.

SCHIERING, C., KRAUSGRUBER, T., CHOMKA, A., FRÖHLICH, A., ADELMANN, K., WOHLFERT, E. A., POTT, J., GRISERI, T., BOLLRATH, J., HEGAZY, A. N., HARRISON, O., OWENS, B. M. J., LÖHNING, M., BELKAID, Y., FALLON, P. G. & POWRIE, F. 2014. The Alarmin IL-33 Promotes Regulatory T Cell Function in the Intestine *In press*.

SCHMIDT-SUPPRIAN, M., COURTOIS, G., TIAN, J., COYLE, A. J., ISRAEL, A., RAJEWSKY, K. & PASPARAKIS, M. 2003. Mature T cells depend on signaling through the IKK complex. *Immunity,* 19**,** 377-89.

SCHMIDT-SUPPRIAN, M., TIAN, J., GRANT, E. P., PASPARAKIS, M., MAEHR, R., OVAA, H., PLOEGH, H. L., COYLE, A. J. & RAJEWSKY, K. 2004. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. *Proc Natl Acad Sci U S A,* 101**,** 4566-71.

SCHMITZ, J., OWYANG, A., OLDHAM, E., SONG, Y., MURPHY, E., MCCLANAHAN, T. K., ZURAWSKI, G., MOSHREFI, M., QIN, J., LI, X., GORMAN, D. M., BAZAN, J. F. & KASTELEIN, R. A. 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity,* 23**,** 479-90.

SCHULZ, E. G., MARIANI, L., RADBRUCH, A. & HOFER, T. 2009a. Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity,* 30**,** 673-83.

SCHULZ, O., JAENSSON, E., PERSSON, E. K., LIU, X., WORBS, T., AGACE, W. W. & PABST, O. 2009b. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med,* 206**,** 3101-14.

SCZESNAK, A., SEGATA, N., QIN, X., GEVERS, D., PETROSINO, J. F., HUTTENHOWER, C., LITTMAN, D. R. & IVANOV, II 2011. The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. *Cell Host Microbe,* 10**,** 260-72.

SEDHOM, M. A., PICHERY, M., MURDOCH, J. R., FOLIGNE, B., ORTEGA, N., NORMAND, S., MERTZ, K., SANMUGALINGAM, D., BRAULT, L., GRANDJEAN, T., LEFRANCAIS, E., FALLON, P. G., QUESNIAUX, V., PEYRIN-BIROULET, L., CATHOMAS, G., JUNT, T., CHAMAILLARD, M., GIRARD, J. P. & RYFFEL, B. 2013. Neutralisation of the interleukin-33/ST2 pathway ameliorates experimental colitis through enhancement of mucosal healing in mice. *Gut,* 62**,** 1714-23.

SEFIK, E., GEVA-ZATORSKY, N., OH, S., KONNIKOVA, L., ZEMMOUR, D., MCGUIRE, A. M., BURZYN, D., ORTIZ-LOPEZ, A., LOBERA, M., YANG, J., GHOSH, S., EARL, A., SNAPPER, S. B., JUPP, R., KASPER, D., MATHIS, D. & BENOIST, C. 2015. MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. *Science,* 349**,** 993-7.

SEIDELIN, J. B., BJERRUM, J. T., COSKUN, M., WIDJAYA, B., VAINER, B. & NIELSEN, O. H. 2010. IL-33 is upregulated in colonocytes of ulcerative colitis. *Immunol Lett,* 128**,** 80-5.

SELLON, R. K., TONKONOGY, S., SCHULTZ, M., DIELEMAN, L. A., GRENTHER, W., BALISH, E., RENNICK, D. M. & SARTOR, R. B. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun,* 66**,** 5224-31.

SENDER, R., FUCHS, S. & MILO, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol,* 14**,** e1002533.

SENN, K. A., MCCOY, K. D., MALOY, K. J., STARK, G., FROHLI, E., RULICKE, T. & KLEMENZ, R. 2000. T1-deficient and T1-Fc-transgenic mice develop a normal protective Th2-type immune response following infection with Nippostrongylus brasiliensis. *Eur J Immunol,* 30**,** 1929-38.

SEONG, S. Y. & MATZINGER, P. 2004. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol,* 4**,** 469-78.

SESTI-COSTA, R., SILVA, G. K., PROENCA-MODENA, J. L., CARLOS, D., SILVA, M. L., ALVES-FILHO, J. C., ARRUDA, E., LIEW, F. Y. & SILVA, J. S. 2013. The IL-33/ST2 pathway controls coxsackievirus B5-induced experimental pancreatitis. *J Immunol,* 191**,** 283-92.

SHAO, D., PERROS, F., CARAMORI, G., MENG, C., DORMULLER, P., CHOU, P. C., CHURCH, C., PAPI, A., CASOLARI, P., WELSH, D., PEACOCK, A., HUMBERT, M., ADCOCK, I. M. & WORT, S. J. 2014. Nuclear IL-33 regulates soluble ST2 receptor and IL-6 expression in primary human arterial endothelial cells and is decreased in idiopathic pulmonary arterial hypertension. *Biochem Biophys Res Commun,* 451**,** 8-14.

SHAO, J. & SHENG, H. 2010. Amphiregulin promotes intestinal epithelial regeneration: roles of intestinal subepithelial myofibroblasts. *Endocrinology,* 151**,** 3728-37.

SHAW, M. H., KAMADA, N., WARNER, N., KIM, Y. G. & NUNEZ, G. 2011. The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation. *Trends Immunol,* 32**,** 73-9.

SHEVACH, E. M. 2000. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol,* 18**,** 423-49.

SHEVACH, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity,* 30**,** 636-45.

SHIMIZU, J., YAMAZAKI, S., TAKAHASHI, T., ISHIDA, Y. & SAKAGUCHI, S. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol,* 3**,** 135-42.

SHIMPO, M., MORROW, D. A., WEINBERG, E. O., SABATINE, M. S., MURPHY, S. A., ANTMAN, E. M. & LEE, R. T. 2004. Serum levels of the interleukin-1 receptor family member ST2 predict mortality and clinical outcome in acute myocardial infarction. *Circulation,* 109**,** 2186-90.

SHINKAI, Y., KOYASU, S., NAKAYAMA, K., MURPHY, K. M., LOH, D. Y., REINHERZ, E. L. & ALT, F. W. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science,* 259**,** 822-5.

SHOUVAL, D. S., BISWAS, A., GOETTEL, J. A., MCCANN, K., CONAWAY, E., REDHU, N. S., MASCANFRONI, I. D., AL ADHAM, Z., LAVOIE, S., IBOURK, M., NGUYEN, D. D., SAMSOM, J. N., ESCHER, J. C., SOMECH, R., WEISS, B., BEIER, R., CONKLIN, L. S., EBENS, C. L., SANTOS, F. G., FERREIRA, A. R., SHERLOCK, M., BHAN, A. K., MULLER, W., MORA, J. R., QUINTANA, F. J., KLEIN, C., MUISE, A. M., HORWITZ, B. H. & SNAPPER, S. B. 2014. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity,* 40**,** 706-19.

SHULL, M. M., ORMSBY, I., KIER, A. B., PAWLOWSKI, S., DIEBOLD, R. J., YIN, M., ALLEN, R., SIDMAN, C., PROETZEL, G., CALVIN, D. & ET AL. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature,* 359**,** 693-9.

SIEDE, J., FROHLICH, A., DATSI, A., HEGAZY, A. N., VARGA, D. V., HOLECSKA, V., SAITO, H., NAKAE, S. & LOHNING, M. 2016. IL-33 Receptor-Expressing Regulatory T Cells Are Highly Activated, Th2 Biased and Suppress CD4 T Cell Proliferation through IL-10 and TGFbeta Release. *PLoS One,* 11**,** e0161507.

SIMPSON, S. J., SHAH, S., COMISKEY, M., DE JONG, Y. P., WANG, B., MIZOGUCHI, E., BHAN, A. K. & TERHORST, C. 1998. T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin 12/Signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon gamma expression by T cells. *J Exp Med,* 187**,** 1225-34.

SIMS, J. E. & SMITH, D. E. 2010. The IL-1 family: regulators of immunity. *Nat Rev Immunol,* 10**,** 89-102.

SINGH, K., HJORT, M., THORVALDSON, L. & SANDLER, S. 2015. Concomitant analysis of Helios and Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naive mice. *Sci Rep,* 5**,** 7767.

SLACK, E., HAPFELMEIER, S., STECHER, B., VELYKOREDKO, Y., STOEL, M., LAWSON, M. A., GEUKING, M. B., BEUTLER, B., TEDDER, T. F., HARDT, W. D., BERCIK, P., VERDU, E. F., MCCOY, K. D. & MACPHERSON, A. J. 2009. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science,* 325**,** 617-20.

SMITH, P. M., HOWITT, M. R., PANIKOV, N., MICHAUD, M., GALLINI, C. A., BOHLOOLY, Y. M., GLICKMAN, J. N. & GARRETT, W. S. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science,* 341**,** 569-73.

SMITH-GARVIN, J. E., KORETZKY, G. A. & JORDAN, M. S. 2009. T cell activation. *Annu Rev Immunol,* 27**,** 591-619.

SMITHGALL, M. D., COMEAU, M. R., YOON, B. R., KAUFMAN, D., ARMITAGE, R. & SMITH, D. E. 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol,* 20**,** 1019-30.

SOKOL, H., PIGNEUR, B., WATTERLOT, L., LAKHDARI, O., BERMUDEZ-HUMARAN, L. G., GRATADOUX, J. J., BLUGEON, S., BRIDONNEAU, C., FURET, J. P., CORTHIER, G., GRANGETTE, C., VASQUEZ, N., POCHART, P., TRUGNAN, G., THOMAS, G., BLOTTIERE, H. M., DORE, J., MARTEAU, P., SEKSIK, P. & LANGELLA, P. 2008. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A,* 105**,** 16731-6.

SONNENBERG, G. F., FOUSER, L. A. & ARTIS, D. 2011a. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol,* 12**,** 383-90.

SONNENBERG, G. F., MONTICELLI, L. A., ALENGHAT, T., FUNG, T. C., HUTNICK, N. A., KUNISAWA, J., SHIBATA, N., GRUNBERG, S., SINHA, R., ZAHM, A. M., TARDIF, M. R., SATHALIYAWALA, T., KUBOTA, M., FARBER, D. L., COLLMAN, R. G., SHAKED, A., FOUSER, L. A., WEINER, D. B., TESSIER, P. A., FRIEDMAN, J. R., KIYONO, H., BUSHMAN, F. D., CHANG, K. M. & ARTIS, D. 2012. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science,* 336**,** 1321-5.

SONNENBERG, G. F., MONTICELLI, L. A., ELLOSO, M. M., FOUSER, L. A. & ARTIS, D. 2011b. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity,* 34**,** 122-34.

SPITS, H., ARTIS, D., COLONNA, M., DIEFENBACH, A., DI SANTO, J. P., EBERL, G., KOYASU, S., LOCKSLEY, R. M., MCKENZIE, A. N., MEBIUS, R. E., POWRIE, F. & VIVIER, E. 2013. Innate lymphoid cells--a proposal for uniform nomenclature. *Nat Rev Immunol,* 13**,** 145-9.

SPONHEIM, J., POLLHEIMER, J., OLSEN, T., BALOGH, J., HAMMARSTROM, C., LOOS, T., KASPRZYCKA, M., SORENSEN, D. R., NILSEN, H. R., KUCHLER, A. M., VATN, M. H. & HARALDSEN, G. 2010. Inflammatory bowel disease-associated interleukin-33 is preferentially expressed in ulceration-associated myofibroblasts. *Am J Pathol,* 177**,** 2804-15.

SPOONER, C. J., LESCH, J., YAN, D., KHAN, A. A., ABBAS, A., RAMIREZ-CARROZZI, V., ZHOU, M., SORIANO, R., EASTHAM-ANDERSON, J., DIEHL, L., LEE, W. P., MODRUSAN, Z., PAPPU, R., XU, M., DEVOSS, J. & SINGH, H. 2013. Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1. *Nat Immunol,* 14**,** 1229-36.

STEFKA, A. T., FEEHLEY, T., TRIPATHI, P., QIU, J., MCCOY, K., MAZMANIAN, S. K., TJOTA, M. Y., SEO, G. Y., CAO, S., THERIAULT, B. R., ANTONOPOULOS, D. A., ZHOU, L.,

CHANG, E. B., FU, Y. X. & NAGLER, C. R. 2014. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci U S A,* 111**,** 13145-50.
STRITESKY, G. L., MUTHUKRISHNAN, R., SEHRA, S., GOSWAMI, R., PHAM, D., TRAVERS, J., NGUYEN, E. T., LEVY, D. E. & KAPLAN, M. H. 2011. The transcription factor STAT3 is required for T helper 2 cell development. *Immunity,* 34**,** 39-49.
STROBER, W., FUSS, I. J. & BLUMBERG, R. S. 2002. The immunology of mucosal models of inflammation. *Annu Rev Immunol,* 20**,** 495-549.
SU, Z., LIN, J., LU, F., ZHANG, X., ZHANG, L., GANDHI, N. B., DE PAIVA, C. S., PFLUGFELDER, S. C. & LI, D. Q. 2013. Potential autocrine regulation of interleukin-33/ST2 signaling of dendritic cells in allergic inflammation. *Mucosal Immunol,* 6**,** 921-30.
SUN, C. M., HALL, J. A., BLANK, R. B., BOULADOUX, N., OUKKA, M., MORA, J. R. & BELKAID, Y. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med,* 204**,** 1775-85.
SUN, Z., UNUTMAZ, D., ZOU, Y. R., SUNSHINE, M. J., PIERANI, A., BRENNER-MORTON, S., MEBIUS, R. E. & LITTMAN, D. R. 2000. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science,* 288**,** 2369-73.
SUNDNES, O., PIETKA, W., LOOS, T., SPONHEIM, J., RANKIN, A. L., PFLANZ, S., BERTELSEN, V., SITEK, J. C., HOL, J., HARALDSEN, G. & KHNYKIN, D. 2015. Epidermal Expression and Regulation of Interleukin-33 during Homeostasis and Inflammation: Strong Species Differences. *J Invest Dermatol,* 135**,** 1771-80.
SUTMULLER, R. P., DEN BROK, M. H., KRAMER, M., BENNINK, E. J., TOONEN, L. W., KULLBERG, B. J., JOOSTEN, L. A., AKIRA, S., NETEA, M. G. & ADEMA, G. J. 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest,* 116**,** 485-94.
SUZUKAWA, M., IIKURA, M., KOKETSU, R., NAGASE, H., TAMURA, C., KOMIYA, A., NAKAE, S., MATSUSHIMA, K., OHTA, K., YAMAMOTO, K. & YAMAGUCHI, M. 2008. An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J Immunol,* 181**,** 5981-9.
SZUREK, E., CEBULA, A., WOJCIECH, L., PIETRZAK, M., REMPALA, G., KISIELOW, P. & IGNATOWICZ, L. 2015. Differences in Expression Level of Helios and Neuropilin-1 Do Not Distinguish Thymus-Derived from Extrathymically-Induced CD4+Foxp3+ Regulatory T Cells. *PLoS One,* 10**,** e0141161.
TAKEDA, A., HAMANO, S., YAMANAKA, A., HANADA, T., ISHIBASHI, T., MAK, T. W., YOSHIMURA, A. & YOSHIDA, H. 2003. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol,* 170**,** 4886-90.
TAKIMOTO, T., WAKABAYASHI, Y., SEKIYA, T., INOUE, N., MORITA, R., ICHIYAMA, K., TAKAHASHI, R., ASAKAWA, M., MUTO, G., MORI, T., HASEGAWA, E., SAIKA, S., HARA, T., NOMURA, M. & YOSHIMURA, A. 2010. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *J Immunol,* 185**,** 842-55.
TALABOT-AYER, D., CALO, N., VIGNE, S., LAMACCHIA, C., GABAY, C. & PALMER, G. 2012. The mouse interleukin (Il)33 gene is expressed in a cell type- and stimulus-dependent manner from two alternative promoters. *J Leukoc Biol,* 91**,** 119-25.

TALABOT-AYER, D., LAMACCHIA, C., GABAY, C. & PALMER, G. 2009. Interleukin-33 is biologically active independently of caspase-1 cleavage. *J Biol Chem,* 284**,** 19420-6.

TANG, C., KAMIYA, T., LIU, Y., KADOKI, M., KAKUTA, S., OSHIMA, K., HATTORI, M., TAKESHITA, K., KANAI, T., SAIJO, S., OHNO, N. & IWAKURA, Y. 2015. Inhibition of Dectin-1 Signaling Ameliorates Colitis by Inducing Lactobacillus-Mediated Regulatory T Cell Expansion in the Intestine. *Cell Host Microbe,* 18**,** 183-97.

TANOUE, T., ATARASHI, K. & HONDA, K. 2016. Development and maintenance of intestinal regulatory T cells. *Nat Rev Immunol,* 16**,** 295-309.

TARGAN, S. R., HANAUER, S. B., VAN DEVENTER, S. J., MAYER, L., PRESENT, D. H., BRAAKMAN, T., DEWOODY, K. L., SCHAIBLE, T. F. & RUTGEERTS, P. J. 1997. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med,* 337**,** 1029-35.

TAYLOR, S. L., RENSHAW, B. R., GARKA, K. E., SMITH, D. E. & SIMS, J. E. 2002. Genomic organization of the interleukin-1 locus. *Genomics,* 79**,** 726-33.

THORNTON, A. M., KORTY, P. E., TRAN, D. Q., WOHLFERT, E. A., MURRAY, P. E., BELKAID, Y. & SHEVACH, E. M. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol,* 184**,** 3433-41.

TONE, Y., FURUUCHI, K., KOJIMA, Y., TYKOCINSKI, M. L., GREENE, M. I. & TONE, M. 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol,* 9**,** 194-202.

TOTSUKA, T., KANAI, T., MAKITA, S., FUJII, R., NEMOTO, Y., OSHIMA, S., OKAMOTO, R., KOYANAGI, A., AKIBA, H., OKUMURA, K., YAGITA, H. & WATANABE, M. 2005. Regulation of murine chronic colitis by CD4+CD25- programmed death-1+ T cells. *Eur J Immunol,* 35**,** 1773-85.

TOWNSEND, M. J., FALLON, P. G., MATTHEWS, D. J., JOLIN, H. E. & MCKENZIE, A. N. 2000. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med,* 191**,** 1069-76.

TURNQUIST, H. R., CARDINAL, J., MACEDO, C., ROSBOROUGH, B. R., SUMPTER, T. L., GELLER, D. A., METES, D. & THOMSON, A. W. 2010. mTOR and GSK-3 shape the CD4+ T-cell stimulatory and differentiation capacity of myeloid DCs after exposure to LPS. *Blood,* 115**,** 4758-69.

TURNQUIST, H. R., SUMPTER, T. L., TSUNG, A., ZAHORCHAK, A. F., NAKAO, A., NAU, G. J., LIEW, F. Y., GELLER, D. A. & THOMSON, A. W. 2008. IL-1beta-driven ST2L expression promotes maturation resistance in rapamycin-conditioned dendritic cells. *J Immunol,* 181**,** 62-72.

TURNQUIST, H. R., ZHAO, Z., ROSBOROUGH, B. R., LIU, Q., CASTELLANETA, A., ISSE, K., WANG, Z., LANG, M., STOLZ, D. B., ZHENG, X. X., DEMETRIS, A. J., LIEW, F. Y., WOOD, K. J. & THOMSON, A. W. 2011. IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. *J Immunol,* 187**,** 4598-610.

UHLIG, H. H., COOMBES, J., MOTTET, C., IZCUE, A., THOMPSON, C., FANGER, A., TANNAPFEL, A., FONTENOT, J. D., RAMSDELL, F. & POWRIE, F. 2006a.

Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol,* 177**,** 5852-60.

UHLIG, H. H., MCKENZIE, B. S., HUE, S., THOMPSON, C., JOYCE-SHAIKH, B., STEPANKOVA, R., ROBINSON, N., BUONOCORE, S., TLASKALOVA-HOGENOVA, H., CUA, D. J. & POWRIE, F. 2006b. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity,* 25**,** 309-18.

USUI, T., PREISS, J. C., KANNO, Y., YAO, Z. J., BREAM, J. H., O'SHEA, J. J. & STROBER, W. 2006. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *J Exp Med,* 203**,** 755-66.

VAISHNAVA, S., BEHRENDT, C. L., ISMAIL, A. S., ECKMANN, L. & HOOPER, L. V. 2008. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A,* 105**,** 20858-63.

VAN DE PAVERT, S. A. & MEBIUS, R. E. 2010. New insights into the development of lymphoid tissues. *Nat Rev Immunol,* 10**,** 664-74.

VAN DER SLUIS, M., DE KONING, B. A., DE BRUIJN, A. C., VELCICH, A., MEIJERINK, J. P., VAN GOUDOEVER, J. B., BULLER, H. A., DEKKER, J., VAN SEUNINGEN, I., RENES, I. B. & EINERHAND, A. W. 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology,* 131**,** 117-29.

VAN SANTEN, H. M., BENOIST, C. & MATHIS, D. 2004. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J Exp Med,* 200**,** 1221-30.

VANG, K. B., YANG, J., MAHMUD, S. A., BURCHILL, M. A., VEGOE, A. L. & FARRAR, M. A. 2008. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. *J Immunol,* 181**,** 3285-90.

VAROL, C., VALLON-EBERHARD, A., ELINAV, E., AYCHEK, T., SHAPIRA, Y., LUCHE, H., FEHLING, H. J., HARDT, W. D., SHAKHAR, G. & JUNG, S. 2009. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity,* 31**,** 502-12.

VASANTHAKUMAR, A., MORO, K., XIN, A., LIAO, Y., GLOURY, R., KAWAMOTO, S., FAGARASAN, S., MIELKE, L. A., AFSHAR-STERLE, S., MASTERS, S. L., NAKAE, S., SAITO, H., WENTWORTH, J. M., LI, P., LIAO, W., LEONARD, W. J., SMYTH, G. K., SHI, W., NUTT, S. L., KOYASU, S. & KALLIES, A. 2015. The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. *Nat Immunol,* 16**,** 276-85.

VEIGA-PARGA, T., SEHRAWAT, S. & ROUSE, B. T. 2013. Role of regulatory T cells during virus infection. *Immunol Rev,* 255**,** 182-96.

VELDHOEN, M., HOCKING, R. J., ATKINS, C. J., LOCKSLEY, R. M. & STOCKINGER, B. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity,* 24**,** 179-89.

VIJAY-KUMAR, M., AITKEN, J. D., CARVALHO, F. A., CULLENDER, T. C., MWANGI, S., SRINIVASAN, S., SITARAMAN, S. V., KNIGHT, R., LEY, R. E. & GEWIRTZ, A. T. 2010. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. *Science,* 328**,** 228-31.

VIJAY-KUMAR, M., SANDERS, C. J., TAYLOR, R. T., KUMAR, A., AITKEN, J. D., SITARAMAN, S. V., NEISH, A. S., UEMATSU, S., AKIRA, S., WILLIAMS, I. R. &

GEWIRTZ, A. T. 2007. Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest,* 117**,** 3909-21.
VILLALTA, S. A., ROSENTHAL, W., MARTINEZ, L., KAUR, A., SPARWASSER, T., TIDBALL, J. G., MARGETA, M., SPENCER, M. J. & BLUESTONE, J. A. 2014. Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. *Sci Transl Med,* 6**,** 258ra142.
VOEHRINGER, D., VAN ROOIJEN, N. & LOCKSLEY, R. M. 2007. Eosinophils develop in distinct stages and are recruited to peripheral sites by alternatively activated macrophages. *J Leukoc Biol,* 81**,** 1434-44.
WADDELL, A., VALLANCE, J. E., MOORE, P. D., HUMMEL, A. T., WU, D., SHANMUKHAPPA, S. K., FEI, L., WASHINGTON, M. K., MINAR, P., COBURN, L. A., NAKAE, S., WILSON, K. T., DENSON, L. A., HOGAN, S. P. & ROSEN, M. J. 2015. IL-33 Signaling Protects from Murine Oxazolone Colitis by Supporting Intestinal Epithelial Function. *Inflamm Bowel Dis,* 21**,** 2737-46.
WALTON, K. L., HOLT, L. & SARTOR, R. B. 2009. Lipopolysaccharide activates innate immune responses in murine intestinal myofibroblasts through multiple signaling pathways. *Am J Physiol Gastrointest Liver Physiol,* 296**,** G601-11.
WALZL, G., MATTHEWS, S., KENDALL, S., GUTIERREZ-RAMOS, J. C., COYLE, A. J., OPENSHAW, P. J. & HUSSELL, T. 2001. Inhibition of T1/ST2 during respiratory syncytial virus infection prevents T helper cell type 2 (Th2)- but not Th1-driven immunopathology. *J Exp Med,* 193**,** 785-92.
WAN, Y. Y., CHI, H., XIE, M., SCHNEIDER, M. D. & FLAVELL, R. A. 2006. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. *Nat Immunol,* 7**,** 851-8.
WANG, S., ZHANG, Y., WANG, Y., YE, P., LI, J., LI, H., DING, Q. & XIA, J. 2016. Amphiregulin Confers Regulatory T Cell Suppressive Function and Tumor Invasion via the EGFR/GSK-3beta/Foxp3 Axis. *J Biol Chem,* 291**,** 21085-21095.
WANG, Y., DEVKOTA, S., MUSCH, M. W., JABRI, B., NAGLER, C., ANTONOPOULOS, D. A., CHERVONSKY, A. & CHANG, E. B. 2010. Regional mucosa-associated microbiota determine physiological expression of TLR2 and TLR4 in murine colon. *PLoS One,* 5**,** e13607.
WANG, Y., SU, M. A. & WAN, Y. Y. 2011. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity,* 35**,** 337-48.
WARD, J. M., ANVER, M. R., HAINES, D. C., MELHORN, J. M., GORELICK, P., YAN, L. & FOX, J. G. 1996. Inflammatory large bowel disease in immunodeficient mice naturally infected with Helicobacter hepaticus. *Lab Anim Sci,* 46**,** 15-20.
WEAVER, C. T., HATTON, R. D., MANGAN, P. R. & HARRINGTON, L. E. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol,* 25**,** 821-52.
WEHKAMP, J., HARDER, J., WEICHENTHAL, M., MUELLER, O., HERRLINGER, K. R., FELLERMANN, K., SCHROEDER, J. M. & STANGE, E. F. 2003. Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis,* 9**,** 215-23.
WEHKAMP, J., SALZMAN, N. H., PORTER, E., NUDING, S., WEICHENTHAL, M., PETRAS, R. E., SHEN, B., SCHAEFFELER, E., SCHWAB, M., LINZMEIER, R., FEATHERS, R. W., CHU, H., LIMA, H., JR., FELLERMANN, K., GANZ, T., STANGE, E. F. & BEVINS, C. L.

2005. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A,* 102**,** 18129-34.

WEISS, J. M., BILATE, A. M., GOBERT, M., DING, Y., CUROTTO DE LAFAILLE, M. A., PARKHURST, C. N., XIONG, H., DOLPADY, J., FREY, A. B., RUOCCO, M. G., YANG, Y., FLOESS, S., HUEHN, J., OH, S., LI, M. O., NIEC, R. E., RUDENSKY, A. Y., DUSTIN, M. L., LITTMAN, D. R. & LAFAILLE, J. J. 2012. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med,* 209**,** 1723-42, S1.

WELLCOME TRUST CASE CONTROL, C. 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature,* 447**,** 661-78.

WESCHE, H., HENZEL, W. J., SHILLINGLAW, W., LI, S. & CAO, Z. 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity,* 7**,** 837-47.

WILDIN, R. S., RAMSDELL, F., PEAKE, J., FARAVELLI, F., CASANOVA, J. L., BUIST, N., LEVY-LAHAD, E., MAZZELLA, M., GOULET, O., PERRONI, L., BRICARELLI, F. D., BYRNE, G., MCEUEN, M., PROLL, S., APPLEBY, M. & BRUNKOW, M. E. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet,* 27**,** 18-20.

WING, K., ONISHI, Y., PRIETO-MARTIN, P., YAMAGUCHI, T., MIYARA, M., FEHERVARI, Z., NOMURA, T. & SAKAGUCHI, S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science,* 322**,** 271-5.

WIRTZ, S., FINOTTO, S., KANZLER, S., LOHSE, A. W., BLESSING, M., LEHR, H. A., GALLE, P. R. & NEURATH, M. F. 1999. Cutting edge: chronic intestinal inflammation in STAT-4 transgenic mice: characterization of disease and adoptive transfer by TNF- plus IFN-gamma-producing CD4+ T cells that respond to bacterial antigens. *J Immunol,* 162**,** 1884-8.

WLODARSKA, M., WILLING, B., KEENEY, K. M., MENENDEZ, A., BERGSTROM, K. S., GILL, N., RUSSELL, S. L., VALLANCE, B. A. & FINLAY, B. B. 2011. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. *Infect Immun,* 79**,** 1536-45.

WOHLFERT, E. A., GRAINGER, J. R., BOULADOUX, N., KONKEL, J. E., OLDENHOVE, G., RIBEIRO, C. H., HALL, J. A., YAGI, R., NAIK, S., BHAIRAVABHOTLA, R., PAUL, W. E., BOSSELUT, R., WEI, G., ZHAO, K., OUKKA, M., ZHU, J. & BELKAID, Y. 2011. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest,* 121**,** 4503-15.

WU, H. J., IVANOV, II, DARCE, J., HATTORI, K., SHIMA, T., UMESAKI, Y., LITTMAN, D. R., BENOIST, C. & MATHIS, D. 2010. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity,* 32**,** 815-27.

XIAO, H., GULEN, M. F., QIN, J., YAO, J., BULEK, K., KISH, D., ALTUNTAS, C. Z., WALD, D., MA, C., ZHOU, H., TUOHY, V. K., FAIRCHILD, R. L., DE LA MOTTE, C., CUA, D., VALLANCE, B. A. & LI, X. 2007. The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. *Immunity,* 26**,** 461-75.

XU, D., CHAN, W. L., LEUNG, B. P., HUANG, F., WHEELER, R., PIEDRAFITA, D., ROBINSON, J. H. & LIEW, F. Y. 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med,* 187**,** 787-94.

XU, D., JIANG, H. R., KEWIN, P., LI, Y., MU, R., FRASER, A. R., PITMAN, N., KUROWSKA-STOLARSKA, M., MCKENZIE, A. N., MCINNES, I. B. & LIEW, F. Y. 2008. IL-33 exacerbates antigen-induced arthritis by activating mast cells. *Proc Natl Acad Sci U S A,* 105**,** 10913-8.

XU, L., KITANI, A., STUELTEN, C., MCGRADY, G., FUSS, I. & STROBER, W. 2010. Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I. *Immunity,* 33**,** 313-25.

YADAV, M., LOUVET, C., DAVINI, D., GARDNER, J. M., MARTINEZ-LLORDELLA, M., BAILEY-BUCKTROUT, S., ANTHONY, B. A., SVERDRUP, F. M., HEAD, R., KUSTER, D. J., RUMINSKI, P., WEISS, D., VON SCHACK, D. & BLUESTONE, J. A. 2012. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med,* 209**,** 1713-22, S1-19.

YAGI, R., JUNTTILA, I. S., WEI, G., URBAN, J. F., JR., ZHAO, K., PAUL, W. E. & ZHU, J. 2010. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma. *Immunity,* 32**,** 507-17.

YAGI, R., ZHONG, C., NORTHRUP, D. L., YU, F., BOULADOUX, N., SPENCER, S., HU, G., BARRON, L., SHARMA, S., NAKAYAMA, T., BELKAID, Y., ZHAO, K. & ZHU, J. 2014. The transcription factor GATA3 is critical for the development of all IL-7Ralpha-expressing innate lymphoid cells. *Immunity,* 40**,** 378-88.

YANAGAWA, Y., MATSUMOTO, M. & TOGASHI, H. 2011a. Adrenoceptor-mediated enhancement of interleukin-33 production by dendritic cells. *Brain Behav Immun,* 25**,** 1427-33.

YANAGAWA, Y., SUZUKI, M., MATSUMOTO, M. & TOGASHI, H. 2011b. Prostaglandin E(2) enhances IL-33 production by dendritic cells. *Immunol Lett,* 141**,** 55-60.

YANG, B. H., HAGEMANN, S., MAMARELI, P., LAUER, U., HOFFMANN, U., BECKSTETTE, M., FOHSE, L., PRINZ, I., PEZOLDT, J., SUERBAUM, S., SPARWASSER, T., HAMANN, A., FLOESS, S., HUEHN, J. & LOCHNER, M. 2016a. Foxp3(+) T cells expressing RORgammat represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol,* 9**,** 444-57.

YANG, Q., LI, G., ZHU, Y., LIU, L., CHEN, E., TURNQUIST, H., ZHANG, X., FINN, O. J., CHEN, X. & LU, B. 2011. IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8+ T cells. *Eur J Immunol,* 41**,** 3351-60.

YANG, S., FUJIKADO, N., KOLODIN, D., BENOIST, C. & MATHIS, D. 2015. Immune tolerance. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance. *Science,* 348**,** 589-94.

YANG, X. O., PANOPOULOS, A. D., NURIEVA, R., CHANG, S. H., WANG, D., WATOWICH, S. S. & DONG, C. 2007a. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem,* 282**,** 9358-63.

YANG, X. O., PAPPU, B. P., NURIEVA, R., AKIMZHANOV, A., KANG, H. S., CHUNG, Y., MA, L., SHAH, B., PANOPOULOS, A. D., SCHLUNS, K. S., WATOWICH, S. S., TIAN, Q., JETTEN, A. M. & DONG, C. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity,* 28**,** 29-39.

YANG, Y., ANDERSSON, P., HOSAKA, K., ZHANG, Y., CAO, R., IWAMOTO, H., YANG, X., NAKAMURA, M., WANG, J., ZHUANG, R., MORIKAWA, H., XUE, Y., BRAUN, H., BEYAERT, R., SAMANI, N., NAKAE, S., HAMS, E., DISSING, S., FALLON, P. G.,

LANGER, R. & CAO, Y. 2016b. The PDGF-BB-SOX7 axis-modulated IL-33 in pericytes and stromal cells promotes metastasis through tumour-associated macrophages. *Nat Commun,* 7**,** 11385.

YANG, Y., OCHANDO, J. C., BROMBERG, J. S. & DING, Y. 2007b. Identification of a distant T-bet enhancer responsive to IL-12/Stat4 and IFNgamma/Stat1 signals. *Blood,* 110**,** 2494-500.

YANG, Y., WANG, J. B., LI, Y. M., ZHAO, Y. U., WANG, R., WU, Q., ZHENG, R. S. & OU, Y. R. 2016c. Role of IL-33 expression in oncogenesis and development of human hepatocellular carcinoma. *Oncol Lett,* 12**,** 429-436.

YAO, Z., KANNO, Y., KERENYI, M., STEPHENS, G., DURANT, L., WATFORD, W. T., LAURENCE, A., ROBINSON, G. W., SHEVACH, E. M., MORIGGL, R., HENNIGHAUSEN, L., WU, C. & O'SHEA, J. J. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood,* 109**,** 4368-75.

YASUDA, K., MUTO, T., KAWAGOE, T., MATSUMOTO, M., SASAKI, Y., MATSUSHITA, K., TAKI, Y., FUTATSUGI-YUMIKURA, S., TSUTSUI, H., ISHII, K. J., YOSHIMOTO, T., AKIRA, S. & NAKANISHI, K. 2012. Contribution of IL-33-activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice. *Proc Natl Acad Sci U S A,* 109**,** 3451-6.

YEN, D., CHEUNG, J., SCHEERENS, H., POULET, F., MCCLANAHAN, T., MCKENZIE, B., KLEINSCHEK, M. A., OWYANG, A., MATTSON, J., BLUMENSCHEIN, W., MURPHY, E., SATHE, M., CUA, D. J., KASTELEIN, R. A. & RENNICK, D. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest,* 116**,** 1310-6.

YOSHIMOTO, T., TAKEDA, K., TANAKA, T., OHKUSU, K., KASHIWAMURA, S., OKAMURA, H., AKIRA, S. & NAKANISHI, K. 1998. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J Immunol,* 161**,** 3400-7.

YU, L. C. & PERDUE, M. H. 2001. Role of mast cells in intestinal mucosal function: studies in models of hypersensitivity and stress. *Immunol Rev,* 179**,** 61-73.

YURCHENKO, E., SHIO, M. T., HUANG, T. C., DA SILVA MARTINS, M., SZYF, M., LEVINGS, M. K., OLIVIER, M. & PICCIRILLO, C. A. 2012. Inflammation-driven reprogramming of CD4+ Foxp3+ regulatory T cells into pathogenic Th1/Th17 T effectors is abrogated by mTOR inhibition in vivo. *PLoS One,* 7**,** e35572.

ZAISS, D. M., GAUSE, W. C., OSBORNE, L. C. & ARTIS, D. 2015. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity,* 42**,** 216-26.

ZAISS, D. M., VAN LOOSDREGT, J., GORLANI, A., BEKKER, C. P., GRONE, A., SIBILIA, M., VAN BERGEN EN HENEGOUWEN, P. M., ROOVERS, R. C., COFFER, P. J. & SIJTS, A. J. 2013. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. *Immunity,* 38**,** 275-84.

ZEUTHEN, L. H., FINK, L. N. & FROKIAER, H. 2008. Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. *Immunology,* 123**,** 197-208.

ZHANG, D. H., COHN, L., RAY, P., BOTTOMLY, K. & RAY, A. 1997. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem,* 272**,** 21597-603.

ZHAO, D. M., THORNTON, A. M., DIPAOLO, R. J. & SHEVACH, E. M. 2006. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood,* 107**,** 3925-32.

ZHAO, J., WEI, J., MIALKI, R. K., MALLAMPALLI, D. F., CHEN, B. B., COON, T., ZOU, C., MALLAMPALLI, R. K. & ZHAO, Y. 2012. F-box protein FBXL19-mediated ubiquitination and degradation of the receptor for IL-33 limits pulmonary inflammation. *Nat Immunol,* 13**,** 651-8.

ZHENG, W. & FLAVELL, R. A. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell,* 89**,** 587-96.

ZHENG, Y., CHAUDHRY, A., KAS, A., DEROOS, P., KIM, J. M., CHU, T. T., CORCORAN, L., TREUTING, P., KLEIN, U. & RUDENSKY, A. Y. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature,* 458**,** 351-6.

ZHENG, Y., JOSEFOWICZ, S., CHAUDHRY, A., PENG, X. P., FORBUSH, K. & RUDENSKY, A. Y. 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature,* 463**,** 808-12.

ZHOU, L., IVANOV, II, SPOLSKI, R., MIN, R., SHENDEROV, K., EGAWA, T., LEVY, D. E., LEONARD, W. J. & LITTMAN, D. R. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol,* 8**,** 967-74.

ZHOU, X., BAILEY-BUCKTROUT, S. L., JEKER, L. T., PENARANDA, C., MARTINEZ-LLORDELLA, M., ASHBY, M., NAKAYAMA, M., ROSENTHAL, W. & BLUESTONE, J. A. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol,* 10**,** 1000-7.

ZHU, J., WANG, Y., YANG, F., SANG, L., ZHAI, J., LI, S., LI, Y., WANG, D., LU, C. & SUN, X. 2015. IL-33 alleviates DSS-induced chronic colitis in C57BL/6 mice colon lamina propria by suppressing Th17 cell response as well as Th1 cell response. *Int Immunopharmacol,* 29**,** 846-53.

ZIELINSKI, C. E., MELE, F., ASCHENBRENNER, D., JARROSSAY, D., RONCHI, F., GATTORNO, M., MONTICELLI, S., LANZAVECCHIA, A. & SALLUSTO, F. 2012. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature,* 484**,** 514-8.

ZIGMOND, E., BERNSHTEIN, B., FRIEDLANDER, G., WALKER, C. R., YONA, S., KIM, K. W., BRENNER, O., KRAUTHGAMER, R., VAROL, C., MULLER, W. & JUNG, S. 2014. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity,* 40**,** 720-33.

ZIMMERMANN, J., KUHL, A. A., WEBER, M., GRUN, J. R., LOFFLER, J., HAFTMANN, C., RIEDEL, R., MASCHMEYER, P., LEHMANN, K., WESTENDORF, K., MASHREGHI, M. F., LOHNING, M., MACK, M., RADBRUCH, A. & CHANG, H. D. 2016. T-bet expression by Th cells promotes type 1 inflammation but is dispensable for colitis. *Mucosal Immunol,* 9**,** 1487-1499.