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The role of CCL17 in murine experimental colitis

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ABBREVIATIONS

µM	micro molar
A	adenine
AA	amino acid
ABTS	2,2'-Azino-di-(3-ethylbenzthiazolin)-6-sulfonsäure
AD	atopic dermatitis
Aldh1a2	aldehyde dehydrogenase 1family, member a2
AP-1	activating protein-1
APC	antigen presenting cell
APC	allophycocyanin
ARE	AU-rich elements
ATG16L1	autophagy-related 16-like 1 gene
BMDCs	bone marrow derived dendritic cells
CARD15	caspase activation recruitment domain 15
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD	Crohn's disease
CD45RB	cluster of differentiation 45 receptor splice variant B
cDNA	copy desoxyribonucleic acid
cm	centimeter
Csf	colony stimulating factor
CFSE	carboxyfluorescin diacetate succinimidyl ester
CTLs	cytotoxic T lymphocytes
CX_3CR1	fractalkine receptor
CXCL	chemokine (C-X-C motif) ligand
DC	dendritic cell
DSS	dextran sulfate sodium
DT	diphtheria toxin
DTT	dithiothreitol

E	eGFP
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead box protein 3
g	gram
G	guanine
GALT	gut-associated lymphoid tissue
Gata3	GATA binding protein 3
GelE	gelatinase E
GI	gastrointestinal
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
GMCSF	granulocyte and macrophage colony stimulating factor
GWASs	genome-wide association studies
h	hour(s)
H&E	hematoxylin and eosin
HCl	hydrochloric acid
HRP	horseradish peroxidase
i.p.	intraperitoneal
IBD	inflammatory bowel diseases
ICOS	inducible T cell costimulator
ICOSL	ICOS ligand
IEL	intraepithelial leukocytes
IFN-α	interferon-alpha
IFN-γ	interferon-gamma
IL	interleukin
IP-10	IFN-γ inducible protein-10
IRFs	interferon regulatory factors

kDa	kilo dalton
LPL	lamina propia leukocytes
LPS	lipopolysaccharide
LysM cre	cre recombinase expression under the control of murine M lysozyme
M cell	microfold cell
MACS	magnetic activated cell sorting
MadCAM-1	mucosal addressin cell adhesion molecule-1
MALT	mucosa-associated lymphoid tissue
MDC	macrophage-derived chemokine
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
min	minutes
mLN	mesenteric lymph node
mM	milli molar
mRNA	messenger ribonucleic acid
n	number
N	normal
NEAA	non essential amino acids
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
ng	nano gram
NLRs	NOD-like receptors
NOD	nucleotide oligomerization domain
NOD2	nucleotide-binding oligomerization domain 2
O/N	overnight
Pam_3Cys	S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-cysteine
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDCs	plasmacytoid DCs
PE	phycoerythrin

PE-Cy	phycoerythrin-cyanine dye
PerCP	peridinin-chlorophyll
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
Poly(I:C)	Polyinosinic:polycytidylic acid
pOVA	ovalbumin peptide
PPs	Peyer's Patches
PRRs	pattern recognition receptors
qPCR	quantitative PCR
RA	retinoic acid
Rag	recombinase activating gene
RIG-I	retinoic acid inducible gene-1
RLR	RIG-I like receptor
rm	recombinant mouse
Rorc	retinoic-acid-receptor-related orphan receptor gamma
RORγt	retinoic-acid-receptor-related orphan receptor gamma t
RT	room temperature
SCID	severely combined immune deficiency
SD	standard deviation
SDS	sodium dodecyl sulfate
SED	subepithelial dome region
SFB	segmental filamentous bacteria
SNP	single nucleotide polymorphysm
SSC	side scatter
STAT	signal transducer and activator of transcription
T	thymine
TARC	thymus and activation regulated chemokine
T-bet	T-box transcription factor TBX21
TCR	T cell receptor
TGF-β	transforming growth factor-beta

T_H	T helper cell
TLR	Toll-like receptor
TMB	3,3′,5,5′-tetramethylbenzidine
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF-α	tumor necrosis factor-alpha
T_{reg}	regulatory T cells
TSLP	thymic stromal lymphopoietin
U	uracil
UC	ulcerative colitis
v/v	volume per volume
w/v	weight per volume
WT	wild type
XBP1	X-box binding protein 1
α4β7	integrin subunit alpha 4 and beta 7

1 INTRODUCTION

1.1 The immune system

The immune system has developed during evolution to counterstrike invading pathogens like viruses, bacteria, parasites and fungi, as well as environmental toxins. It consists of two branches, the innate and the adaptive acquired immune system.

1.1.1 The innate immune system

The innate immune system was long thought to be a fast and mainly unspecific response which is mediated by various hematopoietic cells like granulocytes, macrophages, monocytes, mast cells, natural killer cells and dendritic cells (DCs) as well as non-hematopoietic cells such as epithelial cells, hepatocytes and fibroblasts. However, in 1996 a receptor named Toll, which was discovered in *Drosophila,* was shown to function as pathogen recognition receptor[1]. This activation of innate immune cells by specialized receptors had already been proposed by Charles Janeway in 1989[2]. Shortly thereafter, the groups of Charles Janeway and Bruce Beutler discovered human and mouse "Toll-like receptor 4" (TLR4)[3, 4]. After that, many TLRs were identified and demonstrated to recognize PAMPs (pathogen-associated molecular patterns), specifically expressed by pathogens, allowing the host to discriminate between self and non-self patterns. For example TLR2, which is localized at the outer membrane of cells, was shown to recognize hemagglutinin derived from measles virus[5] or lipophosphoglycan derived from *Leishmania*[6]. TLR4, which is as well localized at the membrane, was shown to recognize lipopolysaccharides (LPS) derived from gram-negative bacteria[4]. However, besides the TLRs, which are expressed on cell surface membranes or on membranes of intracellular vesicles (TLR3, -7 and -9 are intracellularly expressed and recognize viral[7] or bacterial patterns[8, 9], respectively), additional receptor families of the innate immune system were discovered. These belong to the family of nucleotide oligomerization domain (NOD)-like receptors (NLRs), the family of RIG-I (retinoic acid inducible gene 1)-like receptors (RLRs), or to the family of C-type lectins. C-type lectins have been shown to be involved in the recognition of fungi[10], but also in the clearance of dying cells by phagocytes[11]. In contrast to TLRs, NLRs and RLRs were identified to be exclusively localized in the cytosol[12, 13]. As a common feature all four receptor types recognize molecular patterns

derived from pathogens and are therefore designated as pattern recognition receptors (PRRs). Their triggering by specific ligands leads to subsequent downstream activation of signaling cascades resulting in the activation of transcription factors NF-κB, AP-1 and IRFs which induce the production of effector molecules such as cytokines and chemokines[14, 15]. As the present study was conducted in mice, the following characterizations are based on what is known from studies in mice.

1.1.2 The adaptive immune system

While the diversity of the innate immune system is restricted by the ability of the PRRs to recognize molecular patterns of pathogens, which are germ line encoded, the adaptive immune system provides a much higher specificity, achieved by gene rearrangement. Cells of the adaptive immune system are T and B lymphocytes as well as natural killer T cells (NKT cells). Moreover, another special feature of the adaptive immune system is the development of long lasting memory allowing a faster response towards a specific pathogen in case of a repeated challenge.

However, in contrast to the direct activation of the cells of the innate immune system via pattern recognition receptors, adaptive immune cells need to be activated by cells of the innate immune system, presenting the foreign antigen[16]. This is provided by specialized antigen presenting cells (APCs). Besides macrophages and B cells, DCs are a very well characterized type of APCs which are most efficient in presenting antigens. Various subtypes have been identified so far and are associated with specific functions. Thus, DCs can be classified into conventional DCs (cDCs) and plasmacytoid DCs (pDCs)[17]. Although all subsets of DCs express PRRs, cDCs have been shown to be able to express TLR2 and TLR4 and efficiently produce interleukin (IL)-12 and activate T cells, while pDCs can express TLR7 and TLR9 and efficiently produce interferon (IFN)-α and are thus a part of the antiviral response[18, 19]. In general, pDCs differentiate from precursors derived from the bone marrow[20, 21]. cDCs however, either originate from the bone marrow[21], or differentiate from monocytes in the periphery under inflammatory conditions[22-24]. Nonetheless, a common feature of all DCs is to take up antigens, process these antigens and present them to other cells via specialized membrane receptors called major histocompatibility complex (MHC) class I[25] or II[26]. This is achieved by loading peptides derived from antigen processing onto MHC I or II

molecules within the DCs and presenting them on the cell surface[27, 28], subsequently leading to the activation of the adaptive immune system.

T cells, which express a T cell receptor (TCR) on their surface, are the target of this antigen presentation by APCs. The TCR recognizes the combination of MHC molecule and the peptide. However, the interaction of the TCR with MHC I and II requires specific coreceptors – cluster of differentiation (CD)4 and CD8 – expressed by T cells. CD4 is required for the interaction of the TCR with MHCII and CD8 is required for the interaction of the TCR with MHC I[29], respectively. $CD4^+$ T cells are mainly T helper lymphocytes (T_H cells) and regulatory T cells (T_{reg}) cells, while $CD8^+$ T cells are called cytotoxic T lymphocytes (CTLs). T_H cells foremost activate other cell types, whereas T_{reg} cells dampen immune reactions and CTLs kill infected cells.

The specificity and diversity of the adaptive immune response is on the one hand provided by B cells, which produce antibodies that are specific for the recognized antigen. On the other hand, the specificity arises from the TCR which is expressed on T cells, due to the fact that each TCR only interacts with one specific MHC/peptide combination. This specific recognition is achieved by positive and negative selection within the thymus, allowing only those T cells to enter systemic circulation that recognize foreign peptides but not "self" antigens[30-33]. Such selection should prevent the development of autoreactive T cells that harm the body. However, this selection is sometimes leaky and can lead to the development of autoimmune diseases like type I diabetes or multiple sclerosis which are caused by autoreactive T cells that have escaped negative selection in the thymus. To counteract these unwanted reactions against "self"-antigens as well as non-pathological foreign antigens like those derived from food, the immune system has evolved regulatory mechanisms. One of these mechanisms is the development of T_{reg} cells[34-36] which keep the immune response in balance in an antigen-specific manner. This is mainly achieved by producing IL-10[37-39], and transforming growth factor (TGF)-β[40], by direct effects on T cells[41-43], and by downmodulating DC function, e.g. by reducing the expression of costimulatory molecules on DCs via cytotoxic T lymphocyte antigen-4 (CTLA-4) interactions[44].

In general, the activation of $CD4^+$ T_H cells and $CD4^+$ T_{reg} cells by DCs is a multi-step process that needs three general signals (Figure 1). The first signal is the specific

recognition of MHCII/peptide combination by the TCR. Once the TCR has interacted with the MHCII molecules and the immunological synapse has formed[45], downstream signaling is turned on that subsequently activates the T_H cells.

The second signal is the interaction of costimulatory molecules expressed on APCs with their matching receptors expressed on T cells. For example, CD80 or CD86 are expressed on DCs and interact with CD28, expressed on the naïve T cells. This interaction elicits signal transduction in the T cells, resulting in enhanced activating of the T cells[46]. The third signal is the cytokine milieu at the site of APC-T cell interaction. This signal not only accounts for full activation of the T_H cells but also directs the nature of the T_H cells that develop. Thus, depending on the cytokines which are produced by the DCs, different types of T_H cells develop.

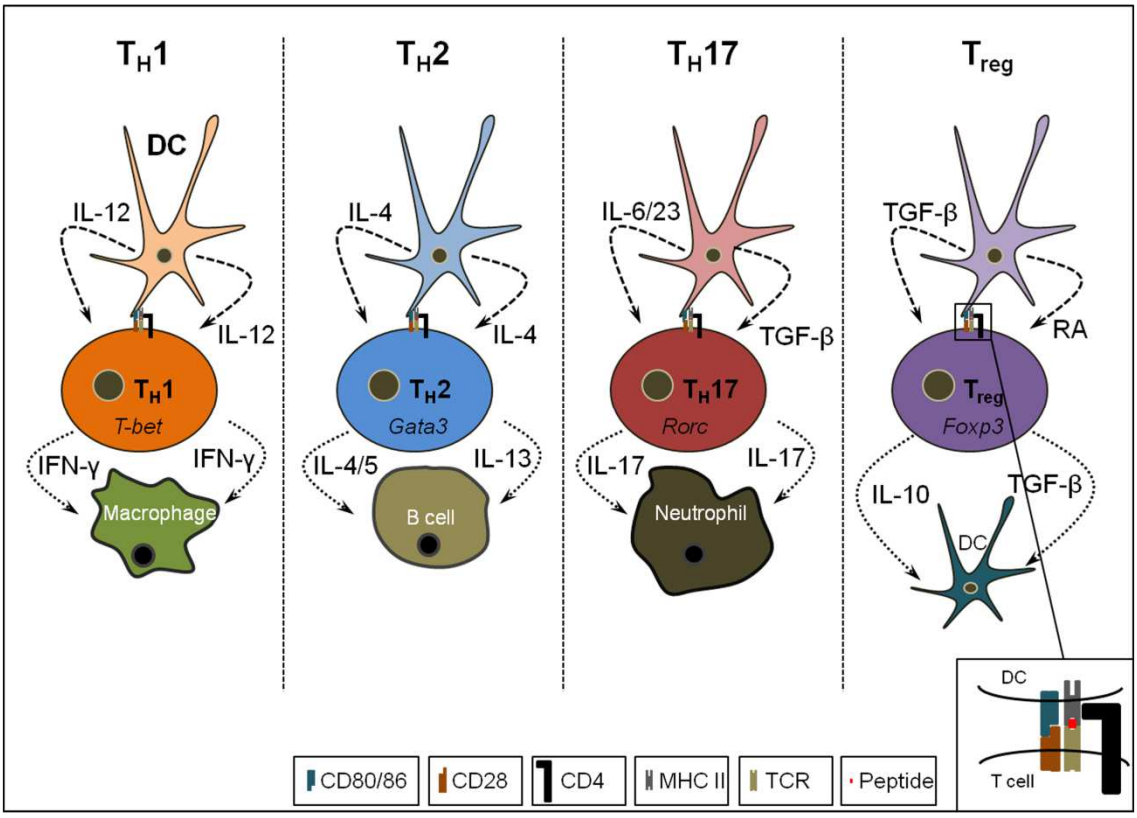

Figure 1: Differentiation of T cells under the control of DCs. DCs activate T cells in three steps. Step one is the interaction of the MHCII/peptide complex on the surface of the DC with the corresponding TCR on the surface of the T cell. The second step is the activation of the T cell by CD28 ligation with CD80/86. The third signal is driving the differentiation of the T cell and is provided by cytokines. Thus, IL-12 is driving T_H1 differentiation, IL-4 is driving T_H2 differentiation, IL-6 in cooperation with IL-23 and TGF-β is driving T_H17 differentiation and TGF-β together with retinoic acid is driving T_{reg} cell differentiation. (CD, cluster of differentiation; DC, dendritic cell; Foxp3, forkhead box protein 3; Gata3, GATA binding protein 3; IFN-γ, interferon gamma; IL, interleukin; RA, retinoic acid; Rorc, RAR-related orphan receptor gamma; T-bet, T-box transcription factor TBX21; TCR, T cell receptor)

These T_H cells can be characterized by specific transcription factors and are currently divided in three categories termed T_H1, T_H2 or T_H17 cells. T_{reg} cells also undergo this activation process and can as well be characterized by a specific transcription factor. In Figure 1, the different activation signals, specific transcription factors and downstream effector functions of the T_H and T_{reg} cells are illustrated.

Thus, it was shown that IL-12 production by macrophages and DCs leads to T_H1 differentiation[47], IL-4 promotes T_H2 differentiation[48] and IL-6 in combination with IL-23 and TGF-β leads to T_H17 differentiation[49, 50], whereas T_{reg} cells develop in the presence of TGF-β in combination with retinoic acid (RA)[51-54]. Further, it was demonstrated that the transcription factor T-box transcription factor TBX21 (*T-bet*) is characteristic for T_H1 cells[55], GATA binding protein 3 (*Gata3*) for T_H2 cells[56, 57], and retinoic-acid-receptor-related orphan receptor gamma (*Rorc*) for T_H17 cells[58]. Forkhead box P3 (*Foxp3*) has been shown to be crucial for T_{reg} cells[59, 60]. Each T_H subtype then activates or regulates different target cells. T_H1 cells activate macrophages and other phagocytes by production of IL-2 and interferon (IFN)-γ[61]. T_H2 cells provide B cell help, mainly by IL-4, IL-5 and IL-13 production[62] and T_H17 cells have been shown to activate and attract neutrophils by producing IL-17[63]. On the contrary, as mentioned above, the main function of T_{reg} cells is to suppress immune responses. This is not only achieved by regulating DCs, but also by direct downregulation of proinflammatory T cell responses mediated by $T_H1/17$ cells. These functions are mediated by both, soluble factors like IL-10 or TGF-β as well as by direct cell-cell contact for example via CTLA-4 expression on T cells.

Like $CD4^+$ T_H cells and $CD4^+$ T_{reg} cells, $CD8^+$ CTLs are also activated by APCs. However, as MHC I is expressed ubiquitously, literally all cells can be the target for CTLs. As mentioned above, the function of CTLs is to induce apoptosis in infected cells[64], which are presenting peptides of the invading pathogen on MHC I, leading eventually to the eradication of the pathogen itself.

The third key players of the adaptive immune response are B cells, which together with T cells are the mediators of long lasting memory of the immune system. Their dominating function is the production of specific antibodies that make pathogens detectable for phagocytes and professional killer cells (NK cells, macrophages), which

finally eradicate the invading pathogens. However, they are not in the focus of the work described here, and are therefore not further highlighted.

1.2 Gut-associated lymphoid tissue (GALT)

Securing the areas of the body with close contact to the enviroment, such as the skin and the lung is especially demanding for the immune system. Threats, such as viruses, fungi and bacteria, are most prominent, yet a constant inflammatory response should be avoided. The largest interface is at the gastrointestinal (GI) mucosa with approximately 400 m^2 of size[65]. The gut-associated lymphoid tissue (GALT) is localized along the GI tract which consists of the mouth, the esophagus, the stomach, the small and the large bowel. For a long time, the only function addressed to the GI tract was digestion and absorption of nutrients. However, research, especially in the last two decades, has revealed that the GI tract is the largest secondary lymphoid organ within the human body, which might harbor up to 60 % of all T cells[66]. Apart from the classical immune cells of the innate and the adaptive immune system which are originating from the bone marrow, it has been shown that the epithelial cells themselves play an important role in the immune response. On the one hand they form a tight physical barrier which protects against pathogens and are known to produce mucus that keeps pathogens from getting in contact with the epithelium[67, 68]. But on the other hand, epithelial cells have also been reported to produce immune mediators and to express surface molecules for interaction with immune cells. For example they can produce thymic stromal lymphopoietin (TSLP)[69, 70], a protein belonging to the family of cytokines, which has been shown to induce chemokine (C-C motif) ligand 17 (CCL17) production by DCs[71, 72]. As well, the expression of MHCII molecules on epithelial cells and the consequential presentation of antigens to T cells has been demonstrated[73-75]. However, to protect against harmful pathogens, while preventing inflammation or autoimmunity, the activation of classical immune cells like T cells, DCs, macrophages, neutrophils and granulocytes has to be tightly regulated in the GALT.

In the small intestine (Figure 2), this regulation takes place in special structural features called Peyer's Patches (PPs). These PPs are covered by epithelial cells, providing a barrier to the gut lumen. However, specialized epithelial cells covering the PPs, called

microfold cells (M cells) are able to directly take up antigens from the lumen[76] and transfer them to DCs residing in the subepithelial dome region (SED) of the PPs, lying directly beneath the epithelial cells[77]. These antigens are then either subsequently presented to T_H cells within the PPs or antigen loaded DCs might migrate to mesenteric lymph nodes (mLNs), draining the intestine, where they subsequently present antigen to T_H cells. T_H cell responses in the colon are primarily triggered within the mLNs. Primed T_H cells then circulate to the lamina propia (LP), lying beneath the epithelial layer in the gut. There, they either elicit an immune response or act in a regulatory fashion, depending on the response the respective antigen has induced. For the homing of naïve T_H cells to the mLNs, CD62L (L-selectin) is very important[78].

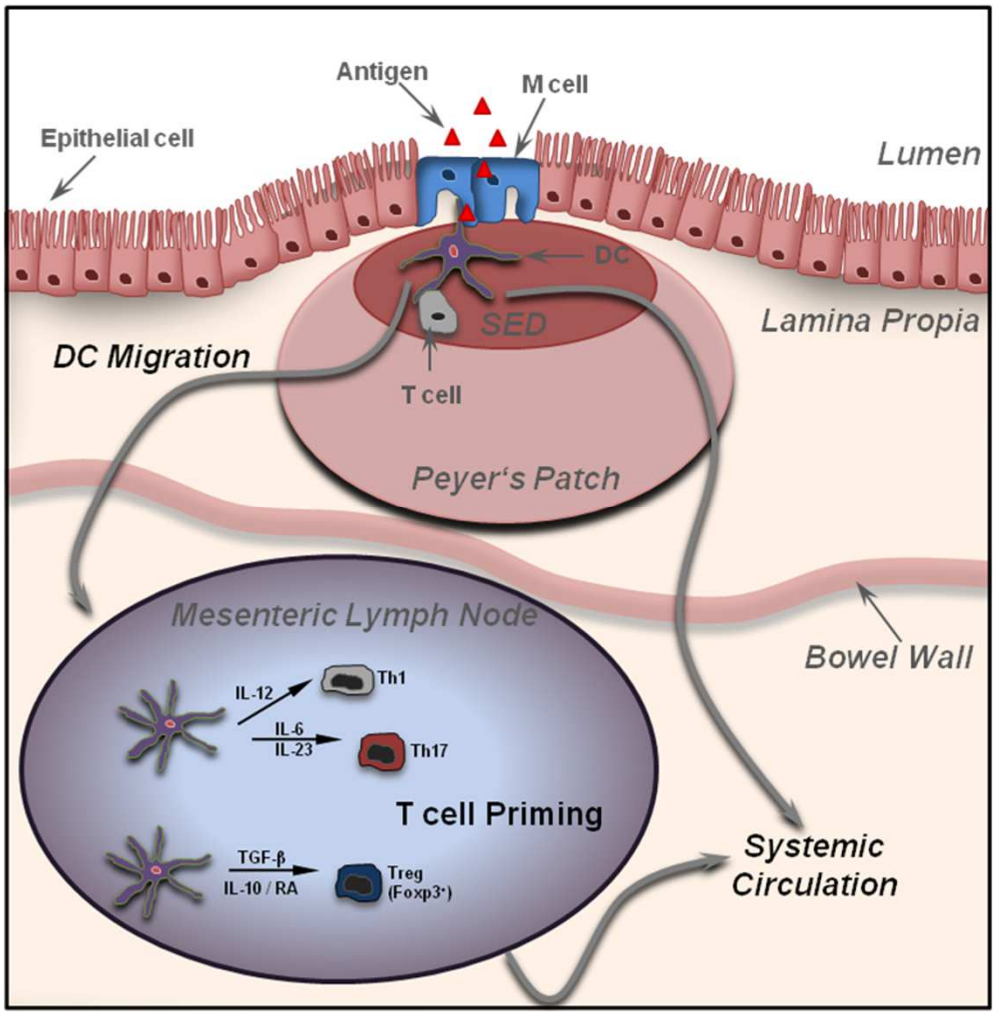

Figure 2: Organization of the immune system in the small intestine. Luminal antigens are sampled by specialized microfold (M) cells and presented to dendritic cells (DCs) in the subepithelial dome (SED) region of the Peyer's Patches (PP). Subsequently, DCs present antigen to T cells either within the PP or after migration into mesenteric lymph nodes (mLNs). T cells, primed by DCs, then enter systemic circulation. Depending on the antigen stimulus, DCs either elicit proinflammatory T cell responses by interleukin (IL) -12, or IL-6/-23 production, or regulatory responses by IL-10/TGF-β. Macrophages also participate in this process.

By binding to GlyCAM-1 (Glycosylation-dependent cell adhesion molecule-1) or MadCAM-1 (mucosal addressin cell adhesion molecule-1) it mediates, in cooperation with α4β7, the entry from the bloodstream into the mLNs in high endothelial venules[79-81]. In the mLNs these naïve T cells are then eventually primed by DCs and differentiate into effector T_H cells or T_{reg} cells. Then they reenter the circulation and migrate to the intestine where they conduct their functions.

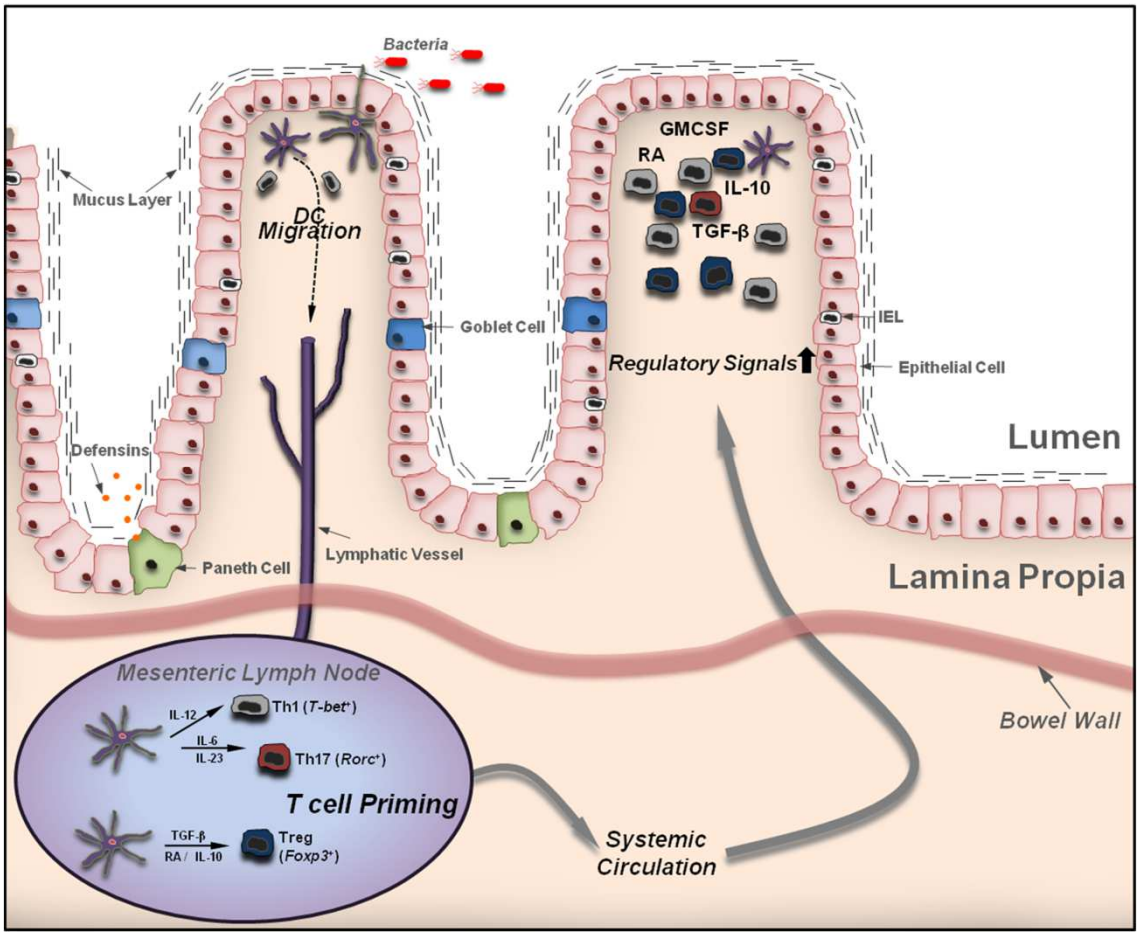

Figure 3: Organization of the immune system in the large intestine. Mucus, produced by goblet cells and epithelial cells provides a physical barrier, keeping potentially invading pathogens away from the epithelial layer which itself functions as a second physical barrier, tightly held together by tight junction proteins. Specialized CX_3CR1 expressing DCs can sense into the lumen by breaking up the tight junctions. Thus, they can sample luminal contents (antigens), and by circulating to the mLNs, present these antigens to T cells. In the mLNs, depending on the nature of the antigen, the T cells are subsequently primed to become either proinflammatory T cells (T_H1/T_H17) or regulatory T cells (T_{reg}). Primed T cells then circulate to the lamina propia and execute their functions. In the steady state, the balance between proinflammatory and regulatory signals is in a state of equilibrium and no excessive inflammation takes place. This is mediated by the production of regulatory factors like granulocyte and macrophage colony stimulating factor **(**GMCSF), retinoic acid (RA), IL-10 and TGF-β.

Like in the small bowel, the epithelial cells in the colon (Figure 3) from a tight barrier. This is achieved by expression of tight junction proteins, which keep the epithelial cells

in close contact. However, a specialized subset of DCs has been reported to extend dendrites between these tight junctions and is characterized by the expression of the chemokine receptor CX_3CR1 (fractalkine receptor) in the ileum[82]. These DCs sense with one of their dendrites into the gut lumen where they take up antigens. A recent report, however, describes CX_3CR1^+ cells as colonic macrophages[83]. Another subtype of DCs within the intestine is characterized by the expression of the the integrin αE (CD103) which has been reported to bind to E-Cadherin[84], one of the tight junction proteins. CD103 has been shown to be specific for DCs that migrate from the gut to the mLNs to present antigen[85].

Under steady state conditions, these $CD103^+$ DCs produce TGF-β and retinoic acid and preferentially induce T_{reg} cells, thus contributing to the homeostasis[86]. Additionally, they induce the gut homing molecules C-C chemokine receptor 9 (CCR9) and α4β7 on T cells[87, 88]. However, under inflammatory conditions, this tolerogenic property of $CD103^+$ DCs has been shown to be lost[85]. Moreover, a subset of intestinal DCs expressing E-Cadherin itself has been reported to promote intestinal inflammation[89].

In the intestine, the recruitment of immune cells from the gut to the mLNs is mainly mediated by the interaction of CCR 7 with CCL19/21[90]. CCR7 is expressed on the immune cells and CCL19/21 are produced by stromal cells in the T cell area of mLNs[91-93], leading to a directed migration of the $CCR7^+$ cells towards the CCL19/21-producing cells in the T cell area of mLNs.

Within the gut two major compartments have been described. One is associated to the epithelial layer and cells within this fraction are termed intraepithelial leukocytes (IEL). In this compartment, mostly $CD8\alpha\alpha^+$ T cells[94] , but also $\gamma\delta^+$ T cells[95] reside. Underneath, in the LP fraction, so called lamina propia leukocytes (LPLs) execute their functions. In the small intestine, T cells that have been primed within the PPs or the mLNs migrate to the LP or IEL-fraction via CCR9-CCL25 recruitment[96]. CCR9 is expressed on the T cells and CCL25 is produced by epithelial cells in the small intestine. In contrast, the recruitment to the LPL compartment in the large bowel is widely unknown. At least the α4β7 integrin has been suggested to play a role in T cell homing to the colon during inflammatory processes[97]. However, α4β7 could only be shown to be accountable for a part of the T cell homing to the colon.

1.3 Chronic intestinal inflammation / inflammatory bowel diseases (IBD)

In the steady state, the immune system in the GI tract has to be tightly regulated to prevent unspecific responses which might have deleterious effects. On the one hand, it has to deal with about $1*10^{11}$ to $1x*10^{14}$ commensal bacteria per gram of luminal content, that usually do not induce inflammation[98, 99]. On the other hand, it has to quickly eradicate invading pathogens and terminate this immune response afterwards to avoid an excessive immune response that might be harmful for the host. In contrast to those pathogenic bacteria that elicit an immune reaction, commensal bacteria live in symbiosis with the host and do not induce proinflammatory reactions. However, under pathological conditions, this regulation is disrupted or completely missing, leading to the development of chronic intestinal inflammations termed inflammatory bowel diseases (IBD). The two most prominent phenotypes of IBD are ulcerative colitis (UC) and Crohn's disease (CD).

1.3.1.1 Genetic factors driving IBD

IBD is an idiopathic disease with multiple factors described to be relevant for disease onset. Approximately 1 in 250 adults in Western countries is affected[100]. Both, genetic susceptibility caused by gene mutations, as well as environmental factors like bacteria (described in the following) or food constituents, have been shown to be required for IBD development. In genome-wide association studies (GWASs) several mutated genes have been identified to increase the risk to develop IBD. Most of these risk alleles are specific for UC or CD, however some have been identified to be relevant for both. In 2001, the *NOD2/CARD15* (nucleotide-binding oligomerization domain 2/caspase activation recruitment domain 15) gene was the first to be identified as a susceptibility gene for CD[101]. *NOD2/CARD15* is a member of intracellular NLR family and accounts for innate immune recognition of peptidoglycans from bacterial cell walls[102]. Highest expression is detected in Paneth cells in the crypt region of the intestinal epithelium and reported to be upreguleted by tumor necrosis factor (TNF)-α[103]. Along this line, in patients carrying *NOD2/CARD15* variants, a reduced secretion of α-defensins, which are produced by Paneth cells and demonstrated to have antibiotic effects, has been detected[104]. Other variants of the innate immune system, for example *TLR4*[105] or

CARD9[106], have been associated with increased risk for CD. As well, several parts of the adaptive immune system have been linked to the development of IBD. For instance, ICOSL (inducible T cell costimulatory ligand), which is expressed by intestinal epithelial cells and interacts with ICOS (inducible T cell costimulatory receptor) expressed on T cells, has been identified in a locus that contributes to the genetic susceptibility for CD[107]. In this context of T cell involvement in intestinal inflammation, IL-23-receptor (*IL-23R*) mutations have been identified to be associated with CD[108, 109]. IL-23R signaling plays a crucial role in the development and maintenance of T_H17 cells. In line with this involvement of T_H17 cells, variations of *CCR6*, which is expressed on T_H17 cells, have also been shown to predispose to CD[107]. In these cases, the recruitment of T_H17 cells to the colonic epithelium where CCL20, the chemokine binding to CCR6, is expressed could be influenced. In addition, loci coding for genes involved in autophagy have been identified by GWAS to be associated with IBD. For example, a variant of *ATG16L1* (autophagy-related 16-like 1 gene) has been shown to be associated with CD[110]. An important role of ATG16L1 in intestinal inflammatory processes has been confirmed in both human and murine studies with an involvement of Paneth cells and an associated lack of lysozyme[111, 112]. Moreover, XBP1 (X-box binding protein 1), a protein involved in endoplasmic reticulum (ER) stress responses has been associated with both, CD and UC[113, 114]. However, the identification of gene variants that were associated with the development of UC or CD alone, are not sufficient to account for disease onset. Altogether, the concordance rate for CD in monozygotic twins is only 30-35 %. And in UC patients the rate drops to 10-15 %[115]. This fosters the idea that environmental factors, which are discussed in the following, might be even more important in IBD development than the genetic susceptibility.

1.3.1.2 Environmental factors driving IBD

Western life style *per se* has been associated with a higher risk to develop IBD[116]. In particular, this has been attributed to the intake of special nutritional components like low fiber and high sugar/high animal fat[117, 118]. In addition, the increase of incidence in Asian countries and Eastern Europe has been associated with a adoption of a more Western life style in these regions[119, 120]. In a study conducted in the United Kingdom, a higher rate of relapse of treated UC patients has been correlated to red meat and alcohol consumption[121]. As well, an increase in the incidence for both, UC and CD in a

genetically stable population like that of Iceland in the last 50 years, can only be explained by changes in environmental factors[122].

Commensal bacteria within the gut are probably one of the most important environmental factors shaping the immune system in the intestine. This has been shown in several animal models by mono-association studies or bacteria-induced colitis models and an important role for intestinal microbiota has also been shown in patients treated with antibiotics that benefit from the treatment. Thus, it was demonstrated in mouse models that *Lactobacilli* on the one hand protected IL-10-deficient mice from *Helicobacter hepaticus* induced colitis development[123] and reduced susceptibility to chemically induced colitis[124, 125]. On the other hand, a bacterial product, the metalloprotease GelE (gelatinase E) derived from the commensal bacterium *Enterococcus faecalis*, was shown to interfere with epithelial barrier function during inflammation and thus increases severity of experimental colitis in mice[126].

Other environmental factors like cigarette smoking have also been implicated in both phenotypes of IBD. However, smoking seems to have adverse effects in UC and CD. In UC patients, non-smokers and former smokers have an increased disease activity, while in CD patients smoking increases the risk for a new flare [127].

Although most epidemiological studies give insights into risk factors driving IBD, functional analyses of the immune dysregulation underlying IBD is necessary for the development of new therapeutic drugs. This can be achieved by analysis of specimen obtained from intestinal biopsies or surgery of IBD patients as well as from animal models.

1.3.1.3 Dysregulation of the intestinal immune response in IBD

In spite of the fact that up to date the role of environmental, genetic or other factors leading to IBD, has not been clarified, research in the last two decades has shed much light on the molecular and cellular mechanisms accounting for the disease. As manifold as the genetic and environmental factors are that have been found to influence disease induction and maintenance, as manifold are the dysregulations of the immune system in the gut that are associated with IBD. Although much data from human studies are

available, mostly from specimen obtained from surgery or endoscopic biopsies, most functional data is derived from mouse models, which have been established to resemble humans' IBD. For instance, chemical induction of apoptosis in epithelial cells via dextran sulfate sodium (DSS) application in the drinking water has been shown to result in an inflammation in the intestine of mice, resembling UC. Another well established model has been introduced by Fiona Powrie *et al*. in 1993[128], in which mice lacking B and T cells were reconstituted with naïve $CD4^+/CD45RB^{high}$ T cells. The lack of mature B and T cells is either due to a defective recombination of the T and B cell receptors in severely combined immune deficiency (SCID) mice[129] or due to the lack of the recombination activation gene (*Rag*) in either *Rag1* or *Rag2* knockout mice ($Rag1/2^{-/-}$)[130, 131]. After transfer of naïve $CD4^+/CD45RB^{high}$ T cells, these mice develop an intestinal inflammation, foremost in the colon. This T cell transfer model of colitis has been modified several times using different subtypes of T cells for the transfer. For example Wirtz *et al*. used $CD4^+/CD62L^+$ splenic T cells for the transfer[132]. These T cells also express high levels of CD45RB, but are selected for the expression of CD62L, reported to be involved in mechanisms essential for the entry into the mLNs[78]. The use of genetically engineered mice, like $Rag1^{-/-}$ mice, for investigations on the immune response during intestinal inflammation led to the discovery of several fundamental mechanisms underlying IBD. Thus, pathways of both, the innate and the adaptive immune system were identified to be essential to either initiate experimental colitis, or to have a protective effect. Among many others, major players of the innate immune system that are involved in IBD are the TNF-α and the IL-6-STAT3 (signal transducer and activator of transcription 3) pathway. TNF-α mRNA stability is controlled by AU-rich elements (ARE) and mice lacking these elements ($Tnf^{\Delta ARE}$) overexpress TNF-α, which leads to a CD-like transmural intestinal inflammation[133]. This inflammation could be shown to be ameliorated in TNF receptor 2 deficient ($Tnfr2^{-/-}$)/$Tnf^{\Delta ARE}$ mice, and was absence in $Tnfr1^{-/-}/Tnf^{\Delta ARE}$ mice. An effect of TNF-α on the adaptive immune system could be demonstrated in $Rag1^{-/-}Tnf^{\Delta ARE}$ mice. These mice only develop mild, superficial inflammation in the gut, raising the thought that T and B cells are necessary to induce transmural inflammation. Although no mutation that links TNF-α to UC or CD is known, anti-TNF-α antibodies have been shown to be effective therapeutics for IBD[134], especially for CD.

IL-6, which signals through cell surface bound IL-6R, or through soluble IL-6R[135], has also been implicated in IBD[136]. Its signaling cascade either leads to STAT3 or STAT1 activation. IL-6 has been shown to be upregulated in both, CD and UC patients and anti-IL-6R therapy has been shown to be beneficial at least in patients with active CD[137]. In contrast, mice lacking the downstream signaling molecule STAT3 specifically in macrophages (*Stat3*$^{LysM\text{-}cre}$), developed spontaneous enterocolitis[138]. This observation showed that one pathway can lead to adverse effects in a cell type specific manner. Many other innate immune pathways have been identified to be involved in IBD development and maintenance. However, the majority of these pathways lead in the end to the activation of adaptive immune responses. This is especially obvious in IBD as these diseases are described to be chronic and relapsing. This was shown for IL-12 and IL-23. These cytokines are heterodimers, sharing much similarity, as both consist in part of the IL-12/23p40 subunit. IL-12p40 couples with the subunit IL-12p35 to form the bioactive IL-12p70 and IL-12p40 together with IL-23p19 forms the IL-23 heterodimer. Both are produced by DCs after PRR-mediated stimulation. IL-12 was originally shown to play a role in 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis[139], another chemically induced colitis model. Subsequently, studies revealed that IL-12p40 neutralization resulted in decreased IFN-γ production by $CD4^+$ T cells, linking innate to adaptive immune mechanisms during intestinal inflammation. Further evidence for the IL-12/T_H1 axis in IBD emerged when IFN-γ abrogation in SCID mice reconstituted with $CD45RB^{high}$ T cells resulted in diminished disease activity[140]. Moreover, it was demonstrated that *T-bet* deficient T cells, lacking the transcription factor driving T_H1 development, were no longer able to induce colitis in mice[141]. Although the data from mouse models were very promising, only minor effects of IL-12p40 or IFN-γ neutralization in human trials were observed[142, 143].

Like IL-12, IL-23 was also shown to be crucially involved in intestinal inflammatory processes. Thus, in an innate colitis model, induced by the injection of anti-CD40 antibodies in *Rag1*$^{-/-}$ mice, IL-23 was shown to be essential for intestinal pathology[144]. In a similar model, where intestinal inflammation is induced in *Rag1*$^{-/-}$ mice by oral gavage of the pathogenic bacterium *Helicobacter hepaticus*, IL-23 was linked to the induction of IL-17 by non-T cells[145]. Furthermore, in the transfer model of colitis, T cell mediated inflammation was also dependent on IL-23 and IL-23 expression on T cells. IL-23 was further shown to be essential for the expansion of T_H17 cells, linking innate

to adaptive immune responses in the context of IBD[146]. T_H17 cells reside within the small intestinal lamina propia even under steady state conditions. The frequency of T_H17 cells in this compartment is dependent on the colonization status of the mice, demonstrated by reduced numbers of T_H17 cells in germ-free mice. Moreover, the newly identified segmental filamentous bacteria (SFB), residing in the gut, were shown to be able to directly induce T_H17 differentiation[147]. Further, in humans' Crohn's disease, IL-17 secreting T cells were markedly increased in the disease-affected mucosa[148]. In general, T_H17 cells secrete two types of IL-17: IL-17 (IL-17A) and IL-17F[149]. IL-17A and IL-17F seem to play opposing roles during the immune response in the intestine. On the one hand, recent studies suggest a protective role of IL-17A. These studies showed that the transfer of IL-17A-deficient $CD45RB^{high}$ $CD4^+$ T cells, as well as the transfer of IL-17R-deficient T cells, surprisingly increased disease severity[150]. On the other hand, IL-17F-deficient mice were protected from DSS induced colitis[151], and IL-17A had a proinflammatory role in TNBS induced colitis[152]. Additionally, it was shown that *Rorc*-deficient T cells, lacking the transcription factor retinoic-acid-receptor-related orphan receptor gamma t (RORγt), characteristic for T_H17 cells, were no longer able to induce colitis after transfer into *Rag1*$^{-/-}$ mice[153]. Taken together, these controversial results suggest that more research will be needed to decipher the role of IL-17 in IBD development.

Counteracting the proinflammatory responses elicited by DCs and $T_H1/17$ cells, T_{reg} cells have been in the focus of extensive studies in recent years. In mice, these $CD4^+$ T cells are characterized by high expression of the IL-2Rα chain (CD25) and the transcription factor forkhead box protein 3 (Foxp3)[37, 154, 155]. Several studies investigated the mechanisms how T_{reg} cells suppress effector cell functions. Hence, it was demonstrated that the suppressive capacity of T_{reg} cells especially in the intestine foremost relies on the production of IL-10 and TGF-β, as well as direct cell-to-cell contact[37-40, 42, 43]. T_{reg} cells have further been shown to be able to reverse an already established intestinal inflammation in the $CD45RB^{high}$ T cell transfer model and even to prevent its onset[140, 156].

1.3.1.4 Chemokines in IBD

Apart from the different cellular responses elicited in mLNs and the gut, fundamental processes that are shaping the intestinal immune system are recruitment and recirculation of the immune cells. These processes are controlled by upregulation of adhesion molecules on the cell surface, or by expression of chemokines by cells in the destination area. Chemokines are small (7-15 kDa), structurally related molecules. Over 40 have been identified and can be grouped in four subfamilies depending on the arrangement of cysteine residues (C, CC, CXC or CX3C)[157, 158]. First evidence that chemokines are involved in IBD has been provided in 1992 and 1993 when 3 studies described that IL-8 (CXCL-8) is upregulated in UC and CD patients[159, 160]. In the following years, more attention was paid to the potential involvement of chemokines in the inflammatory processes that promote IBD. Hence, IL-8 was not only shown to be upregulated in IBD patients, but strikingly correlated with the expression of TNF-α[161].

In mouse models, many chemokines and their cognate receptors have been identified to be crucially involved in IBD development. For example the receptor CXCR3, and its ligands CXCL9, CXCL10 (IFN-γ inducible protein 10 (IP-10)) and CXCL11 which have been shown to be upregulated in IBD patients[162, 163], were functionally analyzed in mouse studies. Here, mouse experiments revealed that treatment with antibodies against IP-10 abrogated spontaneous colitis in IL-$10^{-/-}$ mice[164].

In line with this, CCL2 (monocyte chemotactic protein-1 (MCP-1)) and its receptor CCR2 have also been shown to be upregulated in IBD patients[165, 166]. Again, mouse experiments illuminated that CCR2 deficiency leads to reduced colitis activity in DSS treated animals and that blocking CCL2 had beneficial effects on both, inflammatory processes, as well as colitis associated cancer development[167, 168].

CCL5 (regulated upon activation, normal T-cell expressed, and secreted (RANTES)), which binds to CCR1 and CCR5 is induced in human IBD[165]. Subsequent studies in mice and rats revealed that CCR5 deficiency ameliorated colitis and that this chemokine/chemokine-receptor trio was associated with the recruitment of inflammatory cells such as $CCR5^{+}$ T cells, monocytes and neutrophils into the colonic mucosa of colitogenic animals[169]

CCL20 (liver activation regulated chemokine (LARC)), acting via its receptor CCR6[170], has additionally been reported to be upregulated in patients suffering from IBD[171]. Recent studies in mice revealed that anti-CCL20 antibody treatment attenuated disease activity, and CCR6-deficient mice showed reduced disease onset, suggesting a proinflammatory role for the CCL20/CCR6 axis[172].

A special role has been identified for the chemokine decoy receptor D6, which, although binding many chemokines, does not elicit downstream signaling. Thus, it acts as a sink for many proinflammatory chemokines[173]. Hence, an increased susceptibility of mice lacking the receptor D6, along with increased levels of the chemokines CCL2, CCL3, CCL5, CXCL1 and CXCL2 could be observed. In accordance with that, elevated recruitment of leukocytes to the mucosa correlated with increased disease severity[174].

1.4 Chemokine (C-C motif) ligand 17 (CCL17)

CCL17, which is also known as TARC (thymus and activation regulated chemokine), clusters with the chemokines fractalkine (CX_3CL1) and macrophage-derived chemokine (MDC) on human chromosome 16q13[175]. CCL17 has been reported to be produced by keratinocytes[176], distinct subsets of DCs[177] as well as macrophages[178]. In human keratinocytes, CCL17 was shown to be inducible by IFN-γ and TNF-α stimulation[176]. In contrast, in murine Langerhans cells, CCL17 was reported to be upregulated by TNF-α and IL-4 stimulation and blocked by IFN-γ[179]. Furthermore, stimulation of mice with TLR-ligands LPS and Pam_3Cys has been shown to result in CCL17 production in mLNDCs[177]. However, the data from reporter mice describe that DCs outside the spleen are the only sorce for CCL17 under *in vivo* conditions[177]. The major function of CCL17 is the recruitment of CCR4 expressing T cells[180, 181]. Although some publications suggested that CCL17 also binds to CCR8[180], CCR4 remains the receptor repeatedly demonstrated to be the target of CCL17[182]. Besides the expression on T cells, CCR4 is also expressed on macrophages[183], DCs[184], and NK cells[185, 186]. T cell subsets that express CCR4 are on the one hand effector T cells: T_H17 cells[187], T_H2 cells[188] and CTLs[189]; and on the other hand T_{reg} cells[180, 181]. In addition to CCL17, CCR4 also binds CCL22[190]. CCR4 mediated recruitment of target cells via CCL17 or CCL22 has been

reported in the skin[191] and lung[178, 192], as well as the microenvironment of tumors[193-195]. Most studies in humans and mice specifically suggest a role for CCL17 in attracting $CCR4^+$ T_H2 cells to the skin; however, one study also demonstrated that CCL17 induced the migration of DCs from the skin to the draining lymph nodes in a CCR4-independent but CCR7/CXCR4-dependent manner[196].

Data, which is implicating a role for CCL17 in disease development, is derived from studies on atopic dermatitis, or on chronic diseases like cancer. In atopic dermatitis (AD), CCL17 was shown to be highly upregulated selectively in lesions and suggested to recruit $CCR4^+$ lymphocytes to these skin lesions[197-199]. Highly elevated serum levels of CCL17 were detected in AD patients when compared to healthy control subjects[200, 201]. Hence, CCL17 was suggested as a marker for the severity of AD[202]. CCL17 was as well is implicated in the atopic disease asthma and associated lung inflammation by recruiting T_H2 cells[203]. In the microenvironment of tumors, high expression of CCL17 is reported, and CCR4-CCL17/CCL22 dependent T_{reg} cell recruitment is described in gastric cancer[204] as well as esophageal squamous cell carcinoma[205]. Further studies implicated a role for CCL17 in CCR4-dependent lung metastasis from breast cancer[194]. More recently, CCL17 has also been implicated in the progression of atherosclerosis[206]. In this mouse study, the authors also demonstrated a beneficial role of a CCL17-blocking antibody. Another study reported that Hepatitis C virus induced CCL17/CCL22 production and thereby mediated T_{reg} cell recruitment to the site of infection[207]. These results suggest that CCL17 can have both, proinflammatory and regulatory functions.

CCL17 was shown to be upregulated on mRNA level in the acute phase of T cell transfer induced colitis, as well as in the chronic phase of spontaneous colitis in IL-10-deficient mice[208], and in a TNF-α driven spontaneous colitis model in mice[209]. Additionally, CCL17 was shown to be downregulated in mice when T cell transfer induced colitis was reversed by transfer of $CD4^+/CD25^+$ T cells[210]. One publication also demonstrated an upregulation of CCL17 in patients with active Crohn's disease but not in UC patients[211]. The upregulation was detected in inflamed compared to non-inflamed tissue and compared to healthy controls. However, analysis of single nucleotide polymorphisms (SNPs) did not reveal genetic variations linking CCL17 to human's IBD[175].

Despite the fact that only very little data is available, concerning a putative functional role for CCL17 in the context of IBD, the manifold investigations relating CCL17 to acute inflammatory processes give reason to analyze experimental colitis development in mice in the absence of CCL17.

2 AIMS OF THE STUDY

In recent years, chemokines, which are mediators of the immune response, responsible for attracting immune cells, have been shown to play crucial roles in shaping the outcome and intensity of intestinal inflammations. CCL17 (also named TARC) is produced by DCs in peripheral lymphoid and non-lymphoid organs, including the intestine, and has been shown to be involved in the recruitment of effector T cells as well as T_{reg} cells. Although human as well as mouse studies demonstrated an upregulation of CCL17 during inflammation in the gut, functional studies were missing. Thus, the role of CCL17 in the context of IBD remained elusive.

The central aim of this study was to answer the question if CCL17 plays a role in intestinal inflammation.

For this purpose the enhanced green fluorescent protein (eGFP (E)) knockin model was used. In this model, eGPF is inserted into the second exon of the *Ccl17* locus. This results in a reporter system in heterozygous knockin mice (*Ccl17*$^{WT/E}$), whereas homozygous knockin mice completely lack CCL17 expression and express eGFP instead (*Ccl17*$^{E/E}$). Intestinal inflammation, resembling human's IBD, was either induced chemically, by adding dextran sulfate sodium (DSS) to the drinking water, or immunologically, by adoptively transferring naïve T cells into lymphopenic mice initially lacking T cells. Bodyweight assessment, cytokine profiling on mRNA level, as well as histological scoring of the colonic inflammation were performed to monitor colitis development. Furthermore, in the T cell transfer model, direct effects on infiltrating cell types were analyzed by fluorescent activating cell sorting (FACS). To investigate specific effects of CCL17 on the differentiation of DCs or T cells, *in vitro* and *ex vivo* assays were performed. To get insights into longterm regulatory processes in *Ccl17*-deficient mice T_{reg} cells were depleted from the T cells before transfer.

Taken together, this study was designed to elucidate the function of CCL17 during colitis development and analyze in detail the mechanism of action in intestinal inflammation.

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Equipment

Device / Software	Manufacturer/ Distributor
Analytical balance	Ohaus (Pine Brook, NJ, USA)
Balance	Ohaus (Pine Brrok, NJ, USA)
Cell strainer (100 µm)	BD Biosciences (Heidelberg, Germany)
Centrifuge 5418	Eppendorf (Hamburg, Germany)
Centrifuge Biofuge Fresco	Heraeus (Hanau, Germany)
Centriuge 5810R	Eppendorf (Hamburg, Germany)
FlowJo Software	Tree Star (Olten, Switzerland)
Freezer –20 °C	Siemens (München, Germany)
Freezer –80 °C	Kendro (Langenselbold, Germany)
Fridge	Liebherr (Bulle, Switzerland)
Fully Enclosed Tissue Processor Leica ASP300 S	Leica Microsystems (Wetzlar, Germany)
Gallios Flow Cytometer	Beckman Coulter (Krefeld, Germany)
Glass wool	Roth (Karlsruhe, Germany)
HM 355 S automatic microtome	Thermo Scientific (Langenselbold, Germany)
Ice machine	Ziegra (Isernhagen, Germany)
Incubator Hera Cell 240	Heraeus (Hanau, Germany)
Kaluza Software	Beckman Coulter (Krefeld, Germany)
Laminar flow Hera Safe	Kendro (Langenselbold, Germany)
MACS Multi Stand	Miltenyi Biotec (Bergisch Gladbach, Germany)

MACS Seperation Colomns (LS / MS)	Miltenyi Biotec (Bergisch Gladbach, Germany)
Magnetic stirrer	Heidolph (Schwabach, Germany)
Microscope Optech IB	Exacta Optech (München, Germany)
Microscope Slides	Thermo Scientific (Langenselbold, Germany)
MIDI /MINI MACS Magnets	Miltenyi Biotec (Bergisch Gladbach, Germany)
Multipipette plus	Eppendorf (Hamburg, Germany)
Multiskan EX Microplate Photometer	Thermo Scientific (Langenselbold, Germany)
Nanodrop ND-1000 Spectrophotometer	Peqlab (Erlangen, Germany)
Neubauer counting chamber	Roth (Karlsruhe, Germany)
Nitrogen freezing tank MVE 6000	MVE (Marietta, GA, USA)
Nunc-Immuno™ Plates	Nunc GmbH & Co. KG (Langenselbold, Germany)
PCR cycler Mastercycler	Eppendorf (Hamburg, Germany)
Petri-dish (10 cm)	Greiner bio-one (Frickenhausen, Germany)
pH-meter	WTW (Weilheim, Germany)
Pipetboy acu	Integra Biosciences (Fernwald, Germany)
Pipettes	Gilson (Middleton, WI, USA)
TaqMan StepOne Plus	Applied Biosystems (Carlsbad, CA, USA)
Thermo Scientific Ascent Software	Thermo Scientific (Langenselbold, Germany)
Thermomixer	Eppendorf (Hamburg, Germany)
Vortexer Genie 2	Scientific Industries (Bohemia,NY, USA)
Water Bath	GFL (Burgwedel, Germany)

3.1.2 Reagents

Name	Manufacturer/ Distributor
2-Propanol	J.T. Baker (Deventer, Netherlands)
4 % (v/v) formaldehyde (Roti®-Histofix)	Carl Roth, Karlsruhe, Germany
ABTS	2,2'-Azino-di-(3-ethylbenzthiazolin) -6-sulfonsäure
Acetic acid	Merck (Darmstadt, Germany)
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Invitrogen (Karlsruhe, Germany)
Citric acid	Roth (Karlsruhe, Germany)
Diphteriatoxin (DT)	Calbiochem (Darmstadt, Germany)
Disodium phosphate	Fluka (Seelze, Germany)
Dithiothreitol (DTT)	Roth (Karlsruhe, Germany)
dNTP mix	Promega (Mannheim, Germany)
EDTA (0.5 M, pH 8.0)	Invitrogen (Karlsruhe, Germany)
Eosin	Roth (Karlsruhe, Germany)
Ethanol (for use in molecular biology)	Merck (Darmstadt, Germany)
Ethanol absolute (EtOH)	J.T. Baker (Deventer, Netherlands)
Ethidiumbromide (10 mg/ml)	Invitrogen (Karlsruhe, Germany)
Fc-block (mHB197 supernatnat)	Own production
Fetal Calf Serum (FCS)	Biochrom (Berlin, Germany)
Formaldehyde (37 %)	Roth (Karlsruhe, Germany)
Glutamax-I (100 x)	Invitrogen (Karlsruhe, Germany)
Golgi-Plug	BD Bioscience (Heidelberg, Germany)
Golgi-Stop	BD Bioscience (Heidelberg, Germany)
Hematoxylin	Roth (Karlsruhe, Germany)

Hepes	Sigma-Aldrich (Seelze, Germany)
Hepes buffer solution (1 M)	Invitrogen (Karlsruhe, Germany)
Hydrochloric acid (HCl)	Merck (Darmstadt, Germany)
Hydrogen peroxide (30 % (v/v))	Sigma-Aldrich (Seelze, Germany)
Ionomycin	Sigma-Aldrich (Seelze, Germany)
Isofluran (Forene 100 % (v/v))	Abbott (Wiesbaden, Germany)
L2000 Pam_3Cys-SKKKK (Pam_3Cys)	EMC (Tübingen, Germany)
Lipoplysaccharide from *E.coli* 0111:B4 (LPS)	Sigma-Aldrich (Seelze, Germany)
Magnesium chloride ($MgCl_2$ x 6 H_2O)	Roth (Karlsruhe, Germany)
Magnesium sulphate (MgSO4 x 7 H_2O)	Roth (Karlsruhe, Germany)
Methanol	J.T. Baker (Deventer, Netherlands)
Non essential amino acids (NEAA) (100 x)	PAA (Pasching, Austria)
Ovalbumin peptide, AA sequence 323-339: ISQAVHAAHAEINEAGR (pOVA)	Genscript, Piscataway, USA
PBS (w/o Ca^{2+} and Mg^{2+}) solution	PAA (Pasching, Austria)
PBS powder (w/o Ca^{2+} and Mg^{2+})	Invitrogen (Karlsruhe, Germany)
Penicillin/Streptomycin (100 x)	PAA (Pasching, Austria)
Phenol	Roth (Karlsruhe, Germany)
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich (Seelze, Germany)
Poly(I:C)	Amersham GE Healthcare (München, Germany)
Propidium iodide (PI)	Sigma-Aldrich (Seelze, Germany)
Recombinant mouse GM-CSF	Peprotech (Hamburg, Germany)
Red Blood Cell Lysis Buffer	Sigma-Aldrich (Seelze, Germany)
RPMI 1640	Invitrogen (Karlsruhe, Germany)
SDS	Sigma-Aldrich (Seelze, Germany)
Sodium acetate ($C_2H_3NaO_2$ x 3 H_2O)	Roth (Karlsruhe, Germany)

Sodium pyruvate	PAA (Pasching, Austria)
Thioglycolate (Thioglycol)	Fisher Scientific (Schwerte, Germany)
TMB	Fisher Scientific (Schwerte, Germany)
TRIzol reagent	Invitrogen (Karlsruhe, Germany)
β-Mercaptoethanol	Sigma-Aldrich (Seelze, Germany)

3.1.3 Kits and enzymes

Name	Manufacturer/ Distributor
CD11c (N418) Isolation Kit	Miltenyi Biotec (Bergisch Gladbach, Germany)
CD4 CD62L T cell Isolation Kit II	Miltenyi Biotec (Bergisch Gladbach, Germany)
Collagenase D	Roche (Mannheim, Germany)
DNase I	Roche (Mannheim, Germany)
Fix & Perm Cell Permeabilization Kit	Invitrogen (Karlsruhe, Germany)
Horseradish peroxidase	GE Healthcare (Munich, Germany)
Mouse Regulatory T Cell Staining Kit #1	NatuTec GmbH (Frankfurt, Germany)
Superscript III reverse transcriptase	Invitrogen (Karlsruhe, Germany)
TaqMan Gene Expression Master Mix	Applied Biosystems (Foster City, USA)
Mouse IFN-γ Duo Set	R&D Systems (Wiesbaden-Nordenstadt, Germany)

3.1.4 Antibodies for fluorescent activated cell sorting (FACS) and enzyme linked immunosorbent assay (ELISA)

Name (Antigen)	Conjugate	Application	Manufacturer/ Distributor
CD103	APC, PE	Flow cytometry	BD Biosciences (Heidelberg, Germany)
CD11b	APC, PE, PerCP-Cy5	Flow cytometry	BD Biosciences (Heidelberg, Germany)

CD11c	APC, PE-Cy7	Flow cytometry	eBioscience (San Diego, CA USA)
CD3ε	Purified, APC780	Flow cytometry, coculture	BD Biosciences (Heidelberg, Germany)
CD4	eFluor450	Flow cytometry	eBioscience (San Diego, CA USA)
CD40	PE	Flow cytometry	BD Biosciences (Heidelberg, Germany)
CD80	PE, APC	Flow cytometry	BD Biosciences (Heidelberg, Germany)
CD86	PE	Flow cytometry	BD Biosciences (Heidelberg, Germany)
CD8α	APC, APC780, eFluor450	Flow cytometry	BD Biosciences (Heidelberg, Germany)
F4/80	APC	Flow cytometry	BD Biosciences (Heidelberg, Germany)
Foxp3	PE	Flow cytometry (intracellular staining)	eBioscience (San Diego, CA, USA)
HRP	avidin	ELISA	eBioscience (San Diego, CA, USA)
HRP	streptavidin	ELISA	GE Healthcare (München, Germany)
IFN-γ	PE	Flow cytometry (intracellular staining)	eBioscience (San Diego, CA, USA)
IFN-γ	unconjugated	ELISA	R&D Systems (Wiesbaden-Nordenstadt, Germany)
IFN-γ	biotinylated	ELISA	R&D Systems (Wiesbaden-Nordenstadt, Germany)
IL-12p40/70	unconjugated	ELISA	BD Biosciences (Heidelberg, Germany)
IL-12p4ß/70	biotinylated	ELISA	BD Biosciences (Heidelberg, Germany)
IL-17A	APC	Flow cytometry (intracellular staining)	eBioscience (San Diego, CA, USA)
IL-17A	unconjugated	ELISA	eBioscience (San Diego, CA, USA)
IL-17A	biotinylated	ELISA	eBioscience (San Diego, CA, USA)
MHCII ($I\text{-}A^b$)	eFluor450	Flow cytometry	BD Biosciences (Heidelberg, Germany)

3.1.5 Media and buffers

3.1.5.1 Media for cell culture

DC medium	RPMI 1640	
	10 % (v/v)	FCS (heat inactivated)
	1 % (v/v)	Glutamax-I
	1 % (v/v)	Sodium pyruvate
	1 % (v/v)	NEAA
	1 % (v/v)	Penicillin/Streptomycin
	0.05 mM	β-Mercaptoethanol

Restimulation medium	DC medium
	20 ng/ml Phorbol 12-myristate 13-acetate (PMA)
	1 µg/ml Ionomycin
	0.2 % v/v Golgi Plug
	0.14 % v/v Golgi Stop

Digestion medium	RPMI 1640
	0.5 µg/ml Collagenase D (II)
	0.1 µg/ml DNase I grade II (D)

3.1.5.2 Buffers for enzyme linked immunosorbent assay (ELISA)

Coating Buffer	PBS (1x)	
Blocking Buffer	PBS (1x)	
	10 % (v/v)	FCS
Dilution Buffer	PBS (1x)	
	10 % (v/v)	FCS
Washing Buffer	PBS (1x)	
	0.5 % (v/v)	Tween-20

ELISA Substrate	0.1 M	Citric acid pH 4.0
	0.02 % (v/v)	ABTS (50 mg/ml)
	1:1000 30 %	hydrogen peroxide
Stop Solution	1 % (w/v)	SDS in ddH_2O

3.1.5.3 Buffer for magnetic activated cell sorting (MACS)

MACS-buffer	PBS (1x)
	2 % (v/v) FCS + 2 µM EDTA

3.1.5.4 Buffer for fluorescent activated cell sorting (FACS)

FACS-buffer	PBS (1x)
	5 % (v/v) FCS

3.2 Methods

3.2.1 Mice

All mice were used on C57BL/6 background, backcrossed for at least 10 generations. A list of the mice used is provided in the following. For T cell transfer experiments, *Ccl17*$^{eGFP/eGFP}$ and *Ccr4*$^{-/-}$ mice were backcrossed to *Rag1*$^{-/-}$ mice. *Ccl17*$^{eGFP/eGFP}$ (*Ccl17*$^{E/E}$) mice were a kind gift of Irmgard Förster (Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, now at Molecular Immunology, Institut für Umweltmedizinische Forschung an der Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The eGFP knockin strategy is illustrated in Figure 4. Experiments were performed in accordance with the German animal care and ethics legislation and had been approved by the local government authorities.

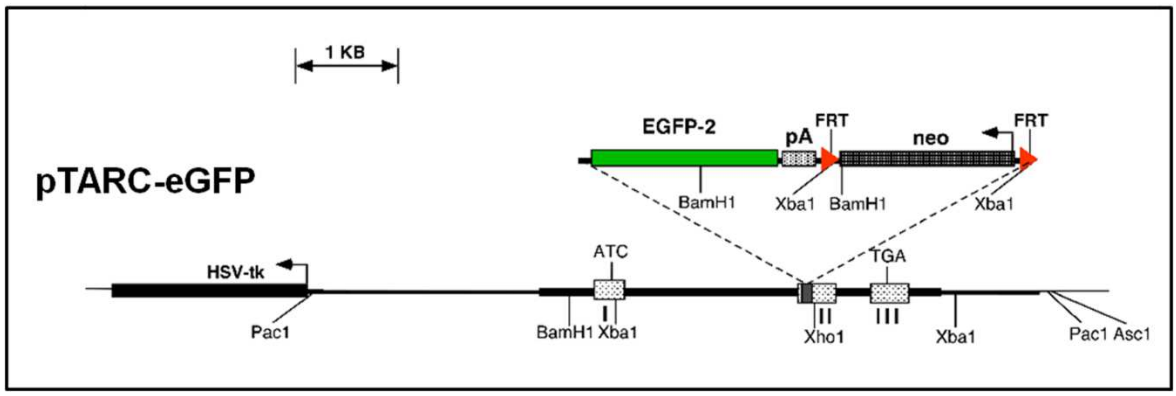

Figure 4: eGFP knockin model. eGFP (illustrated in green) was inserted into the second exon of the *Ccl17* locus leading to the expression of eGFP instead of CCL17. Thus, heterozygous mice can be used as reporter mice, whereas homozygous mice completely lack CCL17 expression, expressing eGFP instead. (Modified from Alferink J. *et al.* J Exp Med (2003))

Table 1: Characteristics of mice used throughout this study.

Genotype	Phenotype	Experimental Use
Ccl17$^{E/E}$	Express eGFP instead of CCL17[177]	BMDC generation, DSS colitis
Ccl17$^{E/WT}$	CCL17 producing cells also produce eGFP[177]	BMDC generation, DSS colitis
Ccr4$^{-/-}$	Lack CCR4	BMDC generation
DEREG 23.2	Express eGFP and DTR under the control of Foxp3 promoter[212]	T_{reg} cell depletion before T cell transfer
Rag1$^{-/-}$	Lack B and T cells	Recipient mice in T cell transfer colitis

3.2.2 Colitis induction

Colitis was induced in two different ways: (1) chemically, by adding DSS into the drinking water; (2) by transferring T cells.

DSS colitis was induced by administration of 4 % (w/v) DSS in the drinking water for 5 days followed by drinking water alone. T cell transfer colitis was induced in mice on *Rag1*$^{-/-}$ background (lymphopenic mice) by adoptively transferring $3*10^5$ $CD4^+/CD62L^+$ double positive spleen T cells intraperitoneal (i.p.). The purity of T cells was routinely over 90 %, determined by FACS analysis. Bodyweight measurement, severity of diarrhea and blood in the feces were used as indicators of colitis induction. Mice were sacrificed when they had lost 20 % of their bodyweight. In both models, diarrhea and blood in the feces, as well as histological analysis, correlated with bodyweight loss.

3.2.3 Isolation of colon lamina propia leukocytes (LPL) and intraepithelial leukocytes (IEL)

Mice were sacrificed by cervical dislocation. Colons were removed and flushed with ice cold PBS. The whole colon was cut longitudinally and about 3 mm long pieces were washed three times in ice cold PBS for subsequent incubation under stirring in 50 ml PBS containing 2 mM DTT and 5 mM EDTA at 37 °C for 30 min. Colon pieces were then passed through a cell strainer (100 µm) and the filtrate was used to isolate the IEL fraction by further filtrating with glass wool. The remaining colon pieces were incubated for 30 min at 37 °C in digestion medium and pressed through a cell strainer (100 µm). Single cell suspensions were regarded as LPL fraction. For intracellular cytokine staining, LPL and IEL fractions were stimulated for 6 h in stimulation medium prior to staining.

3.2.4 Generation of bone marrow derived recombinant mouse (rm)GMCSF cultured dendritic cells (BMDCs)

Bone marrow of *femur* and *tibia* was flushed out and single cell suspensions were prepared. Red blood cells were removed using red blood cell lysis buffer at RT for 5 min. $6*10^5$ cells/ml were cultured for 3 days in 10 ml DC medium containing 20 ng/ml rmGMCSF in 10 cm petri-dishes. At day 3 of culture, 10 ml of DC medium containing 20 ng/ml rmGMCSF were added. At day 5, 10 ml cell suspension were removed and

centrifuged for 5 min at 1500 rpm at 4 °C. The cell pellet was resuspended in 10 ml fresh medium containing 20 ng/ml rmGMCSF, added to the remaining 10 ml in the petri-dish and cultured for another 2 days. On day 7, cells were routinely control stained for CD11c, CD11b and CD86. $CD11c^+/CD11b^+$ double positive cells were regarded as DCs and this fraction was routinely > 80 %.

3.2.5 Enzyme linked immunosorbent assay (ELISA)

ELISA was performed to detect cytokines in the supernatant of stimulated DCs and cocultures of DCs and T cells. IL-17A and IFN-γ were measured using ELISA kits according to the manufacturers' protocols. IL-12p40/p70 was detected using matched antibody pairs. The following protocol for IL-12p40/70 represents the logic for all three ELISAs provided in Table 2.

Plates were coated overnight with capture antibody diluted in PBS (1:2000). After washing steps plates were incubated with blocking buffer for 1 h and washed again. Next, standards and samples were diluted in dilution buffer and added to the plate for 2 h, followed by another washing step. Then biotinylated detection antibody was added for 2 h and plates were washed. After washing, streptavidin conjugated horseradish peroxidase (HRP) was added for 45 min. After an additional washing step ABTS was added as substrate until coloring appeared. To stop the enzymatic reaction 100 µl 1 % SDS was added. Analysis of the reaction was performed using a Photometer. Specific dilutions and incubation periods for each assay is provided in the following list (Table 2).

Table 2: Cytokine ELISAs.

	IFN-γ	IL-17A	IL12p40
Capture antibody	1:180 in PBS (50 µl; overnight)	1:250 in coating buffer (100 µl; overnight)	1:2000 in PBS (50 µl; overnight)
Washing 1	3 x	5 x	3 x
Blocking	PBS + 10 % FCS (1 h)	1 x Assay Diluent (1 h)	PBS + 10 % FCS (1 h)
Washing 2	3 x	1 x	1 x
Standard incubation	4000 pg/ml in 20 mM Tris/150 mM NaCl + 0.1 % BSA + 0.05 % Tween20 (Reagent	500 pg/ml in 1 x Assay Diluent (100 µl; 2 h)	8000 pg/ml in PBS + 0.05 Tween20 + 10 % FCS (100 µl; 2 h)

	Diluent) (60 µl; 2 h)		
Sample incubation	1:20 in Reagent Diluent (60 µl; 2h)	1:10 in 1 x Assay Diluent (100 µl; 2 h)	1:4 in PBS + 0.05 (v/v) Tween20 + 10 % FCS (100 µl; 2 h)
Washing 3	3 x	5 x	4 x
Detection antibody	1:180 in Reagent Diluent (50 µl; 2 h)	1:250 in 1 x Assay Diluent (100 µl; 2 h)	1:1500 in Wash buffer + 10 % FCS (100 µl; 2h)
Washing 4	3 x	5 x	4 x
Streptavidin-HRP	1:1000 in Reagent Diluent (50 µl; 20 min)	1:250 in 1 x Assay Diluent (100 µl; 30 min)	1:2000 in Wash buffer + 10 % FCS (50 µl; 45 min)
Washing 5	3 x	7 x	4 x
Substrate	10 ml 0.1 M Citric Acid + 1 mg/ml ABTS + 10 µl 30 % H_2O_2 (100 µl)	1 x TMB (100 µl)	10 ml 0.1 M Citric Acid + 1 mg/ml ABTS + 10 µl 30 % H_2O_2 (100 µl)
Stop-solution	1 % SDS (100 µl)	2 N H_2SO_4 (100 µl)	1 % SDS (100 µl)

3.2.6 Cell isolation and magnetic activated cell sorting (MACS)

Mice were sacrificed by cervical dislocation and spleens were removed. $CD4^+/CD62L^+$ T cells were isolated from splenocytes using the CD4 CD62L T cell Isolation Kit II, according to the manufacturer's protocol. Single cell suspensions of splenocytes were generated by pressing spleens through cell strainers (100 µm). After centrifugation, cell pellets were subsequently magnetically labeled by incubating antibodies coupled to magnetic beads. Magnetic separation was performed according to the manufacturer's protocol. First, $CD4^+$ T cells were negatively selected, followed by positive selection of $CD62L^+$ $CD4^+$ T cells.

For isolation of DCs from mesenteric lymph nodes (mLNs), the mLNs were removed, flushed and incubated in digestion medium for 30 min and passed through a 100 µm cell strainer. Single cell suspensions were then MACS sorted using CD11c (N418) MicroBeads according to the manufacturer's protocol.

3.2.7 Fluorescence activated cell sorting (FACS)

For FACS analysis, cells were stained using fluorescently labeled antibodies directed against CD11c, CD11b, CD103, CD3ε, CD4, CD62L, CD86, CD40, α4β7 and MHCII. Single cell suspensions were washed once in ice cold FACS buffer. Subsequently, cells were incubated for 20 min in FACS-buffer containing the antibodies in a final dilution of 1:200. To avoid unspecific binding of the antibodies to Fc receptors, the FACS-buffer was mixed 1:1 with the supernatant of the mHB197 cell line, producing blocking antibodies (anti-CD16/anti-CD32). Cells were then washed two times in FACS buffer and resuspended in FACS buffer for analysis. Propidium iodide (PI) was added to exclude dead cells from analysis.

For intracellular cytokine staining, T cells were stimulated for 6 h in stimulation medium prior to staining. Staining with anti-IFN-γ or anti-IL-17A antibodies was performed using Fix & Perm Cell Permeabilization Kit. Intracellular Foxp3 staining was performed using Mouse Regulatory T Cell Staining Kit #1 according to the manufacturer's protocol.

For intracellular stainings, cells were stained in the first step for membrane bound markers as described above. After the second washing step, cells were then incubated in fixation medium as described in the manufacturer's protocol (either Fix & Perm Cell Permeabilization Kit, or Mouse Regulatory T Cell Staining Kit #1) and washed. Afterwards, cells were stained with the antibodies in permeabilization medium of the respective kit for 20 min at 4 °C (for Foxp3) or at room temperature (for cytokines). After two washing steps, cells were analyzed without the addition of PI.

A Gallios flowcytometer was used for analysis. Either Kaluza analysis software or FlowJo software were used to analyze data and to generate plots and histograms.

3.2.8 *In vitro* stimulation and coculture

For *in vitro* experiments, $1*10^6$ BMDCs/ml were stimulated with 100 ng/ml LPS or Pam_3Cys in 6 well dishes (total volume 3 ml). After 6 h, cell suspensions were centrifuged for 5 min at 1500 rpm at 4 °C. Supernatants were used to detect secreted cytokines by ELISA. For RNA isolation the cell pellets were resuspended in TRIzol.

For *in vitro* coculture experiments, $1*10^4$ BMDCs were cocultured with $1*10^5$ $CD4^+/CD62L^+$ T cells, isolated from splenocytes of OTII transgenic mice, for 4 days in the presence of 100 ng/ml LPS and 5 µg/ml OVA-peptide (pOVA). In *ex vivo* experiments, $1*10^4$ mLNDCs were cocultured with $1*10^5$ $CD4^+/CD62L^+$ T cells, isolated from spleens of OTII transgenic mice, for 5 days in the presence of 100 ng/ml LPS and 5 µg/ml pOVA. In both coculture systems LPS and pOVA were added at the beginning. For *in vitro* Foxp3 T_{reg} coculture experiments, 5 µg/ml CD3ε, 5 ng/ml TGF-β and 200 U/ml IL-2 were added to the medium and cells were analyzed after 4 days by FACS analysis. For *ex vivo* coculture experiments, DCs were MACS isolated from mLNs of mice pretreated with 200 µg LPS overnight. To monitor T cell proliferation in *in vitro* coculture experiments, T cells were labled with 5 µM CFSE for 10 min at 37 °C prior to coculture. CFSE labled T cells were washed three times before added to the coculture.

3.2.9 RNA isolation and real-time quantitative PCR (qPCR)

Total RNA was isolated from whole tissue of the proximal colon using TRIzol reagent according to the manufacturer's protocol. An about 1 mm long piece of the proximal colon was taken up in 0.5 ml TRIzol reagent. cDNA was generated from total RNA using Superscript III according to the manufacturer's protocol. qPCR was run on cDNA using a TaqMan StepOne Plus instrument. For the reactrion 10 µl TaqMan gene expression master mix were mixed with 9 µl cDNA and 1 µl probe sets. The following primers and probe sets were ordered from Applied Biosystems: *IL-6* (Mm00446190_M1), *IL-12p35* (Mm00434169_M1), *IL-17a* (Mm00439619_M1), *IL-22* (Mm00444241_M1), *IL-23p19* (Mm00518984_M1), *TNFα* (Mm00443260_g1), *IFNγ* (Mm99999071_M1), *TGFβ* (Mm03024053_M1), *Aldh1a2* (Mm00501306_M1), *HPRT1* (Mm00446968_M1) and *Csf2* (GMCSF) (Mm01290062_M1). *HPRT1* was used as housekeeping gene for normalization. Quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method[213].

3.2.10 Histology

Mice were sacrificed by cervical dislocation. Colons were removed and flushed with ice cold PBS. Whole colon tissues were subsequently fixed o/n in 4 % (v/v) formaldehyde (Roti-Histofix). Fixed colon tissues were then transferred to 70 % ethanol, and

subsequently dehydrated. Fixed and dehydrated colon tissues were then embedded in paraffin and 4 µm thick slices were cut. Next, colon tissue slices were air dried and stained using hematoxylin and eosin (H&E).

Histological scoring was performed on formalin fixed, paraffin embedded sections of the colon. 4 µm thin slices were stained with hematoxylin and eosin. Scoring was performed in a blinded fashion by Hans-Anton Lehr (IUP Institut universitaire de pathologie de Lausanne, Lausanne, Switzerland) as previously published[214].

3.2.11 Peritoneal lavage

To induce inflammation in the peritoneum, 1 ml 4 % thioglycolate was injected i.p.. To identify the populations of infiltrating immune cells, the peritoneal cavity was flushed with 5 ml of ice cold PBS 4 days after thioglycolate injection. By gently massaging the belly of the mice, cells were suspended. The cell suspension was then extracted by using a syringe. Analysis of the peritoneal lavage cells was performed using FACS analysis as described in section 3.2.6.

3.2.12 Statistical analysis

Mean values and standard deviations are shown in the diagrams. Statistical significance was tested using Student's t-test or Dunn's test. One Way ANOVA was used to determine the variance within the samples which were compared. *P*-values below 0.05 were considered to indicate statistically significant differences.

4 RESULTS

4.1 Induction of acute and chronic intestinal inflammation in mice

To induce acute intestinal inflammation in mice, the well characterized model using DSS in the drinking water was used as described in the Materials & Methods section 3.2.2.

A longer lasting inflammation was induced by transferring naïve T cells into lymphopenic mice lacking the recombination activating gene 1 (*Rag1*$^{-/-}$). The absence of *Rag1* results in a lack of mature B and T cells. Additionally, these mice were crossed with mice bearing the additional genotype of interest (*Ccl17*$^{E/E}$ or *Ccr4*$^{-/-}$). Unless otherwise stated, 3*10^5 CD4$^+$/CD62L$^+$ splenocytes, isolated from C57BL/6 wild type (WT) mice were adoptively transferred by intraperitoneal (i.p.) injection to induce intestinal inflammation. The transferred T cells were isolated as described in Material & Methods section 3.2.6. As shown in Figure 5, the MACS isolated T cells had routinely a purity of over 90 %, determined by FACS analysis. Over 95 % of these CD4$^+$ T cells were CD62L$^+$, including a population of about 6-8 % Foxp3$^+$ regulatory T cells (T_{reg}).

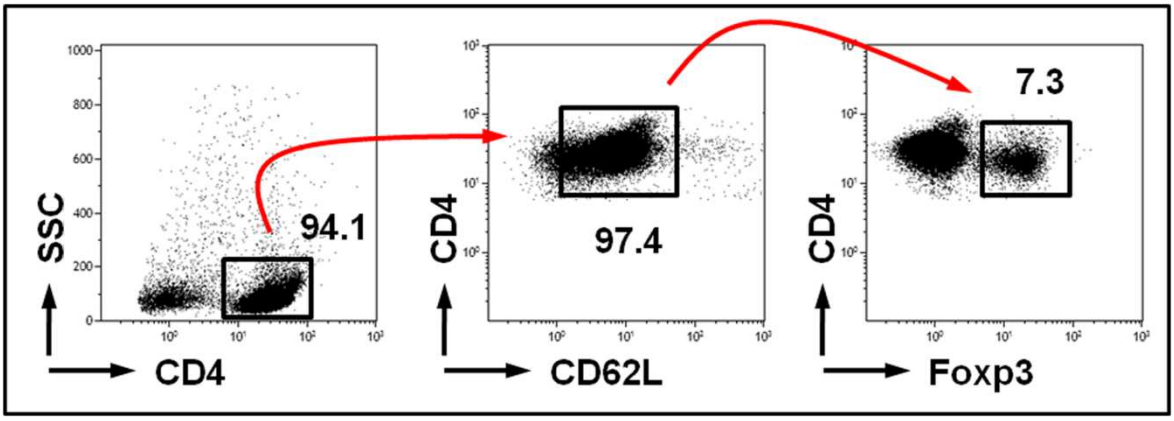

Figure 5: FACS analysis of MACS sorted CD4$^+$/CD62L$^+$ T cells from splenocytes. MACS sorted T cells were stained for CD4 and CD62L on the surface and for Foxp3 intracellularly. Numbers indicate percentages of the gated cells. (SSC = side scatter)

After injection of T cells, bodyweight, as well as consistency of feces was monitored to follow colitis induction.

4.2 Induction of CCL17 during acute and chronic intestinal inflammation

To investigate if CCL17 plays a role during intestinal inflammation it was important to demonstrate that the chemokine is indeed upregulated after induction of acute and chronic intestinal inflammation in the mesenteric lymph nodes, draining the intestine, and the colon, the site of inflammation. To illustrate the regulation of CCL17 an enhanced green fluorescent protein (eGFP) knockin mouse model was used which was created by Alferink J., *et al.* [177]. In this mouse model, heterozygous knockin mice (*Ccl17*$^{E/WT}$) can be used as reporter mice without any reduction of expression and functionality of the protein. In contrast, a homozygous knockin led to a complete loss of expression and function, resembling knockout mice (*Ccl17*$^{E/E}$, see also Material & Methods Figure 4).

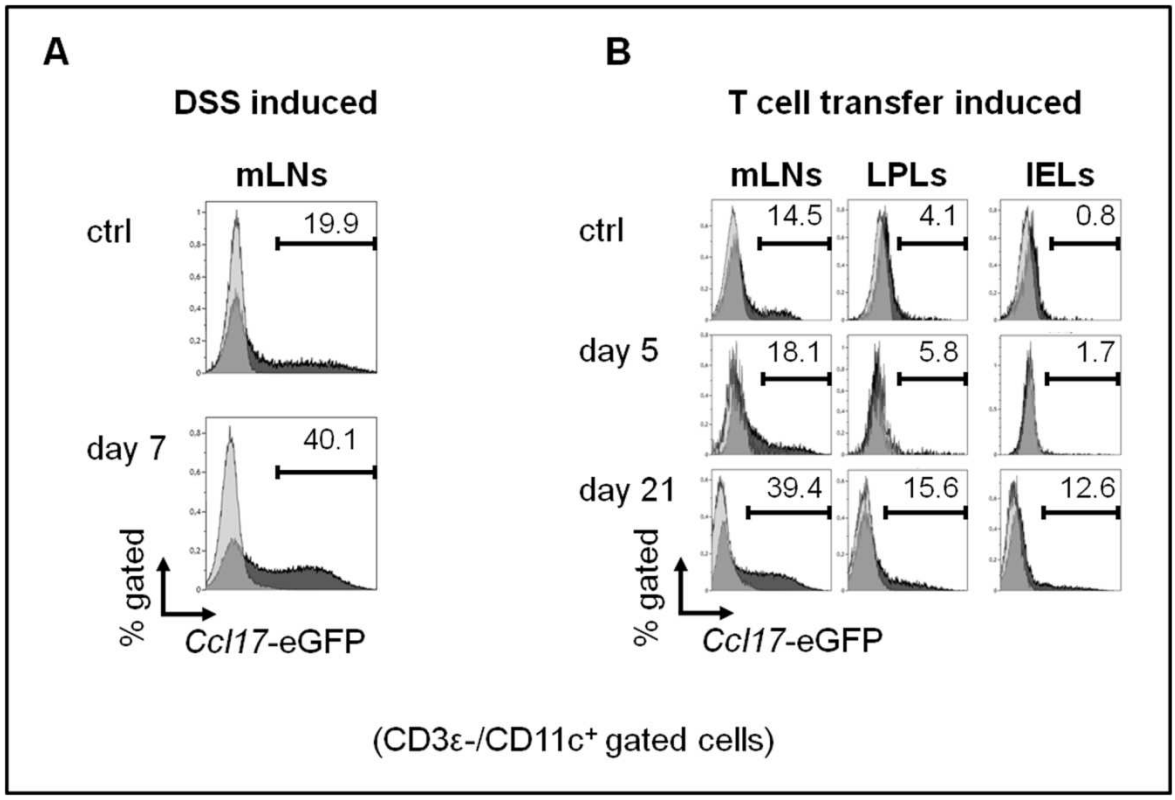

Figure 6: CCL17 induction after colitis induction, monitored by eGFP induction in DCs. CCL17 expression is monitored by eGFP expression in CD3ε$^-$/CD11c$^+$ gated dendritic cells (DCs). (**A**) Percentage of CCL17$^+$ mLN DCs in untreated control (ctrl) mice and in mice treated with 4 % (v/v) DSS in the drinking water for 5 days, followed by 2 days of water alone. (**B**) Percentage of CCL17$^+$ DCs in *Rag1*$^{-/-}$ control (ctrl) mice and in *Rag1*$^{-/-}$ mice 5 days and 21 days after T cell transfer in the indicated organs and fractions in lymphopenic mice. Light gray histograms represent unstained control. (Numbers represent percentages of CCL17$^+$ cells. One representative staining of 5 is shown)

As shown in Figure 6, CCL17 was measured in CD3ε$^-$/CD11c$^+$ gated DCs, either after colitis induction with 4 % (v/v) DSS in the drinking water (Figure 6A) or induction by T cell transfer (Figure 6B). In the DSS model, CCL17 expression was markedly increased in mLNs after 7 days of treatment compared to the basal expression detected before treatment.

Basal expression could also be detected in mice lacking B and T cells (PBS injected *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice (ctrl)). However, no upregulation of CCL17 was measurable 5 days after transfer of T cells in mLNDCs, and DCs of the IEL and LPL fraction of the colon. In contrast, a significant upregulation of CCL17 in these three fractions could be measured 21 days after transfer, correlating with disease induction. In both models, CCL17 induction is shown in CCL17$^{E/E}$ mice and expression in untreated mice in mLNs was comparable between the models. In Figure 7, representative FACS plots show that eGFP$^+$ cells in the colon had high expression levels of the DC markers CD11c, CD11b and MHCII, and only a small amount expressed F4/80 at low levels.

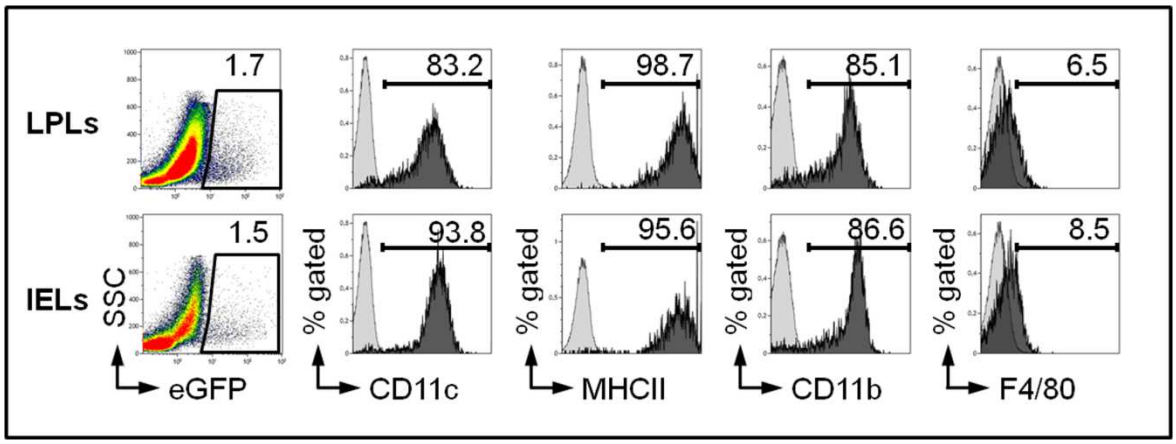

Figure 7: Phenotypic analysis of eGFP$^+$ cells of the colon. 21 days after T cell transfer into *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice, eGFP$^+$ cells of the indicated colon fractions were analyzed for the expression of CD11c, MHCII, CD11b and F4/80. Numbers indicate percentage of gated cells. (SSC = side scatter)

4.3 Reduced colitis induction in mice lacking *Ccl17*

To analyze if CCL17 expression has an impact on the severity of DSS induced colitis, disease development was monitored by bodyweight measurement, measurement of the colon length and histological scoring in CCL17 competent mice (C57BL/6 (WT)) as compared to mice lacking CCL17 (*Ccl17*$^{E/E}$). As shown in Figure 8A, *Ccl17*$^{E/E}$ mice lost significantly less bodyweight than WT mice after DSS treatment (% bodyweight at day 7: *Ccl17*$^{E/E}$ mice: 93.8 ± 2.7; mean ± SD (n = 13); WT mice: 84.1 ± 4.3 (n = 13)). Additionally, as shown in Figure 8B, *Ccl17*$^{E/E}$ mice had significantly longer colons than WT mice after DSS treatment (5.2 ± 0.3 *vs*. 4.5 ± 0.4, respectively (n = 8)). As shown in Figure 8C, a significantly lower colonic inflammation, determined by histological scoring, could be detected in *Ccl17*$^{E/E}$ mice (4.2 ± 2.1 (WT; n = 13) *vs*. 2.1 ± 2.2 (*Ccl17*$^{E/E}$; n = 13)).

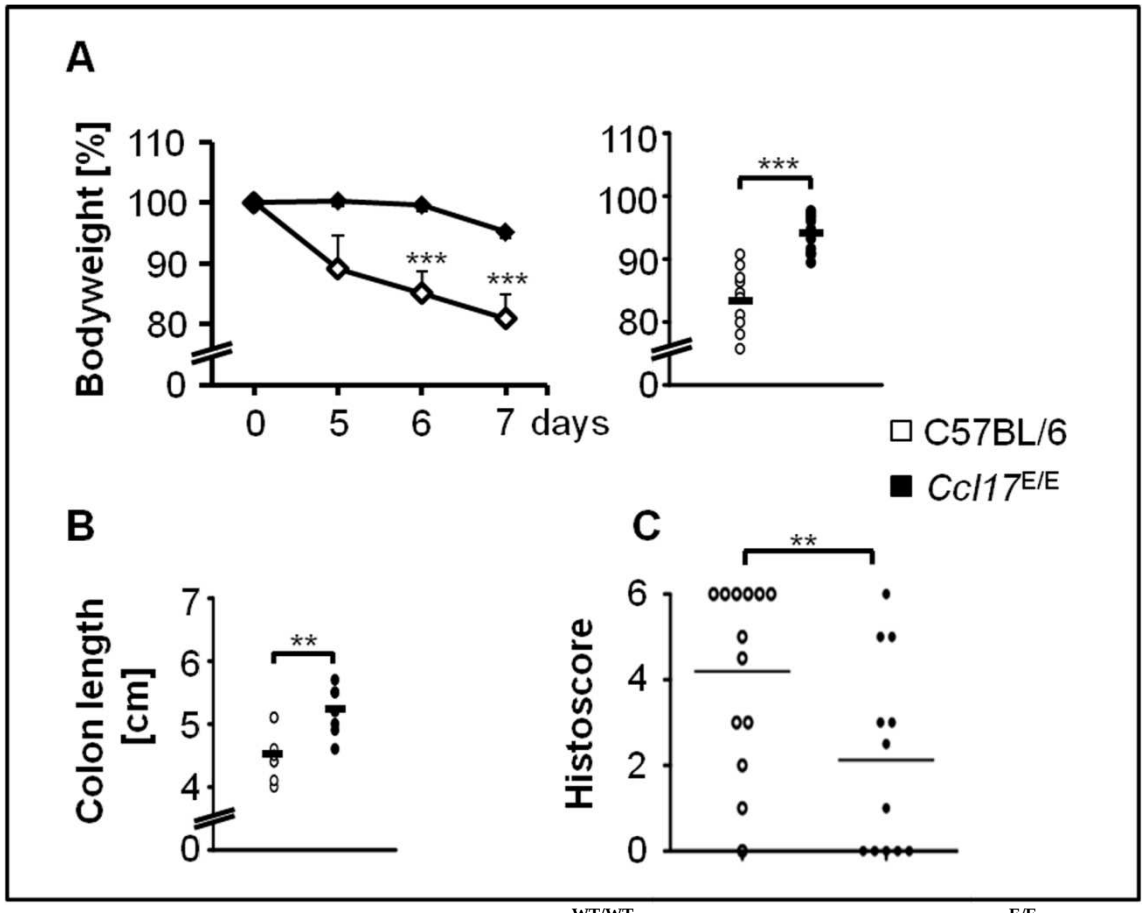

Figure 8: Development of DSS colitis in *Ccl17*$^{WT/WT}$ (C57BL/6) compared to *Ccl17*$^{E/E}$ mice. (**A**) Bodyweight was measured at day 1, 5, 6 and 7 after DSS mediated colitis induction. Left panel: representative bodyweight development of one experiment (n = 4). Right panel: pooled bodyweights on day 7 of 4 experiments (n = 13). (**B**) Colon length at day 7 (n = 8) (**C**) Pooled histoscore of 3 experiments (n = 13). (** P < 0.01; ***P < 0.001; Student's t-test)

To get further insights into the underlying mechanisms leading to the less severe phenotype of DSS colitis in *Ccl17*$^{E/E}$ mice, cytokine patterns in the mLNs as well as colon tissue were analyzed. Although, as shown in Figure 9A, no differences were detectable in the mRNA expression levels of TNF-α, IL-6 and IL-23, neither in the mLNs nor in the colon, a significantly stronger induction of IL-12p35 in the mLNs, and of IFN-γ in the colon could be detected in *Ccl17*$^{WT/WT}$ mice (Figure 9B).

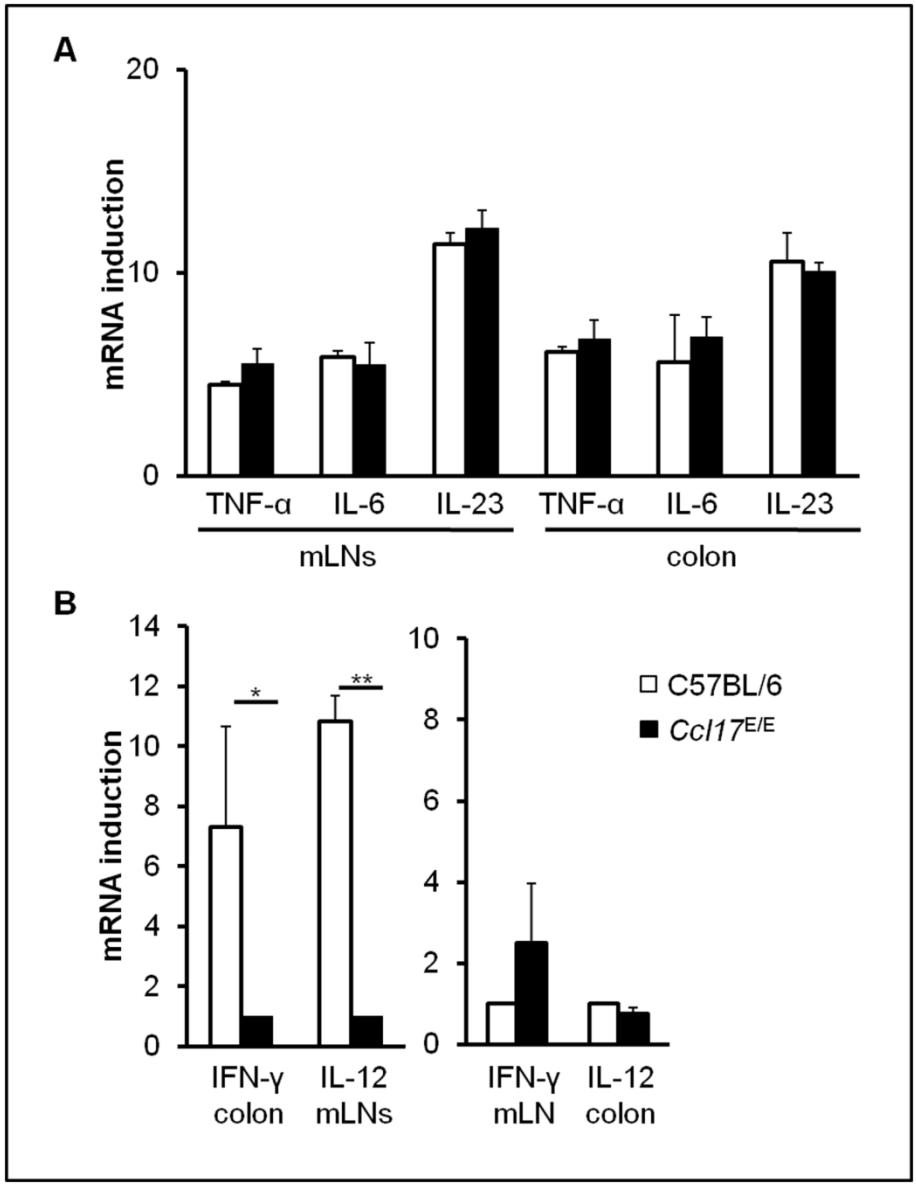

Figure 9: mRNA induction of cytokines after DSS treatment in mLNs and colon tissue. Quantitative real-time PCR was performed on mRNA isolated from single cell suspensions of isolated mLNs or whole colon tissue as described in Materials & Methods section 3.2.9. (**A**) Induction of TNF-α, IL-6 and IL-23 in mLNs and colon tissue. (**B**) Induction of IL-12p35 and IFN-γ in mLNs and colon tissue. (*$P < 0.05$; **$P < 0.01$; Student's *t*-test; ($n = 4$)) One of three independent experiments with 4 mice per group is shown.

Taken together, these data demonstrate that the lack of CCL17 expression leads to reduced colonic inflammation after DSS treatment.

Besides the effects seen in the acute model using DSS, it was of great interest to investigate if CCL17 also has an impact on chronic intestinal inflammation in mice. For this purpose, the T cell transfer model, described earlier, was used. Like in the DSS model, lymphopenic mice lacking CCL17 expression (*Rag1*$^{-/-}$*Ccl17*$^{E/E}$) lost significantly less bodyweight than mice expressing CCL17 (*Rag1*$^{-/-}$) after colitis induction (Figure 10A), with a mean percentage of 81.1 ± 8.1 in *Rag1*$^{-/-}$ mice compared to 100.1 ± 6.4 in *Rag1*$^{-/-}$ *Ccl17*$^{E/E}$ mice on day 21.

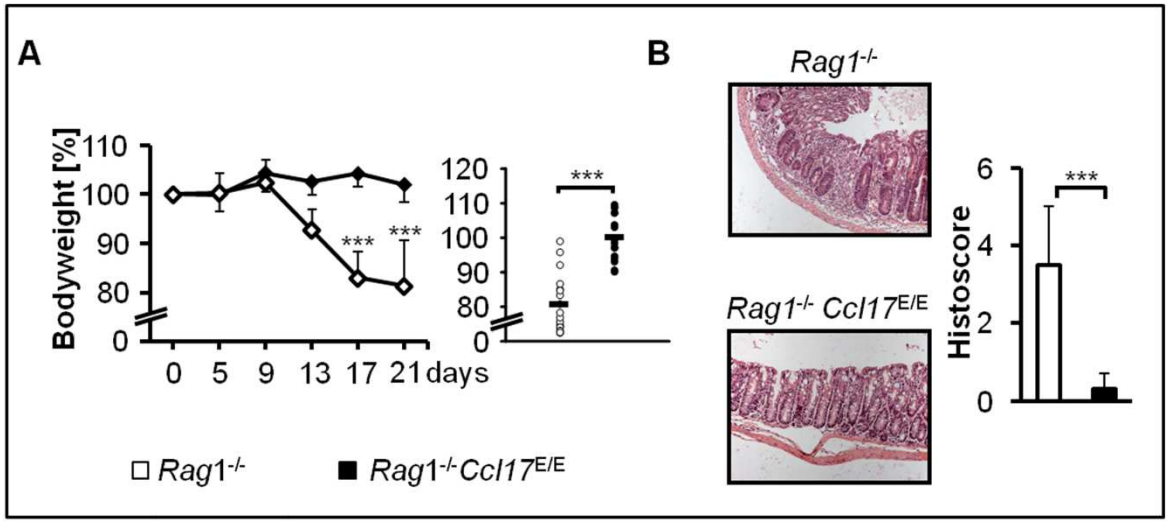

Figure 10: Development of T cell transfer colitis in *Rag1*$^{-/-}$ compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. (**A**) Bodyweight was measured throughout colitis development at the indicated time points after T cell transfer. Left panel: bodyweight development of one representative experiment (n = 3). Right panel: pooled endpoint data of 4 experiments (n = 12). (**B**) Left panel: Representative pictures of H&E stained paraffin embedded colon sections of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. Right panel: Representative histoscore of *Rag1*$^{-/-}$ compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice (n = 3; cumulative score). (***P < 0.001; Student's t-test)

Additionally, *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice developed significantly milder colonic inflammation compared to *Rag1*$^{-/-}$ mice, shown by histological scoring. In the left panel of Figure 10B, representative hematoxylin & eosin (H&E) stains show that less infiltration of immune cells, less crypt loss, less disruption of the mucus layer and less loss of goblet cells was detectable in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. This was represented by an overall significantly lower histoscore of 0.3 ± 0.4 in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice compared to 3.5 ± 1.5 in *Rag1*$^{-/-}$ mice.

Deeper insights into the differences found on macroscopic scale could be provided by analysis of mRNA expression levels of cytokines in mLNs as well as colon tissue. As shown in Figure 11, there was no remarkable induction of the proinflammatory cytokines

IFN-γ, IL-17, IL-6, TNF-α, and IL-22, 5 days after transfer of T cells in both mouse strains, as well as no differences between the two mouse strains.

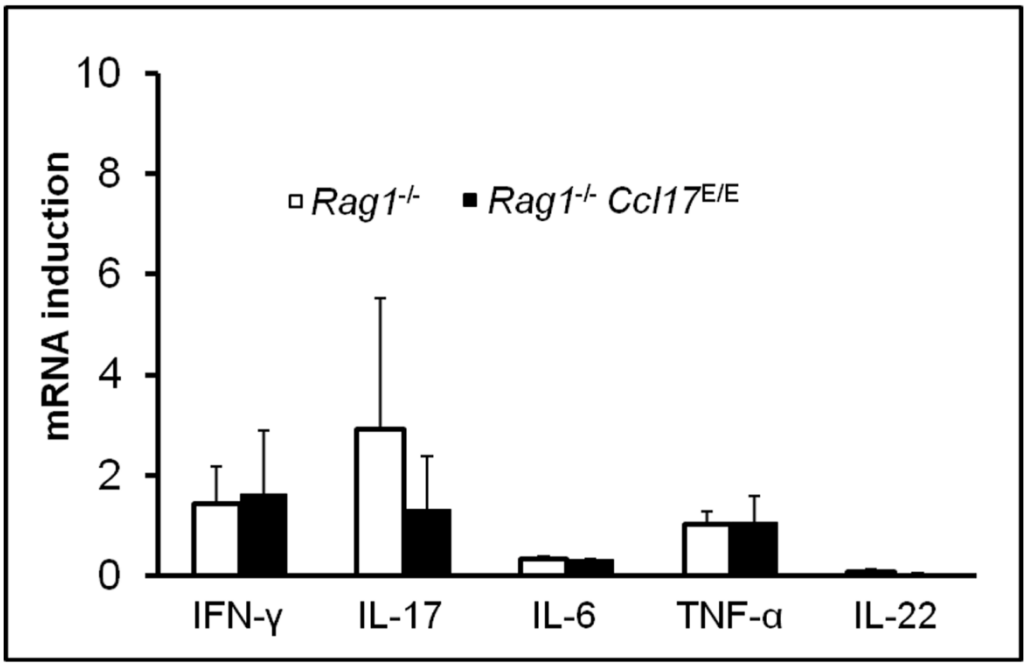

Figure 11: mRNA expression of cytokines in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice 5 days after T cell transfer. mRNA was extracted from colon tissue and real-time qPCR was performed as described in Material & Methods section 3.2.9. Induction of mRNA is calculated relative to induction in PBS treated mice. (mean ± SD of *n* = 3 mice)

This lack of induction of proinflammatory cytokines was in line with the development of bodyweight, as up to this time point, mice of both strains still gained weight. As well, there were no further signs of inflammation like diarrhea or visible blood in the feces detectable at this time. In contrast, as shown in Figure 12A, a stronger induction of IL-12 and IFN-γ mRNA could be detected in mLNs of *Rag1*$^{-/-}$ compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice when experiments had to be terminated due to weight loss of up to 20 % in the *Rag1*$^{-/-}$ group.

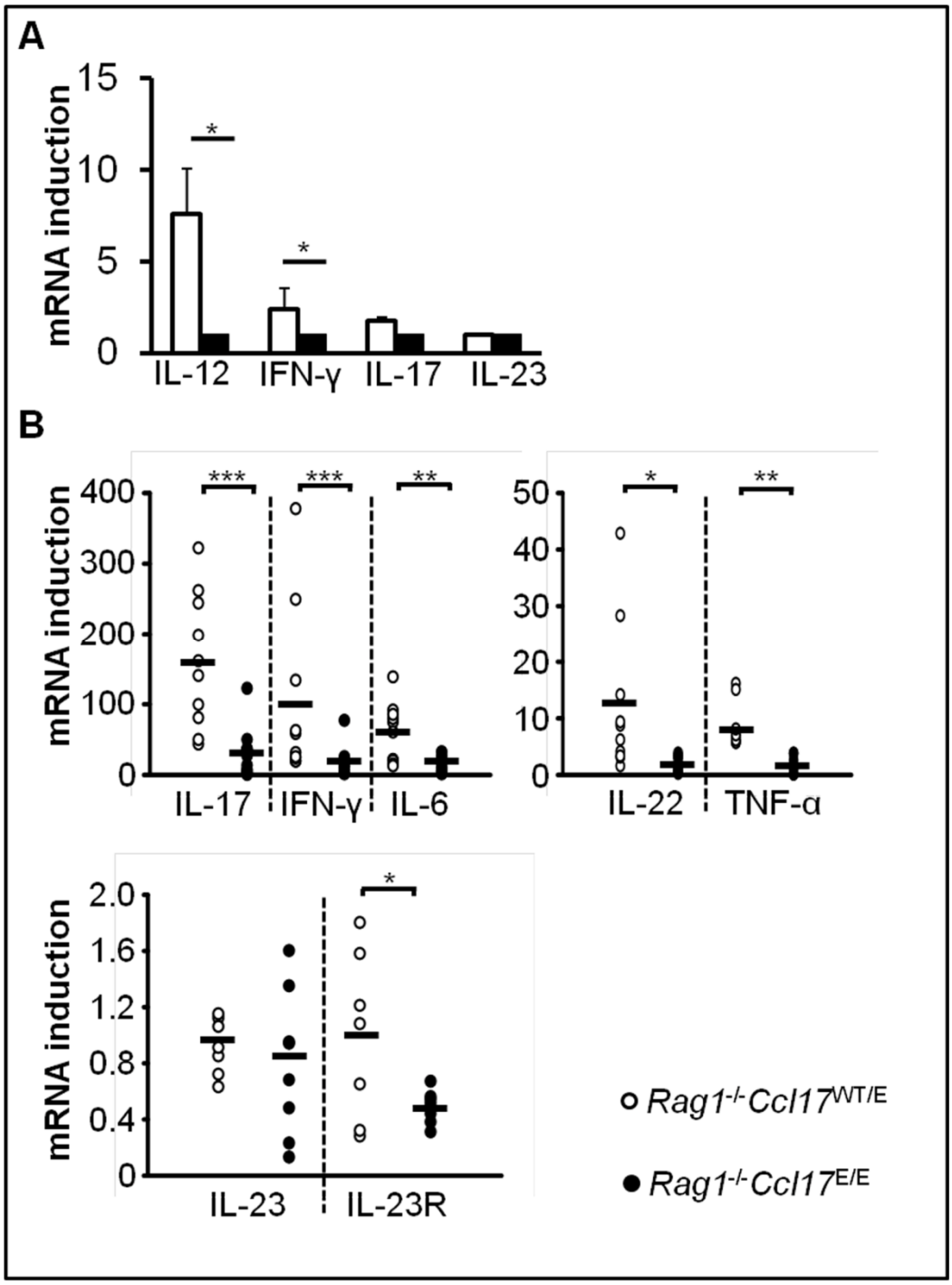

Figure 12: mRNA expression of cytokines in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice 21 days after T cell transfer. mRNA was extracted from single cell suspensions of mLNs or colon tissue and real-time qPCR was performed as described in Material & Methods section 3.2.9. (**A**) Expression of indicated cytokines in mLNs of *Rag1*$^{-/-}$ mice relative to expression in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. (*$P < 0.05$; ($n = 3$) Student's *t*-test) (**B**) Expression of indicated cytokines in colon tissue of *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice relative to expression in PBS treated mice. Pooled data of at least three independent experiments are displayed. Each dot represents one individual mouse and black crossbars indicate the mean *$P < 0.05$; **$P < 0.01$; **$P < 0.001$; ($n = 9$-12) Student's *t*-test

Further, as shown in Figure 12B, a significant induction of IFN-γ, IL-12, IL-6, IL-22 and TNF-α could be detected at that time point (days 19-21) in colon tissue of *Rag1*$^{-/-}$ mice,

which was significantly higher than in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. IL-23 and IL-23R mRNA levels in *Rag1*$^{-/-}$ mice were still comparable to the expression in PBS control mice; however, expression levels of IL-23R significantly deceased in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice.

Taken together, lymphopenic mice lacking CCL17 expression showed reduced susceptibility to colitis induction by transfer of naïve T cells. In both models, DSS treatment and T cell transfer, protection from colitis was demonstrated by bodyweight development and histological analysis, as well as cytokine profiling in mLNs and colon tissue.

4.4 Protection from severe colitis in lymphopenic mice after transfer of *Ccr4*$^{-/-}$ T cells

As mentioned in the introduction, it was shown that the predominant cause of action of CCL17 is to attract T cells to sites of inflammation via CCR4 interaction[180, 181]. For that reason, the T cell transfer model was considered to be most suitable to investigate effects of CCL17 on colitis development. Therefore, the following *in vivo* studies were conducted exclusively in the T cell transfer model of colitis.

By demonstrating on clinical, histological as well as cytokine production level that mice lacking CCL17 are protected from both, acute and chronic intestinal inflammation, first insights into the role of CCL17 during intestinal inflammation were gained. However, one would expect that, especially in the T cell transfer model, a recruitment defect of the transferred T cells, expressing the receptor for CCL17 (CCR4), would be the main underlying effect causing the observed differences. To investigate this hypothesis, T cells isolated from donor mice deficient of CCR4 (*Ccr4*$^{-/-}$) were transferred into *Rag1*$^{-/-}$ in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. Yet, as shown in Figure 13A, the difference and overall outcome of colitis induction was not affected when *Ccr4*$^{-/-}$ T cells were transferred instead of WT T cells, indicating that CCR4 expression on the transferred T cells is neither influencing the colitis induction in *Rag1*$^{-/-}$ nor the protection in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. Along this line, no difference in the recruitment of CD4^{+} T cells to the mLNs could be detected 5 days after transfer of WT T cells (Figure 13B). 21 days after T cell transfer, when colitis was most active in *Rag1*$^{-/-}$ mice, there were also no differences in the frequency of T cells in the mLNs as well as the IEL and LPL fraction of the colon detectable (Figure 13C). These data indicate that a recruitment defect of the transferred T cells is not the underlying effect leading to the protection of *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice.

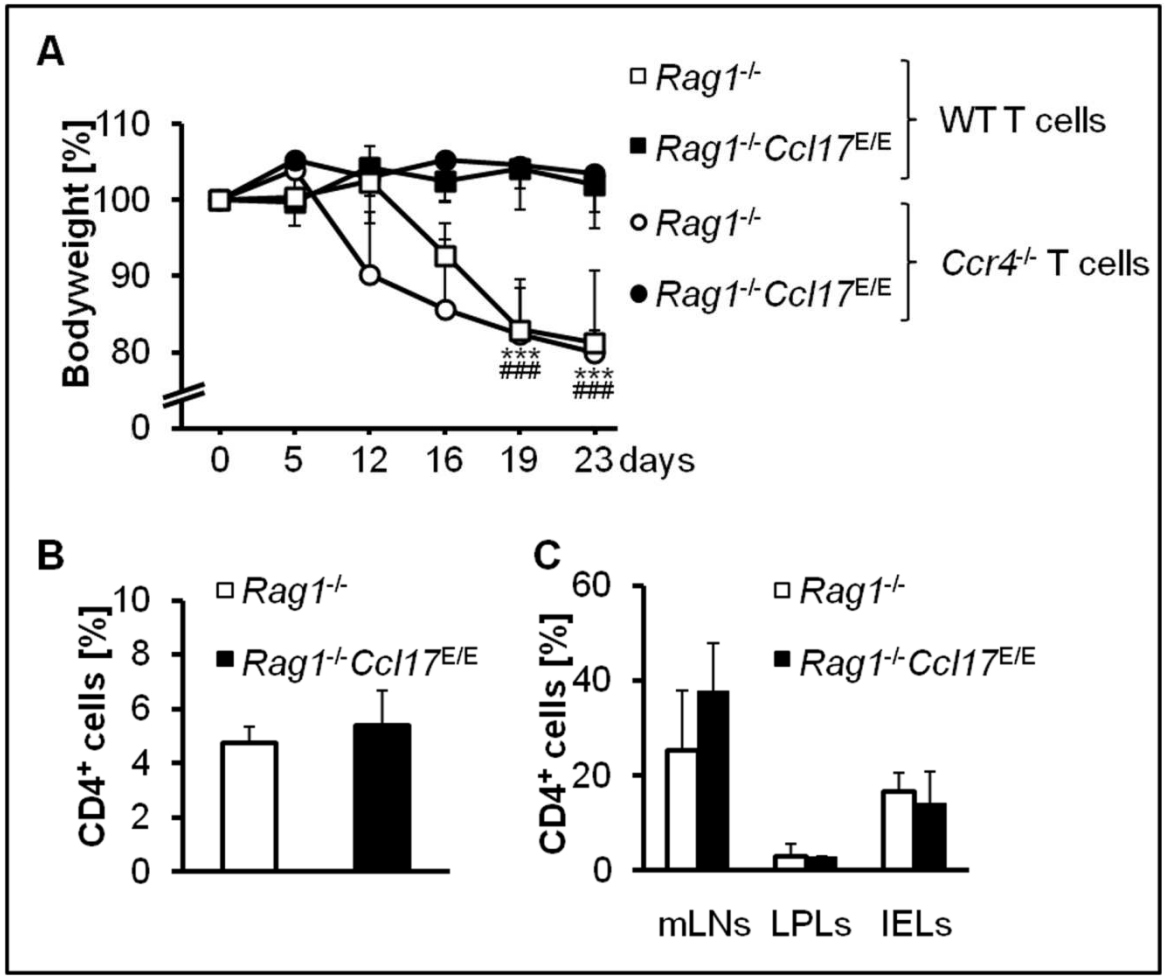

Figure 13: Influence of CCR4 expression on transferred T cells and distribution of T cells after transfer. (**A**) Course of bodyweight of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice after transfer of *Ccr4*$^{-/-}$ T cells or WT T cells. ($^{###}P < 0.001$(WT T cell transfer); $***P < 0.001$(*Ccr4*$^{-/-}$ T cell transfer); ($n = 4$) Student's *t*-test) (**B** and **C**) Percentage of CD4^{+} T cells after transfer of WT T cells in indicated organs and fractions 5 days (**B**) and 21 days (**C**) after transfer. **B** and **C**: one representative of at least three independent experiments is shown ($n = 3$).

In contrast, when WT T cells were transferred into *Rag1*$^{-/-}$*Ccr4*$^{-/-}$ mice, a significantly reduced colitis induction compared to *Rag1*$^{-/-}$ mice could be detected (Figure 14). This protection of *Rag1*$^{-/-}$*Ccr4*$^{-/-}$ mice indicated that a different cell type than T cells which failed to respond to CCL17 in the absence of CCR4 was probably responsible for protection of *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice.

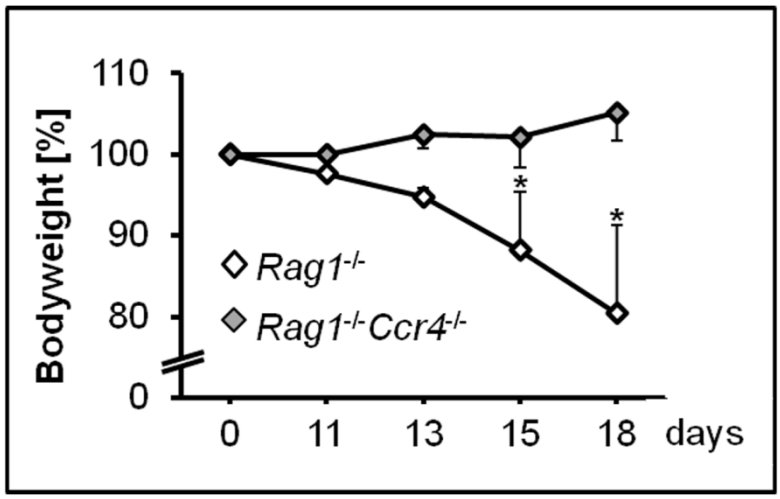

Figure 14: Course of bodyweight of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr4*$^{-/-}$ mice after transfer of WT T cells. WT T cells were transferred into *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr4*$^{-/-}$ mice and bodyweight was measured at the indicated time points after transfer. **P* < 0.05; (*n* = 4)

Thus, the possibility that a different recruitment of DCs might be this factor was tested. For this purpose, the distribution of DCs in untreated mice was assessed. In Figure 15A it is shown that no initial differences in the presence of DCs were detectable in mLNs as well as IEL and LPL fractions of the colon in the steady state. Along this line, although a general increase in frequencies of DCs was detected 21 days after T cell transfer, no difference between *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice was measurable at this time point (Figure 15B), indicating that the lack of CCL17 did not affect the recruitment of total DCs in both, mLNs or colon fractions. Furthermore, peritoneal lavage was used to investigate if differences in the recruitment of DCs, or other effector cells of the innate immune response like CD11b^{+} macrophages might be occurring in the absence of CCL17. However, as shown in Figure 15C, using this method no differences were observed between WT and *Ccl17*$^{E/E}$ mice.

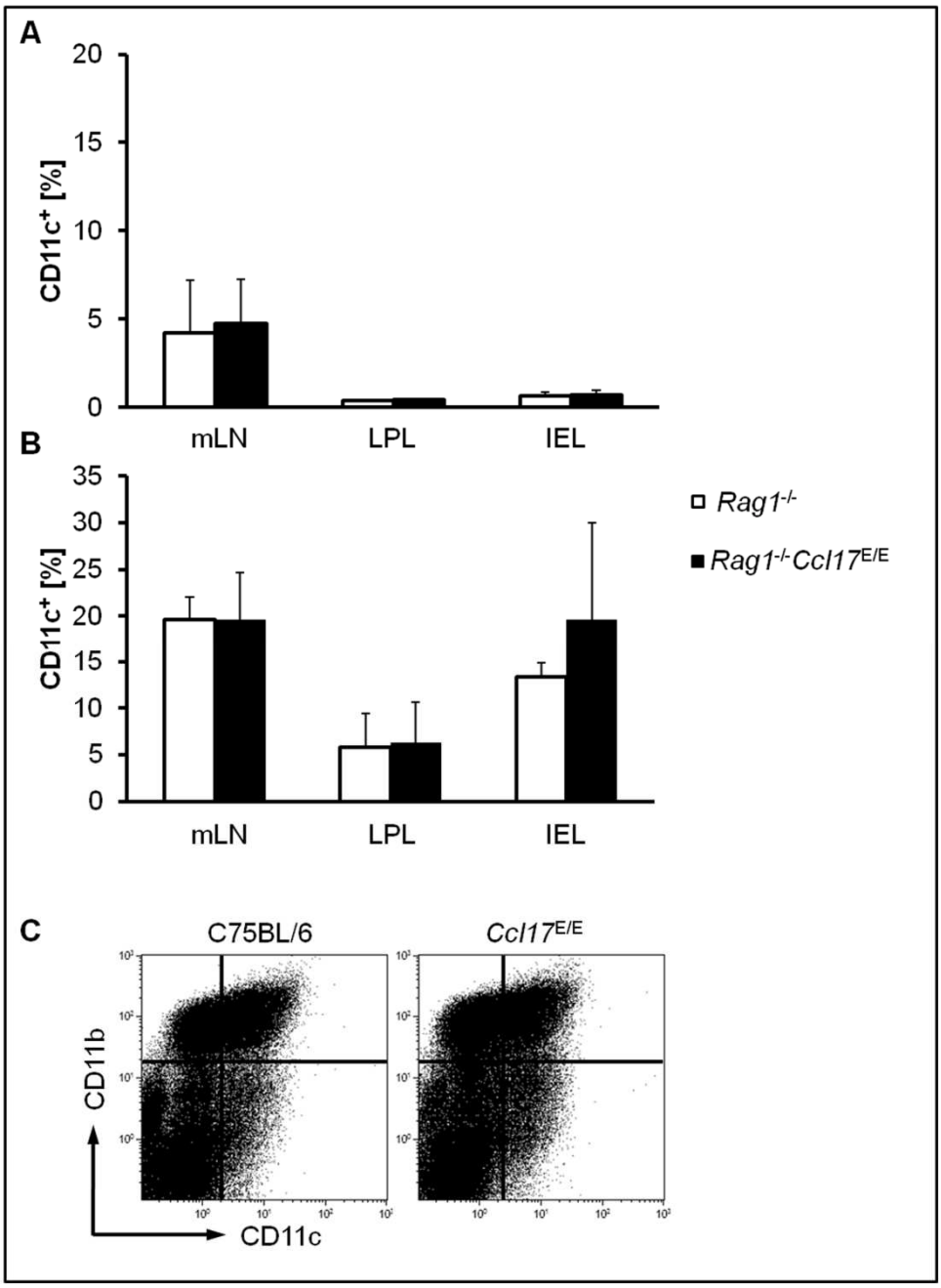

Figure 15: Steady state distribution and recruitment of DCs in WT and CCL17 deficient mice. (**A** and **B**) Percentage of $CD11c^+$ dendritic cells in steady state mice (**A**) (n = 4) and *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice 21 days after T cell transfer (**B**) (n = 3). (**C**) FACS plots of peritoneal lavage cell isolates, stained with CD11c and CD11b, 4 days after 1 ml 4 % thioglycolate had been injected intraperitoneally.

In summary, altered recruitment of total DCs or transferred T cells is not the mechanism that accounts for the protection of mice lacking CCL17. This was demonstrated by: (1) transfer of *Ccr4*$^{-/-}$ T cells; (2) analysis of the distribution of DCs in the mLNs and colon fractions; and (3) by peritoneal lavage to test general recruitment of immune cells to sites of inflammation upon thioglycolate treatment.

4.5 Enhancement of DC-cytokine production by CCL17

In recent publications, it was suggested that chemokines can act like cytokines by directly inducing activation of target cells expressing the corresponding receptor. For example, CX_3CL1 and CCL2 have been shown to induce IL-12 production in macrophages and bone marrow derived dendritic cells (BMDCs), respectively[215, 216]. Since I showed that the recruitment of T cell via CCR4, the receptor for CCL17 did not influence colitis induction in the T cell transfer model, and no difference in the recruitment of DCs could be detected, the hypothesis that CCL17 might directly induce the production of cytokines was tested.

Hence, BMDCs, expressing the receptor CCR4, were stimulated with LPS (TLR4 ligand) or Pam_3Cys (TLR2 ligand). As shown in Figure 16, no differences in the expression of the costimulatory molecules CD40, CD80 and CD86, as well as MHCII-expression could be detected after stimulation with LPS for 24 h in both groups.

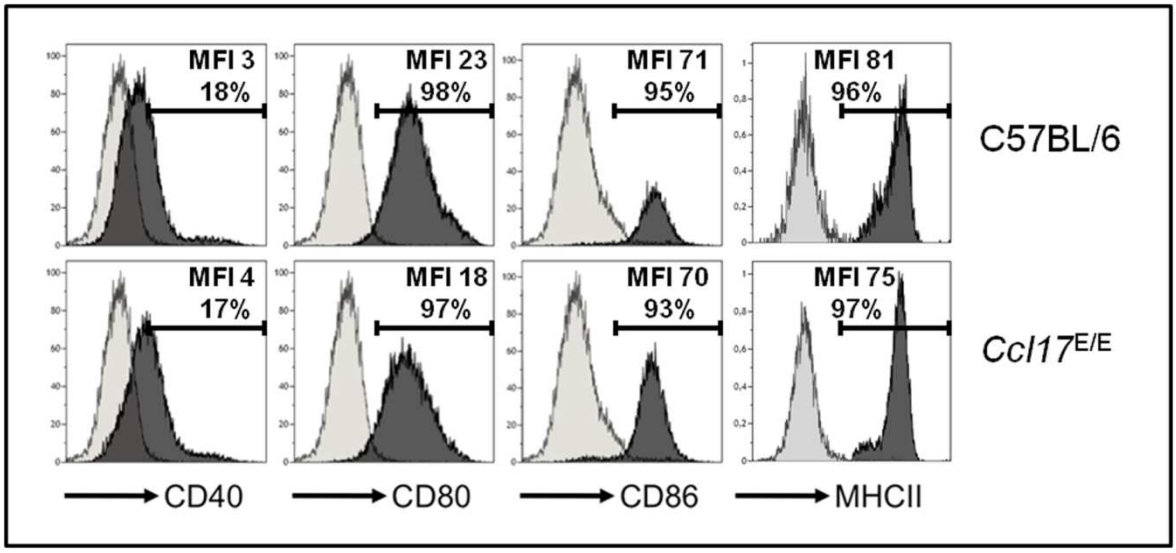

Figure 16: Expression of costimulatory molecules on BMDCs stimulated with LPS. BMDCs were stimulated with 100 ng/ml LPS for 24 h. Expression of the costimulatory molecules CD40, CD80 and CD86, as well as MHCII was measured by FACS analysis. Light gray histograms represent fluorescence minus 1 staining. Dark gray histograms represent staining of the indicated markers. MFI of the markers of the positive cells is indicated in each histogram, including the percentage of positive cells. Representative FACS analyses of three independent experiments are displayed.

However, when BMDCs were stimulated with LPS or Pam_3Cys for 6 h, markedly reduced induction of IL-12p35, IL-23p19 and IL-10 mRNA could be detected in BMDCs isolated from *Ccl17*$^{E/E}$ mice (*Ccl17*$^{E/E}$ DCs) compared to BMDCs isolated from WT mice (Figure 17).

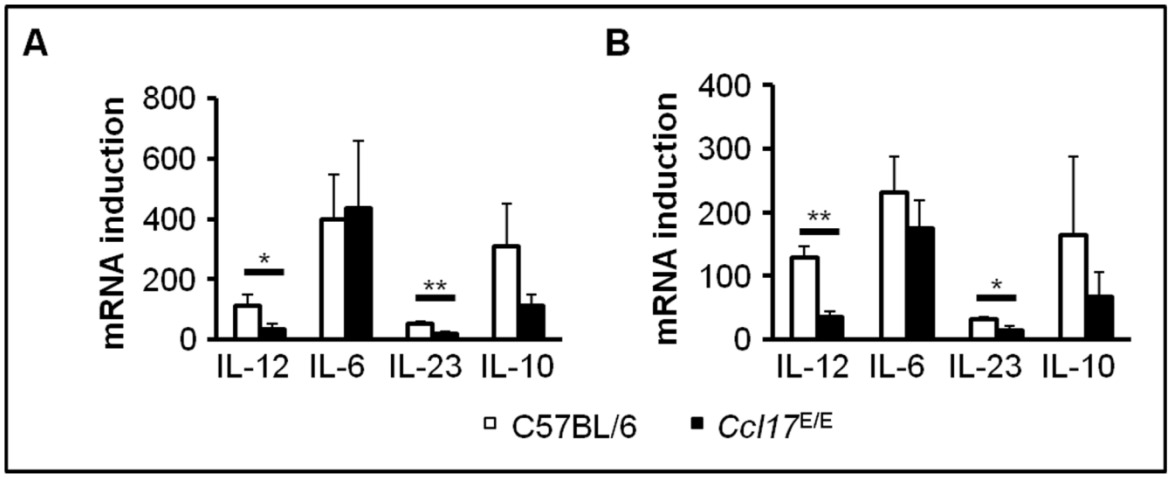

Figure 17: Induction of cytokine mRNA after stimulation of BMDCs with TLR2/4 agonists. BMDCs were stimulated with either 100 ng/ml LPS (**A**) or 100 ng/ml Pam_3Cys (**B**) for 6 h. mRNA was isolated using TRIzol. Real-time qPCR was performed as described in Material & Methods section 3.2.8 and induction levels were calculated relative to unstimulated controls. mean ± SD is shown ($n = 3$); ($^{*}P < 0.05$; $^{**}P < 0.01$)

Moreover, when *Ccl17*$^{E/E}$ DCs were stimulated with LPS in the presence of recombinant mouse (rm) CCL17, a dose dependent induction of IL-12p40 by rmCCL17 could be detected. Yet, this induction was only detectable when LPS was present (Figure 18). In conclusion, these stimulation experiments suggest that CCL17 has an autocrine effect on the DCs which were producing the chemokine in the first place.

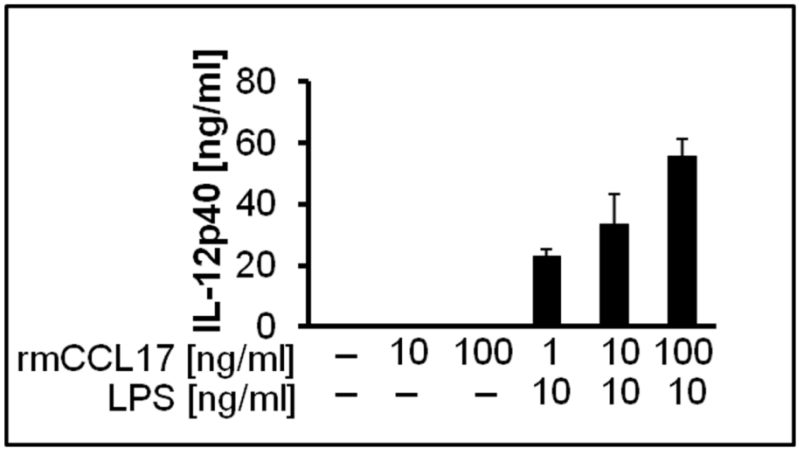

Figure 18: Stimulation of *Ccl17*$^{E/E}$ DCs with combinations of rmCCL17 and LPS. *Ccl17*$^{E/E}$-DCs were stimulated with or without 10 ng/ml LPS and in the presence of increasing amounts of rmCCL17 as indicated. Mean ± SD of stimulations performed in triplicates are displayed. One of two independent experiments is shown.

This autocrine effect of rmCCL17, driving the induction and production of cytokines, might be directly mediated via its receptor CCR4. To test this, LPS-induced induction of cytokines in BMDCs isolated from *Ccr4*$^{-/-}$ mice (*Ccr4*$^{-/-}$ BMDCs) was compared to induction in WT BMDCs. As shown in Figure 19A, a reduced induction of IL-23p19 and IL-10 mRNA in *Ccr4*$^{-/-}$ BMDCs compared to WT BMDCs could indeed be detected. However, this was not true for IL-12p35 mRNA at this timepoint. But, as shown in Figure 19B, a reduced production of IL-12p40 was detected in *Ccr4*$^{-/-}$ BMDCs compared to WT BMDCs.

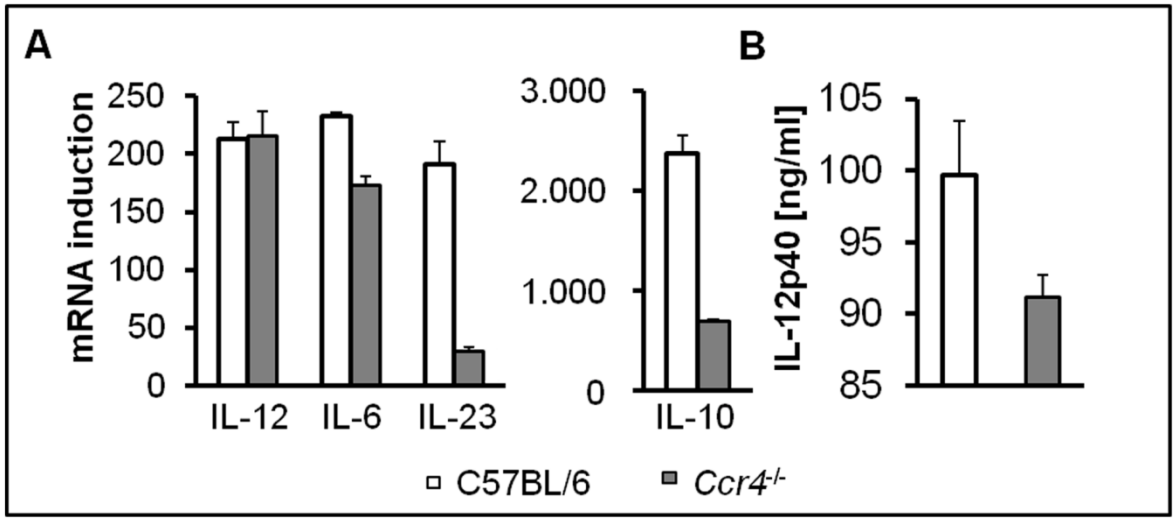

Figure 19: Comparison of cytokine production/induction in *Ccr4*$^{-/-}$ BMDCs compared to WT BMDCs. *Ccr4*$^{-/-}$ BMDCs and WT BMDCs were stimulated with 100 ng/ml LPS for 6 h (**A**) for mRNA induction of the indicated cytokines, or for 24 h (**B**) for production of IL-12p40. Mean ± SD of stimulations performed in triplicates are displayed. One representative of two independent experiments is shown.

Taken together, the *in vitro* stimulation experiments using BMDCs showed that CCL17 can act in an autocrine manner on the DCs themselves. This autocrine effect drives cytokine production by DCs, and is, at least in part, dependent on CCR4 expression on the DCs.

4.6 T cell activation by WT and *Ccl17*$^{E/E}$ DCs

The autocrine effect of CCL17 seen in *in vitro* stimulation experiments using BMDCs (depicted in Figure 17, Figure 18 and Figure 19) demonstrates that CCL17 is driving the production of proinflammatory cytokines. T cells are activated and influenced by DCs and the cytokines secreted by them. Therefore, coculture experiments were performed to get insights whether the reduced induction of cytokines in *Ccl17*$^{E/E}$ DCs affects the differentiation and activation of T cells.

Indeed, as shown in Figure 20A, reduced amounts of IFN-γ were detected in supernatants of T cells cocultivated with *Ccl17*$^{E/E}$ BMDCs. This was in line with a slightly reduced induction of *T-bet*, a transcription factor crucial for T_H1 T cell differentiation (Figure 20B). In contrast, no difference in IL-17 production could be detected in T cell cocultured with WT compared to *Ccl17*$^{E/E}$ BMDCs (Figure 20C).

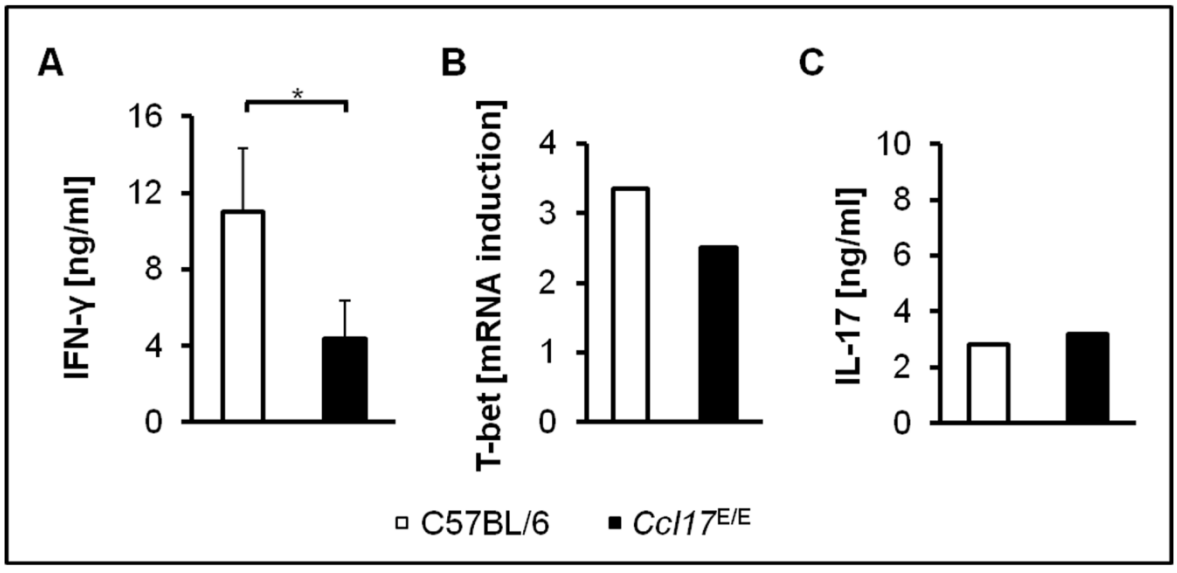

Figure 20: Differentiation of T cells cocultivated either with WT-BMDCs or *Ccl17*$^{E/E}$-BMDCs. CD4$^+$/CD62L$^+$ MACS isolated T cells were cocultivated with either WT-BMDCs or *Ccl17*$^{E/E}$-BMDCs in the presence of 5 µg/ml pOVA and 100 ng/ml LPS for 4 days. (**A**) IFN-γ production was measured in coculture supernatants after 4 days of culture by ELISA. (**B**) mRNA expression levels of cell pellets were calculated relative to expression level in untreated CD4$^+$/CD62L$^+$ MACS isolated T cells. (**C**) IL-17 production was determined in coculture supernatants after 4 days of culture by ELISA. (**A**) Mean ± SD of 4 experiments is displayed. (**B** and **C**) One representative of two independent experiments is shown. (*$P < 0.05$; Student's *t*-test)

If the autocrine effect of CCL17 also affects the differentiation of IL-17 producing T_H17 cells can only be investigated in *in vitro* coculture systems with BMDCs or splenic DCs when additional cytokines, driving T_H17 differentiation, are added. However, as it was

hypothesized that the autocrine loop of CCL17 affects the cytokine production itself, a different approach was chosen. DCs isolated directly from mLNs (mLNDCs) of either WT (WT mLNDCs) or *Ccl17*$^{E/E}$ mice (*Ccl17*$^{E/E}$ mLNDCs), pretreated with LPS for 16 h, were cocultivated with T cells. In Figure 21A, it is shown that T cells cocultivated with WT mLNDCs produce higher amounts of IL-17 than T cells cocultivated with *Ccl17*$^{E/E}$ mLNDCs. Additionally, these T cells show a higher expression of *rorc* encoding RORγt, the transcription factor driving T_H17 differentiation (Figure 21B). In contrast to the *in vitro* coculture system, no effect on IFN-γ production could be detected in this *ex vivo* setting (Figure 21C).

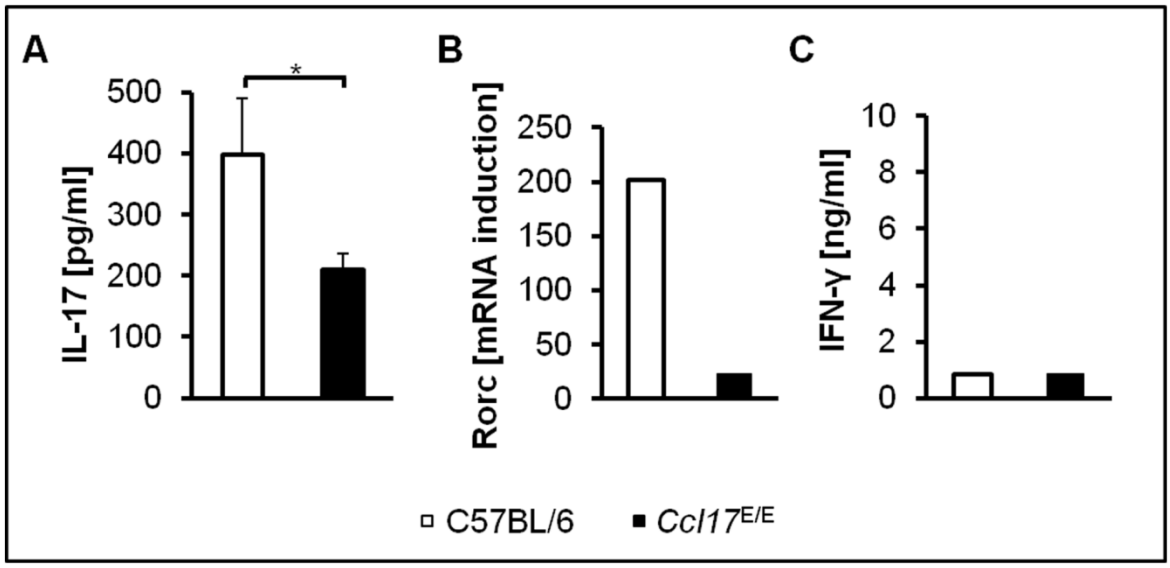

Figure 21: Differentiation of T cell cocultivated with either WT mLNDCs or *Ccl17*$^{E/E}$ mLNDCs. CD4$^+$/CD62L$^+$ MACS isolated T cells were cocultivated with either WT mLNDCs or *Ccl17*$^{E/E}$ mLNDCs in the presence of 5 µg/ml pOVA and 100 ng/ml LPS for 4 days. (**A**) IL-17 production was measured in coculture supernatants after 4 days of culture by ELISA. (**B**) mRNA expression levels of cells were calculated relative to the expression level in untreated CD4$^+$/CD62L$^+$ MACS isolated T cells. (**C**) IFN-γ production was measured in coculture supernatants after 4 days of culture by ELISA. (**A**) Mean ± SD of 4 experiments is displayed. (**B** and **C**) One representative of two independent experiments is shown. (*P < 0.05; Student's *t*-test)

These *in* vitro results which show that CCL17 drives T cell differentiation, most probably by influencing cytokine production of DCs (as shown in Figure 17, Figure 18 and Figure 19) gave first insights into the mechanisms responsible for the colitogenic effect of CCL17. Further evidence that this actually takes place *in vivo* is provided by the increased mRNA levels of IL-17 and IFN-γ found in *Rag1*$^{-/-}$ mice compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice (Figure 12). More evidence that this difference is due to an enforced T_H1/T_H17 differentiation of the transferred T cells in CCL17 competent mice could be obtained by

direct analysis of the T cells *in vivo*. In fact, as demonstrated by FACS analysis (Figure 22A), increased percentages of IFN-γ and IL-17 producing T cells could be detected in *Rag1*$^{-/-}$*Ccl17*$^{E/WT}$ mice, compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice.

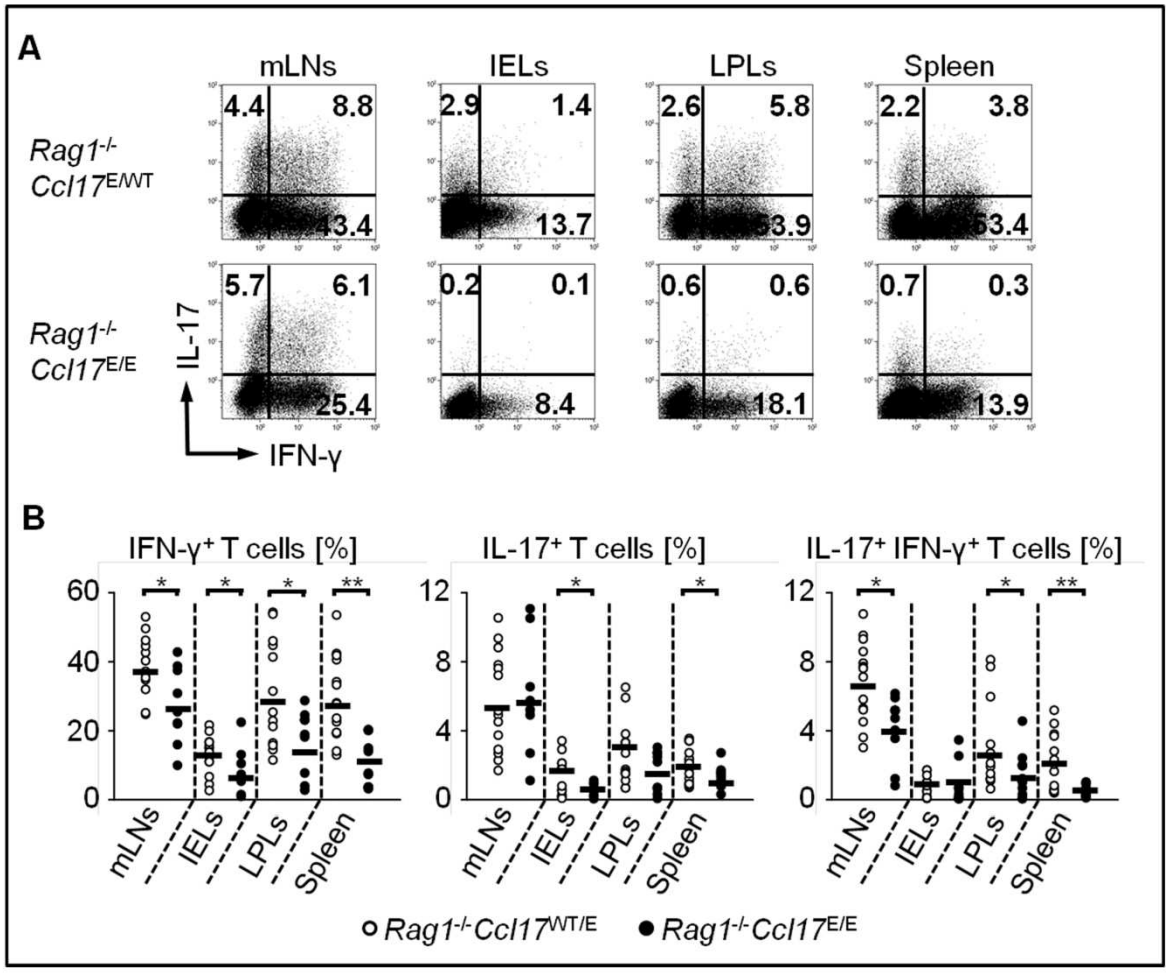

Figure 22: Intracellular IL-17 and IFN-γ staining after T cell transfer. (**A**) Cells of the mLNs, colonic fractions (IELs & LPLs) and the spleen were stained for the membrane bound T cell markers CD4 and CD3ε and intracellularly for the cytokines IFN-γ and IL-17. CD4/CD3ε double positive gated T cells are shown in the representative dot plots with percentages indicated in the respective quadrants. (**B**) Data of three independent experiments were pooled and statistical analysis was performed (n = 9-12; $^{*}P < 0.05$; $^{**}P < 0.01$).

Especially, IFN-γ single positive T cells were significantly more prominent in mLNs (38.4 ± 8.2 *vs*. 27.1 ± 10.3), the IEL fraction (13.1 ± 5.4 vs. 8.0 ± 6.2) and the LPL fraction (30.6 ± 14.8 *vs*. 16.7 ± 9.0) of the colon and the spleen (29.5 ± 11.5 *vs*. 10.4 ± 6.5) of *Rag1*$^{-/-}$ *Ccl17*$^{E/WT}$ mice than *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. IL-17 single positive cells were only present in higher numbers in IEL fractions of the colon (1.3 ± 0.7 *vs*. 0.5 ± 0.2) and the spleen (2.0 ± 0.8 *vs*. 1.1 ± 0.3) of *Rag1*$^{-/-}$*Ccl17*$^{E/WT}$ mice. However, IL-17/IFN-γ double positive cells were significantly more prominent in the mLNs (6.8 ± 2.2 *vs*. 4.1 ± 1.7), the spleen (2.1 ±

1.5 *vs.* 0.4 ± 0.2) and the LPL fraction of the colon (3.1 ± 2.4 *vs.* 1.3 ± 1.2) of *Rag1*$^{-/-}$ *Ccl17*$^{E/WT}$ mice, than *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice (Figure 22B).

Taken together, the results obtained throughout the study lead to the model that CCL17 is produced by DCs, acts on DCs in an autocrine and/or paracrine loop leading to the production of proinflammatory cytokines, which are finally inducing T_H1/T_H17 differentiation of naïve T cells (Figure 23).

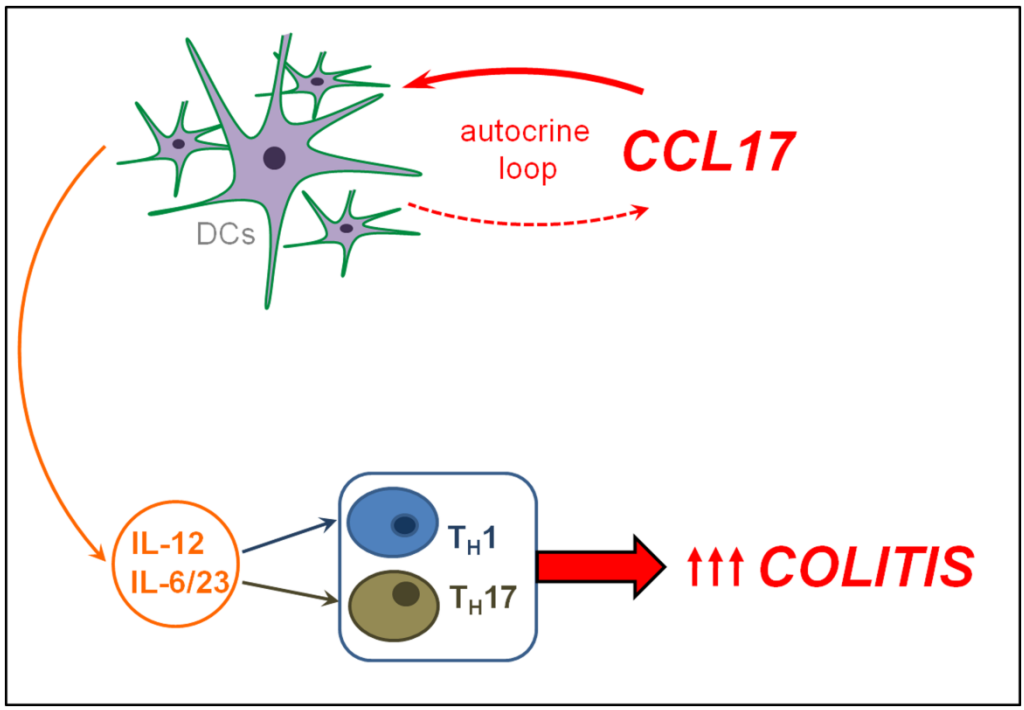

Figure 23: Proposed model of colitis driving effect of CCL17. CCL17, which is produced by DCs acts in an autocrine loop on the DCs themselves. This is driving the production of the cytokines IL-12 and IL-6/23, leading to an increased differentiation of inflammatory T_H1/T_H17 cells. All over, this chain of events is amplifying the induction of colitis.

4.7 Foxp3-dependent longterm protection of *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice

So far, it could be demonstrated that *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice are protected from severe colitis at the timepoint, when *Rag1*$^{-/-}$ mice were severely ill. This was shown by analysis of bodyweight and histology, as well as immunological analysis (mRNA expression analysis and intracellular staining of cytokines). However, the differences might only be due to a delayed onset of colitis in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. To investigate this possibility, longterm experiments were performed. As demonstrated by bodyweight assessment (Figure 24), *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice were protected from severe colitis up to 47 days after T cell transfer. Although the mice slightly lost bodyweight, they did not reach sacrificing criteria, namely a loss of 20 % of their bodyweight. In addition, no other signs of severe colitis like visible blood in the feces or heavy diarrhea developed.

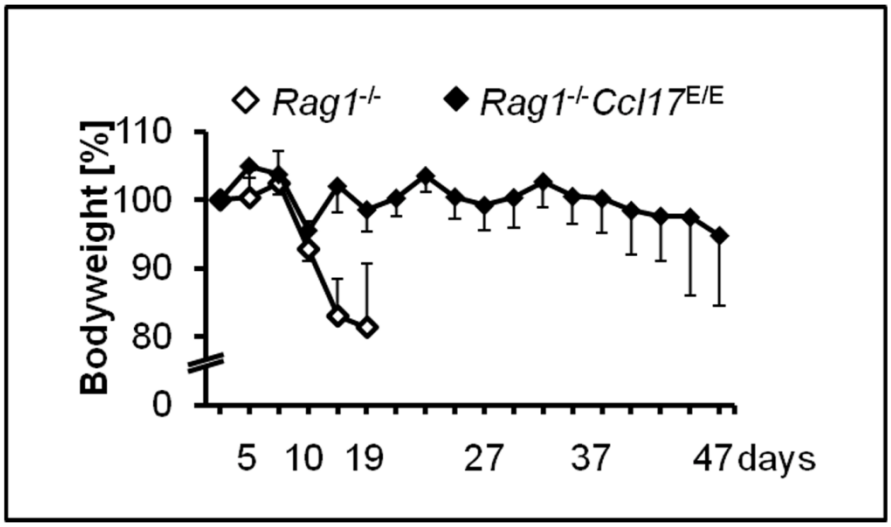

Figure 24: Course of bodyweight in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. Colitis was induced in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice by T cell transfer. Colitis activity was monitored by bodyweight assessment. *n* = 3 mice per group.

Taken together, these results indicate that *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice are indeed protected from severe colitis.

It is known that regulatory T cells (T_{reg} cells) are very important in keeping immune responses in balance. For example, T_{reg} cells limit the proinflammatory responses of T_H1 as well as T_H17 cells, leading to an overall downregulation of proinflammatory mediators, which results in a termination of immune responses. Effectively, T_{reg} cells can limit tissue

damage caused by an overreactive immune response. Foxp3-expressing T_{reg} cells are very well characterized, especially in this context of intestinal immune regulation. As shown in Figure 5, a fraction of around 6-8 % of Foxp3$^+$ T_{reg} cells was detectable in CD4$^+$/CD62L$^+$ MACS sorted T cells which were transferred. This fraction was stable after one day of transfer in mLNs, but very rapidly decreased in *Rag1*$^{-/-}$ mice to 1.4 % ± 0.2 (day 21). In contrast, this fraction only decreased to a significantly lower extend in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice to 3.8 % ± 1.2. Moreover, the percentage of Foxp3$^+$ T_{reg} cells further increased in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice to 14.8 % ± 2.4 at day 47 (Figure 25A).

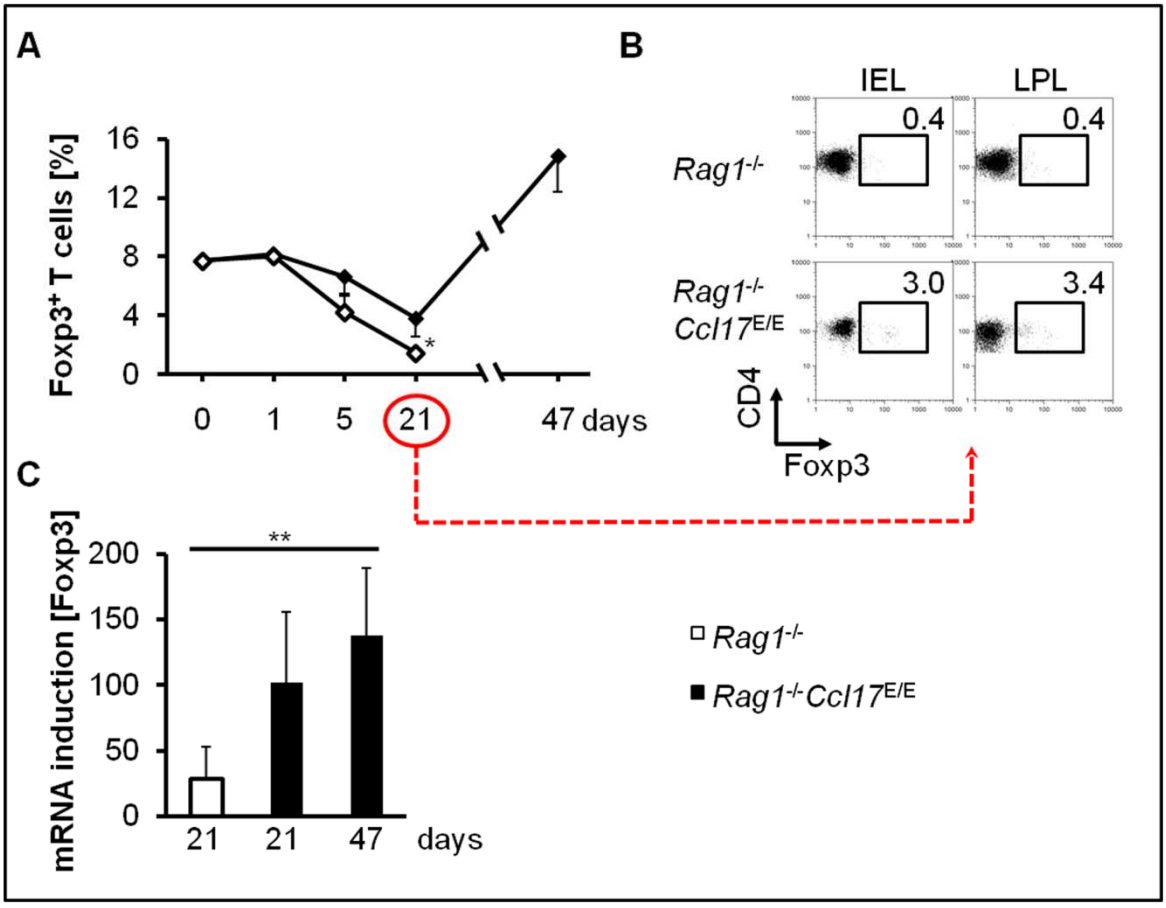

Figure 25: Foxp3$^+$ T_{reg} cells in mLNs as well as colon fractions and Foxp3 mRNA expression in the colon of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice reconstituted with T cells. (**A**) Percentage of Foxp3$^+$ T cells before transfer (day 0) and at indicated time points. Percentages were determined by FACS analysis. (n = 3) (**B**) Representative FACS plots of intracellular Foxp3 expression in T cells in IEL and LPL fractions of the colon. (**C**) Foxp3 mRNA expression in the colon relative to PBS control mice in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice at the indicated time points. (n = 5). (*P < 0.05; **P < 0.01)

The differences observed on day 21 after transfer in the mLNs could also be detected in the colon fraction by FACS analysis (Figure 25B). Higher mRNA expression levels of *Foxp3* were detected in the colon (after 21 days), with a significant increase on day 47 (Figure 25C), consistent with expansion of the T_{reg} population at this site. Moreover higher levels

of mRNA of *tgf-β1*, *csf2* (GMCSF) and *aldh1a2* (enzyme converting retinaldehyde to retinoic acid) could be detected in colon tissue of *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice 21 days after T cell transfer (Figure 26).

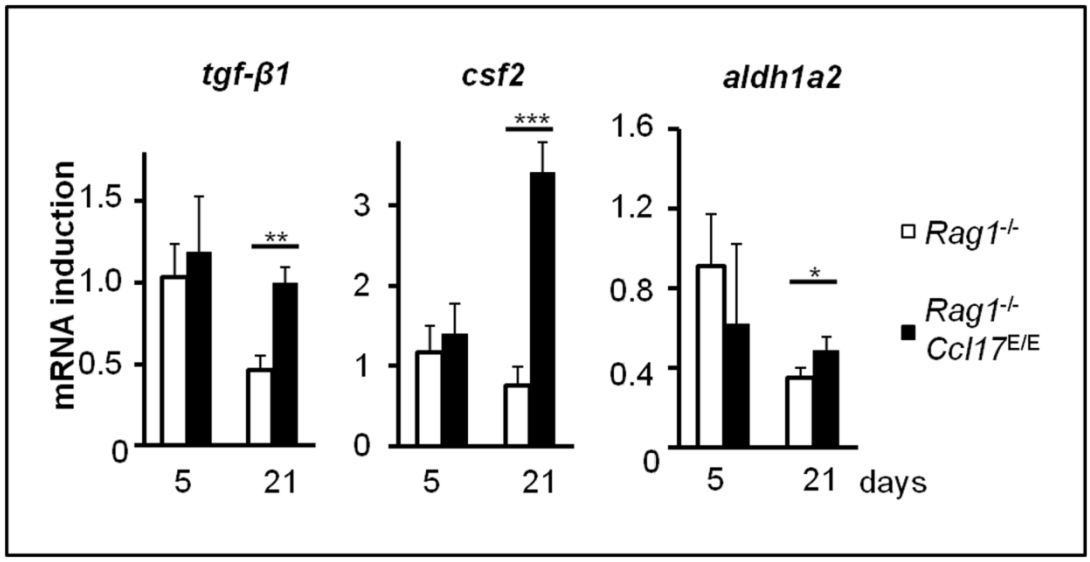

Figure 26: Expression of Foxp3-driving transcription factors in colon tissue of *Rag1*$^{-/-}$and *Rag1*$^{-/-}$ *Ccl17*$^{E/E}$ mice. mRNA was extracted from colon tissue and real-time qPCR was performed as described in Material & Methods section 3.2.9. Induction of mRNA is calculated relative to the induction in PBS treated mice. Mean ± SD of *n* = 3 mice is displayed.

The factors described and analyzed above have been shown to be produced by CD103^{+} DCs, which were described to have a high capacity to induce Foxp3^{+} T_{reg} cells[86, 217]. Analysis of CD103 expression on DCs in the IEL and LPL fractions of the colon revealed that higher frequencies were present in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice in the LPL fraction (65.7 % *vs*. 20.8 %) as well as the IEL fraction (92.6 % *vs*. 45.3 %) when compared to the frequencies in *Rag1*$^{-/-}$ mice (Figure 27) at day 47 in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice compared to day 21 in *Rag1*$^{-/-}$ mice.

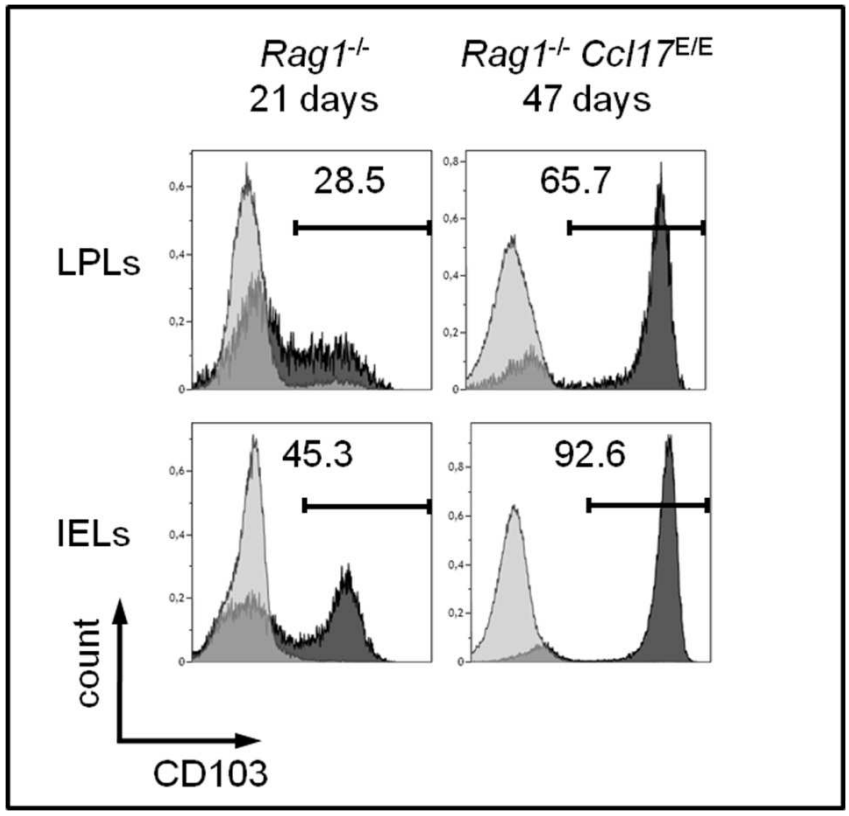

Figure 27: CD103 expression on DCs of *Rag1*$^{-/-}$and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. CD103 expression on CD3ε^{-}/CD11c^{+} colonic DCs was determined by FACS analysis at the indicated time points after T cell transfer into *Rag1*$^{-/-}$and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. Light gray histograms represent negative control staining. Numbers indicate percentage of CD103^{+} cells. One representative staining from 4 mice in each group is shown.

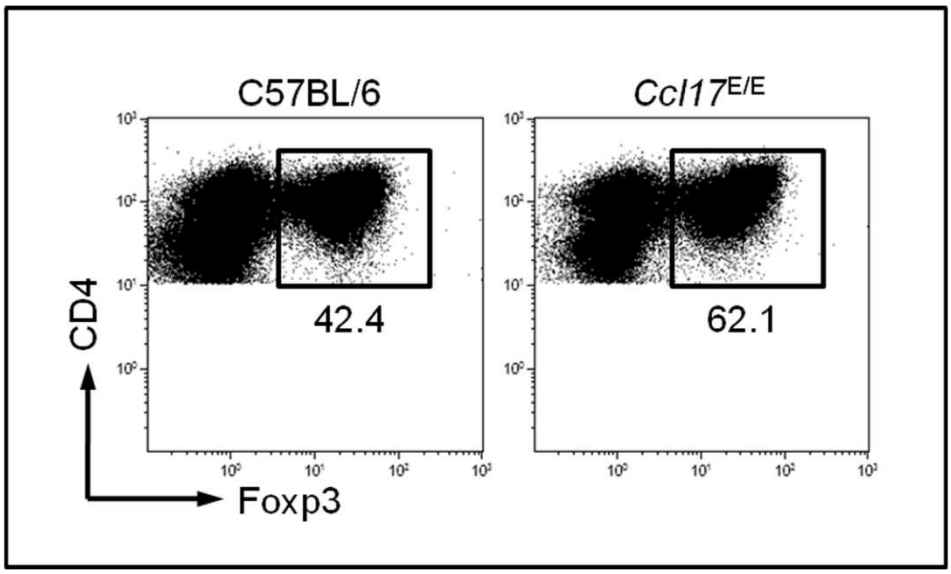

Figure 28: Foxp3 expression in T cells cocultivated with mLNDCs under T_{reg} driving conditions. CD4^{+}/CD62L^{+} MACS isolated T cells were cocultivated with either WT mLNDCs or *Ccl17*$^{E/E}$ mLNDCs in the presence of 5 µg/ml α-CD3ε, 200 U/ml IL-2 and 5 ng/ml TGF-β for 4 days. Foxp3 expression in CD4^{+} T cells is shown in the dot plots. Numbers represent percentage of gated Foxp3^{+} T cells. One of 4 independent experiments with comparable differences is shown.

Furthermore, *ex vivo* coculture of $CD4^+/CD62L^+$ T cells with mLNDCs revealed a higher capacity of mLNDCs isolated from *Ccl17*$^{E/E}$ mice to induce Foxp3 expression in T cells under T_{reg} driving conditions (5 µg/ml α-CD3ε, 200 U/ml IL-2 and 5 ng/ml TGF-β added to the coculture) (Figure 28).

Additionally, a higher steady state mRNA expression of *Foxp3* could be detected in mLNs of untreated *Ccl17*$^{E/E}$ compared to C57BL/6 mice (Figure 29).

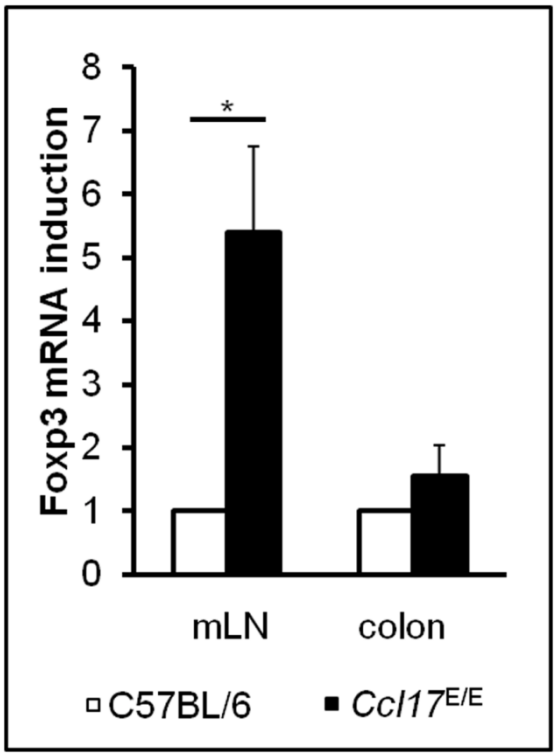

Figure 29: Steady state *Foxp3* mRNA expression in C57BL/6 *vs.* *Ccl17*$^{E/E}$ mice. Real-time qPCR was performed from single cell suspensions of mLNs and whole colon tissue on total RNA isolated using TRIzol. Expression in *Ccl17*$^{E/E}$ mice is calculated relative to the expression in C57BL/6 mice. (*$P < 0.05$; $n = 3$)

The observed expansion of the $Foxp3^+$ T_{reg} population in the colon and the mLNs, might be responsible for the prevention of severe colitis in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice in longterm experiments. To investigate this, Foxp3-depleted T cells were transferred into *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. For this purpose, DEREG 23.2 mice were used. These mice express the diphteria toxin (DT)-receptor in $Foxp3^+$ cells. Thus, by injecting DT all $Foxp3^+$ cells are depleted. As shown in Figure 30, only 0.6 % of the initially 6-8 % $Foxp3^+$ cells remained in the MACS sorted T cells after DT treatment.

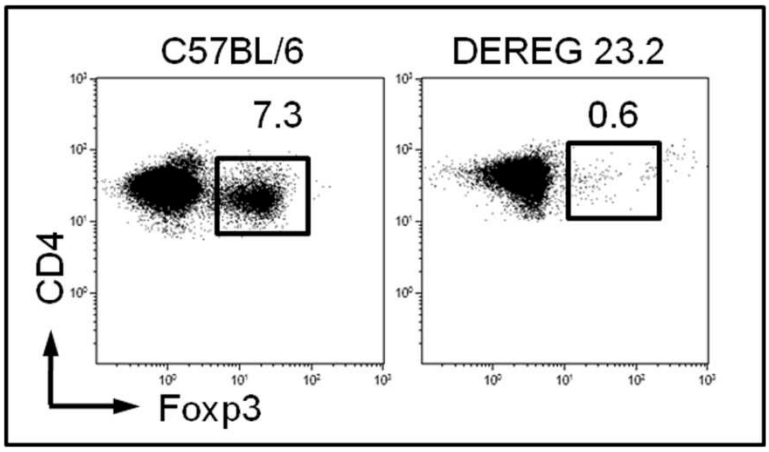

Figure 30: CD4$^+$/CD62L$^+$ MACS sort of WT and diphtheria toxin treated DEREG 23.2 mice. Left panel: Percentage of Foxp3$^+$ CD4$^+$/CD62L$^+$ T cells after MACS sorting from WT mice. Right panel: Percentage of Foxp3$^+$ CD4$^+$/CD62L$^+$ T cells after MACS sorting from DEREG 23.2 mice 36 h after i.p. injection of 500 ng diphtheria toxin.

As shown in Figure 31, the transfer of these Foxp3-depleted T cells resulted in a more rapid colitis induction in *Rag1*$^{-/-}$ mice compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice (difference at day 17), which was in accordance to the previous differences detected when T_{reg} containing T cells were transferred. However, *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice were no longer protected from severe colitis at later timepoints, demonstrated by a weight loss to 85.2 % ± 3.9 of the initial bodyweight at day 25, which was significantly less bodyweight compared to the bodyweight of *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice (100.4 % ± 3.1; $P = 0.006$) which had received T cells containing initially 6-8 % Foxp3$^+$ T cells.

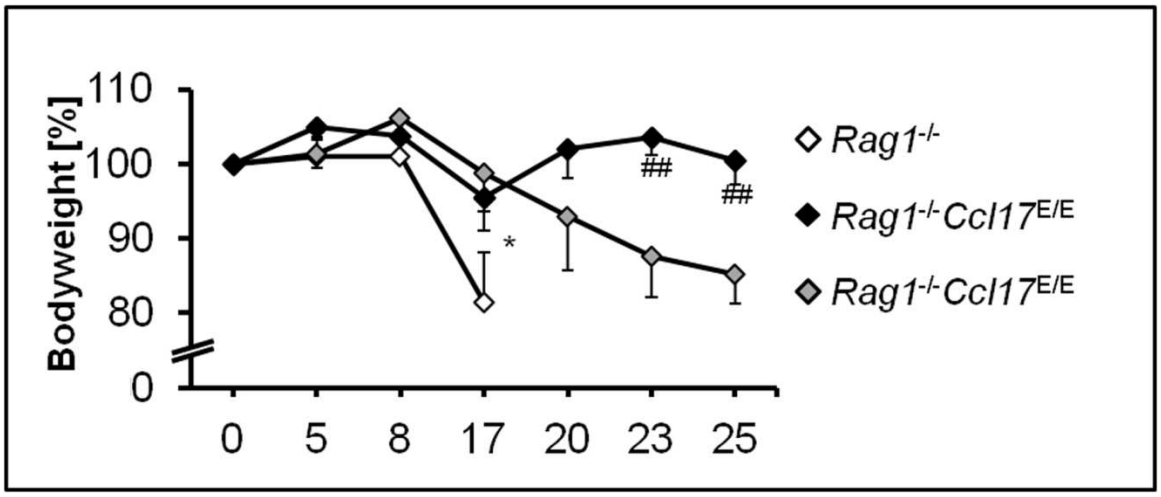

Figure 31: Course of bodyweight of *Rag1*$^{-/-}$and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice reconstituted with Foxp3-depleted T cells or *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice reconstituted with WT T cells. White filled diamonds represent *Rag1*$^{-/-}$ mice reconstituted with Foxp3-depleted T cells; gray filled diamonds represent *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice reconstituted with Foxp3-depleted T cells; black filled diamonds represent *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice reconstituted with WT T cells. *$P < 0.05$; *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ *vs.* *Rag1*$^{-/-}$ mice, both reconstituted with Foxp3-depleted T cells; ##$P < 0.01$; *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice reconstituted with Foxp3-depleted T cells *vs.* *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice reconstituted with WT T cells.

In line with the similar loss of bodyweight, no differences in the levels of mRNA induction of cytokines could be detected any more (day 17 for *Rag1*$^{-/-}$ mice; day 25 for *Rag1*$^{-/-}$ *Ccl17*$^{E/E}$ mice; Figure 32).

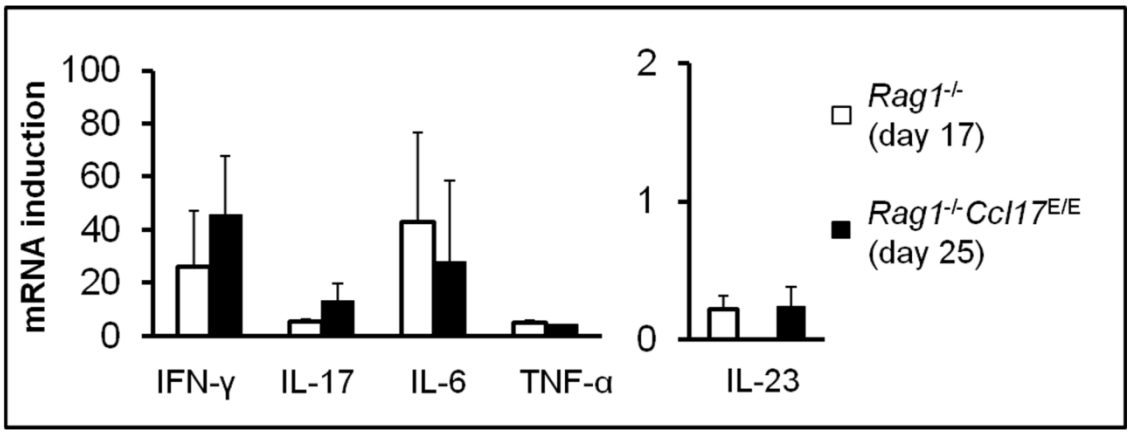

Figure 32: mRNA expression of cytokines in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice after transfer of Foxp3-depleted T cells. mRNA was extracted from colon tissue and real-time qPCR was performed as described in Material & Methods section 3.2.9. Induction of mRNA is relative to the induction in PBS treated mice. *Rag1*$^{-/-}$ mice were sacrificed after 17 days and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice after 25. Mean ± SD of n = 3 mice is displayed.

To further investigate if the high frequencies of Foxp3$^+$ T cells detected in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice after 47 days (Figure 25A and C) are generated by *de novo* induction of T_{reg} cells rather than an expansion of the cotransferred population, Foxp3 expression was analyzed in T cells after transfer of Foxp3-depleted T cells. One would expect an increase in frequencies of Foxp3$^+$ T_{reg} cells if a *de novo* induction is responsible for the high frequencies found at day 47. However, as shown in Figure 33, no increase could be detected, indicating that an expansion of the cotransferred T_{reg} cells accounts for the high frequencies observed at day 47.

On the basis of the results presented here, it can be concluded that CCL17 has a dual function in experimental colitis in mice. First, it is driving the proinflammatory mechanisms leading to tissue damage.

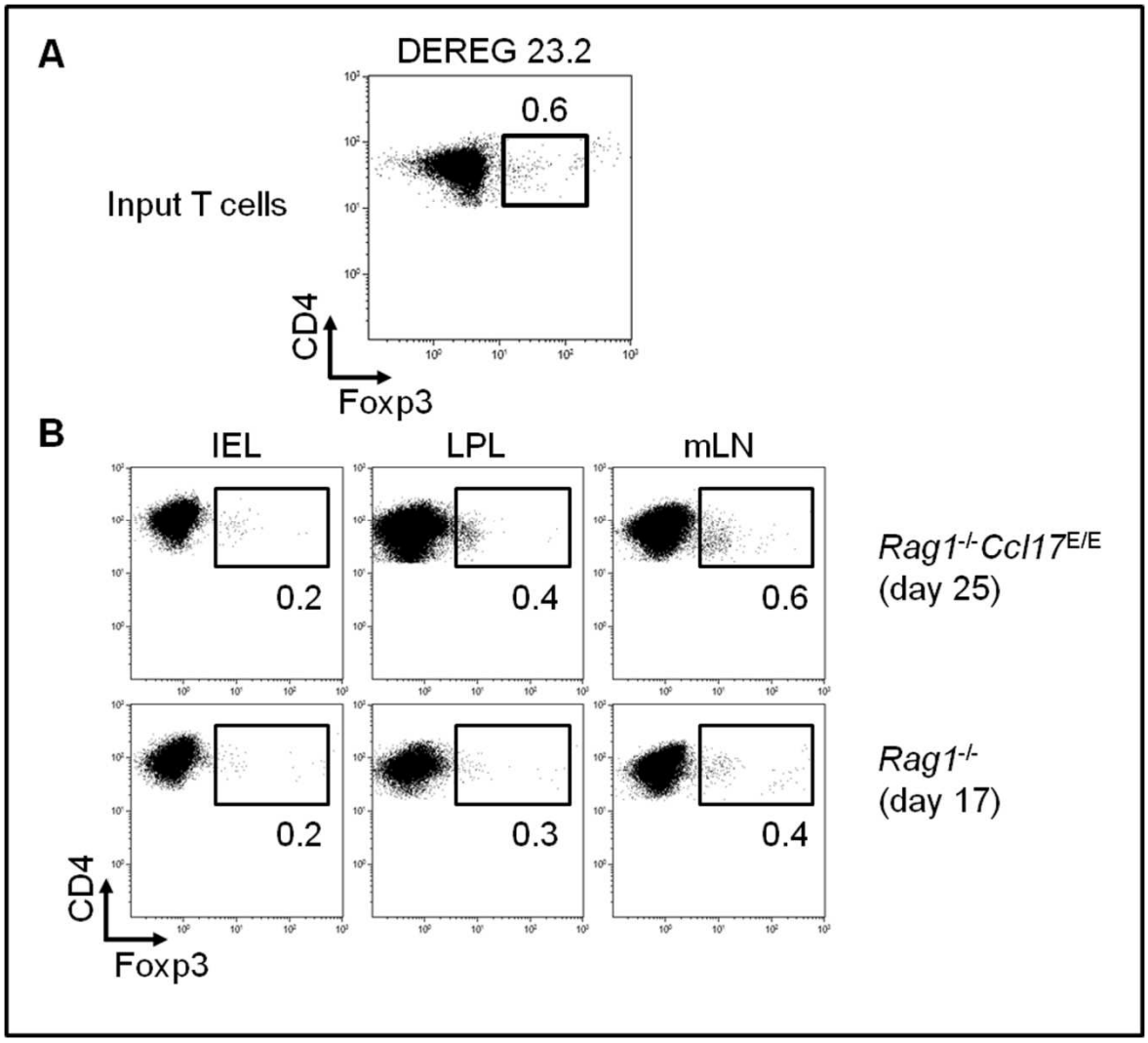

Figure 33: Frequencies of Foxp3-expressing T_{reg} cells after transfer of Foxp3-depleted T cells. (**A**) FACS analysis of $CD4^+/CD62L^+$ MACS sorted T cells isolated from DEREG 23.2 mice 36 h after i.p injection of 500 ng/ml DT (gated on $CD4^+$ cells). (**B**) FACS analysis of Foxp3-expression of $CD4^+$ gated T cells isolated from colon fractions or mLNs at the indicated time points. Dot plots are representative of 3 individual mice.

This action is achieved by an autocrine loop, in which CCL17 is produced by DCs and acts on DCs enhancing the capacity of DCs to produce cytokines, such as IL-12 and IL-23, which is in turn enforcing T_H1/T_H17 differentiation of T cells, which finally leads to an enhanced colitis development. Secondly, CCL17 is preventing the induction and expansion of Foxp3-expressing T_{reg} cells. This is most probably again caused by the autocrine loop, which in this case prevents the maintenance of Foxp3 expression by modulating the cytokine milieu. This effect affects colitis development by inhibiting the immune regulatory mechanisms Foxp3-expressing T_{reg} cells are mediating.

5 DISCUSSION

Research in the last two decades has shed much light on pathophysiological mechanisms influencing the course of IBD. However, up to date, no defined single trigger could be identified. Nonetheless, intensive research led to the development of new therapeutic approaches that are efficient in keeping patients in remission. One of the most successful attempts was and still is the use of anti-TNF-α antibodies, blocking TNF-α signaling and subsequent downstream activation of the immune system. Although this treatment does not eliminate the putative cause of the disease, clinical remission and mucosal healing can be achieved. Nevertheless, not all patients respond to the treatment with anti-TNF-α antibodies and with some patients the therapeutic success vanishes over time. Therefore, more research is needed (1) to find new treatments for those patients that do not respond to the drugs which are available at the moment and (2) to define the molecular and cellular mechanisms that lead to disease onset for a prevention of the disease in the first place.

CCL17, a C-C chemokine that is predominantly produced by conventional DCs, has been shown to be upregulated in atopic diseases like dermatitis[197-202] and astma[203], as well as in the microenvironment of tumors and has been described to attract T_H2 cells and T_{reg} cells in these cases, respectively. Few publications show that CCL17 is also induced during Crohn's disease in patients[211], or experimental colitis in mice[208-210]. Thus, it remains to be investigated whether CCL17 might play a role for IBD development and thus might be a potential target for treatment of IBD patients.

To address the question if CCL17 has a role during colitis development in mice, I used an eGFP knock in mouse model. In this model, eGFP is inserted into the second exon of the *Ccl17* locus. In heterozygous mice (*Ccl17*$^{E/WT}$) this results in a reporter system in which all cells that express CCL17 are also expressing eGFP. In homozygous knock in mice (*Ccl17*$^{E/E}$), all cells that would express CCL17 then express eGFP instead, resulting in a global loss of CCL17 production. This mouse model was established by the group of Irmgard Förster and published in 2003[177]. To investigate the role of CCL17 in murine IBD models, colitis was induced in mice in two ways. First, by adding DSS, a chemical agent, to the drinking water and secondly, by transferring naïve $CD4^+/CD62L^+$ T cells into lymphopenic mice lacking the *Rag1* gene (*Rag1*$^{-/-}$). In both models, *Ccl17*-deficient mice were protected from severe colitis. Colitis activity was measured by bodyweight

assessment and histological analysis of colonic inflammation. Furthermore, cytokine profiling was performed on mRNA level in the mLNs as well as colon tissue. In both models, proinflammatory cytokines were markedly reduced in *Ccl17*-deficient mice. Additionally, in the T cell transfer model infiltration of effector T cells (T_H1/T_H17) into the spleen, the mLNs and colon fractions was reduced in *Ccl17*-deficient mice. Functional analysis of the CCL17 producing DCs was performed in *in vitro* stimulation experiments, and the effects of CCL17 on T cell differentiation were examined in *in vitro* and *ex vivo* coculture setups, demonstrating a proinflammatory role for CCL17. Longterm immune regulation in *Ccl17*-deficient mice was assessed and functionally addressed using DEREG 23.2 mice (described in section 3.2.1), revealing that T_{reg} cells are essential for longterm protection in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice.

5.1 Colitis induction method

Colitis can be induced in mice in several ways. On the one hand there are model systems in which a genetic manipulation of the mice leads to spontaneous colitis development over time. This is the case in *Tnf*$^{\Delta ARE}$ mice, that lack mRNA stability control of TNF-α mRNA, leading to uncontrolled overproduction of TNF-α and subsequent induction of inflammation foremost in the ileal part of the intestine[133]. On the other hand, colitis can be induced in mice by causing injury to the epithelium, resulting in bacterial infiltration and consequential induction of inflammatory processes. This can be achieved by adding DSS to the drinking water. Another approach uses the transfer of naïve T cells into lymphopenic mice, which lack T cells

In this thesis, the DSS and the T cell transfer induced colitis models were used to investigate the role of CCL17 in intestinal inflammation. These two models will be discussed in the following.

5.1.1 T cell transfer colitis

In 2000, it was shown by Atreya *et al.*, that the transfer of $CD4^+/CD62L^+$ T cells into recipient mice led to a rapid loss of bodyweight (~ 20 % after 18 days) as well as severe colonic inflammation[136]. In contrast to the model system previously published by Powrie *et al.* in 1993, in which the transfer of $CD4^+/CD45RB^{high}$ T cells led to a loss of bodyweight

within 12 weeks[128], the $CD4^+/CD62L^+$ T cells transfer model established by Atreya *et al.* is faster. This might be caused by a more effective homing and entry of the transferred T cells into mLNs and colon lamina propia via CD62L-MadCAM or CD62L-GlyCAM interactions at high endothelial venules, which are crucial in this process[78]. Another difference between the models is the percentage of $Foxp3^+$ T_{reg} cells that are initially cotransferred. In the $CD4^+/CD45RB^{high}$ T cells transferred by Powrie *et al.* very few T_{reg} cells are present initially (< 0.5 %)[218], while the $CD4^+/CD62L^+$ T cells harbor a fraction of around 6 to 8 % T_{reg} cells. This is of special interest because T_{reg} cells have been shown to be able to inhibit colitis induction by T cell transfer, when about 30 % of the transferred T cells are T_{reg} cells[140], or to reverse an established colitis when transferred at later time points[37]. However, as demonstrated in *Rag1*$^{-/-}$ mice, the cotransfer of around 6 to 8 % T_{reg} cells did not prevent colitis induction in the experiments conducted during this project. For instance, this might be caused by a rapid decrease in the proportion of $Foxp3^+$ T_{reg} cells or even conversion of T_{reg} cells into effector T cells, detected in the mLNs and colon fractions of *Rag1*$^{-/-}$ mice, or simply because the numbers were too low to efficiently suppress the induction and proliferation of proinflammatory cells like T_H1/T_H17 cells. In general, the cotransferred T_{reg} cells are not expanded along with effector T cells and thus, their frequency decreases, allowing colitis development. This model, however, offers the chance to assess the role of T_{reg} cells in T cell mediated colitis.

5.1.2 Dextran sulfate sodium (DSS) colitis

While one published protocol suggests to add 2 % (w/v) DSS to the drinking water for 8 days, which results in colonic inflammation with a loss of bodyweight first detectable 8 days after starting DSS application[219], I used a dose of 4 % (w/v) DSS for a period of 5 days. Recent publications reported that colitis induction by DSS varies a lot between mouse strains[220] and colonization state of the mice[221, 222]. In our experiments, using 4 % (w/v) DSS for 5 days led to reproducible results. This system is characterized by a rapid loss of bodyweight starting already 5 days after initial DSS application. Consequently C57BL/6 wild type mice lost up to 20 % bodyweight after approximately 7 days and had to be sacrificed, according to German animal care and ethics legislation.

The two models, the T cell transfer colitis model and the DSS model, were used to investigate the role of CCL17 in murine experimental colitis. The T cell transfer model

was chosen because the function of CCL17 was reported to be the attraction of T cells[194, 197-199, 203, 204]. Thus, potential effects of CCL17 on T cells during colitis development could be investigated in a more sophisticated way using this model. The DSS model was used to investigate if CCL17 might have other effects apart from effects on T cells, for example such as on innate immune regulation during intestinal inflammation, which can be investigated in the DSS model, as this model of intestinal inflammation was described to be independent of the adaptive immune system[223].

5.2 CCL17-dependent colitis activity

5.2.1 Clinical and histological assessment of colitis activity

In order to investigate the role of CCL17 during induction and maintenance of intestinal inflammation in mice, it was of fundamental importance to demonstrate that CCL17 is indeed upregulated on protein level during inflammatory processes in the intestine, as well as the mLNs, draining the intestine. Previous reports had shown that *Ccl17* mRNA expression is upregulated in the colon tissue during murine experimental colitis[208-210]. Using the reporter mouse system I could show that CCL17 expression in DCs is in fact upregulated in both models, the DSS and the T cell transfer induced colitis. While a basal CCL17 expression was measurable already before colitis induction, a strong increase was measured during active disease, reflected in a higher percentage of DCs expressing CCL17-eGFP. This correlation was clearly demonstrated in the T cell transfer model, where no CCL17 upregulation was detectable 5 days after transfer when no signs of illness were visible. Furthermore, I could show that CCL17 is exclusively produced by $CD11c^+/CD11b^+/MHCII^+/F4/80^{low/-}$ DCs in the colon, which is in line with previous reports, showing that CCL17 is produced by $CD11b^+$ cutaneous[196] and mLN[177] DCs, as well as in DCs within atherosclerotic lesions[206].

In both colitis models, CCL17 was crucial for the induction of severe colonic inflammation. This could be demonstrated by higher loss of bodyweight, stronger inflammation in the colon, as well as higher induction of proinflammatory cytokines in mice capable of CCL17 production. Assessment of bodyweight was the primary clinical endpoint applied to assess disease activity throughout the experiments. In general, bodyweight loss in murine models of colitis is caused by heavy diarrhea, resulting in water

loss, accompanied by a loss of the integrity of the intestinal tract[219, 224]. In the DSS model, mice lacking CCL17 are not completely protected from induction of this inflammatory processes. This is reflected by the finding that *Ccl17*$^{E/E}$ mice also lose weight during the experiment (93.8 % ± 2.7 % of initial bodyweight at day 7). Similarly, in the T cell transfer model of colitis, *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice do not gain weight (100.1 ± 6.4 at day 21 after T cell transfer), as would be expected in completely healthy animals and inflammatory cytokine production was induced, although at much lower levels than in *Rag1*$^{-/-}$ mice.

In the DSS as well as the T cell transfer model, histological scoring of colon sections was performed to demonstrate that reduced colitis activity was in fact the basis for reduced weight loss in *Ccl17*$^{E/E}$ mice. While nearly no signs of inflammation were observed in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice 21 days after T cell transfer, *Ccl17*-deficient mice exposed to DSS showed signs of inflammation at day 7. This difference can be caused by the different impact the two models have. In the DSS model, DSS initially causes damage to the intestinal epithelial layer by inducing apoptosis in epithelial cells.[219]. This leads to infiltration of bacteria into the mucosa due to barrier break down with subsequently acute inflammation as the result. While CCL17 seems to promote the resulting inflammatory processes leading to bodyweight loss, the initial damage, which is also reflected in the histological score, cannot be entirely prevented by the absence of CCL17. In comparison, the T cell transfer model of colitis is driven by activation of the naïve T cells transferred into recipient mice, reflecting an immunologically induced model. Thus, histological changes could be prevented in *Ccl17*-deficient mice in the T cell transfer model where inflammatory cytokines and chemokines are the driving factors of inflammation.

5.2.2 Role of cytokines and T cell differentiation

In the DSS model, especially TNF-α[225, 226], IL-6[227], but also IL-12[228], IFN-γ[228] and IL-23[229] have been implicated to drive disease severity. However, neither TNF-α nor IL-6 nor IL-23 were differentially regulated on mRNA levels in C57BL/6 compared to *Ccl17*$^{E/E}$ mice treated with DSS. Yet, IL-12 in the mLNs as well as IFN-γ in the colon were significantly stronger induced in C57BL/6 mice, but not *vice versa*. On the one hand, one might argue that the increased levels of IL-12 in the mLNs mediate an enhanced activation of T_H1 cells. These T_H1 cells might then migrate to the colon, the site of inflammation, where they produce IFN-γ. Although acute DSS colitis can be induced in the absence of T and B

cells[223], one report showed reduced susceptibility of *Rag1*$^{-/-}$ mice to low dose DSS (1.5 % w/v)[230]. In line with this report, recent publications clearly implicate a role for T_H1/T_H17 cells in this model[231]. On the other hand, high levels of IFN-γ could also be produced by macrophages, which have been reported to be infiltrating the colonic mucosa during acute DSS induced colitis in mice and to produce high amounts of the cytokine[232]. Another source for IFN-γ might also be NK cells[233], which have been reported to be present in the intestinal lamina propria[234].

Since the T cell transfer model resembles a more chronic inflammation, it is suitable to investigate longterm effects on T cell differentiation as well as regulatory mechanisms. In this model, many cytokines could be demonstrated to be upregulated in colon and mLNs during colitis as observed in the DSS model. About 21 days (± 2 days) after T cell transfer, when *Rag1*$^{-/-}$ mice had to be sacrificed due to severe weight loss and showed severe intestinal inflammation, higher levels of IL-12p35 and IFN-γ could be detected in mLNs of *Rag1*$^{-/-}$ mice, which have both been implicated to drive colitis development[235]. However, although IL-17A and IL-23 have also been associated with colitis induction[236], *IL-17A* and *IL-23* mRNA levels in mLNs were at comparable levels in both groups. In colon tissue, however, mRNA levels of *IL-6, IL-17, IL-22, IFN-γ* and *TNF-α*, as well as *IL-23R* were either much stronger induced, or at least not downregulated, respectively, in *Rag1*$^{-/-}$ compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice.

As mentioned above, *TNF-α* has been shown to be upregulated and crucial in the DSS model of colitis. Furthermore, induction of TNF-α has also been implicated in T cell transfer colitis and treatment with anti-TNF-α antibodies in this setting resulted in reduced T cell infiltrates in colon tissue as well as reduced signs of disease[237]. In line with these results, reduced inflammation in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice correlated with reduced induction of *TNF-α* mRNA in colon tissue in my study.

Although recent reports show that IL-12 is not the essential factor driving intestinal pathology[144, 145], the T_H1 differentiation pathway, which is triggered by IL-12[47], strongly supports the finding that the elevated *IFN-γ* mRNA levels in the mLNs as well as the colon, are caused by induction of T_H1 cells. Indeed, high frequencies of IFN-γ producing T cells could be detected in both organs as well as the spleen, which were strongly reduced in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice.

Another very important T cell differentiation pathway in experimental colitis is the development of IL-17 producing T_H17 cells as well as IFN-γ/IL-17 double producing T cells. Both cell types, T cells exclusively producing IL-17 or both IFN-γ/IL-17, have been shown to depend on IL-23 signaling via its receptor IL-23R on T cells[146, 218, 238]. Although IL-23 mRNA was not differentially regulated in *Rag1*$^{-/-}$ mice compared to *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice, diminished levels of IL-23R expression were detected in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice, correlating with lower percentages of both, T cells exclusively producing IL-17 and IFN-γ/IL-17 double producing T cells. However, the role of T_H17 cells has been a matter of debate lately and is controversially discussed. Recent reports suggested that IL-17A has a protective role in T cell transfer colitis[150] and DSS colitis[151], while IL-17F-deficient mice showed reduced susceptibility to DSS colitis[151]. In my experiments, reduced proportions of both, T cells exclusively producing IL-17 as well as IFN-γ/IL-17 double producing T cells could be detected in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice, correlating with diminished signs of inflammation. These results provide further evidence that these cell types are mediating the inflammation in *Rag1*$^{-/-}$ mice and demonstrate that CCL17 is critically involved in the regulation of T_H1/T_H17 cell differentiation and/or maintenance.

As the deficiency of CCL17 resulted in reduced percentages of T_H1, T_H17 as well as IFN-γ/IL-17 double producing T cells, it might be argued that CCL17 has either a direct or indirect influence on T cell differentiation. To get insight into this question, *in vitro* and *ex vivo* experiments were performed. Although no differences in the expression of the common maturation markers of DCs could be detected in WT DCs compared to *Ccl17*$^{E/E}$ DCs, reduced cytokine mRNA expression of IL-12p35 and IL-23 could be measured in *Ccl17*$^{E/E}$ DCs after stimulation with TLR ligands. Recombinant mouse CCL17 directly enhanced LPS triggered IL-12p40 production by *Ccl17*$^{E/E}$ DCs in a dose dependent manner, suggesting that signaling downstream of TLRs is amplified by an autocrine action of this chemokine. IL-12p40 production in response to LPS was also reduced in *Ccr4*$^{-/-}$ DCs, emphasizing that CCL17 truly acts in an autocrine manner on DCs. Moreover, in cocultivation experiments using BMDCs or mLNDCs and T cells, a higher induction of T_H1 and T_H17 cells could be detected when T cells were cultured with WT DCs than *Ccl17*-deficient DCs. This effect can be explained by the greater capacity of WT DCs to produce the proinflammatory cytokines IL-12 and IL-23, which have been shown to drive T_H1 and T_H17 differentiation, respectively[47, 50]. The novel finding that the DC-specific chemokine CCL17 acts on DCs in an autocrine manner to enhance inflammatory cytokine

production is consistent with other reports showing a similar enhancing activity of the chemokines CCL2 and CX_3CR1 (fractalkine), accompanied by reduced NF-κB expression[215, 216].

5.2.3 Recruitment of inflammatory cells

Apart from a reduced induction of pathogenic T cells in the mLNs and the colon, an altered recruitment of inflammatory cells to the colon would seem reasonable as an explanation for the differences detected in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. This is likely, as the predominant action attributed to chemokines is the attraction of cells bearing the corresponding chemokine receptor. In previous studies, CCL17 has been shown to be produced by cutaneous DCs[177] and to direct recruitment of T cells to the skin[239]

Thus, the possibility that the reduced inflammation detected in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice might be due to a lack of recruitment of the transferred CCR4 expression T cells or DCs to mLNs and/or the colon was investigated. However, there was no difference detectable in the percentages of T cells in mLNs as well as the colon at various time points. This was also true for DCs in untreated *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice, despite a massive increase in the frequencies of DCs mLNs as well as colon fractions. Furthermore, no differences between C57BL/6 and *Ccl17*$^{E/E}$ could be detected with regard to recruitment of innate immune cells to the peritoneal cavity after induction of inflammation. In 2007, Yuan *et al.* showed that *Ccr4*$^{-/-}$ T_{reg} cells failed to accumulate in mLNs at early stages after transfer and argued that this lack of recruitment was causative for the loss of protection from induction of T cell transfer colitis[240]. Thus, the possibility that the transfer of *Ccr4*$^{-/-}$ $CD4^+/CD62L^+$ T cells might alter the outcome of disease induction was analyzed. However, no alteration in disease induction between the transfer of WT or *Ccr4*$^{-/-}$ T cells could be detected. Quite contrary to this assumption, the lack of CCR4 in recipient mice even prevented disease induction, promoting the argument that CCR4 expression on innate immune cells in recipient mice is required to induce strong inflammation after T cell transfer. This result supports the interpretation that CCL17 drives pathogenic T cell differentiation via its autocrine action on the DCs, which produce CCL17.

5.3 Longterm regulation of inflammation in the absence of CCL17

While the results discussed above, clearly demonstrate a proinflammatory role of CCL17 during the development of murine colitis by driving T_H1/T_H17 differentiation, another very important aspect, the regulation of intestinal homeostasis by T_{reg} cells, has not been addressed so far. To get insights into this possibility, the T cell transfer model was used. The advantage of T cell transfer model is that the amount of T_{reg} cells transferred into *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice can be manipulated.

And in fact, investigation of this aspect revealed that the fraction of T_{reg} cells which were cotransferred with the $CD4^+/CD62L^+$ T cells into *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice developed differently over time. Interestingly, a decrease in the percentage of T_{reg} cells could be detected in both groups, whereas after 21 days, this decrease was significantly less pronounced in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice, suggesting that T_{reg} cells are maintained in the absence of CCL17. This difference correlated with higher mRNA levels of *tgf-β* (TGF-β), *csf2* (GMCSF) and *aldh1a2* (Aldh1a2) 21 days after T cell transfer in colon tissue of *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. TGF-β has been shown to directly drive the proliferation and maintenance of Foxp3-expressing T_{reg} cells in the gut[86]. This report also showed that retinoic acid (RA) is crucial in this process, which was confirmed later on[241]. The production of RA by DCs has in turn been shown to be mediated by the aldehyde dehydrogenase 1A isoform 2 (Aldh1a2) and GMCSF (*csf2*) directly triggers the expression of *aldh1a2*[242]. Furthermore, GMCSF has been implicated to support T_{reg} expansion, function and tolerance induction[243, 244], whereas IL-23 and IL-6 have been shown to counteract $Foxp3^+$ T_{reg} generation[50, 146, 218]. Both, IL-6 and IL-23 are much stronger induced in *Rag1*$^{-/-}$ mice 21 days after T cell transfer, correlating with lower percentages of T_{reg} cells. The role of CCL17 as a key player in this scenario is strongly supported by the higher capacity of *Ccl17*$^{E/E}$ DCs to induce T_{reg} cells *in vitro*. In line with this, high percentages of T_{reg} cells could be detected in mLNs 47 days after T cell transfer in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice, correlating with higher levels of $CD103^+$ DCs in the colon, which have been described to promote T_{reg} cells[86, 245]. Additionally, significantly higher levels of *Foxp3* mRNA could be detected at that time point in the colon, compared to the levels detected 21 days after transfer in *Rag1*$^{-/-}$ mice, reflecting an actual increase in T_{reg} cell numbers. Whether the high percentage of T_{reg} cells at day 47 is due to *de novo* generation or due to expansion of the T_{reg} cells that were still

detected on day 21 was addressed by transferring T_{reg}-depleted T cells. Thus, it could be shown that at day 25 after T cell transfer, nearly no T_{reg} cells could be detected in mLNs as well as colon tissue, indicating that the high percentage at day 47 is due to expansion of the T_{reg} cells. Since *Rag1*$^{-/-}$ mice had to be sacrificed when they had lost 20 % of their bodyweight, T_{reg} cells could not be investigated in *Rag1*$^{-/-}$ mice at that late timepoint. However, the transfer of T_{reg}-depleted T cells clearly demonstrated that T_{reg} cells were essential for longterm survival of *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice. However, *Rag1*$^{-/-}$ mice still lost bodyweight more rapidly, indicating that the proinflammatory effect of CCL17 discussed above might be independent of the effects on T_{reg} cells.

A recent study by Weber *et al.*[206] investigated the role of CCL17 in an atherosclerosis model, using as well the *Ccl17*$^{E/E}$ mouse model. Similar to the results I obtained, *Ccl17*-deficient mice showed reduced signs of disease in this model. This was accompanied by higher percentages of Foxp3^{+} T_{reg} cells, detected in peripheral lymph nodes and in atherosclerotic aortas of *Ccl17*$^{E/E}$*Apoe*$^{-/-}$ mice, compared to *Ccl17*$^{+/+}$*Apoe*$^{-/-}$ mice. It was also shown that Foxp3^{+} T_{reg} cells expanded to a higher extent in *Ccl17*$^{E/E}$ mice and higher proportions of Foxp3^{+} T_{reg} cells were detected in anti-CCL17 treated *Ccl17*$^{E/E}$ mice in the atherosclerosis model. This report supports the finding that the longterm protection from colitis detected in *Rag1*$^{-/-}$*Ccl17*$^{E/E}$ mice is indeed mediated by expansion and maintenance of Foxp3^{+} T_{reg} cells in the absence of CCL17. Thus, CCL17 is a proinflammatory mediator and regulator of T_{reg} cell homeostasis and as such may be a promising target for IBD therapy.

5.4 Proposed model for the role of CCL17 in intestinal inflammation

The role of CCL17 during intestinal inflammation in mice as revealed in this study can be summarized in a three-step model.

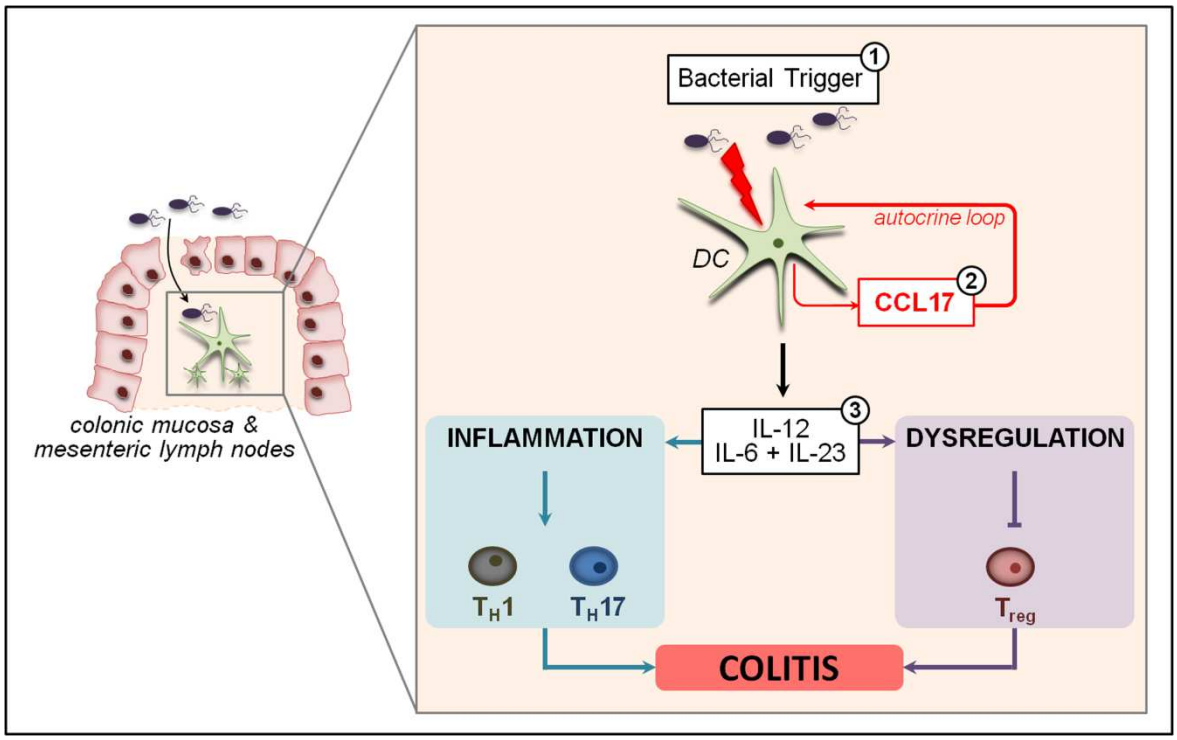

Figure 34: Proposed model of the dual function of CCL17 in experimental murine colitis. At first, DCs are stimulated by bacterial products via their TLRs (i.e. LPS stimulated TLR4) in the colonic mucosa and the mesenteric lymph nodes leading to the production of CCL17 by DCs. CCL17 then acts in an autocrine or paracrine loop on the DCs themselves resulting in an enhanced production of proinflammatory cytokines, such as IL-12 and IL-6/23. On the one hand, this stimulates the development of T_H1 and T_H17 T cells, which are directly driving colitis development. On the other hand, the production of proinflammatory cytokines counteracts the induction of regulatory mechanisms, such as preventing the expansion of Foxp3-expressing T_{reg} cells. This absence of T_{reg} cells in turn results in the lack of regulatory signals, again favoring colitis development.

The first step is the activation of dendritic cells (DCs) in the colonic mucosa or the mesenteric lymph nodes by bacterial triggers via TLR2 or TLR4 activation, leading to the upregulation of CCL17. The second step is an enhanced induction of proinflammatory cytokines by an autocrine or paracrine loop of CCL17, acting on the DCs via CCR4The third step is then on the one hand the induction of T_H1/T_H17 differentiation by the resulting proinflammatory cytokine milieu and on the other hand the inhibition of T_{reg} expansion and maintenance by this milieu.

Finally, these steps, driven by CCL17, are promoting intestinal inflammation, leading to colitis development.

In conclusion of the data obtained in this study it can be anticipated that the chemokine CCL17 might be a promising new target for treatment of IBD patients. Moreover, targeting CCL17 might even be favorable in comparison to other drugs, such as anti-TNF-α antibodies which are already available. This can be argued because (1) although CCL17 is expressed in DCs constitutively, it is not expressed ubiquitously in all organs, thus blocking CCL17 with antibodies would not affect the whole organismy, avoiding side-effects like increased risk of infections. (2) CCL17 is reported to be upregulated and crucial only in a limited number of diseases in a locally restricted manner, thus blocking CCL17 would probably not affect the immune response implicated in various other diseases. (3) CCL17 might also be favorable because, although CCR8 is suggested as additional receptor for CCL17, CCR4 is the only receptor that was confirmed for CCL17. Consequently the cell types that would be affected by a blockade of CCL17 are limited to those expressing CCR4 (and maybe CCR8), again reducing the potential for side-effects by CCL17 targeting.

In summary, this study demonstrates for the first time a fundamental role of the chemokine CCL17 in the development of murine experimental colitis. These data provide evidence that CCL17 might be a new target for the treatment of IBD.

6 SUMMARY

Inflammatory bowel disease is an idiopathic disease of the gastrointestinal tract and has two major forms, ulcerative colitis (UC) and Crohn's disease (CD). Its incidence is rising, especially in Northern Europe and North America, while the lowest incidence is seen in continental Asia. In the last two decades, much effort has been made to identify factors driving the disease. However, up to date, no defined trigger could be identified to be essential for disease onset. Nonetheless, intensive investigation led to the development of various drugs with the main goal to induce and maintain remission. One of the most effective targets is TNF-α, although treatment with anti-TNF-α antibodies is only effective in about 50 % of the patients. In recent years, targeting leukocyte recruitment has come into focus for the treatment of IBD. This approach seems to be preferable, because specific blocking of recruitment of immune players to the site of inflammation, where subsequent damage takes place, could reverse these processes in the gut while leaving protective inflammatory processes of the host against invading pathogens unaltered. Crucial mediators in such homing processes to the gut are for example α4β7, an integrin involved in entry processes at mLNs and in the gut mucosa, as well as CCL25-mediated attraction of immune cells via CCR9 to the gut. Subsequent studies, using humanized monoclonal antibodies targeting these processes are ongoing. CCL17, a chemokine demonstrated to be upregulated in inflammatory processes in the skin as well as in the intestine has been shown to be overexpressen on mRNA level in the intestinal mucosa of active CD patients, as well as in murine colitis models, thus implicating a potential role as novel player for the development of colitis. However, functional data, dissecting whether CCL17 could play a disease promoting or protective role, or if CCL17 is just a bystander of general inflammation was missing when this study was started.

In the present study, I could show that CCL17 expression in DCs is upregulated during intestinal inflammation in colon tissue in two murine models of colitis, DSS induced colitis as well as T cell transfer colitis. Furthermore, I demonstrated that the absence of CCL17 in these models led to a significant decrease in disease severity. This was detected by bodyweight assessment and histological scoring of colon tissue as well as cytokine mRNA levels in the colon. Moreover, this was accompanied by reduced T_H1/T_H17 differentiation of infiltrating T cells in the T cell transfer colitis model. Using *Ccr4*$^{-/-}$ T cells to initiate T cell transfer colitis demonstrated that disease induction was not dependent on CCR4-

CCL17 mediated recruitment of T cells to mLNs or the colon, as this did not alter disease induction compared to WT T cells. Quite contrary to this, CCR4 expression in the host was essential for the induction of T cell transfer colitis. In *in vitro* and *ex vivo* culture systems, this effect could be ascribed to an autocrine or paracrine mechanism of CCL17, amplifying proinflammatory cytokine production by DCs, which led to increased T_H1/T_H17 differentiation in coculture setups. Additionally, longterm protection of CCL17-deficient mice from disease induction by T cell transfer was shown to be mediated by T_{reg} cells, which are initially maintained and then greatly expanded in CCL17-deficient mice.

Taken together, the data obtained throughout this study clearly demonstrate a causal proinflammatory role of CCL17 in murine experimental colitis.

7 REFERENCES

1. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996;86:973-83.

2. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989;54 Pt 1:1-13.

3. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394-7.

4. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085-8.

5. Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, Wagner H, Kirschning CJ, Ter Meulen V, Schneider-Schaulies S. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. J Virol 2002;76:8729-36.

6. de Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, McConville MJ, Handman E, Schofield L. MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. Eur J Immunol 2003;33:2822-31.

7. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.

8. Nishiya T, DeFranco AL. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. J Biol Chem 2004;279:19008-17.

9. Lee J, Chuang TH, Redecke V, She L, Pitha PM, Carson DA, Raz E, Cottam HB. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. Proc Natl Acad Sci U S A 2003;100:6646-51.

10. Yamasaki S, Matsumoto M, Takeuchi O, Matsuzawa T, Ishikawa E, Sakuma M, Tateno H, Uno J, Hirabayashi J, Mikami Y, Takeda K, Akira S, Saito T. C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. Proc Natl Acad Sci U S A 2009;106:1897-902.

11. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med 2001;194:781-95.

12. Inohara N, Ogura Y, Chen FF, Muto A, Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. J Biol Chem 2001;276:2551-4.

13. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730-7.

14. Weinmann AS, Mitchell DM, Sanjabi S, Bradley MN, Hoffmann A, Liou HC, Smale ST. Nucleosome remodeling at the IL-12 p40 promoter is a TLR-dependent, Rel-independent event. Nat Immunol 2001;2:51-7.

15. Dziarski R, Gupta D. Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. J Endotoxin Res 2000;6:401-5.

16. Parish CR, O'Neill ER. Dependence of the adaptive immune response on innate immunity: some questions answered but new paradoxes emerge. Immunol Cell Biol 1997;75:523-7.

17. Segura E, Villadangos JA. Antigen presentation by dendritic cells in vivo. Curr Opin Immunol 2009;21:105-10.

18. Schreibelt G, Tel J, Sliepen KH, Benitez-Ribas D, Figdor CG, Adema GJ, de Vries IJ. Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy. Cancer Immunol Immunother 2010;59:1573-82.

19. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 2008;8:594-606.

20. Harman BC, Miller JP, Nikbakht N, Gerstein R, Allman D. Mouse plasmacytoid dendritic cells derive exclusively from estrogen-resistant myeloid progenitors. Blood 2006;108:878-85.

21. D'Amico A, Wu L. The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. J Exp Med 2003;198:293-303.

22. Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, Liu K, Jakubzick C, Ingersoll MA, Leboeuf M, Stanley ER, Nussenzweig M, Lira SA, Randolph GJ, Merad M. Origin of the lamina propria dendritic cell network. Immunity 2009;31:513-25.

23. Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O'Keeffe M, Shortman K. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. Nat Immunol 2006;7:663-71.

24. Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, Kalchenko V, Geissmann F, Jung S. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. J Exp Med 2007;204:171-80.

25. Gromme M, Neefjes J. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Mol Immunol 2002;39:181-202.

26. Villadangos JA. Presentation of antigens by MHC class II molecules: getting the most out of them. Mol Immunol 2001;38:329-46.

27. Gorbulev S, Abele R, Tampe R. Allosteric crosstalk between peptide-binding, transport, and ATP hydrolysis of the ABC transporter TAP. Proc Natl Acad Sci U S A 2001;98:3732-7.

28. Hiltbold EM, Roche PA. Trafficking of MHC class II molecules in the late secretory pathway. Curr Opin Immunol 2002;14:30-5.

29. Littman DR. The structure of the CD4 and CD8 genes. Annu Rev Immunol 1987;5:561-84.

30. Zerrahn J, Held W, Raulet DH. The MHC reactivity of the T cell repertoire prior to positive and negative selection. Cell 1997;88:627-36.

31. Cosgrove D, Chan SH, Waltzinger C, Benoist C, Mathis D. The thymic compartment responsible for positive selection of CD4+ T cells. Int Immunol 1992;4:707-10.

32. Kishimoto H, Sprent J. Negative selection in the thymus includes semimature T cells. J Exp Med 1997;185:263-71.

33. Alberola-Ila J, Hogquist KA, Swan KA, Bevan MJ, Perlmutter RM. Positive and negative selection invoke distinct signaling pathways. J Exp Med 1996;184:9-18.

34. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. Immunity 2009;30:616-25.

35. Barnes MJ, Powrie F. Regulatory T cells reinforce intestinal homeostasis. Immunity 2009;31:401-11.

36. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol 2010;11:7-13.

37. Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, Tannapfel A, Fontenot JD, Ramsdell F, Powrie F. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. J Immunol 2006;177:5852-60.

38. Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 2003;197:111-9.

39. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR, Jr., Muller W, Rudensky AY. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 2008;28:546-58.

40. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity 2007;26:579-91.

41. Baatar D, Olkhanud P, Sumitomo K, Taub D, Gress R, Biragyn A. Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4+ Tregs and unprimed CCR4- Tregs, regulate effector T cells using FasL. J Immunol 2007;178:4891-900.

42. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol 2007;8:1353-62.

43. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 2004;21:589-601.

44. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology 2006;118:240-9.

45. Shaw AS, Dustin ML. Making the T cell receptor go the distance: a topological view of T cell activation. Immunity 1997;6:361-9.

46. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annu Rev Immunol 1996;14:233-58.

47. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 1993;260:547-9.

48. Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. J Immunol 1990;145:3796-806.

49. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006;24:179-89.

50. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235-8.

51. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. Immunity 2006;25:455-71.

52. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. Nat Immunol 2008;9:632-40.

53. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, Belkaid Y, Mathis D, Benoist C. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity 2008;29:758-70.

54. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775-85.

55. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000;100:655-69.

56. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89:587-96.

57. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem 1997;272:21597-603.

58. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121-33.

59. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-6.

60. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.

61. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009;27:451-83.

62. Boom WH, Liano D, Abbas AK. Heterogeneity of helper/inducer T lymphocytes. II. Effects of interleukin 4- and interleukin 2-producing T cell clones on resting B lymphocytes. J Exp Med 1988;167:1350-63.

63. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity 2004;21:467-76.

64. Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, Slot JW, Geuze HJ. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med 1991;173:1099-109.

65. Mowat AM, Viney JL. The anatomical basis of intestinal immunity. Immunol Rev 1997;156:145-66.

66. Guy-Grand D, Vassalli P. Gut intraepithelial T lymphocytes. Curr Opin Immunol 1993;5:247-52.

67. Faure M, Moennoz D, Montigon F, Mettraux C, Mercier S, Schiffrin EJ, Obled C, Breuille D, Boza J. Mucin production and composition is altered in dextran sulfate sodium-induced colitis in rats. Dig Dis Sci 2003;48:1366-73.

68. Willemsen LE, Koetsier MA, van Deventer SJ, van Tol EA. Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts. Gut 2003;52:1442-7.

69. Zeuthen LH, Fink LN, Frokiaer H. Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. Immunology 2008;123:197-208.

70. Fontenot D, He H, Hanabuchi S, Nehete PN, Zhang M, Chang M, Nehete B, Wang YH, Ma ZM, Lee HC, Ziegler SF, Courtney AN, Miller CJ, Sun SC, Liu YJ, Sastry KJ. TSLP production by epithelial cells exposed to immunodeficiency virus triggers DC-mediated mucosal infection of CD4+ T cells. Proc Natl Acad Sci U S A 2009;106:16776-81.

71. Hanabuchi S, Ito T, Park WR, Watanabe N, Shaw JL, Roman E, Arima K, Wang YH, Voo KS, Cao W, Liu YJ. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3+ regulatory T cells in human thymus. J Immunol 2010;184:2999-3007.

72. Lin Y, Wang W, Jin H, Zhong Y, Di J, Zeng S, Saito S. Comparison of murine thymic stromal lymphopoietin- and polyinosinic polycytidylic acid-mediated placental dendritic cell activation. J Reprod Immunol 2009;79:119-28.

73. Hundorfean G, Zimmer KP, Strobel S, Gebert A, Ludwig D, Buning J. Luminal antigens access late endosomes of intestinal epithelial cells enriched in MHC I and MHC II molecules: in vivo study in Crohn's ileitis. Am J Physiol Gastrointest Liver Physiol 2007;293:G798-808.

74. Buning J, Hundorfean G, Schmitz M, Zimmer KP, Strobel S, Gebert A, Ludwig D. Antigen targeting to MHC class II-enriched late endosomes in colonic epithelial cells: trafficking of luminal antigens studied in vivo in Crohn's colitis patients. Faseb J 2006;20:359-61.

75. Zimmer KP, Buning J, Weber P, Kaiserlian D, Strobel S. Modulation of antigen trafficking to MHC class II-positive late endosomes of enterocytes. Gastroenterology 2000;118:128-37.

76. Neutra MR, Kraehenbuhl JP. Transepithelial transport and mucosal defence I: the role of M cells. Trends Cell Biol 1992;2:134-8.

77. Kelsall BL, Strober W. Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. J Exp Med 1996;183:237-47.

78. Dutt S, Ermann J, Tseng D, Liu YP, George TI, Fathman CG, Strober S. L-selectin and beta7 integrin on donor CD4 T cells are required for the early migration to host mesenteric lymph nodes and acute colitis of graft-versus-host disease. Blood 2005;106:4009-15.

79. Wagner N, Lohler J, Tedder TF, Rajewsky K, Muller W, Steeber DA. L-selectin and beta7 integrin synergistically mediate lymphocyte migration to mesenteric lymph nodes. Eur J Immunol 1998;28:3832-9.

80. Steeber DA, Tang ML, Zhang XQ, Muller W, Wagner N, Tedder TF. Efficient lymphocyte migration across high endothelial venules of mouse Peyer's patches requires overlapping expression of L-selectin and beta7 integrin. J Immunol 1998;161:6638-47.

81. Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, Weissman IL, Hamann A, Butcher EC. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 1993;74:185-95.

82. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 2005;307:254-8.

83. Rivollier A, He J, Kole A, Valatas V, Kelsall BL. Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. J Exp Med 2012;209:139-55.

84. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature 1994;372:190-3.

85. Laffont S, Siddiqui KR, Powrie F. Intestinal inflammation abrogates the tolerogenic properties

of MLN CD103+ dendritic cells. Eur J Immunol 2010;40:1877-83.

86. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007;204:1757-64.

87. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity 2004;21:527-38.

88. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med 2008;205:2139-49.

89. Siddiqui KR, Laffont S, Powrie F. E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. Immunity 2010;32:557-67.

90. Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, Guo Z, Umemoto E, Ebisuno Y, Yang BG, Seoh JY, Lipp M, Kiyono H, Miyasaka M. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. J Immunol 2006;176:803-10.

91. Luther SA, Tang HL, Hyman PL, Farr AG, Cyster JG. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. Proc Natl Acad Sci U S A 2000;97:12694-9.

92. Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, Nakano H. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J Exp Med 1999;189:451-60.

93. Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD, Williams LT. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. Proc Natl Acad Sci U S A 1998;95:258-63.

94. Konkel JE, Maruyama T, Carpenter AC, Xiong Y, Zamarron BF, Hall BE, Kulkarni AB, Zhang P, Bosselut R, Chen W. Control of the development of CD8alphaalpha+ intestinal intraepithelial lymphocytes by TGF-beta. Nat Immunol 2011;12:312-9.

95. Ismail AS, Severson KM, Vaishnava S, Behrendt CL, Yu X, Benjamin JL, Ruhn KA, Hou B, DeFranco AL, Yarovinsky F, Hooper LV. Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. Proc Natl Acad Sci U S A 2011;108:8743-8.

96. Wurbel MA, Malissen M, Guy-Grand D, Meffre E, Nussenzweig MC, Richelme M, Carrier A, Malissen B. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. Blood 2001;98:2626-32.

97. Wang C, Hanly EK, Wheeler LW, Kaur M, McDonald KG, Newberry RD. Effect of alpha4beta7 blockade on intestinal lymphocyte subsets and lymphoid tissue development. Inflamm Bowel Dis 2010;16:1751-62.

98. Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A 1998;95:6578-83.

99. Eckburg PB, Lepp PW, Relman DA. Archaea and their potential role in human disease. Infect Immun 2003;71:591-6.

100. Stone MA, Mayberry JF, Baker R. Prevalence and management of inflammatory bowel disease: a cross-sectional study from central England. Eur J Gastroenterol Hepatol 2003;15:1275-80.

101. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.

102. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-72.

103. Rosenstiel P, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D, Schreiber S. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. Gastroenterology 2003;124:1001-9.

104. Bevins CL, Stange EF, Wehkamp J. Decreased Paneth cell defensin expression in ileal Crohn's disease is independent of inflammation, but linked to the NOD2 1007fs genotype. Gut 2009;58:882-3; discussion 883-4.

105. Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, Quertinmont E, Abramowicz M, Van Gossum A, Deviere J, Rutgeerts P. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut 2004;53:987-92.

106. Zhernakova A, Festen EM, Franke L, Trynka G, van Diemen CC, Monsuur AJ, Bevova M, Nijmeijer RM, van 't Slot R, Heijmans R, Boezen HM, van Heel DA, van Bodegraven AA, Stokkers PC, Wijmenga C, Crusius JB, Weersma RK. Genetic analysis of innate immunity in Crohn's disease and ulcerative colitis identifies two susceptibility loci harboring CARD9 and IL18RAP. Am J Hum Genet 2008;82:1202-10.

107. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M,

Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 2008;40:955-62.

108. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Abraham C, Regueiro M, Griffiths A, Dassopoulos T, Bitton A, Yang H, Targan S, Datta LW, Kistner EO, Schumm LP, Lee AT, Gregersen PK, Barmada MM, Rotter JI, Nicolae DL, Cho JH. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006;314:1461-3.

109. Civitavecchia G, Renda MC, Ruggeri RF, Maggio A, Renna S, Orlando A, Cottone M. IL-23R determines susceptibility in Crohn's disease in a Mediterranean area. Inflamm Bowel Dis 2009;15:317-8.

110. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Gunther S, Prescott NJ, Onnie CM, Hasler R, Sipos B, Folsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 2007;39:207-11.

111. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, Kishi C, Kc W, Carrero JA, Hunt S, Stone CD, Brunt EM, Xavier RJ, Sleckman BP, Li E, Mizushima N, Stappenbeck TS, Virgin HWt. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 2008;456:259-63.

112. Klionsky DJ. Crohn's disease, autophagy, and the Paneth cell. N Engl J Med 2009;360:1785-6.

113. Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tilg H, Nieuwenhuis EE, Higgins DE, Schreiber S, Glimcher LH, Blumberg RS. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell 2008;134:743-56.

114. Shkoda A, Ruiz PA, Daniel H, Kim SC, Rogler G, Sartor RB, Haller D. Interleukin-10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation. Gastroenterology 2007;132:190-207.

115. Spehlmann ME, Begun AZ, Burghardt J, Lepage P, Raedler A, Schreiber S. Epidemiology of inflammatory bowel disease in a German twin cohort: results of a nationwide study. Inflamm Bowel Dis 2008;14:968-76.

116. Halme L, Paavola-Sakki P, Turunen U, Lappalainen M, Farkkila M, Kontula K. Family and twin studies in inflammatory bowel disease. World J Gastroenterol 2006;12:3668-72.

117. O'Sullivan M, O'Morain C. Nutrition in inflammatory bowel disease. Best Pract Res Clin Gastroenterol 2006;20:561-73.

118. Yamamoto T, Nakahigashi M, Saniabadi AR. Review article: diet and inflammatory bowel disease--epidemiology and treatment. Aliment Pharmacol Ther 2009;30:99-112.

119. Lakatos PL. Recent trends in the epidemiology of inflammatory bowel diseases: up or down? World J Gastroenterol 2006;12:6102-8.

120. Thia KT, Loftus EV, Jr., Sandborn WJ, Yang SK. An update on the epidemiology of inflammatory bowel disease in Asia. Am J Gastroenterol 2008;103:3167-82.

121. Jowett SL, Seal CJ, Pearce MS, Phillips E, Gregory W, Barton JR, Welfare MR. Influence of dietary factors on the clinical course of ulcerative colitis: a prospective cohort study. Gut 2004;53:1479-84.

122. Bjornsson S, Johannsson JH. Inflammatory bowel disease in Iceland, 1990-1994: a prospective, nationwide, epidemiological study. Eur J Gastroenterol Hepatol 2000;12:31-8.

123. Pena JA, Rogers AB, Ge Z, Ng V, Li SY, Fox JG, Versalovic J. Probiotic Lactobacillus spp. diminish Helicobacter hepaticus-induced inflammatory bowel disease in interleukin-10-deficient mice. Infect Immun 2005;73:912-20.

124. Mane J, Loren V, Pedrosa E, Ojanguren I, Xaus J, Cabre E, Domenech E, Gassull MA. Lactobacillus fermentum CECT 5716 prevents and reverts intestinal damage on TNBS-induced colitis in mice. Inflamm Bowel Dis 2009;15:1155-63.

125. Llopis M, Antolin M, Guarner F, Salas A, Malagelada JR. Mucosal colonisation with Lactobacillus casei mitigates barrier injury induced by exposure to trinitronbenzene sulphonic acid. Gut 2005;54:955-9.

126. Steck N, Hoffmann M, Sava IG, Kim SC, Hahne H, Tonkonogy SL, Mair K, Krueger D, Pruteanu M, Shanahan F, Vogelmann R, Schemann M, Kuster B, Sartor RB, Haller D. Enterococcus faecalis metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. Gastroenterology 2011;141:959-71.

127. Birrenbach T, Bocker U. Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications. Inflamm Bowel Dis 2004;10:848-59.

128. Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol 1993;5:1461-71.

129. Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. Nature 1983;301:527-30.

130. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 1992;68:869-77.

131. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 1992;68:855-67.

132. Wirtz S, Becker C, Blumberg R, Galle PR, Neurath MF. Treatment of T cell-dependent experimental colitis in SCID mice by local administration of an adenovirus expressing IL-18 antisense mRNA. J Immunol 2002;168:411-20.

133. Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity 1999;10:387-98.

134. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 2007;369:1641-57.

135. Kishimoto T. Interleukin-6: from basic science to medicine--40 years in immunology. Annu Rev Immunol 2005;23:1-21.

136. Atreya R, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, Schutz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schurmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S, Neurath MF. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat Med 2000;6:583-8.

137. Ito H, Takazoe M, Fukuda Y, Hibi T, Kusugami K, Andoh A, Matsumoto T, Yamamura T, Azuma J, Nishimoto N, Yoshizaki K, Shimoyama T, Kishimoto T. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. Gastroenterology 2004;126:989-96; discussion 947.

138. Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, Akira S. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity 1999;10:39-49.

139. Neurath MF, Fuss I, Kelsall BL, Stuber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med 1995;182:1281-90.

140. Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity 1994;1:553-62.

141. Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, Mizoguchi A, Mizoguchi E, Mudter J, Galle PR, Bhan A, Autschbach F, Sullivan BM, Szabo SJ, Glimcher LH, Blumberg RS. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. J Exp Med 2002;195:1129-43.

142. Reinisch W, de Villiers W, Bene L, Simon L, Racz I, Katz S, Altorjay I, Feagan B, Riff D, Bernstein CN, Hommes D, Rutgeerts P, Cortot A, Gaspari M, Cheng M, Pearce T, Sands BE. Fontolizumab in moderate to severe Crohn's disease: a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study. Inflamm Bowel Dis 2010;16:233-42.

143. Mannon PJ, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, Dolin B, Goodman N, Groden C, Hornung RL, Quezado M, Yang Z, Neurath MF, Salfeld J, Veldman GM, Schwertschlag U, Strober W. Anti-interleukin-12 antibody for active Crohn's disease. N Engl J Med 2004;351:2069-79.

144. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, Stepankova R, Robinson N, Buonocore S, Tlaskalova-Hogenova H, Cua DJ, Powrie F. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. Immunity 2006;25:309-18.

145. Kullberg MC, Jankovic D, Feng CG, Hue S, Gorelick PL, McKenzie BS, Cua DJ, Powrie F, Cheever AW, Maloy KJ, Sher A. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. J Exp Med 2006;203:2485-94.

146. Ahern PP, Schiering C, Buonocore S, McGeachy MJ, Cua DJ, Maloy KJ, Powrie F. Interleukin-23 drives intestinal inflammation through direct activity on T cells. Immunity 2010;33:279-88.

147. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, Rochet V, Pisi A, De Paepe M, Brandi G, Eberl G, Snel J, Kelly D, Cerf-Bensussan N. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity 2009;31:677-89.

148. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S. Phenotypic and functional features of human Th17 cells. J Exp Med 2007;204:1849-61.

149. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133-41.

150. O'Connor W, Jr., Kamanaka M, Booth CJ, Town T, Nakae S, Iwakura Y, Kolls JK, Flavell RA. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. Nat Immunol 2009;10:603-9.

151. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang YH, Schluns KS, Broaddus RR, Zhu Z, Dong C. Regulation of inflammatory responses by IL-17F. J Exp Med 2008;205:1063-75.

152. Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. Inflamm Bowel Dis 2006;12:382-8.

153. Leppkes M, Becker C, Ivanov, II, Hirth S, Wirtz S, Neufert C, Pouly S, Murphy AJ, Valenzuela DM, Yancopoulos GD, Becher B, Littman DR, Neurath MF. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. Gastroenterology 2009;136:257-67.

154. Zheng Y, Rudensky AY. Foxp3 in control of the regulatory T cell lineage. Nat Immunol 2007;8:457-62.

155. Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA, Powrie F. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J Exp Med 2005;201:737-46.

156. Powrie F, Correa-Oliveira R, Mauze S, Coffman RL. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. J Exp Med 1994;179:589-600.

157. Rollins BJ. Chemokines. Blood 1997;90:909-28.

158. Rossi D, Zlotnik A. The biology of chemokines and their receptors. Annu Rev Immunol 2000;18:217-42.

159. Raab Y, Gerdin B, Ahlstedt S, Hallgren R. Neutrophil mucosal involvement is accompanied

by enhanced local production of interleukin-8 in ulcerative colitis. Gut 1993;34:1203-6.

160. Izzo RS, Witkon K, Chen AI, Hadjiyane C, Weinstein MI, Pellecchia C. Neutrophil-activating peptide (interleukin-8) in colonic mucosa from patients with Crohn's disease. Scand J Gastroenterol 1993;28:296-300.

161. Mitsuyama K, Toyonaga A, Sasaki E, Watanabe K, Tateishi H, Nishiyama T, Saiki T, Ikeda H, Tsuruta O, Tanikawa K. IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. Clin Exp Immunol 1994;96:432-6.

162. Yuan YH, ten Hove T, The FO, Slors JF, van Deventer SJ, te Velde AA. Chemokine receptor CXCR3 expression in inflammatory bowel disease. Inflamm Bowel Dis 2001;7:281-6.

163. Uguccioni M, Gionchetti P, Robbiani DF, Rizzello F, Peruzzo S, Campieri M, Baggiolini M. Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. Am J Pathol 1999;155:331-6.

164. Singh UP, Singh S, Taub DD, Lillard JW, Jr. Inhibition of IFN-gamma-inducible protein-10 abrogates colitis in IL-10-/- mice. J Immunol 2003;171:1401-6.

165. Mazzucchelli L, Hauser C, Zgraggen K, Wagner HE, Hess MW, Laissue JA, Mueller C. Differential in situ expression of the genes encoding the chemokines MCP-1 and RANTES in human inflammatory bowel disease. J Pathol 1996;178:201-6.

166. Reinecker HC, Loh EY, Ringler DJ, Mehta A, Rombeau JL, MacDermott RP. Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. Gastroenterology 1995;108:40-50.

167. Tokuyama H, Ueha S, Kurachi M, Matsushima K, Moriyasu F, Blumberg RS, Kakimi K. The simultaneous blockade of chemokine receptors CCR2, CCR5 and CXCR3 by a non-peptide chemokine receptor antagonist protects mice from dextran sodium sulfate-mediated colitis. Int Immunol 2005;17:1023-34.

168. Popivanova BK, Kostadinova FI, Furuichi K, Shamekh MM, Kondo T, Wada T, Egashira K, Mukaida N. Blockade of a chemokine, CCL2, reduces chronic colitis-associated carcinogenesis in mice. Cancer Res 2009;69:7884-92.

169. Ajuebor MN, Hogaboam CM, Kunkel SL, Proudfoot AE, Wallace JL. The chemokine RANTES is a crucial mediator of the progression from acute to chronic colitis in the rat. J Immunol 2001;166:552-8.

170. Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. J Immunol 1999;162:186-94.

171. Puleston J, Cooper M, Murch S, Bid K, Makh S, Ashwood P, Bingham AH, Green H, Moss P, Dhillon A, Morris R, Strobel S, Gelinas R, Pounder RE, Platt A. A distinct subset of chemokines dominates the mucosal chemokine response in inflammatory bowel disease. Aliment Pharmacol Ther 2005;21:109-20.

172. Teramoto K, Miura S, Tsuzuki Y, Hokari R, Watanabe C, Inamura T, Ogawa T, Hosoe N, Nagata H, Ishii H, Hibi T. Increased lymphocyte trafficking to colonic microvessels is dependent on MAdCAM-1 and C-C chemokine mLARC/CCL20 in DSS-induced mice colitis. Clin Exp Immunol 2005;139:421-8.

173. Locati M, Torre YM, Galliera E, Bonecchi R, Bodduluri H, Vago G, Vecchi A, Mantovani A. Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. Cytokine Growth Factor Rev 2005;16:679-86.

174. Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C, Arena V, Fantini M, Roncalli M, Malesci A, Mantovani A, Locati M, Danese S. The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6. Gut 2010;59:197-206.

175. Fisher SA, Moody A, Mirza MM, Cuthbert AP, Hampe J, Macpherson A, Sanderson J, Forbes A, Mansfield J, Schreiber S, Lewis CM, Mathew CG. Genetic variation at the chromosome 16 chemokine gene cluster: development of a strategy for association studies in complex disease. Ann Hum Genet 2003;67:377-90.

176. Komine M, Kakinuma T, Kagami S, Hanakawa Y, Hashimoto K, Tamaki K. Mechanism of thymus- and activation-regulated chemokine (TARC)/CCL17 production and its modulation by roxithromycin. J Invest Dermatol 2005;125:491-8.

177. Alferink J, Lieberam I, Reindl W, Behrens A, Weiss S, Huser N, Gerauer K, Ross R, Reske-Kunz AB, Ahmad-Nejad P, Wagner H, Forster I. Compartmentalized production of CCL17 in vivo: strong inducibility in peripheral dendritic cells contrasts selective absence from the spleen. J Exp Med 2003;197:585-99.

178. Jakubzick C, Wen H, Matsukawa A, Keller M, Kunkel SL, Hogaboam CM. Role of CCR4 ligands, CCL17 and CCL22, during Schistosoma mansoni egg-induced pulmonary granuloma formation in mice. Am J Pathol 2004;165:1211-21.

179. Xiao T, Fujita H, Saeki H, Mitsui H, Sugaya M, Tada Y, Kakinuma T, Torii H, Nakamura K, Asahina A, Tamaki K. Thymus and activation-regulated chemokine (TARC/CCL17) produced by mouse epidermal Langerhans cells is upregulated by TNF-alpha and IL-4 and downregulated by IFN-gamma. Cytokine 2003;23:126-32.

180. Bernardini G, Hedrick J, Sozzani S, Luini W, Spinetti G, Weiss M, Menon S, Zlotnik A, Mantovani A, Santoni A, Napolitano M. Identification of the CC chemokines TARC and macrophage inflammatory protein-1 beta as novel functional ligands for the CCR8 receptor. Eur J Immunol 1998;28:582-8.

181. Bayry J, Tchilian EZ, Davies MN, Forbes EK, Draper SJ, Kaveri SV, Hill AV, Kazatchkine MD, Beverley PC, Flower DR, Tough DF. In silico identified CCR4 antagonists target regulatory T cells and exert adjuvant activity in vaccination. Proc Natl Acad Sci U S A 2008;105:10221-6.

182. Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological

ligand for CC chemokine receptor 4. J Biol Chem 1997;272:15036-42.

183. Ness TL, Ewing JL, Hogaboam CM, Kunkel SL. CCR4 is a key modulator of innate immune responses. J Immunol 2006;177:7531-9.

184. Sallusto F, Lanzavecchia A. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. J Exp Med 1999;189:611-4.

185. Inngjerdingen M, Damaj B, Maghazachi AA. Expression and regulation of chemokine receptors in human natural killer cells. Blood 2001;97:367-75.

186. Inngjerdingen M, Damaj B, Maghazachi AA. Human NK cells express CC chemokine receptors 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309. J Immunol 2000;164:4048-54.

187. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 2007;8:639-46.

188. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999;11:81-8.

189. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, Rossjohn J, Perlmutter P, Cao J, Godfrey DI, Savage PB, Knolle PA, Kolanus W, Forster I, Kurts C. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. Nat Immunol 2010;11:313-20.

190. Bonecchi R, Sozzani S, Stine JT, Luini W, D'Amico G, Allavena P, Chantry D, Mantovani A. Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. Blood 1998;92:2668-71.

191. Reiss Y, Proudfoot AE, Power CA, Campbell JJ, Butcher EC. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. J Exp Med 2001;194:1541-7.

192. Vijayanand P, Durkin K, Hartmann G, Morjaria J, Seumois G, Staples KJ, Hall D, Bessant C, Bartholomew M, Howarth PH, Friedmann PS, Djukanovic R. Chemokine receptor 4 plays a key role in T cell recruitment into the airways of asthmatic patients. J Immunol 2010;184:4568-74.

193. Izraely S, Klein A, Sagi-Assif O, Meshel T, Tsarfaty G, Hoon DS, Witz IP. Chemokine-chemokine receptor axes in melanoma brain metastasis. Immunol Lett 2010;130:107-14.

194. Olkhanud PB, Baatar D, Bodogai M, Hakim F, Gress R, Anderson RL, Deng J, Xu M, Briest S, Biragyn A. Breast cancer lung metastasis requires expression of chemokine receptor CCR4 and regulatory T cells. Cancer Res 2009;69:5996-6004.

195. Baatar D, Olkhanud P, Newton D, Sumitomo K, Biragyn A. CCR4-expressing T cell tumors can be specifically controlled via delivery of toxins to chemokine receptors. J Immunol 2007;179:1996-2004.

196. Stutte S, Quast T, Gerbitzki N, Savinko T, Novak N, Reifenberger J, Homey B, Kolanus W, Alenius H, Forster I. Requirement of CCL17 for CCR7- and CXCR4-dependent migration of cutaneous dendritic cells. Proc Natl Acad Sci U S A 2010;107:8736-41.

197. Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y, Imai T, Yoshie O, Irimura T, Mizutani H, Matsushima K. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J Clin Invest 1999;104:1097-105.

198. Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol 2000;115:640-6.

199. Zheng X, Nakamura K, Furukawa H, Nishibu A, Takahashi M, Tojo M, Kaneko F, Kakinuma T, Tamaki K. Demonstration of TARC and CCR4 mRNA expression and distribution using in situ RT-PCR in the lesional skin of atopic dermatitis. J Dermatol 2003;30:26-32.

200. Nakazato J, Kishida M, Kuroiwa R, Fujiwara J, Shimoda M, Shinomiya N. Serum levels of Th2 chemokines, CCL17, CCL22, and CCL27, were the important markers of severity in infantile atopic dermatitis. Pediatr Allergy Immunol 2008;19:605-13.

201. Miyahara H, Okazaki N, Nagakura T, Korematsu S, Izumi T. Elevated umbilical cord serum TARC/CCL17 levels predict the development of atopic dermatitis in infancy. Clin Exp Allergy 2011;41:186-91.

202. Morita E, Takahashi H, Niihara H, Dekio I, Sumikawa Y, Murakami Y, Matsunaka H. Stratum corneum TARC level is a new indicator of lesional skin inflammation in atopic dermatitis. Allergy 2010;65:1166-72.

203. Perros F, Hoogsteden HC, Coyle AJ, Lambrecht BN, Hammad H. Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation. Allergy 2009;64:995-1002.

204. Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H, Fujii H. CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. Int J Cancer 2008;122:2286-93.

205. Maruyama T, Kono K, Izawa S, Mizukami Y, Kawaguchi Y, Mimura K, Watanabe M, Fujii H. CCL17 and CCL22 chemokines within tumor microenvironment are related to infiltration of regulatory T cells in esophageal squamous cell carcinoma. Dis Esophagus 2010;23:422-9.

206. Weber C, Meiler S, Doring Y, Koch M, Drechsler M, Megens RT, Rowinska Z, Bidzhekov K, Fecher C, Ribechini E, van Zandvoort MA, Binder CJ, Jelinek I, Hristov M, Boon L, Jung S, Korn T, Lutz MB, Forster I, Zenke M, Hieronymus T, Junt T, Zernecke A. CCL17-expressing dendritic cells drive atherosclerosis by restraining regulatory T

cell homeostasis in mice. J Clin Invest 2011;121:2898-910.

207. Riezu-Boj JI, Larrea E, Aldabe R, Guembe L, Casares N, Galeano E, Echeverria I, Sarobe P, Herrero I, Sangro B, Prieto J, Lasarte JJ. Hepatitis C virus induces the expression of CCL17 and CCL22 chemokines that attract regulatory T cells to the site of infection. J Hepatol;54:422-31.

208. Scheerens H, Hessel E, de Waal-Malefyt R, Leach MW, Rennick D. Characterization of chemokines and chemokine receptors in two murine models of inflammatory bowel disease: IL-10-/- mice and Rag-2-/- mice reconstituted with CD4+CD45RBhigh T cells. Eur J Immunol 2001;31:1465-74.

209. Viejo-Borbolla A, Martin AP, Muniz LR, Shang L, Marchesi F, Thirunarayanan N, Harpaz N, Garcia RA, Apostolaki M, Furtado GC, Mayer L, Kollias G, Alcami A, Lira SA. Attenuation of TNF-driven murine ileitis by intestinal expression of the viral immunomodulator CrmD. Mucosal Immunol 2010;3:633-44.

210. Kristensen NN, Brudzewsky D, Gad M, Claesson MH. Chemokines involved in protection from colitis by CD4+CD25+ regulatory T cells. Inflamm Bowel Dis 2006;12:612-8.

211. Jugde F, Alizadeh M, Boissier C, Chantry D, Siproudhis L, Corbinais S, Quelvennec E, Dyard F, Campion JP, Gosselin M, Bretagne JF, Semana G, Heresbach D. Quantitation of chemokines (MDC, TARC) expression in mucosa from Crohn's disease and ulcerative colitis. Eur Cytokine Netw 2001;12:468-77.

212. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, Hamann A, Wagner H, Huehn J, Sparwasser T. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med 2007;204:57-63.

213. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.

214. Siegmund B, Rieder F, Albrich S, Wolf K, Bidlingmaier C, Firestein GS, Boyle D, Lehr HA, Loher F, Hartmann G, Endres S, Eigler A. Adenosine kinase inhibitor GP515 improves experimental colitis in mice. J Pharmacol Exp Ther 2001;296:99-105.

215. Ishida Y, Hayashi T, Goto T, Kimura A, Akimoto S, Mukaida N, Kondo T. Essential involvement of CX3CR1-mediated signals in the bactericidal host defense during septic peritonitis. J Immunol 2008;181:4208-18.

216. Jimenez F, Quinones MP, Martinez HG, Estrada CA, Clark K, Garavito E, Ibarra J, Melby PC, Ahuja SS. CCR2 plays a critical role in dendritic cell maturation: possible role of CCL2 and NF-kappa B. J Immunol 2010;184:5571-81.

217. Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Forster R, Agace WW. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. J Exp Med 2005;202:1063-73.

218. Izcue A, Hue S, Buonocore S, Arancibia-Carcamo CV, Ahern PP, Iwakura Y, Maloy KJ, Powrie F. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. Immunity 2008;28:559-70.

219. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. Nat Protoc 2007;2:541-6.

220. Nakanishi M, Tazawa H, Tsuchiya N, Sugimura T, Tanaka T, Nakagama H. Mouse strain differences in inflammatory responses of colonic mucosa induced by dextran sulfate sodium cause differential susceptibility to PhIP-induced large bowel carcinogenesis. Cancer Sci 2007;98:1157-63.

221. Natividad JM, Petit V, Huang X, de Palma G, Jury J, Sanz Y, Philpott D, Garcia Rodenas CL, McCoy KD, Verdu EF. Commensal and probiotic bacteria influence intestinal barrier function and susceptibility to colitis in Nod1(-/-) ;Nod2(-/-) Mice. Inflamm Bowel Dis. 2011; doi: 10.1002/ibd.22848

222. Liu Z, Ramer-Tait AE, Henderson AL, Demirkale CY, Nettleton D, Wang C, Hostetter JM, Jergens AE, Wannemuehler MJ. Helicobacter bilis colonization enhances susceptibility to Typhlocolitis following an inflammatory trigger. Dig Dis Sci 2011;56:2838-48.

223. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology 1994;107:1643-52.

224. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 1990;98:694-702.

225. Dharmani P, Leung P, Chadee K. Tumor necrosis factor-alpha and Muc2 mucin play major roles in disease onset and progression in dextran sodium sulphate-induced colitis. PLoS One;6:e25058.

226. Vandenbroucke K, de Haard H, Beirnaert E, Dreier T, Lauwereys M, Huyck L, Van Huysse J, Demetter P, Steidler L, Remaut E, Cuvelier C, Rottiers P. Orally administered L. lactis secreting an anti-TNF Nanobody demonstrate efficacy in chronic colitis. Mucosal Immunol 2010;3:49-56.

227. Naito Y, Takagi T, Uchiyama K, Kuroda M, Kokura S, Ichikawa H, Yanagisawa R, Inoue K, Takano H, Satoh M, Yoshida N, Okanoue T, Yoshikawa T. Reduced intestinal inflammation induced by dextran sodium sulfate in interleukin-6-deficient mice. Int J Mol Med 2004;14:191-6.

228. Hans W, Scholmerich J, Gross V, Falk W. Interleukin-12 induced interferon-gamma increases inflammation in acute dextran sulfate sodium induced colitis in mice. Eur Cytokine Netw 2000;11:67-74.

229. Cox JH, Kljavin NM, Ota N, Leonard J, Roose-Girma M, Diehl L, Ouyang W, Ghilardi N. Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. Mucosal Immunol 2012;5:99-109.

230. Kim TW, Seo JN, Suh YH, Park HJ, Kim JH, Kim JY, Oh KI. Involvement of lymphocytes in dextran sulfate sodium-induced experimental colitis. World J Gastroenterol 2006;12:302-5.

231. Brown JB, Cheresh P, Zhang Z, Ryu H, Managlia E, Barrett TA. P-selectin glycoprotein ligand-1 is needed for sequential recruitment of T-helper 1 (Th1) and local generation of Th17 T cells in dextran sodium sulfate (DSS) colitis. Inflamm Bowel Dis. 2011; doi: 10.1002/ibd.21779.

232. Yan F, Wang L, Shi Y, Cao H, Liu L, Washington MK, Chaturvedi R, Israel DA, Wang B, Peek RM, Jr., Wilson KT, Polk DB. Berberine promotes recovery of colitis and inhibits inflammatory responses in colonic macrophages and epithelial cells in DSS-treated mice. Am J Physiol Gastrointest Liver Physiol. 2011; doi: 10.1152/ajpgi.00312.

233. Tassi I, Cella M, Presti R, Colucci A, Gilfillan S, Littman DR, Colonna M. NK cell-activating receptors require PKC-theta for sustained signaling, transcriptional activation, and IFN-gamma secretion. Blood 2008;112:4109-16.

234. Shi FD, Ljunggren HG, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. Nat Rev Immunol 2011;11:658-71.

235. Feng T, Qin H, Wang L, Benveniste EN, Elson CO, Cong Y. Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production. J Immunol 2011;186:6313-8.

236. Morrison PJ, Ballantyne SJ, Kullberg MC. Interleukin-23 and T helper 17-type responses in intestinal inflammation: from cytokines to T-cell plasticity. Immunology 2011;133:397-408.

237. Terabe F, Fujimoto M, Serada S, Shinzaki S, Iijima H, Tsujii M, Hayashi N, Nomura S, Kawahata H, Jang MH, Miyasaka M, Mihara M, Ohsugi Y, Kishimoto T, Naka T. Comparative analysis of the effects of anti-IL-6 receptor mAb and anti-TNF mAb treatment on CD4+ T-cell responses in murine colitis. Inflamm Bowel Dis 2011;17:491-502

.

238. Buonocore S, Ahern PP, Uhlig HH, Ivanov, II, Littman DR, Maloy KJ, Powrie F. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature 2010;464:1371-5.

239. Wang X, Fujita M, Prado R, Tousson A, Hsu HC, Schottelius A, Kelly DR, Yang PA, Wu Q, Chen J, Xu H, Elmets CA, Mountz JD, Edwards CK, 3rd. Visualizing CD4 T-cell migration into inflamed skin and its inhibition by CCR4/CCR10 blockades using in vivo imaging model. Br J Dermatol 2010;162:487-96.

240. Yuan Q, Bromley SK, Means TK, Jones KJ, Hayashi F, Bhan AK, Luster AD. CCR4-dependent regulatory T cell function in inflammatory bowel disease. J Exp Med 2007;204:1327-34.

241. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, Noelle RJ, Cheroutre H. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. Immunity 2009;30:471-2; author reply 472-3.

242. Yokota A, Takeuchi H, Maeda N, Ohoka Y, Kato C, Song SY, Iwata M. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. Int Immunol 2009;21:361-77.

243. Cheatem D, Ganesh BB, Gangi E, Vasu C, Prabhakar BS. Modulation of dendritic cells using granulocyte-macrophage colony-stimulating factor (GM-CSF) delays type 1 diabetes by enhancing CD4+CD25+ regulatory T cell function. Clin Immunol 2009;131:260-70.

244. Kared H, Leforban B, Montandon R, Renand A, Layseca Espinosa E, Chatenoud L, Rosenstein Y, Schneider E, Dy M, Zavala F. Role of GM-CSF in tolerance induction by mobilized hematopoietic progenitors. Blood 2008;112:2575-8.

245. Siddiqui KR, Powrie F. CD103+ GALT DCs promote Foxp3+ regulatory T cells. Mucosal Immunol 2008;1 Suppl 1:S34-8.

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9 CURRICULUM VITAE

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CONFERENCES

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