Regulation of T cell effector functions in the intestinal mucosa

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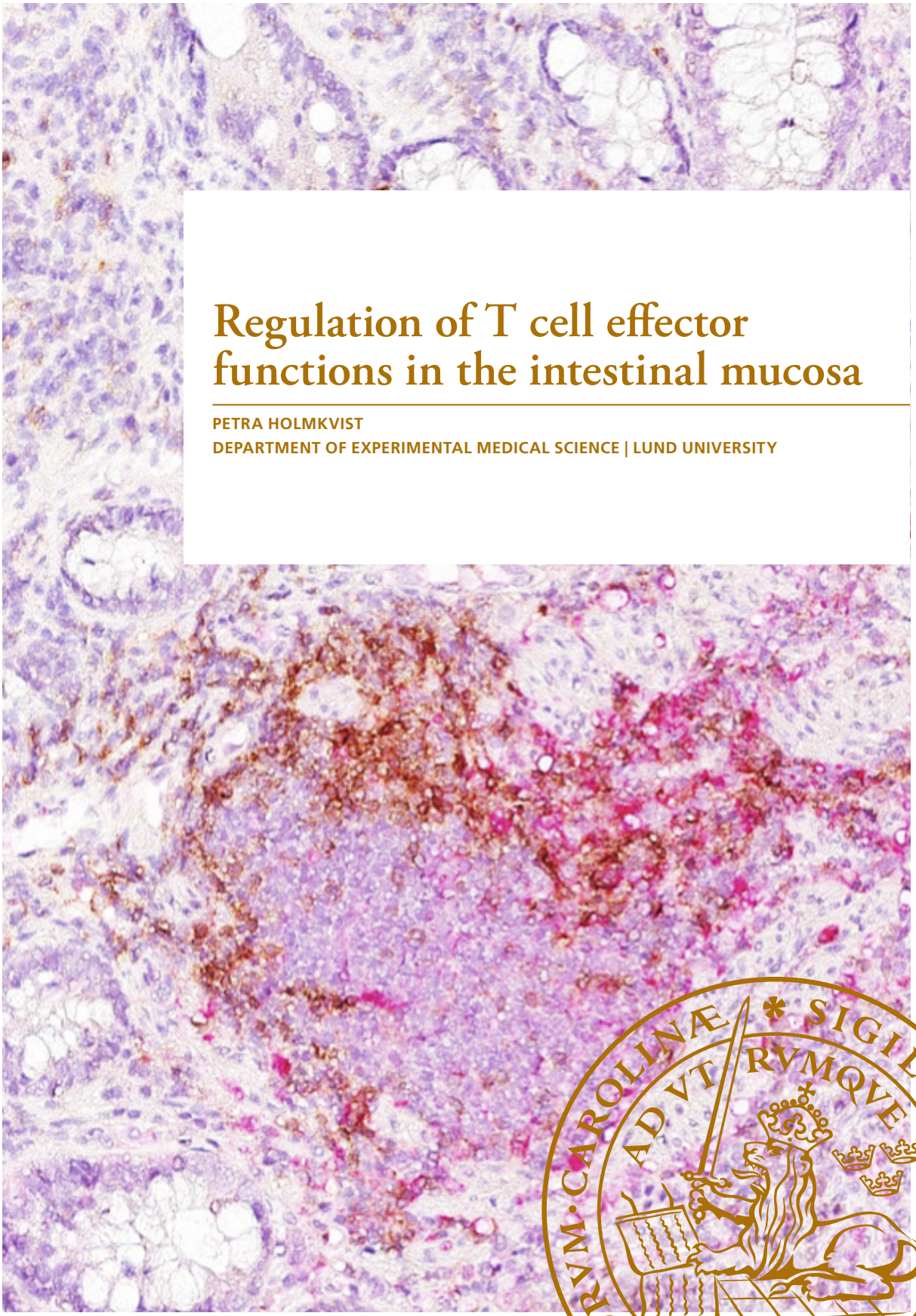

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Abstract
T lymphocytes are a critical cellular component of the adaptive immune response. They are generated in the thymus from bone marrow derived progenitors, where they undergo commitment to the T cell lineage and differentiate and mature into naïve $CD4^+$ and $CD8^+$ T cells. Naïve $CD4^+$ and $CD8^+$ T cells activation takes place in lymphoid organs after recognition of cognate antigen in the context of major histocompatibility complex (MHC) molecules on antigen presenting cells (APCs). The effector functions of $CD8^+$ T cells includes the MHC-I dependent recognition and lysis of target cells (e.g. virally infected cells) through the release of degranulating cytotoxic mediators but $CD8^+$ T cells can also acquire suppressive functions ($CD8^+$ regulatory T cells (Tregs). Activation of naïve $CD4^+$ T cells leads to the generation of T helper cell subsets (Th, e.g. T helper (Th1), Th2, Th17 and $CD4^+$ Tregs) and the type of Th cell that is generated during an immune response is largely dependent on the cytokine signals these cells receive during their initial activation in the lymph node.. The main function of $CD4^+$ Th cells is to produce cytokines that that impact on the differentiation, recruitment and functionality of other immune and stromal cells, and as such these cells play a central role in shaping immune responses. Following an immune response some antigen-specific T cells remain as memory T cells. Memory T cells are long-lived and serve to protect us against re-infection with the same pathogen. The overall aim of this thesis was to study mechanisms regulating differentiation and functionality of intestinal $CD4^+$ and $CD8^+$ T cells.

Memory $CD4^+$ T cells is found in the circulation as well as in tissues. In addition to responding to re-infections in an antigen dependent manner these circulating memory $CD4^+$ T cells can produce interferon (IFN)-γ in response to cytokines in the absence of T cell receptor (TCR) stimulation, suggesting that they may participate in immune responses in an innate-like manner. We could show that IL-15 or tumor necrosis factor (TNF)-like cytokine 1A(TL1a) induced production of IL-5, IL-6, IL-13, IL-22, granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN-γ and TNF-α in a subset of memory $CD4^+$ T cells co-expressing death receptor 3 (DR3) and IL-18Rα in presence of IL-12 and IL-18. TL1a synergized with IL-15 to further enhance the levels of all cytokines measured and the cytokine-induced cytokine production was severely diminished in the absence of IL-18. The intestine is a large reservoir of memory $CD4^+$ T cells and we could show that a large proportion of these cells co-expressed IL-18Rα and DR3 in both humans and mice and these cells produced cytokines in response to cytokine stimulation. These results suggest that both human and murine memory $IL\text{-}18R\alpha^+DR3^+$ $CD4^+$ T cells may contribute to antigen-independent innate-like responses in the intestine.

We also found that $CD4^+$ T cells expressing IL-18Rα and DR3 were present in the inflamed small intestine in patients with Crohn's disease (CD), a chronic inflammatory disease that can affect all areas of the gastrointestinal tract, where theu co-localized with IL-18-expressing cells in intestinal lymphoid aggregates. This suggests that cytokine-activated $CD4^+$ T cells might contribute to the high levels of proinflammatory cytokines observed in these patients. Bioactive IL-18 mRNA transcripts are increased in mucosal samples obtained from CD compared to controls. The role of IL-18 in intestinal inflammation has been thoroughly studied in various animal models of IBD and while most of these mice are protected from severe intestinal inflammation in absence of IL-18 there are also studies suggesting a protective role for IL-18. Although IL-18 is a pleotropic cytokine all of these studies was carried out by systemically blocking IL-18 signalling and did not take into account what cells that were affected. In the $CD45RB^{high}$ $CD4^+$ T cell transfer model of IBD we could observe that all $CD4^+$ T cells recovered from mesenteric lymph node and colon lamina propria (LP) expressed IL-18Rα in the setting of colitis. To identify a potential role of IL-18 signalling in $CD4^+$ T cells ability to induce colitis in this model we transferred *il-18r*$^{-/-}$ $CD45RB^{high}CD4^+$ T cells into *rag-1*$^{-/-}$ mice. The number of IFN-γ producing $CD4^+$ T cells recovered from the colitic colon LP were significantly reduced compared to their wild type counterparts and *il-18r*$^{-/-}$ $CD4^+$ T cells in the setting of colitis failed to produce cytokines in response to cytokine stimulation. Despite these findings *il-18r*$^{-/-}$ $CD4^+$ T cells were equally proficient at inducing colitis as those $CD4^+$ T cells from wild type mice. These results suggesting that IL-18 signaling in $CD4^+$ T cells is not critical for their ability to drive disease pathology in the $CD45RB^{high}$ transfer model of colitis.

The intestine is daily exposed to large amounts of antigens and is the major entry site for intracellular bacteria whose control requires the induction of protective $CD8^+$ T cell responses. In the intestinal fatty acid-binding protein promoter truncated ovalbumin (iFABP-tOVA) mouse in which a surrogate antigen is expressed in the small intestinal epithelium we assessed mechanisms regulating mucosal $CD8^+$ T cell priming and differentiation in the steady state and inflammatory setting. $CD8^+$ T cells were found to differentiate into two distinct subsets, $CD107a/b^+$ cytotoxic T cells (CTLs) and $FoxP3^+CD8^+$ T cells. $FoxP3^+CD8^+$ T cells but not CTLs required chemokine receptor 9 (CCR9) for effective homing to and expansion within the small intestinal mucosa. Further, we found that IRF8, but not IRF4, expression by intestinal dendritic cells (DCs) was critical for the development of the $FoxP3^+$ subset in steady state but not the inflammatory setting. Collectively these findings broaden our understanding of the mechanisms regulating $CD8^+$ T cell responses in the intestinal mucosa and have potential implications for mucosa vaccine design.

Key words
T cells, memory, intestine, cytokines, differentiation, IL-18, inflammation, antigen presentation

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On the cover: Image showing the location of $CD4^+$ (brown) and IL-18-expressing (red) cells in inflamed small intestinal tissue obtained from a patient with Crohn's Disease. Staining was performed at the Unit of Airway Inflammation, Lund University.

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To present and future members of my family

Laughter is timeless,

Imagination has no age,

And dreams are forever

-Walt Disney

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Papers included in this thesis

Paper I

A major population of mucosal memory CD4$^+$ T cells, co-expressing IL-18Rα and DR3, display innate lymphocyte functionality

Petra Holmkvist, Kirstine Roepstorff, Heli Uronen-Hansson, Caroline Sandén, Sigurdur Gudjonsson, Oliver Patschan, Olof Grip, Jan Marsal, Artur Schmidtchen, Lars Hornum, Jonas S. Erjefält, Katarina Håkansson and William W. Agace
Mucosal Immunology, advance online publication 1 October 2014. doi:10.1038/mi.2014.87

Paper II

IL-18Rα deficient CD4$^+$ T cells induce intestinal inflammation in the CD45RB^{high} transfer model of experimental colitis, despite reduced IFN-γ production and a failure to secrete cytokines in response to cytokine stimulation

Petra Holmkvist, Aymeric Rivollier, Karin Hägerbrand and William W. Agace
In manuscript

Paper III

IRF8-dependent DCs play a key role in the regulation of CD8 T cell responses to epithelial derived antigen in the steady state but not inflammatory setting

Thorsten Joeris, Petra Holmkvist, Katarzyna Luda, Emma K. Persson and William W. Agace
In manuscript

Abbreviations

APC	antigen presenting cell
ATP	adenosine triphosphate
Batf3	basic leucine zipper transcription factor, ATF-like 3
Bcl6	B cell lymphoma 6
BCR	B cell receptor
BP	binding protein
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Crohn's disease
CsA	cyclosporine A
CTLA-4	cytotoxic T lymphocyte-associated molecule-4
CTLs	cytotoxic T lymphocytes
DAMP	damage-associated molecular patterns
DC	dendritic cell
DR3	death receptor 3
DSS	dextran sodium sulphate
EAE	experimental autoimmune encephalomyelitis
EC	epithelial compartment
ER	endoplasmatic reticulum
FAE	follicle-associated epithelium
FoxP3	forkhead box P3
GADD	growth arrest and DNA damage protein
GAGs	glycosaminoglycans
GALT	gut-associated lymphoid tissues
GATA-3	GATA-binding protein 3

GM-CSF	granulocyte-macrophage colony-stimulating factor
GVHD	graft versus host disease
HEV	high endothelial venule
HIV	human immunodeficiency virus
IBD	inflammatory bowel disease
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
Id2	inhibitor of DNA binding 2
IEL	intraepithelial lymphocyte
iFABP	intestinal fatty acid binding protein promoter
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate like lymphocyte
IRF	IFN regulatory factor
iTregs	induced Tregs
LP	lamina propria
LPMC	LP mononuclear cell
LPS	lipopolysaccharide
mAB	monoclonal antibody
MAdCAM-1	mucosal addressin cell-adhesion molecule-1
MAPK	mitogen-activated protein kinase
MEKK4	MAPK-extracellular signal-regulated kinase kinase 4
MHC	major histocompatibility complexes
MHC I	MHC class I
MHC II	MHC class II
MLN	mesenteric lymph node
NK	natural killer cell
nTregs	natural Tregs
OVA	Ovalbumin

PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptors
r	recombinant
Rag	recombination-activating gene
RLR	RIG-I-like receptor
RORγt	retinoic acid receptor-related orphan receptor gamma-T
S1PR1	sphingosine-1-phosphate receptor 1
SCID	severely combined immunodeficient
SED	sub epithelial dome
SFB	segmented filamentous bacteria
SILFs	solitary isolated lymphoid follicles
STAT	Signal Transducers and Activators of Transcription
T-bet	T-box transcription factor
Tc	T cytotoxic
Tcm	central memory T
TCR	T cell receptor
Tem	Effector memory T
Tfh	follicular Th
TGF	transforming growth factor
Th	T helper
TL1a	TNF-like ligand 1A
TNBS	2,4,6-trinitrobenzene sulfonic acid
Tr1	T regulatory type 1 cell
Tregs	regulatory T cells
Trm	tissue resident memory T cells
Tscm	stem cell memory T cells
tTregs	thymic T cells
UC	Ulcerative colitis
VCAM-1	vascular cell adhesion molecule-1
WT	wild type

1. The immune system

The immune system is a complex composition of multiple cell types and tissue structures that protects the body against disease. The body is constantly in contact with the surrounding environment and the immune system is daily dealing with a large load of foreign substances, called antigens. An important task of the immune system is to generate tolerogenic immune responses to food antigens as well as to the numerous commensal microorganisms that inhabit our body surfaces (intestine, skin, upper airways) while maintaining the ability to provide protective immune responses to potential pathogens. Inappropriate immune responses can lead to chronic inflammatory and autoimmune diseases, allergies and chronic infections. The immune system can be broadly divided into two compartments, the innate and adaptive immune system.

1.1 Innate immune system

The innate immune system is the body's first line of defence. The body is partly separated from the external environment by physical barriers such as the skin and the single cell layer of epithelial cells that line mucosal surfaces. However mucosal barriers must be kept semi-permeable since the body needs to take up for example oxygen and nutrients from the surroundings, making the mucosal sites particularly vulnerable for invading infectious agents like viruses, fungi, bacteria and protozoa.

Epithelial cells lining the intestinal mucosa not only form a physical barrier but also include specialized epithelial lineages including Paneth cells, that produce anti-microbial peptides like α-defensins and lysosomes, and goblet cells that produce mucin that makes up the thick gel-like mucus protecting the underlying epithelium. Both Paneth cells and goblet cells are important factors in mucosal homeostasis, excluding bacteria and other foreign material from the epithelial cell layer, and defects in their functions are associated with inflammatory bowel disease (IBD), a chronic inflammation in the intestine [1].

If a pathogen breaks through these barriers there is a range of cells like dendritic cells (DCs), macrophages, innate like lymphocytes (ILCs) and granulocytes underneath that sense the presence of pathogens and respond rapidly (within hours). A major mechanism by which innate immune cells, including the epithelial cells [2], sense and respond to the presence of pathogens is through their recognition of conserved structures on the pathogen termed pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Toll-like receptors (TLRs), nucleotide-binding oligomerization domain–like receptors (NLRs), C-type lectins (CLRs) and RIG-I-like receptors (RLRs) are families of PRRs. In addition to recognizing pathogens the PRRs can also detect host-derived damage-associated molecular patterns (DAMPs). These DAMPs are cellular contents like adenosine triphosphate (ATP) and uric acid that get released when a cell is injured. The different PRRs can be expressed either on the cell surface or internally. For example the TLR family members TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cells surface while TLR3, TLR7, TLR8 and TLR9 are expressed on endosomal compartments within the cell. The different PRRs are specialized to recognize different PAMPs. For example TLR3 recognize double stranded RNA, TLR4 lipopolysaccharide (LPS), TLR5 flagellin and TLR7 single stranded RNA [3].

Activation of innate immune cells through PRRs leads, among other things, to production of a range of small signalling molecules, called cytokines and chemokines. Cytokines shape the immediate cells that express the appropriate receptors and can for example change the responding cells activation-status, cytokine production and receptor expression. Chemokines serves to attract cells to tissues and also to relocate cells within tissues.

ILCs are a group of innate cells recently proposed to be classified into 3 different groups depending on their effector cytokine properties and transcription factors regulating their development and function [4]. While the members within ILCs have diverse phenotypes and functions they share similarities like the need for the transcriptional repressor inhibitor of DNA binding 2 (Id2) and expression of the common cytokine receptor γ chain (IL-2Rγ) for their development [5]. Two of the most studied cell types within the ILCs are the natural killer cells (NKs) belonging to ILC group 1. NKs produce interferon (IFN)-γ upon activation and are important mediators of early responses against viruses.

In addition to produce signalling molecules, phagocytes like DCs and macrophages engulf pathogens, through a mechanism called phagocytosis, and degrade the pathogen internally. Peptides, i.e. antigens, of the internally destroyed pathogen are then displayed on major histocompatibility complexes (MHC) on the phagocytes surface. These antigen presenting cells (APCs) present the antigen:MHC complexes to cells within the adaptive immune system and take part in these cells subsequent activation and thus provide an important link between the innate and adaptive immune system.

1.2 Cells of the adaptive immune system

The adaptive immune system is only present in vertebrates and participates in immune responses in a more efficient and specific way than the innate immune system. T and B lymphocytes are the central cell populations within the adaptive immune system and each cell express receptors with unique specificity against a single antigen. However since each T and B cell only express receptors recognizing one specific antigen and since the body is in contact with a broad range of foreign particles it usually takes several days to find and activate the adaptive immune cells with specificity to a particular antigen.

Upon activation of T and B lymphocytes these cells contribute to homeostasis in different ways, B lymphocytes produce antibodies that bind and neutralize antigens while $CD8^+$ T cells can develop into cytotoxic T cells that kill cells presenting their cognate antigen on MHC class I (MHC I) molecules and $CD4^+$ T cells produce cytokines and express receptors that enhance the activation and function of a variety of cells including $CD8^+$ T and B lymphocytes.

An important function of the adaptive immune system is to generate an immunological memory. A small subset of pathogen specific T and B cells persist after the clearance of a pathogen and in case of a second infection by the same pathogen these memory cells are activated and the infection is cleared more rapidly.

T cell generation

Uncommitted T cell precursors are generated in the bone marrow and migrate, via the circulation, into the thymus where they undergo commitment to the T cell lineage and differentiate and mature into naïve $CD4^+$ and $CD8^+$ T cells.

In the thymus each T cell gets equipped with a unique T cell receptor (TCR) that only recognize its cognate antigen when presented on MHC molecules. The TCRs are formed by random recombination of gene segments (V(D)J). There are multiple V, D and J segments and the unique specificity of TCRs is partly dependent on the large number of VDJ combinations that can form but also involve the random addition and removal of nucleotides that take place during the joining of these gene segments. Several enzymes take part in this recombination process including recombination-activating gene-1 and -2 (Rag-1 and Rag-2). After successful rearrangement of the TCR all T cells go through positive and negative selection. First T cells must bind MHC molecules on thymic epithelial cells presenting self-derived antigens with an affinity strong enough to get a survival signal (positive selection). Next T cells encounter APCs expressing self-derived antigens on MHC molecules and those T cells expressing TCRs with high affinity towards the antigen:MHC complex will receive apoptotic signals. These selection processes serves both to assure that T cells leaving the thymus express functional TCRs that can recognize MHC molecules as well as eliminating self-reactive T cells, i.e. those T cells with TCRs with high affinity towards self-derived peptides. In thymus T cells will also commit to either the CD4 or CD8 T cell linage that recognize antigens presented on distinct MHC molecules; $CD4^+$ T cells recognize antigen presented on MHC II while $CD8^+$ T cells needs the antigen to be presented on MHC I. Naïve $CD4^+$ and $CD8^+$ T cells with functional TCRs leave the thymus and enter the circulation where they can enter secondary lymph nodes.

B cell generation

Immature B cells develop in the bone marrow and recognize and bind their cognate antigen with their B cell receptors (BCRs). The BCRs are membrane bound immunoglobulins (Igs) that are generated by recombination of gene segments. In absence of their cognate antigen immature B cells circulate in blood and lymph nodes. In contrast to T cells

that need their antigen to be presented on MHC molecules, B cells recognize their antigen in its native form. Upon binding to the BCR antigen is internalized, degraded and presented on MHC II on the B cell surface. $CD4^+$ T cells that recognize the antigen:MHC II complex provide help to B cells that go through several differentiation steps including Ig class-switch and somatic hypermutation of the BCR to finally mature into plasma cells that produce and release high-affinity soluble BCRs, i.e. antibodies. These high-affinity antibodies attach to their cognate antigen to either neutralize it or to make it more accessible to the cells within the innate immune system and thereby limit the spread of the infection. Since the papers included in this thesis is focused on the regulation of T cell responses subsequent chapters will be centred on T cells.

2. T cell activation

Following their generation in the thymus naïve T cells enter the blood and continually circulate between blood and lymphoid organs. Lymphoid organs are dynamic and highly organized tissue structures where circulating naïve T cells meet APCs, primarily DCs, migrating from peripheral tissues. Since there for any given antigen only are a few antigen-specific T cells [6] these lymphoid organs such as spleen and lymph nodes are of high importance to optimize the chances of rare antigen specific T cells to find and interact with APCs (DCs) bearing the correct antigen peptide in the context of MHC molecules. The immune cells reside within different compartments of the lymph node; B cells are located in follicles in the outer cortex of lymph nodes while T cells are distributed in the paracortical areas, also called T cell zones, adjacent to the B cell follicles. The approximation of B and T cell sites is optimal since maturation of B cells is dependent on help from the T cells.

DCs are located throughout peripheral tissue where they constantly sampling their environment for antigens. The recognition of a foreign particle and activation signals leads to internalization of the particle and maturation of the DC. DCs can take up foreign material or damaged cells from the external environment through receptor-mediated phagocytosis. Receptor mediated phagocytosis can be triggered in cells by binding Ig coated targets or by direct binding to the target. Upon the initial interaction with the target a multistep process involving receptor clustering, recruitment and reorganization of actin structures are initiated and drive engulfment of the target antigen. Engulfed particles are kept in internal vesicles, called phagosomes and these merge with lysosomes containing enzymes that mediate the degradation of the engulfed particle. The peptides from internally degraded material are then displayed on the APCs surface on MHC molecules. In the maturation process DCs up regulate the expression of MHC II and co-stimulatory molecules like CD80 and CD86 as well as the CC-chemokine receptor (CCR) 7. The mature antigen presenting DCs migrate through afferent lymphatics and enter draining lymph nodes. CCR7 is thought to be essential for DC mobilization and their subsequent localization in the T cell zones, however little is known about

mechanisms involved in DC trafficking in afferent lymphatics and how they enter lymphatic tissues [7, 8].

Naïve T cells circulating in the blood express CD62L (L-selectin) and CCR7 that direct the migration of T cells to lymph nodes, which they enter through specialized blood vessels called high endothelial venules (HEVs). Naïve T cells have to go through a multistep adhesion process to be able to enter lymphoid tissues and the receptors and ligands known to be involved in this process somewhat differ depending on the location of the lymph node [7, 9]. Naïve T cells transported in the blood flow can with their CD62L transiently bind peripheral node addressins expressed by HEVs causing a rolling movement of the cell along the endothelia. The ligands for CCR7, CC-chemokine ligand (CCL) 21 and/or CCL19, are displayed on HEVs bound to glycosaminoglycans (GAGs) and binding of CCR7 to its ligand causes conformational changes in the cells $\alpha_1\beta_2$ integrins. Activated integrins can then firmly bind to HEVs intercellular adhesion molecule (ICAM)-1 and ICAM-2 causing an arrest of the cells that subsequently transmigrate into the lymph node. Naïve T cells entering mesenteric lymph nodes (MLNs) and Payers patches, lymphoid tissues in the intestine, needs to activate integrin $\alpha_4\beta_7$ that can bind to mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) expressed by these lymph organs HEVs. In the lymph node T cells locate in the T cell zones where they scan through the APCs for their cognate antigen:MHC complex. Most T cells have a TCR that does not recognize the antigen:MHC complex presented on the DCs and these return to the blood stream through efferent lymphatics and continue to circulate in blood and lymphoid tissue until they encounter an APC presenting their cognate antigen:MHC complex. However the T cells that express the specific TCR against the antigen binds to the antigen:MHC complex and go through multiple rounds of clonal proliferation and develop into effector T cells.

Generally three distinct signals are required for the optimal activation of naïve T cells. The T cells recognition of the antigen:MHC complex on APCs is referred to the first signal. The activation of naïve $CD4^+$ T cells involves the recognition of cognate antigen presented on MHC II molecules, while activation of naïve $CD8^+$ T cells require antigen presentation on MHC I molecules. The second signal is mediated through co-stimulatory molecules like CD80 and CD86 expressed by APCs that bind to CD28 expressed on the T cells. However multiple other co-stimulatory molecules and receptors, including members of the tumour necrosis factor (TNF) receptor family, are capable of providing signal 2 to

the responding T cells [10]. The third signal is mediated by cytokines produced by a variety of cells including APCs. The strength by which the TCR binds to antigen:MHC complex, the nature of the co-stimulatory molecules and their receptors as well as the composition of cytokines in the T cell microenvironment provide cues that direct the differentiation of T cells into subsets with appropriate effector functions to handle the antigen presented. After activation the effector cells are ready to participate in the immune response either by migration to affected tissue or by staying in the lymph node to provide help to adjacent cells.

2.1 $CD4^+$ T cell subsets

Effector $CD4^+$ T cells are usually denoted T helper (Th) cells since they possess specialized helper functions and contribute to immune responses mainly by producing cytokines and expressing receptors that can enhance other cells activity and function. There are multiple Th cell subsets that produce different arrays of cytokines and play distinct roles in the regulation of immune responses.

Th1 cells

Mosmann and Coffman [11] defined the Th1 cell subset already in 1986. The major regulator for Th1 differentiation and function are the T-box transcription factor (T-bet) and the cytokine interleukin (IL) -12, produced primarily by APCs, is central to drive Th1 cells differentiation and mediate expression of IL-18Rα [12]. Th1 cell development involves Signal Transducers and Activators of Transcription (STAT) 1 and STAT4 and all IL-12 mediated immune responses, including Th1 cell differentiation, were disrupted in mice that lack STAT4 [13]. Both IL-12 and IL-18 are potent inducers of the production of the primary Th1 effector cytokine, IFN-γ [14].

Th1 cells regulate many central functions within the immune system. For example they are important for clearance of intracellular infections by for example activating macrophages through production of IFN-γ [15]. IFN-γ can further promote the differentiation of more Th1 cells by suppressing the inhibitory effects of IL-4 on Th1 cell differentiation [16]. IFN-γ also possesses important roles in protection against tumour development by for example directly suppressing tumour growth and activating macrophages [17]. In

addition to host protective mechanisms a role for Th1 cells in immunopathological processes are suggested in chronic inflammatory and autoimmune diseases like Crohn's disease (CD) [18] and rheumatism arthritis (RA) [19].

Th2 cells

The major regulator for Th2 cell development are GATA-binding protein 3 (GATA- 3) and except for inducing the differentiation and function of Th2 cells GATA-3 supress STAT-4 expression and thereby inhibit the differentiation of Th1 cells [20]. IL-4 is an important cytokine in Th2 cell differentiation and can be produced by e.g. γδ T cells, ILCs and eosinophils but is also the major product of activated Th2 cells. Except for IL-4 the Th2 cells are associated with the production of IL-5 and IL-13.

Th2 cells are important in immune responses against extracellular parasites like helminth infections by for example regulating responses of B cells and activating mast cells [21]. IL-4 promotes B cells class switch into IgE and IgG1 that activate innate immune cells expressing high-affinity IgG1 and IgE receptors, like mast cells. In addition IL-4 induces the expression of the high-affinity IgE receptor on mast cells that release inflammatory mediators such as histamine when stimulated with IgE [22]. IL-4 is also involved in the upregulation of vascular cell adhesion molecule-1 (VCAM-1) expression on endothelial cells, increasing the infiltration of immune cells to tissues [23]. Finally Th2 cells are believed to play a central role in several immune mediated pathologies, most notably asthma and allergy [24].

Th17 cells

Th17 cells were initially identified in two papers published in 2005 describing a Th cell that produced IL-17A and whose developmental pathways was distinct from Th1 and Th2 cells. Also differentiation of naïve T cells into Th17 cells was inhibited by the presence of IFN-γ and IL-4 [25, 26]. The master regulator in Th17 cells is retinoic acid receptor-related orphan receptor gamma-T (RORγt) and several factors have been implicated in driving the differentiation and maintenance of Th17 cells. IL-6 and transforming growth factor (TGF)-β are thought to play central roles in promoting naïve T cells differentiation into Th17 cells [27, 28]. However Th17 cells can develop in absence of TGF-β *in vitro* in presence of IL-1β,

IL-6 and IL-23 [29]. During $CD4^+$ T cells differentiation into Th17 cells these cells upregulate IL-23Rα and start to produce IL-21. IL-21 and IL-23 are important factors in amplification and stabilization of this subset [27]. Th17 cells are primarily characterised by their expression IL-17A (referred from this point as IL-17) but these cells in different contexts are capable of producing a wide range of additional cytokines including IL-10, IL-17F, IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Th17 cells have a protective role in the clearance of extracellular bacterial and fungal infections by for example producing IL-17 that promotes neutrophils migration and maturation [30]. Th17 cells is one of the sources of IL-22 which is thought to regulate key functions within barrier immunity and integrity by induction of antimicrobial peptides like RegIIIβ and RegIIIγ [31] and induction of genes encoding survival, proliferation and mucus in epithelial cells [32].

In addition to their protective functions Th17 cells are suggested to play critical pro-inflammatory pathogenic roles in a range of autoimmune and chronic inflammatory disorders including multiple sclerosis, RA and IBD [30, 33, 34]. Factors that are licensing Th17 cells with pathogenic properties are currently being investigated. Characteristics of pathogenic Th17 cells notably include production of IFN-γ and expression of IL-18Rα and CXCR3 and are thought to occur when Th17 cells differentiate in absence of TGF-β [29, 33]. Indeed a large proportion of the IL-17 producing cells found in models of colitis and experimental autoimmune encephalomyelitis (EAE) co-produce IFN-γ [33]. Exposure to IL-23 diminishes the concentration of the anti-inflammatory cytokine IL-10 in developing Th17 cells and thus is also a factor that makes these cells pathogenic [35]. A recent work by Lee et al reported that IL-23 actually controls the production of TGF-β3 in developing Th17 cells and that TGF-β3 production was directly driving the pathogenicity of these cells [36]. Polymorphism of the receptor for IL-23 has been genetically linked to many human autoimmune diseases, including psoriasis, IBD and ankylosis spondylitis [36]. Other studies have shown that GM-CSF, which is produced by Th17 cells themselves and transactivated by RORγt, is also required for Th17 cells to acquire pathogenic functions in EAE [37, 38]. Importantly, studies in both humans and mice suggest that IL-17A and IL-17F may play redundant functions in intestinal inflammation [39]. Additional work is required to clearly identify the factors responsible for the licensing of Th17 cells with pathogenic properties and also to define the

molecular signature of pathogenic versus non-pathogenic effector Th17 cells, especially in IBD.

Tfh cells

Follicular Th (Tfh) cells function primarily to expand and differentiate B cells during immune responses. The master regulator of Tfh cells is B cell lymphoma 6 (Bcl6) and after priming of Tfh cells in the T cells zones they lose their expression of CCR7 but gain expression of the receptor CXCR5 that mediate their migration into B cell follicles where the ligand, CXCL13, is expressed. Tfh cells have high levels of surface receptors with critical roles for T-B cell interactions for instance are high levels of CD40L expressed on Tfh that promote proliferation, activation, differentiation and survival of B cells [40, 41]. Tfh cells can produce a variety of cytokines including IL-21 that is important for B cell affinity maturation [40]. In addition Tfh cells, depending on the cytokine environment, can produce IFN-γ, IL-13, IL-5 and IL-4, indicating that Tfh might be a specialized differentiation state of activated Th cells [40].

Treg cells

Th cells with suppressive functions are called regulatory T cells (Tregs) and possess important functions in regulating immune responses and in maintaining tolerance against self-derived antigens as well as to harmless foreign antigens like those derived from the diet. Soluble molecules such as IL-10 and TGF-β as well as cell surface molecules including cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) are believed to underlie the suppressive activity of Tregs [42].

Regulatory $CD4^+$ T can be broadly divided into two major subsets; forkhead box P3 $(FoxP3)^-$ T regulatory type 1 cells (Tr1) and $FoxP3^+$ Treg. $FoxP3^+$ Tregs are further divided into natural Tregs (nTregs) (or thymic T cells (tTregs)) and induced Tregs (iTregs) and FoxP3 is a central transcription factor driving the differentiation and function of these cells. nTregs develop already in the thymus and are thought to develop from T cells expressing TCRs with a high affinity towards self-derived antigens presented. However the affinity of the TCR binding must be low enough to escape the apoptotic signals induced in the negative selection process [43]. iTregs, on the other hand, develop from circulating naïve $CD4^+$ T cells

following their encounter with antigen in the periphery. nTregs and iTregs have been suggested to differ in their protective functions where nTregs protect the body from autoreactive T cells (mediating immune responses against self-derived antigens) while iTregs protect and control immune responses to non-self antigens [43].

The generation of iTregs depends on TGF-β signalling and their differentiation *in vitro* is dependent on IL-2 and the presence of the Vitamin A metabolite retinoic acid further promote this differentiation [44-47]. In both humans and mice it has now been shown that FoxP3$^+$ Tregs are not a homogenous population but can co-express surface markers and transcription factors mirroring the subsets of Th1, Th2 and Th17 cells [48-51]. This suggest that Tregs, like Th1, Th2 and Th17 cells may develop into specialized subsets with diverse functions that are able to co-localize and effectively regulate the particular Th cell subset that is activated in a particular immune response. Indeed Duhen et al [48] could see that the different Treg subsets, Th1-, Th2- and Th17-like Tregs, expressed equivalent chemokine receptors on their surface and produced the same effector cytokine (although at lower levels) as their corresponding Th cell subset. The Treg subsets also differed in their ability to produce IL-10, while Th1- and Th17-like Tregs produce high levels of IL-10 Th2-like Tregs failed to produce this cytokine suggesting that the different Th-like Treg subsets have adopted distinct ways to regulate immune responses [48].

Plasticity in Th cell lineage and functionality

Initially Th cell subsets were thought to be fixed in their defined lineage but growing evidence suggest that effector CD4$^+$ T cells display a degree of plasticity and may alter their cytokine profile, and thus functionality, in response to local environmental queues. For example Hirota et al [34] utilizing IL-17A fate reporter mice to track the fate of Th17 cells *in vivo* observed in experimental autoimmune encephalomyelitis (EAE) that Th17 cells lost their production of IL-17 in the inflamed spinal cord and upregulated production of IFN-γ. This transformation into IFN-γ producing cells was associated with a loss in RORγt and IL-23R expression and a gain in expression of T-bet and IL-12Rβ. However during *Candida albicans* infection Th17 cells rapidly shut down IL-17 production, but instead of gaining a Th1-like phenotype these cells appeared quiescent [34].

2.2 $CD8^+$ T cell subsets

The function of $CD8^+$ T cells is to discriminate between immunological self and non-self. $CD8^+$ T cells screen cells based on the peptide array they present on MHC I molecules. If their TCR detects a foreign or non-self antigen e.g. on tumour or virus infected cells they can lyse their targets using different mechanisms including release of cytotoxic granules or by activating cell-surface death receptors on the target cell [52]. Naïve $CD8^+$ T cells are like all T cells activated into effector cells in lymphoid organs by professional APCs presenting their cognate antigen:MHC I complex. However these APCs must be able to present exogenous antigen on MHC I molecules and have therefor evolved a process termed 'cross-presentation' in which antigenic material is taken up by phagocytosis, processed into peptides, either in the cytosol by the proteasome (cytosolic pathway) or in the phagosome by lysosomal proteolysis (vascular pathway), loaded on MHC I molecules in the phagosome or in the endoplasmatic reticulum (ER) and then transported to the cell surface [53]. Since their main function is cytotoxic killing of infected or tumour cells, it is often simplified that $CD8^+$ T cells only develop into cytotoxic T lymphocytes (CTLs). However, like $CD4^+$ T cells they can develop into different subsets depending on environmental queues [54, 55]. $CD8^+$ T cell subsets include T cytotoxic (Tc) 1, Tc2 and Tc17 cells that mirror their $CD4^+$ Th cell subset counterparts' cytokine production and expression of transcription factors [54]. Tc17 has been observed in multiple sclerosis lesions [56] and Huber et al showed recently that the presence of Tc17 is important to initiate inflammation in the EAE mouse model by supporting Th17 cell pathology [57]. In addition $CD8^+$ T cells primed in the presence of TGF-β have been shown to differentiate into cells with suppressive capacity [46, 58-60] and various subsets of $CD8^+$ suppressor cells ($CD8^+$ Tregs), either $FoxP3^+$ and $FoxP3^-$, have been described [55, 58, 60-64]. The $CD8^+$ T cell suppressor functions have been shown *in vivo* in models of graft versus host disease (GVHD), influenza infection and colitis to be dependent on IL-10 signalling [58-61, 64]. These studies highlight the importance of regulating the presence of suppressive $CD8^+$ T cells cause, while suppressive $CD8^+$ T cells are protective in diseases like GVHD and colitis the presence of these cells in infections hinder the clearance of the infection by suppressing effector T cell functions. In addition, $FoxP3^+CD8^+$ Tregs has also been observed in human diseases such as human immunodeficiency virus (HIV) infection and colorectal carcinoma [65, 66].

3. Initiation of adaptive immune responses in the intestine

The intestine is daily exposed to large amounts of foreign material derived from food, commensal bacteria as well as infectious pathogens. Consistent with its continual expose to foreign antigens the intestinal mucosa contains the largest numbers of immune cells in the body that collectively are thought to play a key role in maintain intestinal homeostasis. The initiation of intestinal adaptive immune responses is believed to occur primarily in lymphoid structures present within or draining the intestinal mucosa. The former are termed gut-associated lymphoid tissues (GALTs) and include Peyer's patches and solitary isolated lymphoid follicles (SILFs), and the latter include the MLNs. GALTs are separated from the intestinal lumen by a single cell layer of epithelia cells termed the follicle-associated epithelium (FAE). This epithelium contains specialized epithelial cells termed M (microfold) cells that can transport particulate luminal antigens such as bacteria and viruses from the intestinal lumen direct to the underlying sub epithelial dome (SED), an area rich in APCs. Having acquired luminal antigen APCs initiate the priming of T cells in GALT. GALT is also a primary site for the generation of IgA producing B cells, which subsequently migrate, via the circulation to the intestinal lamina propria (LP). IgA generated by intestinal lamina propria IgA plasmablasts is actively transported across the intestinal epithelium into the lumen and serves to limit bacterial colonization [67].

In contrast to GALT, intestinal draining lymph nodes receive antigen via intestinal draining afferent lymphatics, transported in intestinal derived migratory DCs, or potentially in free form within the lymph. Of note transport of luminal derived soluble antigen by intestinal DCs into the MLNs appears critical for the establishment of oral tolerance [68].

3.1 Role of antigen presenting cell subsets in the initiation of mucosal adaptive immune responses

Intestinal DCs are located both in intestinal associated lymphoid structures and LP and are thought to play an important role in maintaining intestinal homeostasis by inducing tolerogenic responses against harmless antigens derived from food and commensal bacteria while initiating protective immunity against infectious pathogens. The identification of new markers to separate classical DCs (cDCs) from other intestinal mononuclear phagocytes has lead to a deeper understanding of the particular roles of these cells in the intestinal mucosa [69, 70]. The majority of the DCs in the intestinal LP express the integrin CD103 [71] and these DCs migrate to the draining MLNs through the afferent lymphatic vessels [72-74]. In MLNs the migratory small intestinal DCs are believed to play an important role in both T cell activation against intestinal derived antigens and in the imprinting of T cells with receptors mediating their migration to the gut [69, 70]. The upregulation of the gut-homing receptors chemokine receptor 9 (CCR9) and the integrin $\alpha_4\beta_7$ was found to be dependent on the presence of the Vitamin A metabolite retinoic acid [75]. Further the number of $CD4^+$ T cells recovered from small intestinal LP was severely reduced in Vitamin A deficient mice compared to controls; this while the number of $CD4^+$ T cells located in lung or liver was unaffected [75]. Small intestinal LP derived DCs have the ability to imprint T cells with CCR9 and $\alpha_4\beta_7$ *in vitro* and this is likely due to their capacity to metabolize Vitamin A into retinoic acid [72, 74]. However *in vivo* experiments using lymph node transplantations suggest that MLN-derived stromal cells strongly contribute to the imprinting of T cells with CCR9 and $\alpha_4\beta_7$ as well [76, 77].

Intestinal $CD103^+$ cDCs are further divided into two distinct subsets, $CD103^+CD11b^+$ and $CD103^+CD11b^-$ DCs, that are dependent on different transcription factors for their development/survival; $CD103^+CD11b^-$ cells are dependent on IFN regulatory factor (IRF) 8, basic leucine zipper transcription factor, ATF-like 3 (Batf3) and Id2 while the $CD103^+CD11b^+$ cells are dependent on IRF4 and Notch-2 [69, 70, 78, 79]. The identifications of the diverse development requirements has led to the generation of genetically modified mice that specifically lack or have highly reduced numbers of one of the specific subsets and these mice can be used to identify the *in vivo* role of the particular subsets. The ability of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs to preferentially differentiate

$CD4^+$ T cells into certain Th subsets is under investigation. A potential role of $CD103^+CD11b^+$ DCs in the differentiation of $CD4^+$ T cells into Th17 cells in the MLNs was recently suggested in an *in vivo* study where mice with reduced numbers of $CD103^+CD11b^+$ DCs (due to deletion of *IRF4* in $CD11c^+$ cells) in small intestine and MLNs had significantly reduced numbers of Th17 cells in intestine and MLNs [79]. DCs isolated from these mice had lower levels of IL-6 mRNA compared to controls and, since IL-6 is an important cytokine in Th17 cell differentiation, this may be one of the reasons behind the reduced numbers of Th17 cells encountered in these mice [79]. While a recent paper by Cerovic et al it suggested that intestinal derived $CD103^+CD11b^-$ DC play an important role in the initiation of $CD8^+$ T cell responses to epithelial derived self-antigen in MLNs [80], the role of these IRF8 dependent DCs in intestinal T cell homeostasis remains largely unclear.

4. Memory T cells

While most effector T cells generated in the primary immune response die after the clearance of infection, a few persist as long-lived antigen-specific memory T cells. These memory T cells serve to protect us against re-infection with the same pathogen. Most human memory T cells are though to be generated early in life (before 20 years of age). Between the age of 30 and 65 the frequency of memory T cells does not increase. In mice the mechanisms that maintain memory CD4 and $CD8^+$ T cells differ somewhat; maintenance of $CD4^+$ T cells are thought to be dependent on TCR signalling [81] while maintenance of $CD8^+$ T cells are believed to be independent on TCR signalling but instead rely on the cytokines IL-7 and IL-15 [82]. Interestingly, after 65 years of age newly generated memory T cells in humans appear defective in their ability to expand and achieve full effector functions upon re-infections, while memory T cells formed in youth from the same individual still mount protective recall responses [83-85].

The classical way of identifying memory T cells in mice is by their high expression of CD44 and low expression of CD45RB while in humans the expression of CD45RO and the lack of CD45RA distinguish effector/memory from naïve T cells. However, memory T cells are a heterogeneous population of cells that differ among other things in their migratory capabilities and locations. The central memory T (Tcm) cells circulate in the blood, spleen and through lymph nodes and express markers promoting migration to lymph nodes, including CD62L and CCR7. Effector memory T (Tem) cells can migrate into multiple peripheral tissues and express tissue-specific homing receptors to direct their migration to certain tissues [86, 87]. Recently a third human memory T cell subset was identified in the circulation that had a phenotype similar to naïve T cells ($CD45RA^+CD45RO^-$ expressing CD62L and CCR7) but also expressed the memory markers IL-2Rβ and CD95 [88]. These naïve-like memory T cells were denoted stem cell memory T cells (Tscm) since they had self-renewal capacity and could develop into Tcm and Tem cells *in vitro* [88]. Another member of the memory T cell family that was recently described are the tissue resident memory T cells (Trm) that do not circulate in blood but persist in peripheral tissues [89, 90]. These Trm cells could be separated from

circulating cells by their lack of CCR7 and upregulation of CD69, which all circulating memory T cells in the blood lack [91]. The expression of CD69 is otherwise associated with early T cell activation and might retain recently primed T cells in the lymph nodes by downregulating the expression of the sphingosine-1-phosphate receptor 1 (S1PR1) that is essential for T cell egress from the lymph nodes [92]. Whether CD69 upregulation on Trm cells is involved in their retention in tissues and if, in that case, this is dependent on the regulation of S1PR1 is not known. $CD8^+$ Trm cells also, in addition to CD69, upregulate the expression of CD103, preferentially at mucosal tissues where the CD103 ligand, E cadherin, is expressed on the epithelial cells. The developmental pathways and functions of these diverse memory T cell populations is under intense investigation, as their induction and maintenance forms the basis of effective vaccines. It has been suggested that Tcm cells may be important in systemic infections while Tem and Trm support immune responses in the tissue they repopulate [83].

4.1 Memory T cells in gut

The intestinal mucosa contains the greatest number of memory T cells in the body whose numbers accumulate with age [91]. Intestinal T cells reside throughout the intestinal LP and epithelial compartment (EC) however their phenotype and function differs dramatically between these compartments.

Intraepithelial lymphocytes

The T cells in the epithelium, called intraepithelial lymphocytes (IELs), are mainly $CD8^+$ T cells. IELs are divided into group a, conventional IELs, and group b, unconventional IELs [93] that collectively play important roles in maintaining epithelial integrity and in the defence of mucosal pathogens [94]. Group a IEL consists primarily of conventional $CD8\alpha\beta^+TCR\alpha\beta^+$ T cells and a minor population of $CD4^+TCR\alpha\beta^+$ T cells that are thought to migrate into the epithelium following their activation in intestinal lymph nodes. These cells represent the major IEL population in humans while there proportions in mice are lower and vary with mouse strain. Group b IEL does not express CD8β but express the CD8αα homodimer and either TCRαβ or TCRγδ. The origin and development of group b has been widely disputed and discussed [94].

Lamina propria lymphocytes

T cells occupying the intestinal LP consist of conventional T cells that previously been primed in lymph nodes by APCs presenting their cognate antigen in context of MHC molecules. The majority of the T cells are $CD4^+$ (CD4/CD8 ratio are approximately 2:1) and include Th17, Th1 and Treg subsets, discussed in chapter 2.1. Th17 cells possess important functions in epithelial integrity by their production of IL-22 and mediate clearance of infections by recruitment and activation of neutrophils [95]. The intestinal lamina propria contains populations of both nTregs and iTregs. Small intestinal DCs have *in vitro* been shown to have a high capacity to generate iTregs compared to their splenic counterparts and this was associated with their capacity to produce retinoic acid [44]. In addition $CD103^+$ DCs can promote iTreg differentiation by activating TGF-β, a process that required expression of the integrin α_v subunit [96]. Hadis et al recently demonstrated that iTregs generated in intestinal lymph nodes migrate to the intestinal LP where they undergo expansion, and further suggested that iTreg expansion in the LP was critical for the development of oral tolerance [97]. In addition iTregs appear to be critical regulators in the mucosal homeostasis as in their absence mice develop Th2 mediated intestinal pathology [98]. Recent studies have focused the role of the intestinal microflora in regulating the Th17/Treg balance in the intestinal LP [42, 99]. A special role in induction of Th17 cell development selective for small intestine was found for segmented filamentous bacteria (SFB) [100]. Also the development of Tregs are influenced by the microbiota and Smith et al showed that short-chain fatty acids, that are abundant microbial metabolites, could influence the number and function of colonic Tregs [101]. Thus commensal bacteria have the power to regulate the Th17:Treg balance, important to keep intestinal homeostasis, and could consequently be important factors in the development of intestinal chronic inflammatory diseases like inflammatory bowel disease (IBD) where an aberrant skewing towards the Th17 cell subset is observed [102].

5. Cytokine-induced innate-like responses in T cells

While cytokines are key regulators of both innate and adaptive immune responses, most studies assessing the impact of cytokines on T cells have focused on their role in T cell differentiation in the context of T cell receptor activation. It is however clear that certain cytokines when used in combination have the ability to stimulate memory T cells to produce cytokines in the absence of TCR stimulation [14, 103-107]. For example the cytokines IL-12 and IL-18 induce murine Th1 cells and effector $CD8^+$ T cells, as well as human circulating $CD4^+$ T cells, to produce IFN-γ in absence of TCR activation [14, 103, 105, 108]. The mechanisms by which IL-12/IL-18 induce IFN-γ production in Th cells is distinct from TCR induce IFN-γ production [109, 110]. The first notion of distinct pathways came with a study showing that cyclosporine A (CsA) treatment differently effected cytokine-induced and TCR- induced IFN-γ production; while IL-12 and IL-18 induced IFN-γ was totally resistant to CsA treatment, CsA totally inhibited TCR dependent IFN-γ production [109]. Further IL-12 and IL-18, but not TCR, induced IFN-γ production is dependent on growth arrest and DNA damage protein (GADD) 45β and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase kinase 4 (MEKK4) signalling and is sensitive to p38 MAPK inhibitors [110].

The addition of IL-15 to IL-12 and IL-18 containing cultures could increase the IFN-γ production from circulating human Th cells compared to IL-12 and IL-18 alone [103]. Similarly, TNF-like ligand 1A (TL1a) also increase the production of IFN-γ in circulating human $CD4^+$ T cells cultured with IL-12 and IL-18 in the absence TCR stimulation [108, 111]. In addition to induction of IFN-γ can IL-12, IL-18 and TL1a stimulated circulating $CD4^+$ T cells produce a range of other cytokines including GM-CSF, IL-6, and TNF-α [111].

5.1 Phenotype of cytokine responsive $CD4^+$ T cells

Studies have shown that it is mainly memory $CD4^+$ T cells that possess the ability to respond to cytokine stimulation [103, 104]. Munk et al showed that a minor population of the "naïve" ($CD45RA^+$) $CD4^+$ T cell compartment in human blood could be activated to produce IFN-γ in response to IL-12 and IL-18 stimulation [104], however these cells expressed IL-18Rα and might be Tscm cells. The phenotype of cytokine responsive cells is still a matter of debate. Sattler et al showed that circulating human $CD4^+$ T cells producing IFN-γ in response to a cytokine cocktail containing IL-1β, IL-6, IL-7, IL-8, IL-12, IL-15, IL-17, IL-18, TNF-α and macrophage inflammatory protein (MIP)-1α were mainly $IL\text{-}18R\alpha^+$ [103]. These $IL\text{-}18R\alpha^+$ $CD4^+$ T cells were mainly effector memory ($CCR7^-$) T cells and the authors observed that $IL\text{-}18R\alpha^+$, but not $IL\text{-}18R\alpha^-$, cells expressed functional IL-15R and IL-12R [103]. Two groups have identified the C-type-lectin receptor CD161 as a marker for circulating human $CD4^+$ T cells responding to IL-12, IL-18 and TL1a stimulation by production of IFN-γ [111, 112] as well as other cytokines including TNF-α, IL-6 and GM-CSF [111]. In paper I we further explore the phenotype of human memory $CD4^+$ T cells responsive to combinations of IL-12, IL-18, IL-15 and TL1a.

5.2 Role of cytokine-induced T cells in the early response to infections

While the above studies suggest that T cells can be induced to produce cytokines in the absence of TCR ligation, the *in vivo* role for cytokine-induced T cell responses remains less well explored. Kastenmüller et al identified a population of $CD44^{high}CD8^+$ T cells located in lymph nodes that contributed to the early (4 h post infection) IFN-γ production after oral infection with *Salmonella typhimurium* and after systemic infection with *Pseudomonas aeruginosa* [113]. This innate-like response was highly diminished in IL-18 knockout mice indicating a key role for IL-18-induced IFN-γ production from $CD44^{high}CD8^+$ T cells *in vivo* [113]. In addition splenic cells cultured with *Burkholderia pseudomallei* identified a subset of $CD44^{high}CD8^+$ T cells that within 15 h post infection produced IFN-γ, a response that was inhibited in presence of neutralizing monoclonal antibodies (mAb) to either IL-12 or IL-18 [105]. This rapid IFN-γ response

was also observed in cultured splenic $CD8^+$ T cells when stimulated with *Listeria monocytogenes* and mice infected with *Listeria monocytogenes* had increased percentage of IFN-γ^+CD8^+ T cells compared to uninfected controls 16 h post infection [105]. Recently activated $CD4^+$ T cells in mice infected with *Salmonella thyphimurium* gain the ability to rapidly respond to non-cognate antigen like LPS injections by producing IFN-γ. This non-cognate induction of IFN-γ was largely dependent on $CD4^+$ T cells ability to respond to IL-18 signalling [114, 115]. This non-cognate induction of IFN-γ in recently activated $CD4^+$ T cells was found to occur in absence of MHC II molecules. $CD4^+$ T cells were isolated from *Salmonella thyphimurium* infected mice and were transferred into either wild type (WT) or MHC $II^{-/-}$ mice, when these mice were treated with LPS approximately 30% of the recently activated $CD4^+$ T cells transferred into MHC $II^{-/-}$ mice produced IFN-γ [114]. These results suggest that recently activated $CD4^+$ T cells gain the ability to respond to non-cognate stimuli and this ability is likely to be preserved in the remaining memory cells that are formed since memory T cells can produce IFN-γ in response to IL-12 and IL-18 in absence of TCR activation. Collectively these results indicate that TCR independent cytokine-activation of T cells may play a role in early innate as well as during immune responses to pathogens.

Given that expression of IL-12, IL-18, IL-15 and TL1a are enhanced in a range of inflammatory diseases including Crohn's disease [116-121], it also seems possible that TCR independent activation of memory T cells may contribute to disease pathology.

5.3 Cytokines that regulate TCR independent T cell responses

IL-12

IL-12 is a heterodimeric cytokine that belongs to the IL-12 family of cytokines and is mainly produced by APCs [122]. IL-12 can bind and activate cells expressing the IL-12R that consists of the two receptor subunits IL-12Rβ (shared with IL-23) and IL-12Rβ2 (shared with IL-35)

IL-18

IL-18 belongs to the IL-1 family of cytokines and is produced as a 24kDa precursor, pro-IL-18, by various cell types including DCs, macrophages and intestinal epithelial cells [123]. Pro-IL-18 is kept in the cells cytosol and needs to be cleaved by proteases, like active Caspase-1, to form the active 18kDa IL-18 molecule that can be released from the cell. Caspase-1 gets activated when assembled in cytosolic complexes called inflammasomes that can form when certain cytosolic PRRs including Nod-like receptor 3 (NLRP3) sense the presence of for example viruses and bacteria [123]. Active IL-18 signals through the IL-18R complex that consists of the primary chain IL-18Rα (IL-1R5) and the accessory chain IL-18Rβ (IL-1R7) [124]. IL-18Rα is in $CD4^+$ T cells primarily associated with a Th1 phenotype [125].

IL-15

IL-15 belongs to the common γ chain family of cytokines and IL-15 mRNA has been found in various cell types including DCs, macrophages and epithelial cells [126]. The IL-15R is a trimetric receptor complex that consists of the common γ chain (shared with IL-2, IL-4, IL-7, IL-9 and IL-21), IL-2Rβ (shared with IL-2) and the unique IL-15Rα. It has been suggested that most IL-15 are not secreted from cells but rather bind IL-15Rα in the golgi complex before transported to the cell surface where it is presented to cells expressing the IL-2Rβ/γ-chain complex, a mechanism called trans-presentation [126, 127]. However cells expressing IL-15Rα can present IL-15 to IL-2Rβ/γ complexes located on the same cell, called cis-presentation [128].

TL1a

TNF-like ligand 1A (TL1a) belongs to the TNF family of cytokines and is expressed as a membrane bound trimeric molecule but can become soluble after proteolytic cleavage. A variety of cells can be induced to express TL1a for example human peripheral blood monocytes and monocyte-derived DCs could be induced to express both membrane bound and soluble TL1a upon microbial stimulation or after stimulation with immune complexes [129, 130]. Death receptor 3 (DR3) is a receptor for TL1a and is continuously expressed by T cells [131].

6. Inflammatory bowel disease

Chronic inflammatory disorders occurring in the gut mucosa are collectively called inflammatory bowel diseases (IBD). Ulcerative colitis (UC) and Crohn's disease (CD) are the two most common diseases within IBD. Inflammation in UC is restricted to the colon while CD while most frequently affecting the terminal ileum and proximal colon can affect the entire gastrointestinal tract. Inflammation in UC patients affects the mucosal layer and usually starts at the rectum and then extends along the entire colon. In contrast the lesions in CD are patchy and the inflammation is generally transmural [132]. In Europe 2.5-3 million people are estimated to suffer from some form of IBD [133]. The onset of IBD occurs usually early in life and most patients are diagnosed when they are between 15 and 30 years old, still IBD can appear at any age.

The etiology of IBD is, even after years of intense research, still not entirely known. The current prevailing hypothesis is that the mucosal immune system somehow looses tolerance towards the intestinal microflora resulting in an aberrant immune response against commensal bacteria, enhanced T cell infiltration and tissue destruction. Since this tissue destruction affect the integrity of the epithelial barrier more intestinal bacteria gain access to the intestinal tissue and more immune cells are recruited to this site to deal with the large load of antigens, all together causing a devastating inflammation in the intestine. The cause behind this abnormal immune response seems to involve several factors including genetic factors, in both the innate and adaptive immune system, alterations in barrier function but also environmental factors including the composition of the microbiota and diet [99].

6.1 Animal models of intestinal inflammation

Numerous animal models have been utilized to study inflammation in the intestinal mucosa, and while no single model truly represents a counterpart to human IBD, these models have been useful in identifying several pathways important in the maintenance of intestinal homeostasis and in

driving mucosal inflammation [134]. The models include chemically induced dextran sodium sulphate (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS) and oxazolone colitis models, genetically modified animal models of colitis such as the IL-10$^{-/-}$ and TNFΔARE mouse models [134] and bacterial induced colitis models like *Helicobacter hepaticus* triggered innate immune model [135]. Below follows a summary of the two intestinal inflammatory models utilized in the current thesis.

CD45RB^{hi} CD4^{+} T cell transfer model of colitis

A frequently used model to assess CD4^{+} T cell driven intestinal inflammation is the CD45RB^{high} CD4^{+} T cell transfer model of colitis in which splenic naïve (CD45RB^{high}) CD25^{-}CD4^{+} T cells are transferred into immune-deficient mice e.g. *RAG1*$^{-/-}$, *RAG2*$^{-/-}$ or severely combined immunodeficient (SCID) mice [136]. In this model, transferred naive CD4^{+} T cells are activated in response to intestinal commensal bacteria antigens and initiate a colonic transmural inflammation. This model normally develops colitis within 5-8 weeks after T cell transfer and has been extremely helpful to study early immunological events in intestinal inflammation as well as the perpetuation of the disease, but also to dissect the individual contributions of various T helper cell and regulatory T cells subsets in intestinal homeostasis [39]. The Th1 associated cytokine IFN-γ was found to be an important mediator in the initiation phase in this model [137]. Blocking experiments using mAb showed that blocking IFN-γ early after T cell transfers effectively prevented the development of intestinal inflammation [137]. However T cell-derived IFN-γ appears to be dispensable for disease pathogenesis [138]. The T cell transfer of colitis model has been used to study an eventual role of IL-23 in intestinal inflammation. It was shown that daily injections with recombinant (r)IL-23 accelerated the disease onset compared to saline treated controls [139]. Further this T cell transfer model was used to study the role of IL-12 and IL-23 by using *Rag*$^{-/-}$ mice lacking the IL-12 specific subunit p35, IL-23 specific subunit p19 and the IL-12 and IL-23 shared subunit p40, respectively. The inflammatory scoring of these mice showed that while *Rag*$^{-/-}$ mice lacking p40 and p19 were protected from the severe inflammation, mice lacking p35 had inflammatory scores similar to normal *Rag*$^{-/-}$ mice, suggesting that IL-23, rather than IL-12, is essential for development of colitis in this model [135]. It was later reported that IL-23, through direct signalling into T cells, drives intestinal T cell proliferation, promotes intestinal Th17 cell accumulation,

and enhanced the emergence of an IL-17^{+}IFN-γ^{+} population of T cells. Furthermore, IL-23R signalling in intestinal T cells suppressed the differentiation of Foxp3^{+} cells and T cell IL-10 production [140].

In addition to the increased knowledge of effector cytokines in colitis the T cells transfer model has contributed to our understanding of the role of regulatory T cells in intestinal diseases. Immunodeficient mice co-transferred with both naïve and effector/memory (containing regulatory CD4^{+} T cells) CD4^{+} T cells do not develop colitis [141, 142]. The protective cell subset was restricted to a CD4^{+} T cell expressing CD25 and these cells prevented colitis both when co-transferred with naïve CD4 T cells but also when transferred into mice with already established colitis [143, 144]. The protective mechanism of CD25^{+}CD4^{+} T cells did not entirely rely on IL-10 production since IL-10$^{-/-}$ CD25^{+}CD4^{+} T cells could supress colitis, although not as efficient as their IL-10$^{+/+}$ counterparts [145]. In addition the severe inflammation observed in T cell mediated colitis was attenuated with daily treatment of rIL-10 compared to controls [137]. In paper II we utilized this model to investigate whether the removal of IL-18Rα expression on naïve CD4^{+} T cells influenced their capability to induce inflammation in *Rag-1*$^{-/-}$ mice.

iFABP-tOVA model

The iFABP-tOVA transgenic mouse was generated in the laboratories of Leo Lefrancois and is a model to study CD8^{+} T cell responses directed towards an epithelial derived antigen in the small intestine. In these mice a truncated form of the model antigen Ovalbumin (tOVA) is expressed under control of the intestinal fatty acid binding protein promoter (iFABP) resulting in constitutive expression of cytosolic OVA in mature small intestinal epithelial cells. OT-I cells (transgenic CD8^{+} T cells expressing a OVA-specific TCR) adoptively transferred under steady state conditions initially expand in these mice but do not break the peripheral tolerance, which is maintained by various described mechanisms including induction of split anergy in CTLs and the PD-1:PD-L1 inhibitory pathway [146, 147]. However, if the OT-I cells transferred to IFABP-tOva mice are activated by an inflammatory trigger, such as a bystander infection or an adjuvant, the peripheral tolerance can be broken. This result in the expansion of OT-I derived CTLs, which destroy the OVA-expressing epithelial cells in the small intestine and thus cause severe inflammation [147, 148]. In paper III we

utilize this model to study the phenotype of tolerogenic and inflammatory $CD8^+$ T cells (OT-I) generated towards epithelial derived OVA. In this context, we addressed the role of the gut homing chemokine receptor CCR9 and two cDC subsets, IRF4-dependent ($CD103^+CD11b^+$ intestinal cDCs) and IRF8-dependent ($CD103^+CD11b^-$ intestinal cDCs) in this process.

6.2 Cytokines involved in IBD pathogenesis

The cytokine environment in the inflamed intestine of IBD patients plays key roles in shaping and driving the inflammatory response at this site. The cytokines present in mucosal tissue of IBD patients have been thoroughly studied and enhanced levels of several pro-inflammatory cytokines including TL1a, IL-18, IL-15, IL-12, IL-6, TNF, and IL-23 have been observed in either CD and/or UC patients [149-151]. By studying genetically altered mice and by blocking cytokines or administrating recombinant cytokines in animal models of IBD it is clear that cytokines are important regulators of intestinal inflammation. In addition a major breakthrough in IBD therapy came with a study discovering that a TNF inhibitor, a chimeric antibody called infliximab, was beneficial in CD patients [152]. Since then several additional TNF inhibitors have entered the market including the fully humanized adalimumab (used in patients with CD) and the human anti-TNF mAb golimumab (used in patients with UC) [153]. However approximately 10-40% of patients with CD and up to 50% of patients with UC do not respond to TNF therapy and around 60% loose their responsiveness to the treatments within 12 months. Development of anti-drug antibodies could be one possible reason behind this lost responsiveness. Thus there is still a need to develop new and more efficient drugs. Still the TNF inhibitors show that cytokines is a valuable target in patients with IBD and in the following paragraphs I have chosen to focus on the role of some of the key effector and regulatory cytokines that are though to possess important modulating roles in IBD.

IL-23

IL-23 is a member of the IL-12 family of heterodimeric cytokines and $CD14^+$ macrophages are one of the sources of IL-23 in patients with CD [154]. GWAS reported that polymorphism in the IL-*23R* mediates CD susceptibility [155]. A pathogenic role of IL-23 in controlling T cells

responses in the intestinal inflammation has been described in detail in previous chapters (2.1 and 6.1). However IL-23 is not only affecting the intestinal T cell responses but was found to be driving DSS induced colitis in $Rag^{-/-}$ mice. The IL-23 responsive cells were identified as an ILC that produced large amounts of IL-17 and IFN-γ when activated with IL-23 *in vitro*. In addition blocking the IL-12 and IL-23 shared p40 subunit (ustekinumab) were found in clinical trials to be effective in patients with active CD and particularly in those patients unresponsive to anti-TNF therapy [156].

IL-18

Increased bioactive IL-18 mRNA transcripts was detected in mucosal samples obtained from CD and UC patients compared to controls [116]. Blocking IL-18 signalling in DSS and TNBS induced colitis could prevent the development of severe colitis and weight loss [157-161]. These results could be confirmed using $IL\text{-}18^{-/-}$ mice in which TNBS could not induce sever inflammation [158]. Further Ten Hove et al showed an therapeutic effect of blocking IL-18 by administrate the naturally secreted inhibitor IL-18 binding protein (BP) to mice with already established colitis induced by TNBS treatment [157]. In contrast to these studies indicating that IL-18 have a pathogenic role in intestinal inflammation Takagi et al could show that $IL\text{-}18^{-/-}$ and $IL\text{-}18R^{-/-}$ mice developed a more severe inflammation when treated with DSS than the $IL\text{-}18^{-/+}$ and $IL\text{-}18R^{-/+}$ control mice. Both $IL\text{-}18^{-/-}$ and $IL\text{-}18R^{-/-}$ mice had higher mortality and higher histological scores compared to controls suggesting that IL-18 also might possess protective functions during IBD development [162]. This protective function of IL-18 was suggested to be due to its positive effect for early phase wound healing [162]. Thus it is seems like IL-18 have a dual role in intestinal pathogenesis and might have pathogenic or protective roles depending on what cell type it signals through.

TL1a

Enhanced mRNA levels of TL1a have been observed in mucosal biopsies from both CD and UC patients compared to controls, although the levels of TL1a are higher in biopsies from active CD patients compared to UC patients [111, 118, 120]. Two studies have by flow cytometer analyses shown that the frequency of $DR3^{+}$ T cells is enhanced in mucosal tissue from both CD

and UC patients [118, 120]. In contrast, Jin et al could not detect any increase in DR3 mRNA from biopsies either from CD or UC patients [111]. In paper I we estimated the proportion of $DR3^{+}CD4^{+}$ T cells in inflamed tissues from CD patients and could not detect any increase compared to healthy controls. Thus while it is clear that the levels of TL1a is increased in IBD patients mucosal tissue the levels of DR3 is still controversial.

Blocking TL1a/DR3 interactions has been shown beneficial in both DSS and TNBS induced colitis [163, 164]. Meylan et al blocked TL1a/DR3 interactions through antagonistic anti-TL1a mAb, DR3-Fc or antagonistic DR3 Fab in the TNBS model and observed that TL1a/DR3 blocked mice were significantly protected from weight loss and mortality compared to non-treated animals [163]. Takedatsu et al showed that weekly injections with an anti-TL1a antibody significantly protected from chronic colitis induced by DSS treatment i.e. attenuation of weight loss and colon shortening. In addition anti-TL1a treatment in DSS animals with established colitis enhanced these cells recovery phase with regained body weight and improved histological score compared to non-treated controls [164]. These studies indicate that blocking TL1a/DR3 interactions may have therapeutic benefit in IBD patients.

Multiple GWAS studies carried out on IBD patients with various ethnicities have linked a single-nucleotide polymorphism (SNP) in *TNFSF15* encoding TL1a to IBD susceptibility [165, 166]. T cells and monocytes isolated from patients with the disease-associated SNP have increased expression of TL1a after activation with immune complexes, suggesting that an aberrant expression of TL1a can contribute in the development of IBD [167]. To study the role of TL1a overexpression *in vivo* two groups generated transgenic mice with constitutive expression of TL1a in either the T cells or the $CD11c^{+}$ cells [163, 168]. Both groups observed that transgenic mice developed a spontaneous mild intestinal inflammation mainly affecting the small intestine and in particular the ileum. The small intestinal mucosa had increased IL-13 and IL-5 mRNA [163, 168] levels and neutralizing antibodies of IL-13 was highly effective in reducing pathology [163]. The effect of TL1a was dependent on DR3 since T cells with constitutive expression of TL1a in DR3-deficient mice did not show signs of intestinal inflammation and had normal levels of IL-13 mRNA [163]. These results suggest that overexpression of TL1a can drive IL-13 dependent small intestinal inflammation through DR3.

IL-10 and TGF-β

The importance of IL-10 signalling in regulating inflammatory responses in the intestinal mucosa has been shown both in IL-$10^{-/-}$ mice that develop chronic enterocolitis [169] and in animal models where exogenous administration of rIL-10 attenuates colitis [137]. Loss-of-function mutations in *IL-10* or *IL-10R* are linked to an early onset of IBD [170]. However administration of rIL-10 to patients with CD did not show any clinical improvement [171, 172]. The TGF-β rich environment usually present in the intestinal mucosa promotes the differentiation of regulatory T cells over effector T cells. However Monteleone et al observed that T cells isolated from mucosal tissues from IBD patients had increased expression of the negative regulator of TGF-β1 signalling, smad7, compared to controls [173]. Further stimulating LP mononuclear cells (LPMCs) isolated from CD patients with TGF-β1 had only had minor effects on the IFN-γ and TNF-α production compared to controls, however, in presence of an smad7 inhibitor, TGF-β1 clearly reduced the LPMCs production of IFN-γ and TNF-α. These results suggest that mucosal cells within CD tissue have lost their responsiveness towards TGF-β and blocking smad7 could reverse this. SMAD7 antisense oligonucleotids (GED0301) were recently used in a phase 1 clinical study for patients with active CD were all patients treated with the drug experienced a clinical response [174].

7. Aims of this thesis

The overall aim of this thesis work was to study mechanisms regulating differentiation and functionality of intestinal $CD4^+$ and $CD8^+$ T cells.

The specific aims were:

Paper I. To assess the ability of IL-15 and TL1a to promote IL-12/IL-18 induced cytokine production in human memory $CD4^+$ T cells, identify putative markers of cytokine-responsive cells and determine whether such cells are present in the healthy and inflamed human intestine.

Paper II. To assess whether murine intestinal $CD4^+$ T cells express functional IL-18Rα and determine the role of this receptor in a murine model of T cell mediated colitis.

Paper III. To assess the role of intestinal DC subsets and chemokine receptor CCR9 in $CD8^+$ T cell tolerogenic and effector responses in the murine small intestine

8. Summary of key findings in the papers

Paper I: A major population of mucosal memory CD4$^+$ T cells, co-expressing IL-18Rα and DR3, display innate lymphocyte functionality.

In this paper we demonstrate that TL1a and IL-15, in a TCR independent manner, synergize to induce production of IL-5, IL-6, IL-13, GM-CSF, IFN-γ and TNF-α in human circulating CD45RO$^+$CD4$^+$ T cells in the presence of IL-12 and IL-18. TL1a and IL-15 induced proinflammatory cytokine production was dependent on the presence of IL-18 in the cultures while production of IL-22, GM-CSF and the Th2 associated cytokines IL-5, and IL-13 occurred independently of IL-12. These results indicate the IL-18 has a key role in TL1a and IL-15 mediated innate-activation of T cells.

Having determined a critical role for IL-18 in TL1a and IL-15 induced cytokine production we next sought to assess the phenotype of cytokine responsive cells. We could observe that IL-12/IL-18/IL-15 and TL1a exclusively induced production of IFN-γ, IL-6, GM-CSF and TNF-α in a subset of IL-18Rα$^+$CD45RO$^+$CD4$^+$ T cells that all co-expressed the receptor for TL1a, death receptor 3 (DR3). Both CD161$^+$ and CD161$^-$ IL-18Rα$^+$CD4$^+$ T cells produced IFN-γ after IL-12/IL-18/IL-15 and TL1a stimulation indicating that CD161 expression is not a suitable marker for cytokine responsive memory CD4$^+$ T cells. Instead cytokine responsiveness seems to be restricted to a subset of memory CD4$^+$ T cells expressing IL-18Rα and DR3.

Having demonstrated that IL-15 and TL1a synergized to induce production of proinflammatory cytokines we next measured the memory CD4$^+$ T cells ability to produce the regulatory cytokine IL-10 in response to cytokine stimulation. IL-12 and IL-15 was shown to produce IL-10 independently of IL-18 and TL1a. Although neither TL1a nor IL-18 had any effect on IL-12 and IL-15 induced IL-10 production on their own these two cytokines synergistically downregulated the IL-10 production when added together.

Peripheral tissues contain large numbers of memory $CD4^+$ T cells and we could show that most memory T cells in the healthy and inflamed human intestine co-expressed DR3 and IL-18Rα, and also produce cytokines in response to TCR independent cytokine stimulation. In the inflammatory setting that occur in intestines from patients with Crohn's disease (CD) we could show that the $DR3^+IL\text{-}18R\alpha^+CD4^+$ T cells localized in tertiary lymphoid structures together with IL-18 producing cells. These data suggest that cytokine-activated $DR3^+IL\text{-}18R\alpha^+CD4^+$ T cells might participate in driving the inflammation in CD patients by producing pro-inflammatory cytokines

Collectively these results suggests that cytokine-responsive memory $CD4^+$ T cells might contribute to the early defence against invading microbes but also driving chronic inflammation, in absence of their cognate antigen. Still more studies are required to directly assess the functional role of memory $CD4^+$ T cells.

Paper II: IL-18Ra deficient $CD4^+$ T cells induce intestinal inflammation in the $CD45RB^{hi}$ transfer model of experimental colitis, despite reduced IFN-γ production and a failure to secrete cytokines in response to cytokine stimulation.

Based on our finding from paper I that IL-18 is a key cytokine in driving TCR independent pro-inflammatory cytokine production in human memory $CD4^+$ T cells, in paper II we sought to address the potential role of IL-18 signalling in $CD4^+$ T cells during intestinal homeostasis and in T cell mediated colitis. We found that IL-18Rα and the TL1a receptor, DR3 were co-expressed on a large proportion of murine intestinal $CD4^+$ T cells including IFN-γ, IL-17, and TNF-α producing cells as well as $FoxP3^+$ $CD4^+$ T cells indicating that IL-18 could influence responses in various $CD4^+$ T cell subsets. Intestinal $CD4^+$ T cells from WT but not *il18rα* $^{-/-}$ mice produced GM-CSF, IFN-γ and IL-17 in response to IL-12 and IL-18 with or without addition of IL-15 and TL1a demonstrating that murine intestinal $CD4^+$ T cells can be activated by cytokines and that IL-18Rα plays a central role in the induction of this response.

To study the importance of IL-18Rα expression by $CD4^+$ T cells during T cell driven colitis we used the $CD45RB^{high}$ transfer model of colitis where splenic naïve ($CD45RB^{high}$) $CD4^+$ T cells transferred into *rag-1* $^{-/-}$ mice (that

lack B and T cells) responding to commensal bacteria cause a severe intestinal inflammation in colon. In this model almost all intestinal $CD4^+$ T cells expressed IL-18Rα during established colitis (6-7 weeks after transfer). Further recipients of *il18rα*$^{-/-}$ $CD45RB^{high}$ $CD4^+$ T cells displayed reduced numbers of IFN-γ producing $CD4^+$ T cells in the colonic lamina propria compared with recipients of WT $CD4^+$ T cells. WT $CD4^+$ T cells isolated from the inflamed colon maintained their responsiveness to IL-12/IL-18/IL-15 and TL1a while *il18rα*$^{-/-}$ $CD4^+$ T cells failed to produce cytokines in response to cytokine stimulation. Despite these findings *il18rα*$^{-/-}$ $CD45RB^{high}$ $CD4^+$ T cells were equally efficient at driving colitis. Collectively these results suggest that in this colitis model IL-18 signalling in $CD4^+$ T cells is dispensable for the induction/maintenance of intestinal inflammation.

Paper III: IRF-8-dependent DCs play a key role in the regulation of CD8 T cell responses to epithelial derived antigen in the steady state but not inflammatory setting.

In this study we utilized the iFABP-tOVA mouse model to assess the mechanisms impacting on mucosal $CD8^+$ T cell differentiation and function in the intestinal mucosa. To study the role of antigen-specific $CD8^+$ T cells in steady state conditions OT-I cells was adoptively transferred into these mice, OT-I cells expands but do not break the peripheral tolerance in these mice. However to study OT-I responses in inflammation we disrupted the peripheral tolerance by administrating the TLR7/8 agonist R848. Following transfer of naïve OT-I cells into iFABP-tOVA mice these cells differentiated into two major $CD8^+$ T cell subsets; $CD107a/b^+$ cytotoxic T cells (CTLs) and $FoxP3^+CD8^+$ T cells. Following transfer of OT-I cells alone, the majority of cells converted into $FoxP3^+CD8^+$ T cells, and these accumulated in the MLNs, small intestinal LP and epithelium. In contrast, oral administration of R848 resulted in a massive expansion of CTLs, which destroyed the epithelium leading to a dramatic weight loss of recipient mice. This occurred despite the parallel generation of $FoxP3^+$ OT-I cells. Further phenotypic analysis of responding OT-I cells demonstrated that $FoxP3^+CD8^+$ T cells, but not CTLs, expressed high levels of the gut homing receptor CCR9 and the integrin α_E (CD103)β_7 while the IFN-γ production was restricted to the CTL subset.

Given the selective expression of CCR9 on FoxP3$^+$ Tregs we next assessed what impact CCR9 deficiency had on the distribution and functionality of responding OT-I cells. We observed that transfer of *ccr9*$^{-/-}$ OT-I cells into iFABP-tOVA mice treated with R848 dramatically reduced the absolute number of FoxP3$^+$CD8$^+$ T cells recovered from small intestinal LP. However there was no increase or accumulation of these cells in the MLNs where the numbers of FoxP3$^+$CD8$^+$ T cells were comparable to what is observed after transfer with *ccr9*$^{+/+}$ WT cells. These results indicate that CCR9 deficiency not only affects the homing of these cells, but might also impair their overall expansion. Interestingly, CTLs entered the small intestinal LP with equal efficiency in the absence of CCR9 and in fact significant increased in the numbers in the LP compared to their WT counterparts. These observations indicate that CCR9 neither regulates the expansion nor the homing of CTL in this experimental setting, in line with the low levels of CCR9 expression by this subset.

To determine the role of DC subsets in the generation of the two distinct OT-I subsets we generated chimeric mice lacking either IRF-8 dependent or IRF-4 dependent DCs. iFABP-tOVA mice were lethally irradiated and reconstituted with bone marrow cells from *cd11c*$^{-}$*cre.irf8*$^{fl/fl}$ mice (to generate chimeric mice where the hematopoietic system fails to generate IRF8-dependent DCs) or *irf8*$^{fl/fl}$ (with an intact DC compartment). During steady state conditions the numbers and distribution of CTLs were not affected by the absence of IRF-8 dependent DCs. However we detected a major reduction in the total numbers of FoxP3$^+$CD8$^+$ T cells located in the small intestinal LP and epithelial compartments in cre+ mice compared to cre- controls. In contrast, during inflammation had OT-I cells primed in cre+ chimeric mice comparable numbers of FoxP3$^+$CD8$^+$ T cells and CTLs in small intestinal effector sites to cre- controls suggesting that the lack of IRF-8 dependent DCs can be fully compensated during inflammatory conditions.

Together these results demonstrate a key role for CCR9 and IRF8 dependent DCs in the generation and distribution of FoxP3$^+$CD8$^+$ T cells but not CTLs in response to an epithelial derived antigen in the small intestine. Importantly we demonstrate that IRF8 dependent DCs are dispensable for the FoxP3$^+$CD8$^+$ T cell subset differentiation and distribution in the setting of intestinal inflammation.

9. Discussion

9.1 Cytokine-activation of $CD4^+$ T cells

Phenotype of cytokine responsive cells

The phenotype of cytokine-responsive cells is just starting to emerge. Cytokine-induced responses in human circulating $CD4^+$ T cells are, in the absence of TCR signalling, restricted to a subset of antigen-experienced T cells [103, 104]. Sattler et al further showed that Tem ($CCR7^-$) cells rather than Tcm ($CCR7^+$) cells responded to cytokine stimulation by producing IFN-γ [103]. While the functional role of the diverse memory $CD4^+$ T cell subsets are still to be established these data suggest that Tem, rather than Tcm, can contribute to immune responses in a TCR independent manner.

Two markers have previously been proposed to identify cytokine-responsive $CD4^+$ T cells in human peripheral blood; CD161 [111, 112] and IL-18Rα [103, 104]. We showed in paper I that a subset of $CD4^+$ T cells expressing both IL-18Rα and DR3 exclusively produced IFN-γ, IL-6, GM-CSF and TNF-α after IL-12/IL-18/IL-15 and TL1a stimulation. These results are consistent with our results showing a dependency of IL-18 in IL-15/TL1a induced pro-inflammatory responses. While a large proportion of $IFN\text{-}\gamma^+IL\text{-}18R\alpha^+DR3^+$ $CD4^+$ T cells also expressed CD161 we also observed IFN-γ production in $CD161^-$ $18R\alpha^+DR3^+$ $CD4^+$ T cells suggesting that CD161 is not a selective marker for cytokine responsive cells that rather correlates with $CD4^+$ T cells expression of IL-18Rα and DR3.

Cytokine-responsive cells in the healthy human intestine

Identification of IL-18Rα and DR3 as markers of cytokine-responsive cells we wanted to observe whether cells with such phenotype were present in the intestinal mucosa that serves as a major reservoir of $CD45RO^+$ T cells [91]. The majority of intestinal $CD4^+$ T cells co-expressed IL-18Rα and DR3 and these cells were distributed throughout the healthy human intestinal LP.

After culturing intestinal LPMCs isolated from healthy human intestine with IL-12/IL-18/IL-15 and TL1a for 48 h approximately 60% of the $CD4^+$ T cells produced IFN-γ and this response was independent on TCR activation. Two different memory T cell subsets have been identified in the human intestine; the tissue circulating and tissue resident cells. Whether one of these cells is more sensitive to cytokine-stimulation than the other is still to be determined.

We observed that cytokine responsive IL-18Rα^+DR3$^+$ $CD4^+$ T cells in the human small intestine in addition to IFN-γ also produced IL-6, GM-CSF, TNF-α and IL-22 in response to IL-12/IL-18/IL-15 and TL1a and in peripheral blood it was also shown that memory $CD4^+$ T cells produced IL-5 and IL-13 in response to such stimuli, suggesting that these cytokine-activated cells can support various intestinal immune responses. In addition removal of either TL1a or IL-18 from the LPMC cultures induced the $CD4^+$ T cells to produce IL-10, a response that in peripheral blood memory $CD4^+$ T cells were shown to be independent on the presence of TL1a and IL-18. Thus depending on the intestinal cytokine environment these cells will acquire particular cytokine production in absence of TCR stimulation. Since we don't now the function of these cells we can only speculate that production of IL-12 and IL-15 might contribute to the homeostatic state by producing IL-10 that regulate effector responses, a combination of IL-18/IL-15 and TL1a will promote the production of Th2 associated cytokines (IL-5, IL-13 and GM-CSF) important in immune responses to parasites and IL-12/IL-18/IL-15 and TL1a might promote a strong pro-inflammatory response that can participate in the early innate responses by producing IFN-γ, IL-6, GM-CSF, IL-5, IL-13 and TNF-α that all in different ways support the innate immune system in the clearance of pathogens including activating and recruiting cells to the site of infection. However further studies are needed to confirm if such responses occur *in vivo* and whether such responses are relevant in immune responses.

Determine a role of cytokine-activated memory $CD4^+$ T cells responses in mice might be challenging. Laboratory mice live in germ-restricted environments and have, compared to humans, a short life span. Humans on the other hand are constantly in contact with microbes and have generated a broad palette of various memory cells at 20 years of age that are maintained through out their life time. Even though we in paper II observed that murine intestinal $CD4^+$ T cells in naïve mice expressed DR3 and IL-18Rα these $CD4^+$ T cells were far less responsive (approximately 5%) to cytokine-

induced activation compared to what we in paper I observed in their human counterparts (approximately 60%). These differences might make it difficult to fully understand the role of cytokine-activated $CD4^+$ T cells *in vivo* by using animal models.

9.2 IL-18 signalling in intestinal inflammation

While IL-12/IL-18/IL-15 and TL1a activation of intestinal $CD4^+$ T cells might contribute in the clearance of diverse pathogens, in the setting of a chronic inflammation such pro-inflammatory response might further promote inflammation. We could observe that the proportion of intestinal LP IL-18Rα^+DR3$^+$ $CD4^+$ T cells were maintained in patients with CD and that these cells mainly located in lymphoid structures together with IL-18 producing cells. This suggests that these lymphoid structures could serve as sites of cytokine-induced production of pro-inflammatory cytokines that support the inflammatory process.

While it has been shown that bioactive IL-18 is enhanced in intestinal mucosa in both UC and CD [116] the role of IL-18 signalling is not fully understood. Studies in animal models of colitis where IL-18 signalling was absent either by administration of IL-18 inhibitors or by using IL-$18^{-/-}$/IL-$18R^{-/-}$ mice showed both protective and pathogenic functions of IL-18 in intestinal inflammation [157-162]. IL-18 is a pleiotropic cytokine and the outcome of IL-18 signalling depends both on the cell type and the presence of additional cytokines suggesting that the different outcomes from the animal models could depend on what cells and cytokines are involved in the particular models pathogenesis.

We sought to determine whether IL-18 signalling was important for $CD4^+$ T cells ability to induce/maintain colitis by transferring either *il-18r*$^{-/-}$ or WT CD45RB^{high} $CD4^+$ T cells into *rag-1*$^{-/-}$ mice. Even though we could see that *rag*$^{-/-}$ mice had significantly fewer IFN-$\gamma^+$$CD4^+$ T cells in their intestinal mucosa and were unable to respond to IL-12/IL-18/IL-15 and TL1a stimulation these cells were as efficient as their WT counterparts in inducing/maintaining colitis. This mouse model of colitis has been shown to be dependent on IFN-γ in the initiation phase [136] but the critical IFN-γ production was not dependent on T cells ability to produce IFN-γ [138]. Further studies to establish the role of IL-18 in the different cell types are important to fully understand the role of IL-18 in intestinal inflammation

and whether IL-18 inhibitors that target specific cell types could have therapeutic effects in patients with IBD.

9.3 Regulation of $CD8^+$ T cell responses in intestinal mucosa

Two distinct subset of $CD8^+$ T cells are generated in the iFABP-tOVA mouse

In comparison to the regulatory mechanisms controlling $CD4^+$ Th and Treg differentiation rather little is known about what is regulating their $CD8^+$ counterparts in gut tissue. In paper III we used the iFABP-tOVA mouse model and observed the development of two distinct $CD8^+$ T cells after transfer of OT-I cells, $FoxP3^+$ and $CD107a/b^+$ $CD8^+$ T cells. CD107a/b marks degranulating functional cells and is from this point referred to CTLs and FoxP3 marks $CD8^+$ T cells with suppressive functions [58, 59, 61, 62, 65] and is from this point referred to $CD8^+$ Tregs. Both of the subsets developed under steady state conditions (adoptive transfer of OT-I cells) and in inflammatory conditions (adoptive transfer of OT-I cells and R848 treatment), which made it possible to study both of these subsets in the same host. During inflammatory condition we could observe expansion of both $CD8^+$ T cells subsets however the most drastic expansion was observed in the CTLs, consistent with the selective generation of inflammation in these animals.

We could observe that CTLs and $CD8^+$ Tregs primed in the iFABt-tOVA mice differed in receptor expression as well as in cytokine production. CCR9 and CD103 were highly expressed on $CD8^+$ Tregs while only a minor fraction of CTLs expressed these markers. IFN-γ-secreting OT-I cells were CTLs and most of the CTLs cells expressed Ly-6C [175]. Neither IFN-γ production nor Ly-6C expression could be observed in $CD8^+$ T regs. IL-10 production was enriched in the CCR9 and CD103 population. However although this suggest that the $CD8^+$ Tregs are likely the subset producing IL-10 there is still a minor population of CTLs and also a population of $FoxP3^-CD107^{-/-}$ OT-I cells that express these markers thus further experiments are needed to confirm this. These results collectively suggest that CTLs and $CD8^+$ Tregs cells might differ in homing capacities as well as in effector functions. In steady state conditions iFABP-tOVA mice

tolerize transferred naïve OVA-specific $CD8^+$ T cells and converting these antigen-specific cells into $CD8^+$ Tregs might be important in restricting antigen specific CTLs responses against OVA expressing small intestinal epithelial cells.

The role of homing molecules in the regulation of $CD8^+$ Treg responses

Since we had observed that CTLs and $CD8^+$ Tregs differed in their expression of CCR9 that is required for effective gut-homing of $CD8^+$ T cells in the steady state [176] we carried out experiments using *ccr9*$^{-/-}$ OT-I cells. In steady state conditions both CTLs and $CD8^+$ Tregs were diminished in their homing to the small intestine but the reduction was only significantly different for $CD8^+$ Tregs in the LP compartment. In the setting of inflammation the $CD8^+$ Tregs dependency of CCR9 was strikingly different from CTLs. In the setting of inflammation the CCR9-defiecient $CD8^+$ Tregs were drastically reduced in small intestinal LP while the CTLs accumulation in the intestine were independent of CCR9, consistent with the former cells expression of CCR9. The fact that $CD8^+$ Tregs rather than CTLs were dependent on CCR9 expression during inflammation suggested that blocking CCR9 in intestinal inflammation might not be beneficial but rather the opposite. Indeed we could detect a significant increase in the total number of CCR9 deficient CTLs in small intestinal LP compared to WT CTLs suggesting that $CD8^+$ Tregs may supress the expansion of CTLs at this site during inflammation.

In addition, we could not observe an accumulation of CCR9 deficient $CD8^+$ Tregs in the MLN during inflammatory conditions suggesting that this subset is dependent on signals/antigen presentation in gut mucosa for their expansion, which previously has been observed for $CD4^+$ iTregs [97]. Whether this expansion of Tregs occurs in response to local APCs presenting their cognate antigen or the cytokine environment or other factors is still to be determined.

The role of IRF-4 and IRF-8 dependent intestinal DCs in regulating $CD8^+$ T cell responses

The migratory intestinal $CD103^+$ DCs are divided into two distinct subsets $CD103^+CD11b^+$ and $CD103^+CD11b^-$ DCs and these subsets are dependent on distinct transcriptions factors for their development/survival;

$CD103^+CD11b^+$ DCs are dependent on IRF-4 and $CD103^+CD11b^-$ DCs on IRF8. By generating genetically modified mice where the $CD11c^+$ cells specifically lack either IRF-4 or IRF-8 the individual role of the $CD103^+CD11b^+$ and $CD103^+CD11b^-$ DCs, respectively, can be studied. While $CD103^+CD11b^-$ DCs were shown to be the main migratory subset of DCs in the gut-draining lymph that could cross-present epithelial derived antigen and could induce proliferation of naïve antigen-specific $CD8^+$ T cells under steady state conditions [80] little is known about the contribution of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs in regulation of $CD8^+$ T cell responses in the intestine.

In paper III we used chimeric mice where the mature small intestinal epithelial cells express OVA and the hematopoietic system fails to generate either IRF8-dependent DCs or IRF-4 dependent DCs to study their roles in expansion and differentiation of OT-I cells in steady state and inflammatory conditions. Our results suggest that the IRF-4 dependent DC subset does not play an essential role in the expansion or differentiation of $CD8^+$ T cells responses restricted towards an epithelial derived antigen since the total number and phenotype of CTLs and $CD8^+$ Tregs were preserved in absence of IRF-4 dependent DCs. In contrast, lack of IRF-8 dependent mice caused a marked reduction of the total number of $CD8^+$ Tregs recovered from small intestinal mucosa in steady state while total number of CTLs in small intestinal LP and epithelium was not affected. These results suggest that $CD103^+CD11b^-$ DCs is involved in the regulation of CD8 Tregs responses while it is dispensable for CTLs responses towards epithelial derived antigen. During inflammatory conditions no differences in the total number of either CTLs or $CD8^+$ Tregs could be observed. Thus in the inflammatory setting cells other than IRF8 dependent DCs drive CD8 T cell expansion and differentiation in the intestine.

Future perspectives

Although paper III gives hints of how homing molecules and DC subsets can influence intestinal $CD8^+$ T cell priming, differentiation and distribution many critical questions remain. For example it remains unclear where the priming of $CD8^+$ Tregs and CTLs occur in steady state and inflammatory conditions and what cells prime $CD8^+$ T cell responses in the setting of inflammation.

Some of the future plans include;

1) Address whether the $CD8^+$ Tregs expression of CD103 is important for homing and expansion of Tregs. By using $CD103^{-/-}$ OT-I cells we can use a similar approach as we used for establishing the role of CCR9.

2) Study whether inflammatory monocytes that infiltrate the inflamed intestinal mucosa are responsible for the compensatory expansion observed in $CD8^+$ Tregs during inflammation in absence of IRF-8 dependent DCs. These $CD11c^+$ monocyte-derived DCs have previously, in inflammatory conditions, been shown to be capable to cross-present antigen to $CD8^+$ T cells [177]. To address whether this is the case we plan to generate chimeric iFABP-tOVA mice where $CD11c^+$ specifically lack CCR2 expression. This set-up will exclude infiltrating inflammatory monocytes from the intestine and reveal whether these monocytes impact on $CD8^+$ Tregs expansion during both steady state and inflammatory conditions at this site.

10. Populärvetenskaplig sammanfattning

Dagligen handskas vår kropp med otaliga mängder av främmande partiklar, s.k. antigen, som tagits upp från vår omgivning. De flesta av dessa antigen är för oss helt ofarliga så som matpartiklar och harmlösa bakterier från tarmens egen bakterieflora. Dock finns även sjukdomsalstrande mikroorganismer, s.k. patogener, som kan ta sig förbi våra yttre barriärer så som huden och tarmens epitel, för att sedan infektera vår vävnad. För att skydda kroppen mot angrepp av patogener finns ett medfött och ett adaptivt immunförsvar som bland annat servar oss genom att skydda oss mot infektioner. En viktig uppgift för immunförsvaret är att skilja de harmlösa antigenen som vi vill att vår kropp ska tolerera från skadliga antigen som vi vill att immunförsvaret ska avlägsna. Ett misslyckande kan resultera i kroniska infektioner, allergier och autoimmuna sjukdomar.

Det medfödda immunförsvaret känner igen grupper av infekterande bakterier och virus och aktiveras snabbt, inom ett par timmar, för att döda och förhindra spridningen av patogenen. Det medfödda immunförsvaret inkluderar dendritiska celler som kan ta upp antigen från vår omgivning, bryta ner antigenet till små peptider som den sen kan presentera på sin cellyta på speciella receptorer. Dessa dendritiska celler har sedan en viktig uppgift att aktivera det adaptiva immunförsvaret.

T celler är en av huvudaktörerna i det adaptiva immunförsvaret. De bildas i benmärgen, mognar i thymus och cirkulerar sedan i blodet och lymfnoder som omogna (naiva) T celler. Varje T cell uttrycker en unik T cell receptor som specifikt känner igen ett särskilt antigen när det presenteras av på dendritiska celler. Eftersom det för varje antigen bara finns ett fåtal T celler som uttrycker den specifika T cell receptorn som känner igen antigenet, finns lymfnoder där dendritiska celler som tagit upp antigen och cirkulerande naiva T celler möts. Detta ökar chanserna för T cellerna att träffa på dendritiska celler som presenterar just deras specifika antigen och kan aktivera T cellerna. Då T cellerna aktiveras delar sig T cellerna intensivt och bildar en stor pool av identiska T celler som effektivt kan delta i immunförsvarets respons mot infektioner.

T cellerna delas in i två grupper baserad på deras uttryck av CD4- och CD8-protein på deras yta och dessa har olika sätt att handskas med patogener. $CD8^+$ T celler deltar klassiskt genom att döda celler som infekterats med virus eller bakterier genom att frisläppa giftiga ämnen. $CD4^+$ T celler kallas även hjälparceller och bidrar klassiskt genom att producera små signalsubstanser, s.k. cytokiner, som aktiverar närliggande celler. En viktig funktion hos T cellerna är deras förmåga att skapa ett immunologiskt minne vilket innebär att efter varje gång en naiv T cell aktiverats så kommer det att finnas kvar en liten del antigen-specifika T celler vilka snabbt kan aktiveras om samma patogen skulle dyka upp igen.

I min avhandling har jag studerat vad som reglerar aktiveringen och funktionen av $CD4^+$ och $CD8^+$ T celler både i frisk och inflammerad tarmvävnad.

I den första artikeln fokuserade vi på $CD4^+$ minnes T celler isolerade från humant blod och studerade hur de svarar på cytokin-stimulering i frånvarande av antigen-specifik aktivering. Här kunde vi se att cytokinerna interleukin (IL)-12, IL-18, IL-15 och TNF-like factor 1A (TL1a) kunde stimulera $CD4^+$ minnes T celler att producera cytokiner som vanligtvis produceras i inflammerad och infekterad vävnad. Vi observerade dessutom att samtliga inflammatoriska cytokiner som $CD4^+$ minnes T celler producerade var nästan helt beroende av att IL-18 var närvarande. Många $CD4^+$ minnes T celler finns i tarmen och vi kunde konstatera att dessa också svarar på cytokin-stimulering genom att producera inflammatoriska cytokiner. Detta innebär att $CD4^+$ minnes T celler möjligen kan bidra till avlägsnandet av infekterande patogener men också driva inflammation i tarmen i frånvaro av deras specifika antigen.

I tarmvävnad från patienter med kronisk inflammation i tarmen, s.k. inflammatorisk tarmsjukdom, har det visat sig att IL-18 är uppreglerad. Dock är det oklart om IL-18 har skyddande egenskaper eller bidrar till inflammationen. I den andra artikeln studerade vi hur avsaknandet av IL-18 stimulering i $CD4^+$ T celler från möss påverkade deras möjligheter att inducera inflammation i tarmen i immundefekta möss som saknar det adaptiva immunförsvaret inklusive T celler. Då naiva $CD4^+$ T celler injiceras i dessa möss så kommer de att aktiveras mot mössens bakterieflora och inducera inflammation i tjocktarmen. Även om vi kunde se att $CD4^+$ T celler som inte påverkats av IL-18 hade sämre förmåga att producera den inflammatoriska cytokinen interferon (IFN)-γ, så kunde de fortfarande initiera och driva inflammation i tarmen lika effektivt. Detta innebär att IL-

18 stimulering i denna inflammatoriska djurmodell inte påverkar $CD4^+$ T cellers förmåga att inducera inflammation.

I den tredje artikeln studerade vi regleringen och aktiveringen av $CD8^+$ T celler vars specifika antigen uttrycks i tunntarmens epitelceller. Här kunde vi se att regleringen och distributionen av aktiverade $CD8^+$ T celler kunde påverkas dels av deras förmåga att ta sig in i tarmen men att de också var beroende av specifika dendritiska celler som kunde presentera antigenet som de hämtat från epitelet. Detta bidrar till förståelsen hur $CD8^+$ T celler kan regleras i aktivering och expandering mot ett antigen uttryckt i tunntarmen.

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