Aus dem Institut für Immunologie der Ludwig-Maximilians-Universität München

Direktor: Prof. Thomas Brocker

Genetic Background Profoundly Influences Colitis Susceptibility

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

an der medizinischen Fakultät

der Ludwig-Maximilians-Universität München

vorgelegt von

Ana Ogrinc

aus Ljubljana

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Abbreviations

ABX	mixture of antibiotics
AIRE	autoimmune regulatory
APC	antigen presenting cell
Batf3	basic leucin zipper transcription factor ATF-like 3
Bcl-6	B-cell lymphoma 6
BMDC	bone marrow derived dendritic cell
CBA	Cytometric Bead Array
CD205	cluster of differentiation 205
CD40L	CD40 ligand
cDC	conventional DC
cDNA	complementary DNA
Clec4A	C-type lectin domain family 4 member A
Clec9A	C-type lectin domain family 9 member A
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
Cq	quantitation cycle
DC	dendritic cell
DNA	deoxyribonucleic acid
DSS	dextran sodium sulfate

EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr-Virus
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FoxP3	forkhead box protein P3
IBD	inflammatory bowel disease
IBD	inflammatory bowel disease
Id2	inhibitor of DNA binding 2
IP	intraperitoneal
IRF2	interferon regulatory factor 2
IRF4	interferon regulatory factor 4
IRF8	interferon regulatory factor 8
iTreg	induced regulatory T cell
LP	lamina propria
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MCP1	monocyte chemoattractant protein-1
MFI	mean fluorescence intensity
MHC	major histocompatibility complex

mLN	mesenteric lymph node
mRNA	messenger ribonucleic acid
mTEC	medullary thymic epithelial cell
NFIL3	nuclear factor interleukin 3 regulated
NK	natural killer
Notch2	neurogenic locus notch homolog protein 2
nTreg	natural regulatory T cell
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PMA	phorbol-12-myristat-13-acetat
PRDM1	PR domain zinc finger protein 1
PRDM1	PR domain zinc finger protein 1
PRR	pattern recognition receptor
qPCR	quantitative PCR
RAR	retinoic acid receptor
RBP-J	recombining binding protein suppressor of hairless
RelB	v-rel avian reticuloendotheliosis viral oncogene homolog B
RNA	ribonucleic acid

SCID	severe combined immunodeficiency
SEM	standard error of mean
SNP	single nucleotide polymorphism
TCR	T-cell receptor
TLR	toll-like receptor
Treg	regulatory T cell
UV	ultraviolet
wt	wild type
XCR1	Chemokine XC receptor 1

Contents

1 Summary

Inbred mouse strains are powerful tools to study the complexity of the immune system. However, especially in immunology, the choice of the respective mouse strain might also greatly alter the outcome of a study, as factors such as differential gene mutations, polymorphisms and regulation strongly influence the results.

In this study we analyzed the constitutive CD40-triggering of dendritic cells (DCs), its influence on DC maturation, DC homeostasis and the effects on peripheral tolerance induction. For this purpose we used the DC-LMP1/CD40 mice expressing a constitutive LMP1/CD40 transgene in all CD11c expressing cells, to provide the expressing cell with a constant CD40 stimulation. CD40 stimulation has previously been connected with the maturation of DCs and in our mouse model, when combined with a normal intestinal flora of commensal bacteria, resulted in a fatal B- and T-cell dependent colitis. Surprisingly this held true only for transgenic animals on C57BL/6 genetic background, while animals on F1 (C57BL/6 x BALB/c) and BALB/c genetic backgrounds remained healthy.

Here we analyzed the phenotypical differences between DC-LMP1/CD40 animals of different genetic backgrounds. We demonstrated that transgenic animals on a C57BL/6 genetic background had the strongest reduction of tolerogenic $CD103^+$ DCs causing the absence of induced regulatory T cells (iTregs) and increase of Th1/Th17 effector T cells, which were responsible for colitis. In contrast, DC-LMP1/CD40 mice on BALB/c and F1 backgrounds showed higher frequencies of tolerogenic $CD103^+$ DCs, more iTregs and less or no effector T cells.

We also showed that high levels of IL-1β, which were present only in C57BL/6 DC-LMP1/-CD40 animals, formed a cytokine milieu, which was favorable for the development of the pathogenic Th1/Th17 T cells. In agreement with this, blocking of IL-1β prevented disease development by reducing IL-17^-IFN-γ^+ T cells.

Our data provides evidence that the genetic background contributes to disease development by differentially affecting key immune cell populations such as DCs and iTregs.

2 Zusammenfassung

Durch Inzucht erzeugte Mauslinien sind leistungsstarke Werkzeuge zur Erforschung des Immunsystems. Allerdings bestimmt die Auswahl des genetischen Hintergrundes der verwendeten Mauslinien auch das zu erzielende Ergebnis. Da unterschiedliche Mauslinien unterschiedliche Genmutationen und Polymorphismen tragen, führen identische Experimente in unterschiedlichen Mauslinien unter Umständen zu unterschiedlichen Ergebnissen. In der vorliegenden Studie untersuchen wir den Einfluss des CD40 Signals auf dendritische Zellen (DC), ihre Reifung, Homeostase und periphere Toleranzinduktion. Zu diesem Zweck wurde das DC-LMP1/CD40 Mausmodell verwendet, welches ein konstitutives LMP1/CD40 Transgen in allen CD11c-positiven Zellen exprimiert, welches wiederum die exprimierende Zelle mit kontinuierlicher CD40 Stimulierung versorgt. CD40 Stimulation wurde zuvor bereits mit der Reifung von DC in unserem Mausmodell in Verbindung gebracht. In Kombination mit einer normalen Darmflora führte CD40 Stimulation zu einer tödlichen, B- und T-Zell abhängigen Kolitis. Überraschenderweise war dies nur der Fall nur bei transgenen Tieren des genetischem C57BL/6-Hintergrundes, während Tiere im BALB/c- oder F1 (C57BL/6 x BALB/c) Hintergrund gesund blieben.

Wir analysieren die phänotypischen Unterschiede von DC-LMP1/CD40 Tieren mit unterschiedlichem genetischen Hintergrund und zeigen, dass transgene Tiere im C57BL/6 Hintergrund die geringste Anzahl an toleranten $CD103^+$ DCs haben. Dies führt zu einer starken Reduktion von induzierten regulatorischen T Zellen (iTregs), einer Erhöhung von Th1/Th17 Effektor T-Zellen, welche die Krankheit verursachten. Die Anzahl an toleranten $CD103^+$ DCs war in transgenen Tieren mit anderem genetischen Hintergrund weniger reduziert, was sich wiederum in einem Anstieg an iTregs wiederspiegelte, welche in der Lage waren die Entzündung zu kontrollieren. Außerdem zeigten wir, dass hohe IL-1β Level, welche nur in C57BL/6 DC-LMP1/CD40 Tieren vorzufinden waren, ein Zytokinmilieu erzeugten, welches sich positiv auf die Entwicklung von pathogenen Th1/Th17 T Zellen auswirkte. In Übereinstimmung mit diesen Ergebnissen konnten die Blockierung von IL-1β die Krankheitsentwicklung verhindern

und die Anzahl von IL-17^{-}IFN-γ^{+} T Zellen verringern. Dies veranschaulicht die Bedeutung von IL-1β für die Differenzierung von Th17 T Zellen und legt eine mögliche Verwendung von anti-IL-1β für die Behandlung von Patienten mit chronisch-entzündlichen Darmerkrankungen (IBDs) nahe. Unsere Ergebnise zeigen, dass der genetische Hintergrund zur Krankheitentwicklung beiträgt indem wichtige Immunzellen wie DCs und iTregs unterschiedlich stark beeinflusst werden.

3 Introduction

3.1 The immune system

The immune system is a complex integrated network of organs, tissues, cells and their products that work together to defend an organism from invasion and infection. Its main feature is to differentiate between self and non-self antigens. Self antigens have to be tolerated. While pathogenic bacteria, fungi or protozoa expressing non-self antigens have to be neutralized or destroyed, antigens on commensal bacteria should be tolerated. Disorders in the immune system can result in serious autoimmune diseases when the immune system targets the body's own healthy tissues, or in allergies when the immune system performs an exaggerated response against a harmless material like for example grass pollen, food particles or pet dander. The immune system is built from layered defenses of increasing specificity. There are two arms of the immune response: the innate and the adaptive immune system, which complement each other. The innate immune system is activated when conserved small molecular motifs associated with groups of pathogens, so-called pathogen-associated molecular patterns (PAMPs), are detected with toll-like receptors (TLRs) or others pattern recognition receptors (PRRs) of the innate immune system. This is the so called first line of defense and offers immediate protection, but protection is not specific and it does not lead to immunological memory. The innate immune system consist of mechanical barriers (epithelial surfaces, tight junctions, mucosa), cells that release different inflammatory mediators (natural killer (NK) cells, macrophages, mast cells, innate lymphocytes), cells with phagocytic activity (neutrophils, monocytes, macrophages) and different proteins (proteins of complement system, interleukins, defensins). The innate immune system is fast and broadly effective, but since it is not specific it can only prevent entry and spreading of pathogens to a certain degree. More resistant pathogens which overcome the first line of defense have to face the much more sophisticated, highly specific, adaptive immune system, which is capable of destroying pathogens and neutralizing the toxic substances produced by them. For induction of adaptive immune response

in inactive or resting lymphocytes, special antigen presenting cells (APCs) are needed. Their role is bridging the innate and adaptive immune systems, due to their ability to detect invading pathogens or altered body cells, process them and present their antigens on cell surfaces in the context of major histocompatibility complex (MHC) molecules in combination with necessary accessory co-stimulatory molecules. The so formed message is an activation signal for T cells, while B cells have to recognize the native unprocessed antigen. B cells represents the humoral response, due to their ability to make antibodies, which are secreted in extracellular fluids where they bind and neutralize bacteria and toxins. The cellular response consist of cytotoxic T cells, which recognize and kill infected or otherwise damaged cells, and from T helper cells, which secrete cytokines that stimulate other cells and determine which immune response strategy the body will use against a certain pathogen.

Each B or T cell bears many replicas of a receptor with a unique specificity made in a process of V(D)J recombination, a process of antigen receptor rearrangement that enables the production of a large repertoire of receptors from a limited amount of gene segments, which can collectively recognize many different antigens. The price for the high antigen specificity of the adoptive immune system is a low frequency of precursor cells resulting in a long lag phase from selection of suitable cell clones with a specific antigen receptor and the expansion of these clones to the point where they are able to fight infections. Due to the random assembling of antigen receptors there exists a potential danger for self-reactivity, meaning that lymphocytes bearing receptors for self molecules must be deleted or changed in the early stage of maturation. This process is called immunological tolerance and will be discussed later on. The final big advantage and main hallmark of the adoptive immune system is immunological memory, which enables the system to remember previously encountered pathogens by their antigens and if they are encountered again the antigen specific response will be rapid and powerful.

3.2 Immune tolerance

Antigen receptor rearrangement is a process of somatic recombination in immature lymphocytes which results in a large repertoire of antigen receptors with a potential to fight many different pathogens. With random generation of antigen receptors many useful specificities are generated, but so are many useless and potentially self reactive receptors, which have to be deleted or rearranged.

B cells are continuously produced in bone marrow from hematopoietic precursor cells. Before a newly formed B cell leaves the bone marrow and migrates to the spleen in order to finalize its early development, it is tested for potential autoreactivity [1]. However, B cell development will not be discussed in details, since this is not the focus of this thesis.

The thymus is the primary site of T cell lymphopoiesis from incoming bone-marrow-derived progenitors [2]. Since T helper cells are able to activate B cells and cytotoxic T cells can attract self tissue, potentially self reactive T cells represent big danger and have to be deleted, or their receptors have to be edited in the process of tolerance induction [3].

3.2.1 Central T cell tolerance

The thymus is the primary lymphoid organ where central tolerance takes place. Once a T cell receptor is formed and expressed on the cell surface large numbers of $CD4^+CD8^+$ double-positive thymocytes enter a checking point where T-cell receptor (TCR) affinity for self MHC determines the future fate of developing T cells [4]. If the TCR of a thymocyte does not bind to self MHC, then the thymocyte will undergo the default death pathway, since this recognition is a basic requirement for mature T cell functions [5]. Cells with a low TCR-MHC affinity become $CD4^+$ or $CD8^+$ single positive T cells in a process called positive selection. As cells with a high TCR-MHC affinity are potentially dangerous, they will be eliminated in a process called clonal deletion, or are diverted clonally to become regulatory T cells (Tregs) and in rare cases the specificity of their receptors might be changed in a process called receptor editing [3]. Newly formed thymocytes then enter a process of negative selection where medullary

thymic epithelial cells (mTECs), the APCs in thymus, facilitate tolerance induction against self antigens. An expression of the diverse range of tissue specific self antigens in mTECs is controlled by a transcriptional regulator encoded by the autoimmune regulatory (AIRE) gene in order to remove auto-reactive T cells upon their encounter and recognition of self antigens [6, 7].

3.2.2 Peripheral T cell tolerance

Central T cell tolerance is not able to eliminate self reactive lymphocytes, which are specific for antigens not presented by mTECs, therefore there is a need for second mechanism. Peripheral tolerance controls T cells specific for developmental antigens and antigens displayed during chronic infection as well as T cells specific for non-self non-dangerous antigens like food antigens or commensal bacteria, which have to be tolerated and should not evoke an aggressive immune response [3]. The main APCs in the periphery are DCs, which are strategically positioned at body barriers like skin and mucosal surfaces, or are circulating in the blood stream from where they can be rapidly recruited to sites of challenge in response to chemotactic signals [8]. After having sampled the antigens they migrate through lymphatic vessels in T cell zones in order to present processed antigens to naïve T cells [9]. DCs are migrating to T cell zones in inflammatory and in steady state conditions, but due to the precise regulation of their maturation status, which will be discussed further on, induce immunity only against dangerous antigens whilst tolerating other antigens.

For full activation of a naïve T cell three signals are needed: signal one is mediated through TCR after it binds a peptid presented in context of an MHC molecule, signal two comes from co-stimulatory molecules on DCs, like for example CD80 and CD86, which bind to CD28 on T cells. And the last signal, signal three, is a polarizing signal mediated by different soluble or membrane bounded factors [10]. DCs provide signals two and three only if they have previously detected PAMPs through receptors of the innate immune system. According to that mature DCs induce immunity, because they have encountered a pathogen and conse-

quently up-regulated MHC and co-stimulatory molecules on their surface. While immature DCs provide only signal one to a naïve T cell and therefore induce tolerance [11, 12]. An antigen, presented in low amounts [13] or presented by steady state DC therefore does not induce clonal expansion of T cell with specific TCR for it. T cells which received only signal one go into apoptosis [14], anergy [15] or they become Treg cells [16].

3.2.2.1 Tregs

Tregs are $CD4^+CD25^+$forkhead box protein P3 $(FoxP3)^+$ primary mediators of peripheral immune tolerance. FoxP3 is a key transcription factor in Treg cells and it is required for their development and function [17, 18]. Consequently mutations resulting in a loss of function lead to serious autoimmune diseases in humans and mice [19, 20, 21]. Tregs can suppress T cell activation with the production of inhibitory cytokines, with the secretion of granzymes, which cause cytolysis, or with the modulation of DCs maturation status [22].

Tregs consist of two developmentally different groups of cells. The first group are natural regulatory T cells (nTregs), which develop in the thymus during the process of central T cell tolerance, as a result of recognition of self antigens, and can be identified by expression of Helios [23] or Neuropilin-1 [24]. The second group consists of iTregs, which develop in the periphery from mature $CD4^+$ T cells in the conditions where antigen exposure is not optimal or T cells do not receive co-stimulatory signals from DCs [25]. The iTreg cells are most prominently found in the intestinal organs, but are rare in other tissues. iTreg cells express a key transcription factor nuclear hormone receptor RORγt and are dependent on microbiota exposure, since they are profoundly reduced in germfree or antibiotic treated mice (in contrast Helios positive Tregs are unaffected) [26].

In the gastrointestinal tract the immune system comes in close contact with a complex population of commensal bacteria and food antigens, which are all recognized by innate receptors on DCs, but do not trigger effector T cells and rather induce peripheral iTreg cells. TCRs of iTregs cells are of different specificity than those from nTregs, since they are shaped by local

microflora [27]. The relative contribution of each of the two above described developmental pathways to intestinal Treg population is still not completely understood and the system gets even more complex since there is one more major subset of intestinal Tregs cells, which in addition to Helios also expresses Gata3 and is negative for RORγt [26]. These Tregs are less well described, but it is known that they respond to IL-33, which is an endogenous danger signal produced by epithelial cells [28]. Their intrinsic expression of transcription factor Gata3 was shown to be essential in inflammatory settings, where it controls the FoxP3 expression and promotes the accumulation of Tregs in inflamed tissues [29].

iTregs are crucial for maintaining immune cell homeostasis, because if a T cell specific for some food or commensal antigen does not become an iTreg and it differentiates in effector T cell, then it can potentially induce severe inflammation such as colitis [30]. On the other hand, development of all Tregs has to be precisely controlled, as they can also become a double-edged sword by preventing anti-tumor [31] and anti-infection T cell responses [32].

3.3 Dendritic cells

Dendritic cells are tree-like shaped cells (gr. dendron = tree) and were first described in 1973 by Ralph M. Steinman [33]. Soon it became clear that they share a lot of features with Langerhans cells observed by Paul Langerhans five years earlier. DCs belong to a larger group of cells called professional APCs which in addition to DCs include macrophages, Langerhans cells and B cells [34, 35].

DCs consist of a functionally and developmentally diverse group of cells, which are able to link innate and adaptive immunity. With numerous surface receptors of the innate immunity DCs can efficiently take up antigens, process them, and present their peptides in context of MHC molecules to the cells of the adaptive immune system. They also activate T cells by expressing co-stimulatory molecules and provide additional information about the origin of an antigen, in order to trigger a diverse spectrum of efficient protective responses [36].

What makes DCs special in antigen presentation is their potential for cross-presentation of exogenous antigens in context of MHC class I molecules and therefore acting on CD8 cytotoxic T cells. The latter has been shown to be crucial for immune response against viruses and tolerance to self antigens [37, 38].

In addition to Steiman's conventional DCs (cDCs) a second phenotypically and functionally different group of DCs exist. These morphologically resemble plasma cells and are called plasmacytoid DCs (pDCs). These cells produce large amounts of type 1 interferons in response to viral infections [39], but they won't be further discussed here.

cDCs orgin from hematopoietic stem cells derived common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), which were both shown to be capable of further differentiation towards DC [40], but it has been published that under steady state conditions most of DCs are derived from the myeloid lineage [41]. CMP differentiate via several precursors (granulocyte-macrophage precursor differentiate to macropage/DC progenitor, which give rise to common DC precursor) to preDC and finally to cDC [42, 43], which are then distributed all over the body: thymus, lymphoid organs, afferent lymphatics, nonlymphoid

organs (intestinal cDCs), dermis (Langerhans cells) and peripheral blood [44, 45, 46, 47, 48].

3.3.1 Conventional dendritic cells subsets

cDCs, monocytes and macrophages form the mononuclear phagocyte system, a complex group of cells with different, but specially in inflammation often overlapping, functional and phenotypical characteristics. cDCs are generally defined as $CD45^+$, MHC class II^+, $CD11c^+$ cells, which lack other lineage specific markers [49].

Especially in the mucosa of the intestinal tract it is hard to distinguish between cDCs and macrophages, since gut resident macrophages also express CD11c [50] and high levels of MHC class II molecules [51]. In this case even morphological analysis is not helpful because intestinal macrophages express tight junction proteins and form a transepithelial dendritic projections [52].

Therefore for the delineation of cDCs and macrophages some of additional markers like Flt3 [53, 54], the chemokine receptor CCR7 [55], the zinc finger transcription factor zbtb46 [56], CD115 [57], CD64 [58] or F4/80 [59] are needed.

cDCs can be roughly separated according to their migration ability in tissue-migratory cDCs and lymphoid organ-resident cDCs [60, 61]. Tissue-migratory cDCs migrate via afferent lymph due to the up-regulation of the chemokine receptor CCR7 [55] to the T cell areas of secondary lymphoid organs, where they stimulate naïve T cells [62]. Under inflammatory conditions this trafficking is strongly increased [63]. In contrast lymphoid organ-resident cDCs remain in lymphoid tissues [64].

The second separation of cDCs is based on their surface phenotypes and divides cDCs in two main subsets: $CD8^+/CD103^+$ and $CD11b^+$ expressing cells, which both are present in lymphoid and non-lymphoid tissues [65].

3.3.1.1 CD8$^+$/CD103$^+$ DCs

CD8$^+$ expressing lymphoid-resident DCs were first described in murine lymphoid organs [46]. An equivalent subset in non-lymphoid tissues are migratory DCs expressing CD103$^+$ integrin [66]. CD8$^+$/CD103$^+$ DC subsets have a similar phenotype and transcriptional profile. Their development is dependent on interferon regulatory factor 8 (IRF8), inhibitor of DNA binding 2 (Id2), nuclear factor interleukin 3 regulated (NFIL3) and basic leucin zipper transcription factor ATF-like 3 (Batf3) transcription factors, where the absence of either of these leads to a severe developmental deficiency [67, 68, 69, 70, 71]. They also share a unique transcriptional fingerprint since only these two DC subsets express C-type lectin domain family 9 member A (Clec9A) [72] and chemokine receptor Chemokine XC receptor 1 (XCR1) [73, 74]. In addition they express C-type lectin cluster of differentiation 205 (CD205), a well described endocytic recognition receptor for apoptotic and necrotic self, important in antigen uptake, processing, presentation and antigen cross-presentation [75, 76]. Being able of processing exogenous antigens and cross-presenting them in the context of MHC class I pathway, gives these DC subsets the ability to activate CD8$^+$ T cells and generate cytotoxic T cell immunity against antigens associated with viral infection, intracellular bacteria, parasites and tumors [77]. In order to effectively stimulate T cells, CD8$^+$/CD103$^+$ DCs respond to TLR signals with a high level of bioactive IL-12p70 [78, 79], a cytokine involved in the initiation of the Th1 immune response [80].

CD8$^+$ and CD103$^+$ DC subsets were therefore proposed to be very closely related if not identical [74, 81], but there are also publications which reveal some differences between the two subsets. CD8$^+$ DC were published to express higher levels of TLR3 than CD103$^+$ DCs [82]. While a second study, published in 2014 [66], revealed even more differences. They have shown that the two subsets differ in their ability to influence CD4$^+$ T cell differentiation, where the CD103$^+$ subset is more potent in directing of T cells towards the Th17 phenotype, due to its higher production of IL-1 and IL-6 cytokines upon stimulation. They demonstrated that only CD103$^+$ require GM-CSF for development and function, and that these two DC

subsets differ in the expression of inflammasome components, where $CD8^+$ express higher levels of NLRP1a/b, NLRP3, NLRC4 and NLRP12, and $CD103^+$ express higher levels of NLRP6 and NLRP10.

3.3.1.2 $CD11b^+$ DCs

$CD11b^+$ DC subset is in comparison to $CD8^+$/$CD103^+$ DCs more diverse and less characterized. Cells can be found in nonlymphoid tissue and in lymphoid organs except for the thymus [65]. They are not efficient in cross-presentation, but due to the prominent MHC class II machinery [75] and efficient production of IL-6 [83] and IL-23 [84], preferentially prime T helper cells. Batf3-IRF8-Id2 independent $CD11b^+$ DCs developmentally depend on interferon regulatory factor 2 (IRF2), interferon regulatory factor 4 (IRF4), IKAROS, v-rel avian reticuloendotheliosis viral oncogene homolog B (RelB), neurogenic locus notch homolog protein 2 (Notch2) and recombining binding protein suppressor of hairless (RBP-J) (for review, see [85]). $CD11b^+$ DCs in the spleen can be separated in $CD11b^+Esam^{hi}$ and $CD11b^+Esam^{low}$ populations, where only the $CD11b^+Esam^{hi}$ population is controlled by the Notch2 pathway [86].

3.3.2 Intestinal DCs

The intestinal mucosa represents a huge epithelial surface, where the immune system encounters dietary antigens, commensal bacteria as well as potential pathogens. The selective permeability of mucosa is crucial for nutrition digestion, absorption and waste disposal, but represents a potential danger for pathogen entry. The intestine is a crucial site of innate and adaptive immune regulation where pathogens may induce immune response, while destructive responses against food antigens and commensals must be prevented in order to avoid a breakdown of intestinal homeostasis, which may lead to inflammatory bowel disease (IBD), celiac disease or allergies [87, 88]. This fine tuning of immune responses is possible due to the high plasticity of intestinal DCs which are equally equipped to induce regulatory and

stimulatory immune responses.

Intestinal DCs consist of three phenotypically and developmentally distinct populations which are located in the lamina propria of the small and the large intestine, peyers patches, mesenteric lymph nodes (mLNs) and isolated lymphoid follicles [89]. In contrast to DCs in lymphoid organs the intestinal DCs ($MHCII^+CD11c^+CD64^-$ [58]) are divided in three subsets: $CD103^+CD11b^-$, $CD103^+CD11b^+$ and $CD103^-CD11b^+$ [90].

3.3.2.1 Origins and development of the intestinal DCs

Transmembrane integrin CD103 is an adhesion receptor that mediates retention of lymphocytes within epithelial tissues through binding to E-cadherin [91, 92]. CD103 does not seem to be a marker of a particular DC lineage, but is most likely induced on $CD11b^+$ and $CD11b^-$ DCs during their residence within the intestine [90]. This belief is supported by the fact that two CD103 expressing DC populations have different developmental origins, require distinct transcription factors and display unique gene-expression profiles [93]. With use of Zbtb46-DTR mice (transcription factor selectively expressed by cDCs [94]) it has been demonstrated that some of $CD103^+CD11b^+$ cells actually derive from monocytes, since diphtheria toxin treatment reduced this population only by 50 %, while $CD103^+CD11b^-$ were almost completely absent. Partial monocyte origin of $CD103^+CD11b^+$ DCs was confirmed in the same study with mouse model where monocytes and monocyte derived cells were specifically ablated (diphtheria toxin treated $Lysm^{Cre}$ x $Csf1r^{LsL-DTR}$ mice) [95].

$CD103^+CD11b^-$ are closely related to cross-presenting $CD8^+$ lymphoid-resident DCs. They express the chemokine receptor XCR1 [73, 74] and lectin Clec9a [96], while for their development they require Batf3, Id2, IRF8 and B-cell lymphoma 6 (Bcl-6) [81, 70, 97, 98].

On the other side $CD103^+CD11b^+$ intestinal DCs share features with $CD11b^+$ lymphoid-resident DCs. These cells express lectin C-type lectin domain family 4 member A (Clec4A) [75] and signal regulatory protein-α (SIRPα) [83]. It was published that they are developmentally dependent on Notch2, RBP-J, PR domain zinc finger protein 1 (PRDM1) (also known as

Blimp1) and partially on IRF4 [99, 97]. Since in IRF4-deficient mice $CD103^+CD11b^+$ DCs are approximately 50 % reduced in the intestine and almost completely absent in mLNs [83].

The last intestinal $CD103^-CD11b^+$ DCs subset with intermediate CX_3CR1 expression represent a poorly defined heterogeneous group of cells which, for a long time, was not clear if they are really bona fide DCs or instead macrophaghes [70]. Gautier et al. described CD64 as a good marker for discrimination between macrophages and DCs, as DCs are $CD64^-$ [100]. In contrast to $CD103^+$ DCs, which develop from pre-DCs, $CD103^-CD11b^+CX_3CR1^{int}$ derive from $Gr1^+$ monocytes in a GM-CSF and Flt3 controlled manner [101, 102, 70]. The current opinion is that cells expressing intermediate levels of CX_3CR1 appear to be distinct from CX_3CR1^{hi} macrophages, because they depend on cDCs specific transcription factor Zbtb46 [56], express high levels of CCR7, are efficient in priming naïve T cells and continuously migrate from tissue to the draining mLNs, which is an important functional feature to distinguish DCs from macrophages [103]. Transcription factors required for $CD103^-CD11b^+CD64^-$ DCs development remain to be fully assessed, because old studies did not separate these cells from $CD103^-CD11b^+CD64^+$ macrophages. Surprisingly, intestinal $CD103^-CD11b^+$ DCs are not reduced in IRF4 deficient mice, suggesting that they are not related to $CD103^+CD11b^+$, or that IRF4 is needed for CD103 up-regulation on $CD11b^+$ DCs [84].

3.3.2.2 Tolerogenic and immunogenic intestinal DC subsets

Intestinal $CD103^+$ DCs take up bacterial antigens from the gut lumen [104, 105] or from CX_3CR1^{hi} non-migratory macrophages [106] and continuously migrate to mLNs where they prime naïve T cells or induce tolerance [103]. The ability to induce Treg cells from naïve T cells with TCR specific for harmless dietary antigens and commensal flora is of particular benefit in the intestine. Intestinal $CD103^+$ DCs were shown to be superior in inducing Foxp3 Treg cells, due to their high expression level of enzyme aldehyde dehydrogenase, which converts vitamin A into retinoic acid, and their ability to activate latent TGF-β [107, 108]. Retinoic acid is important in the process of imprinting gut homing CCR9 and $\alpha4\beta7$ receptors on newly induced Tregs or effector T cells [109, 110].

A study which compared $CD103^-$ and $CD103^+$ DCs revealed that the $CD103^+$ subset express higher levels of transforming growth factor-β2 (TGF-β2), tissue plasminogen activator and latent TGF-β-binding protein 3. Proteins which have all been shown to be important for efficient secretion, appropriate localization and conversion of latent TGF-β into its active form [111, 108].

However $CD103^+$ DCs were shown not to be hard-wired to induce tolerance, they can adapt to environmental conditions and in inflammation rather induce IFN-γ producing $CD4^+$ T cells than Tregs. Experiments performed in colitic mice have shown that $CD103^+$ DCs in inflammation lower their expression of tgfb2 and aldh1a2, which dampens their tolerogenic properties [112]. This means that there is no exclusively tolerogenic DC subset, but this result rather supports the observation that phenotypically immature DCs induce tolerance, while DCs which detected a danger signal in the form of so called "pathogen associated molecular patterns" become mature, and mature DCs can regardless of their subset induce immunity [12].

Nevertheless, for a long time it was not clear which subset of $CD103^+$ DCs is responsible for Treg cell induction in the periphery. Mice without one or even both $CD103^+$ DC subsets were long lived and did not show any autoimmunity [113, 81, 83]. The ablation of both $CD103^+$ DC subsets resulted in significantly reduced numbers of lamina propria Tregs, while mLN Tregs were not affected [113]. However, when only $CD103^+CD11b^-$ DCs were missing this had no effect on number of Tregs [81]. The same result was found with the ablation of $CD103^+CD11b^+$ DC subset [113, 83]. All these results seem surprising, but it is important to know that most of these studies analyzed only total Foxp3 Treg cells and not specifically iTreg cells. There was only one study which shows that the ablation of $CD103^+CD11b^+$ DCs does not effect total Treg number, whilst it causes a minor, albeit significant, increase in the proportion of $Helios^+$ $Foxp3^+$ cells within the colon [83].

Only recently in 2016 a study by Esterhazy et al. offered a clear answer via specific depletion of either $CD103^+CD11b^-$ or $CD103^+CD11b^+$ DC subset, followed by analysis of

oral tolerance and Treg cell induction. Targeting $CD103^+CD11b^-$ DCs partially prevented the generation of iTregs but did not effect oral tolerance, indicating the ability of the remaining DCs to compensate and ensure oral tolerance. On the other side, the $CD103^+CD11b^+$ DC subset was shown to be rather inefficient in Treg induction. Transcriptome analysis revealed that the main tolerogenic $CD103^+CD11b^-$ DC subset expresses the highest levels of Aldh1a2 (encoding retinaldehyde dehydrogenase RALDH2), Tgfb2 (encoding cytokine TGF-β2) and Itgb8 (encoding integrin β8), all enzymes crucial for Treg induction and imprinting of gut homing. The $CD103^+CD11b^+$ DC subset was shown to have a lower Treg inducing gene signature with other gene isoforms [114].

A subpopulation of intestinal $CD103^+CD11b^+$ DCs, which represent the majority of DCs in the small intestine [115], was shown to have an important role in the induction of inflammatory intestinal Th17 cells in steady state [86, 113, 83] and upon activation also Th1 cells [103, 116]. Specific ablation of $CD103^+CD11b^+$ cells resulted in a greatly reduced frequency of Th17 cells in lamina propria [86, 113, 83], while they were not affected in mice which are lacking $CD103^+CD11b^-$ DCs [113]. It was shown that $CD103^+CD11b^+$ DCs drive mLN Th17 cell differentiation in an IL-6 dependent way [83].

Most $CD103^-$ DCs in lamina propria express CD11b and have a more inflammatory phenotype already in steady state, due to higher expression of TLR2, TLR3 and T-bet [108]. This phenotype makes them efficient in inducing differentiation of IFN-γ and IL-17-producing effector T cells, even in lack of proper stimulation [103]. It has been shown that they express substantially higher levels of an IL-23p19 messenger ribonucleic acid (mRNA) than $CD103^+$ cells and upon activation produce enhanced levels of IL-6 and TNF-α [108, 117]. $CD103^-CD11b^+$ DCs migrate to mLNs, prime naïve T cells and imprint them with a gut homing phenotype. Newly formed effector T cells can therefore migrate to the gut and mount an effective defense against intestinal pathogens [103].

3.4 CD40 signaling in DCs

The CD40 surface receptor is a transmembrane glycoprotein, with a molecular mass of 48 kDa, initially described on B cells and later shown to be expressed also by DCs, follicular DCs, monocytes, epithelial cells, endothelial cells, hematopoietic progenitor cells and carcinomas [118, 119, 120, 121]. CD40 ligand (CD40L) has a molecular mass of 33 kDa and is mostly expressed by activated $CD4^+$ T cells [119]. However, DCs [122], B cells [123], basophils [119], eosinophils [124] and mast cells [125] have also been shown to express CD40L. CD40 crosslinking in B cells is important for cell proliferation [118, 126, 127] and immunoglobulin class switching [128], while its role in DCs is less well understood. Several studies have reported that CD40 signaling improves the antigen presenting potential of stimulated DCs with an up-regulation of MHC class II and co-stimulatory molecules CD80 and CD86 [129, 130, 131]. CD40L induces trimeric clustering of CD40 molecules on DCs which results in the activation of a non-canonical NF-κB pathway [132] and triggers the production of IL-12-p40 and IL-6 cytokine [133, 134]. However, another study has shown that CD40 signaling alone is not sufficient for complete maturation of DCs, because it only induces secretion of IL-12p40, but not its bioactive heterodimer IL-12p70. While it also does not lead to an increase of CD40 expression [135], which is a marker for the discrimination between immature and mature DCs [136]. Therefore other studies suggest that the complete maturation of DCs requires the combination of CD40 signaling with innate microbial priming signals [137, 138, 139].

Certainly there is some inconsistency between studies which probably originates from the design of experiments. An anti-CD40 monoclonal antibody (mAb) can be applied *in vitro* or *in vivo*. Each method has some advantages and disadvantages. Studies mentioned above were performed *in vitro* where growth factors, serum and plastic materials could have influenced cultured cells. It was published that DCs respond to materials with receptors of innate immunity what results in their maturation and that different materials have different effects on DC maturation [140, 141]. Therefore, a mature phenotype of DCs in some studies might have actually be induced by plastic material or other substances but not only by activation

trough the CD40 pathway.

The other approach are *in vivo* studies, where anti-CD40 is injected in mice and the effects on DCs are studied afterwards. A group lead by Fiona Powrie has shown that CD40 stimulation down-regulate CD103 expression on sorted DCs *in vitro* and could show similar effects *in vivo*, when $Rag^{-/-}$ mice were injected with the anti-CD40 antibody and had less $CD103^{+}MHCII^{+}$ cells in spleens and developed wasting disease with colitis [142]. Since $CD103^{+}$ DCs are supposed to be the most tolerogenic DCs subset important for induction of peripherial iTregs [108, 111], their depletion with a single injection of anti-CD40 antibody seemed to be a promising way to study their role. However, this method is not ideal, as CD40 is not expressed only on DCs but also on many other cell types [118, 119, 120, 121]. Therefore, injection of anti-CD40 antibody has a systemic effect and it can not be concluded that all observed changes are only due to triggering of the CD40 pathway in DCs. This concern was supported by one study showing that CD40 ligation triggers a B cell mediated necroinflammatory response in the liver [143]. It is true that experiments from Powrie et al. [142] were performed in $Rag^{-/-}$ animals, which do not have B and T cells, but anyway the anti-CD40 effect on other cells like epithelial and endothelial cells can not be excluded. Therefore, the loss of $CD103^{+}$ DCs and observed DC maturation after anti-CD40 treatment could be a secondary effect of a cytokine storm from other cell types.

3.5 CD4$^+$ effector T cells

Naïve CD4$^+$ T cells develop in effector T cells after encountering antigens presented in the context of MHC class II molecule on APCs. Depending on cytokines present in the microenvironment during the T cell receptor mediated activation process, naïve T cell can differentiate into several different subsets of T helper cells. This T cell subset provide help to other cells of the immune system with secreting cytokines, which orchestrate immune responses to be efficient for specific pathogens. T helper cells are crucial players of the adaptive immune system, they provide help to B cells and cytotoxic T cells and maximize bactericidal activity of macrophages [144, 145, 146].

Helper T cells can be subdivided into several subsets [147]. Th1 and Th2 cells were discovered in 1986 [148] as subsets with different functions responsible to clear different types of pathogens, expressing a different set of cell surface molecules and possess a characteristic cytokine signature dependent on specific distinct transcription factors. Th1 cells were shown to provide protection against intracellular pathogens [149, 150, 151] and are involved in antitumor immunity [152], while their exaggerated response against self antigens can result in an autoimmunity [153]. Th1 cell differentiation requires cytokine IL-12 and depends on transcription factors T-bet, STAT1 and STAT4 [154]. T-bet controls the expression of Th1 signature cytokine INF-γ [154], while Th1 pattern of cytokine production additionally includes TNF-α, TNF-β and IL-2 [148, 155]. By contrast, differentiation of Th2 cells require cytokine IL-4 and depends on transcription factors STAT6 and GATA-3. The master regulator GATA-3 controls all of the cytokines which form the so called Th2 signature: IL-4, IL-5, IL-9 and IL-13 [156]. Th2 cells mediate eosinophil activation and protective humoral immunity against extracellular parasites, while their overreaction has been connected with different forms of allergic responses [157, 158, 159].

Almost 20 years after Mosmann and Coffman divided T helper cells into two distinct subsets, Harrington et al. [160] described Th17 cells, a third subset of T helper cells involved in

protection against various especially extracellular bacteria infections, which are also involved in many autoimmune diseases [161]. These cells differentiate independently from transcription factors needed for Th1 or Th2 cell development [160] and produce IL-17A, IL-17E (IL-25), IL-17F, IL-21 and IL-22 [162, 163, 164, 165, 166, 167]. However, retinoic acid receptor (RAR)-related orphan receptor gamma t (RORγt) has been shown to be the central transcription factor responsible for Th17 cell polarization and IL-17 transcription [168], while a cytokine pattern required for induction and expansion of Th17 cells seems to be very complex and will be discussed bellow.

Up to now additional CD4 T-cell subsets have been identified: iTreg cells, follicular helper T cells, Th9 cells, Th22 cells and ThGM cells [155, 169, 170].

3.5.1 Pathogenic and protective Th17 cells

Th17 cells in peripheral organs do not have a homogeneous function, as on one hand they were shown to confer host protection against extracellular bacteria, mycobacteria and fungal infections [171], but they are also involved in multiple inflammatory and autoimmune diseases [172]. Apparently the cytokine milieu at the side of inflammation can be responsible for the various functions of Th17 cells including their pathogenicity.

Differentiation of effector Th17 cells from naïve CD4$^+$ T cells requires TGF-β1 and proinflammatory cytokine IL-6 [173]. Exposure to IL-6, which induces IL-23 receptor expression, is necessary for initial Th17 lineage commitment, because TGF-β1 alone would result in a differentiation of iTreg cells [174]. Th17 cells differentiated that way are not pathogenic, but rather protective, due to their co-production of anti-inflammatory IL-10 [175]. Further exposure to IL-23 results in a pathogenic expression signature, which in addition to T-bet, the master regulator of Th1 cells, includes GM-CSF, IL-23R and IL-7R [176, 163]. IL-17 expression of Th17 cells was shown to be transient in some inflammatory settings, because cells after IL-23 conversion can stop expressing IL-17, acquire the ability to express IFN-γ and are therefore called ex-Th17 cells or Th1-like cells [177]. However, the pathogenic Th17

cell phenotype can also be induced in an alternative TGF-β1 independent way, if they are exposed to a combination of IL-1, IL-6 and IL-23 or TGF-β3 and IL-6 [178, 176].

Changes in intestinal microbiota and in diet were shown to alter normal cytokine regulation and affect Th17 differentiation, which offers an explanation for the increased incidence of Th17 induced autoimmune diseases in the literature [179].

3.6 Modeling human diseases in mice

The fact that humans and mice share about 95 % of protein coding genes makes mice a powerful model for studying human biology and disease [180]. Inbred mouse strains are genetically nearly identically, since this is essential for reproducibility of research experiments [181]. Short generation time, short life span, being easy to breed and progress in conventional transgenic and gene knockout technologies have enabled modeling of human diseases in experimental mice [182]. Since mouse strains are genetically and phenotypically diverse, it is not a surprise that they demonstrate variable susceptibility to different diseases [183, 184, 185, 186]. Two of the most common used strains, also used in this project, are BALB/c and C57BL/6 mice. BALB/c are white albino mice with a $H2^d$ MHC haplotype, developed by H.J. Bagg in 1913, while C57BL/6 are black with a $H2^b$ MHC haplotype, developed by C.C. Little in 1921.

3.6.1 Ulcerative colitis

Ulcerative colitis is a chronic relapsing and remitting autoimmune disorder where the large intestine (colon) and rectum (end of colon) are chronically inflamed and ulcers that cause crampy abdominal pain, frequent emptying of the colon and rectal bleeding may be formed [187]. In the past, ulcerative colitis was considered a disease of the Western populations, but in the last decades the incidence has dramatically increased also in other countries paralleling changes in lifestyles and behavior [188, 189]. Risk factors for Ulcerative colitis consist from a complex interaction between genetics, environment and microbiota [190]. By now there is no cure for colitis, only a number of medical treatments with a goal to induce disease remission and prevent long-term complications [191, 192, 193], therefore further studies are needed. Mouse models are indispensable for examining the mechanistic details of the disease which can lead to improvements of pre-clinical drugs and novel design of therapies [194].

3.6.2 Mouse models of ulcerative colitis

Imitating ulcerative colitis in mouse models can contribute to a better understanding of the disease and evaluate a number of potentially helpful therapies. Experimental colitis can be induced with a bacterial infection and with the administration of irritant chemicals, while some genetically manipulated mouse strains even develop colitis spontaneously [194]. The chemical used most commonly for the induction of colitis is dextran sodium sulfate (DSS) [195]. Other chemicals used to induce colitis are administrated intrarectally, such as hapten oxazolone, diluted acetic acid or 2,4,6-trinitrobenzene sulfonic acid [196, 194, 197]. The most commonly used bacterial options to trigger colitis are oral infections with gram-negative *Salmonella typhimurium* or adherent invasive *Escherichia coli* [194].

Experimental colitis can also be induced immunologically by adoptive transfer of naïve $CD4^+$ T cells ($CD4^+CD45RB^{high}$ or $CD4^+CD62L^+$ T cells) from healthy wild-type donor mice into a syngeneic immunodeficient severe combined immunodeficiency (SCID) or $Rag^{-/-}$ recipient, which results in wasting disease, diarrhea and in colonic inflammation [198, 199].

One of the earliest and by far most widely used gene-targeted models of experimental colitis was described in IL-10 deficient mice, which develop spontaneous colitis and cecal inflammation with inflammatory cell infiltration into lamina propria [200, 201]. Other commonly used gene-targeted colitis models are IL-7 transgenic mice [202], TCRα knockout mice [203], Wiskott-Aldrich syndrome protein knockout mice [204], Mdr1a knockout mice [205], IL-2 knockout mice [206] and Guanine nucleotide-binding protein G subunit α-2 knockout mice [207].

3.6.3 The DC-LMP1/CD40 mouse model

To study CD40 signaling without systemic effects of anti-CD40 antibody injection our group generated a model where only DCs receive constant CD40 signaling [208]. We used transgenic mice expressing a conditional LMP1/CD40 transgene previously developed by a group led by Ursula Zimber-Strobel [209]. This model is based on Latent membrane protein 1 (LMP1)

molecule from the Epstein-barr virus, which was published to be a viral mimic of the CD40 molecule [210, 211]. However, LMP1/CD40 mice do not have a complete sequence for viral LMP1 gene, but they express a fusion protein with transmembrane domain of LMP1 fused to intracellular signaling domain of human CD40, which was previously shown to be sufficiently conserved between two species to efficiently signal in mice [212]. This LMP1/CD40 construct has been inserted into the transcriptionally active ROSA26 locus and is protected from expression by a loxP side flanked stop cassette upstream of the LMP1/CD40 coding sequence. Therefore, by breeding this mice to a strain which expresses a Cre recombinase, the stop cassette is removed genetically and transcription of the LMP1/CD40 fusion protein can take place.

In the present study we use mice expressing Cre recombinase under control of the CD11c promotor (Itgax-cre mice[213]) leading to expression of the LMP1/CD40 fusion protein selectively in CD11c positive cells, mostly DCs and macrophages. As shown by previous publications, LMP1/CD40 fusion proteins self-aggregate in cell membrane and deliver a ligand-independent constitutive CD40 signal to cells which express the construct [214, 215]. In the context of the CD11c Cre transgene all DCs receive a constitutive CD40 signal.

CD40 signaling was previously connected with the maturation of DCs [129, 130, 131] and since mature DCs are more prone to induce immunity than tolerance [12], DC-LMP1/CD40 mice develop severe fatal colitis (Fig.3.1 A). Only recently we have published that mice with constitutive triggering of the CD40 signaling in DCs show reduced frequencies of tolerogenic $CD103^+$ DCs in lamina propria (Fig.3.1 B) and in mLNs, that results in a strong reduction of iTregs cells (Fig.3.2 A), increased frequency of intestinal Th1 and Th1/Th17 cells (Fig.3.2 B) and consequently in fatal colitis-like phenotype [208]. However, we also show that development of colitis depends on B and T cells, since $RAG^{-/-}$ DC-LMP1/CD40 mice show long-term survival (Fig.3.1 A) and that only CD40 signaling is not enough for colitis development, since DC-LMP1/CD40 animals treated with a mixture of antibiotics (ABX) also show long-term survival (Fig.3.1 A) and no colon inflammation (not shown) [208]. All these observations from

DC-LMP1/CD40 mouse model support the *in vitro* observations from other studies, which have shown that CD40 signaling alone is not sufficient for complete maturation of DCs [135] and that for this additional bacterial signals are needed [137, 138, 139].

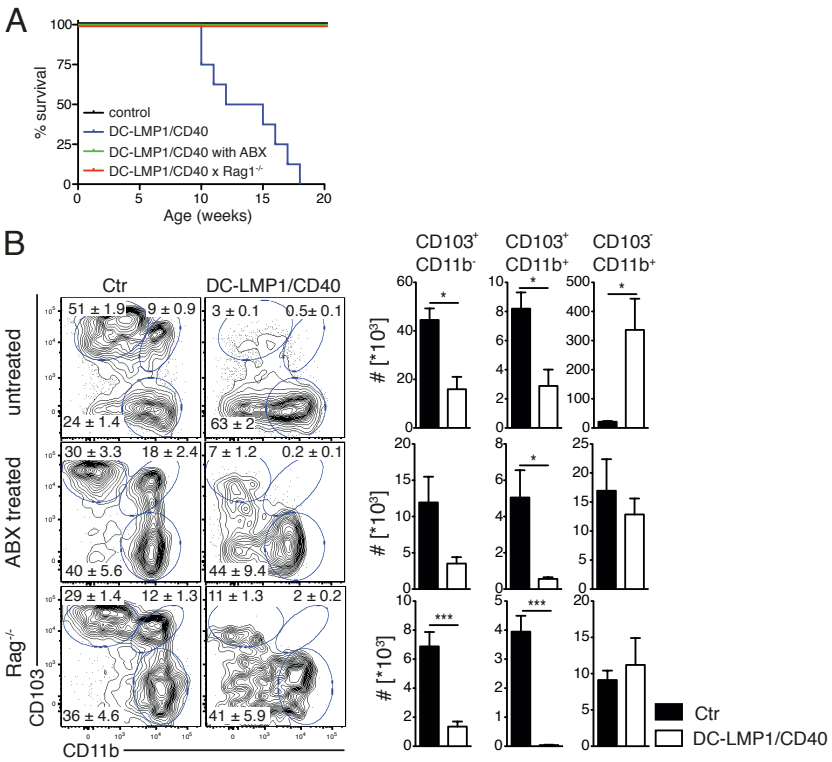

Figure 3.1: DC-LMP1/CD40 animals develop fatal T cell, B cell and bacteria dependent colitis and show reduced frequencies of CD103$^+$ DCs. A) Kaplan-Meier plot showing the survival of control (Ctr) and untreated or ABX-treated DC-LMP1/CD40 animals on C57BL/6 and RAG$^{-/-}$ background (n $\geq$ 6). Figure is adapted from Figure 2 in our recently published paper [208]. **B**) DC subsets in the LP were analyzed. Cells were gated on live, CD45$^+$CD11c$^+$MHCII$^+$CD64$^-$ cells from control, ABX treated or Rag1$^{-/-}$ mice. Representative FACS plots are shown, numbers indicate frequency of DC subsets and bar graphs show absolute numbers per colon. Shown are representative results from at least 2 independent experiments with similar outcome (n = 3). Figure is adapted from Figure 3 in our recently published paper [208].

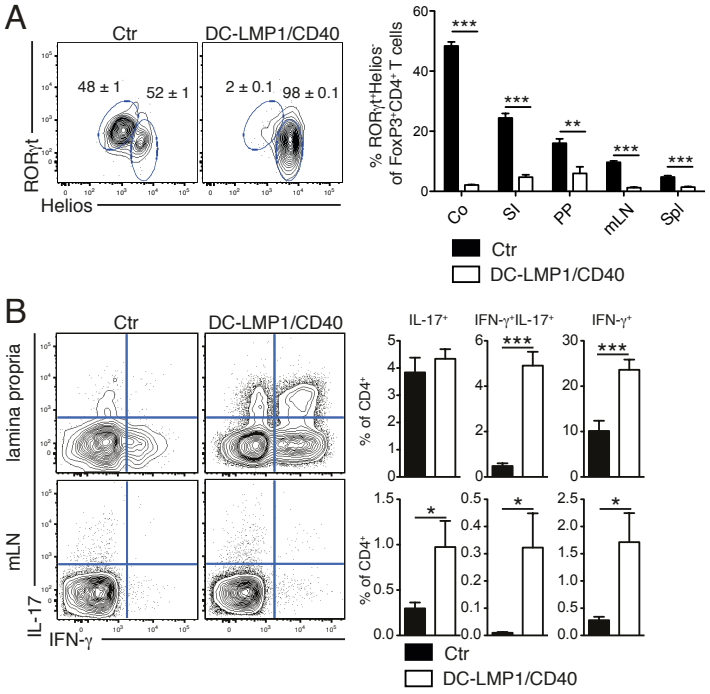

Figure 3.2: Severely impaired iTreg induction and breakdown of T cell tolerance in DC-LMP1/CD40 animals. **A**) Single cell suspension of LP was analyzed for Tregs. Shown are representative FACS-plots of FoxP3$^+$ Tregs found in the LP pre-gated on single, live, CD45$^+$CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ and in the next step subdivided into nTregs (RORγt$^-$Helios$^+$) and iTregs (RORγt$^+$Helios$^-$). Shown is one representative experiment of three with similar outcome (n = 3). Figure is adapted from Figure 4 in our recently published paper [208]. **B**) T cell functionality was analyzed by stimulating single cell suspensions with PMA/Ionomycin and subsequently staining cells intracellular for the production of IL-17 and IFN-γ. Shown are representative FACS-plots with indicated frequencies for LP (gated on single, live, CD45$^+$CD3$^+$CD4$^+$) and mLNs (gated on single, live, CD3$^+$CD4$^+$) as well as pooled statistics from more than 5 experiments (n = 14-18). Figure is adapted from Figure 5 in our recently published paper [208].

3.7 Aim of the thesis

DCs represent a crucial link between innate and adaptive immunity. Depending on maturation status and due to their high level of plasticity, they can induce regulatory or stimulatory immune responses. Our lab has recently been extensively studying the DC-LMP1/CD40 mouse model in which DCs received ligand-free constant CD40 signaling. Just recently we have published that constitutively CD40 triggered DCs lack the intestinal tolerogenic $CD103^+$ DC sub-type. As a consequence strong reduction of iTregs was observed leading to fatal colitis which dependent on the presence of commensals and was caused by increased frequencies of intestinal Th1 and Th1/Th17 cells [208].

The goal of this study was to examine how mouse strain background influences colitis susceptibility, since we observed that only DC-LMP1/CD40 mice on C57BL/6 genetic background developed fatal colitis, while mice carrying the same genetic construct, but having BALB/c or F1 (C57BL/6 x BALB/c) mixed genetic background, did not develop intestinal inflammation. To this end we analyzed the immune phenotypes of transgenic mice in the different backgrounds more thoroughly and compared the frequencies of relevant immune cells as well as their activation, maturation status and cytokine milieus. The goal was to get a more detailed picture of DCs supporting or suppressing mechanisms in the intestine.

4 Material and Methods

4.1 Materials

4.1.1 Devices

Analytic scale (Adventurer, Ohaus Corp., Pine Brooks, NJ, USA), automatic pipettors (Integra Biosciences, Baar, Switzerland), bench centrifuge (Centrifuge 5415 D, Eppendorf, Hamburg, Germany), cell counter (CASY cell counter and analyzer, OMNI life science, Bremen, Germany), centrifuge (Rotixa RP, Hettich, Tuttlingen, Germany), chemical scale (Kern, Albstadt, Germany), ELISA-reader (νmax kinetic microplate reader, Molecular Devices, Biberach, Germany), tissue homogenizer (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA), flow cytometer (FACSCalibur, FACSCantoII and FACSAria, FACSAria Fusion, BD, Heidelberg, Germany), incubator (Hera cell, Heraeus Kendro Laboratory Products, Hanau, Germany), laminar airflow cabinet (Heraeus, Hanau, Germany), magnetic stirrer (Ika Labortechnik, Staufen, Germany), PCR-machine (Biometra, Goettingen, Germany), pH-meter (Inolab, Weilheim, Germany), pipettes (Gilson, Middleton, WI, USA), power supply (Amersham Pharmacia, Piscataway, NJ, USA), Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA), real-time PCR machines (CFX96 Real Time System, BIO-RAD, Hercules, CA, USA; LightCycler 480 System, Roche Diagnostics Deutschland, Mannheim, Germany), vacuum pump (KNF Neuberger, Munzingen, Germany), vortex-Genie2 (Scientific Industries, Bohemia, NY, USA), water bath (Grant Instruments Ltd., Barrington Cambridge, UK).

4.1.2 Consumables

BD Microtainer	BD, Franklin Lakes, NJ, USA
Disposable cell strainer (100 μm nylon)	Falcon a Corning Brand, One Riverfront Plazza, Corning, USA
Disposable injection needle (26 G x 1/2")	Terumo Medical Corporation, Tokyo, Japan

Disposable syringe (1+5 ml) Reaction	Braun, Melsungen, Germany
Disposable glass pasteur pipettes (230 mm)	VWR International bvba, Leuven, Belgium
FrameStar 480/96 for Roche Lightcycler 480	4titude Ltd, Berlin, Germany
Laboratory gloves Latex Gentle Skin Grip	Meditrade GmbH, Kiefersfelden, Germany
Lysing matrix tubes (matrix A)	MP Biomedicals Germany GmbH, Eschwege, Germany
Petri dish (94 x 16 mm)	Greiner Bio-One GmbH, Frickenhausen, Germany
PCR strips tubes (0.2 mL)	VWR International, West , Belgium
Qubit assay tubes (0.5 mL)	Life technologies, Eugene, Oregon, USA
Reaction container 1.5 ml and 2 ml	Eppendorf, Hamburg, Germany
Reaction container 5 ml (FACS)	BD, Franklin Lakes, NJ, USA
Reaction container 15 ml and 50 ml	Greiner, Frickenhausen, Deutschland
Serological pipette, sterile (10 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
TipOne filter tips (10 μl, 200 μl, 1000 μl)	STARLAB GmbH, Hamburg, Germany
Tissue culture plates (96 wells-U, sterile)	VWR International bvba, Leuven, Belgium

4.1.3 Chemicals

Unless stated otherwise, chemicals were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma-Aldrich (St. Louis, MO, USA). All buffers and solutions were prepared using double distilled water.

4.1.4 Buffer and media

ACK:	8.29 g NH_4Cl

	1 g $KHCO_3$
	37.2 mg Na_2EDTA
	H_2O ad 1 L
	pH 7.4
Cresol red buffer:	250 mM KCl
	50 mM Tris-HCl (pH 8.3)
	43 % Glycerol
	2 mM Cresol red
	7.5 mM $MgCl_2$
FACS buffer:	PBS
	2 % FCS
	0.01 % NaN_3
GM-CSF medium:	IMDM
	10 % FCS
	500 mM β-mercaptoethanol
	100 U/ml Penicillin
	100 g/ml Streptomycin
	5 ml Glutamin
	10 ml GM-CSF
HBSS:	137 mM NaCl
	5.4 mM KCl
	0.25 mM Na_2HPO_4
	0.1 g glucose

	0.44 mM KH_2PO_4
	1.3 mM $CaCl_2$
	1.0 mM $MgSO_4$
	4.2 mM $NaHCO_3$
HBSS-EDTA:	HBSS
	8 % FCS
	10 mM EDTA
	10 mM HEPES
PBS:	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	pH 7.4
50x TAE buffer:	242 g Tris-HCl
	57.1 ml 100 % acetic acid (v/v)
	100 ml 0.5 M EDTA (pH 8.0)
	H20 ad 1 L
10x Gitocher:	670 mM Tris pH 8.8
	166 mM NH
	65 mM $MgCl_2$
	0.1 % gelatin
1x Gitocher buffer:	5 µl 10x Gitocher buffer
	2.5 µl 10 % Triton X-100 (v/v)

0.5µl β-mecaptoethanol

3 µl proteinase K (10 mg/ml)

39 µl H_2O

4.1.5 Antibodies

epitope	conjugate	clone	manufacturer
CD3	PE-Cy7	145-2C11	eBioscience
	PerCP	145-2C11	BD Bioscience
	FITC	145-2C11	eBioscience
	Biotin	145-2C11	BioLegend
CD4	APC-Cy7	GK1.5	BioLegend
	BV 510	GK1.5	BioLegend
CD8	BV	53-6.7	BioLegend
CD11b	APC-eFlour780	M1/70	eBioscience
CD11c	PE-Cy7	N418	BioLegend
CD16/CD32		2.4G2	BD Bioscience
CD19	Biotin	1D3	BD Pharmingen
CD25	PC61.5	PerCP-Cy5.5	eBioscience
CD40	Biotin	HM40-3	eBioscience
CD44	Pacific Blue	IM7	BioLegend
	PerCP	IM7	BD Bioscience
CD45	BV 421	30-F11	BioLegend
	PerCP	30-F11	BioLegend
	APC-eFluor780	30-F11	eBioscience
CD62L	APC	MEL-14	BD Bioscience
	PE	MEL-14	eBioscience

Continued on the next page.

epitope	**conjugate**	**clone**	**manufacturer**
CD64	APC	X54-5/7.1	BioLegend
	BV 421	X54-5/7.1	BioLegend
CD69	PE	H1.2F3	BD Bioscience
	PE-Cy7	H1.2F3	BioLegend
CD80	PE	16-10A1	BD Bioscience
CD86	PE	GL-1	eBioscience
CD103	BV 421	M290	BD Horizon
	PE	M290	BD Bioscience
CD154 (CD40L)	PE	MR1	BioLegend
CD152 (CTLA-4)	PE	UC10-4F10-11	BD Pharmingen
CD197(CCR-7)	PE	4B12	eBioscience
F4/80	PE-Cy7	BM8	eBioscience
FoxP3	eFlour660	FJK-16s	eBioscience
	Biotin	FJK-16s	eBioscience
Gata-3	eFlour660	TWAJ	eBioscience
goat a-rabbit	PE		Life technologies
Helios	FITC	22F6	eBioscience
IL-17A	PE	TC11-18H10.1	BD Bioscience
	PE-Cy7	TC11-18H10.1	BioLegend
IFN-g	APC	XMG1.2	eBioscience
	FITC	XMG1.2	eBioscience
KI67	PE	B56	BD Pharmingen
Ly6C	FITC	AL-21	BD Bioscience
	PE-Cy7	AL-21	BD Bioscience
MHCII	FITC	M5/114.15.2	BioLegend
	PerCP	M5/114.15.2	BioLegend

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epitope	conjugate	clone	manufacturer
Rat IgG2a kappa	PE	RTK2758	BioLegend
Rat IgG2b kappa	eFlour660	eB149/10H5	eBioscience
RORgt	PE	AFKJS-9	eBioscience
	PerCP	B2D	eBioscience
Streptavidin	BV 421		BioLegend
	PerCP		BD Bioscience
Tbet	PE-Cy7	eBio4B10	eBioscience
TCR-b	FITC	H57-597	eBioscience
	PerCP	M5/114.15.2	BioLegend

The agonistic anti-CD40 antibody (clone FGK4.5/FGK45, isotype rat IgG2a) for immunizations was purchased from Bio X Cell (West Lebanon, USA) where we also purchased the anti mouse IL-1 beta (clone B122, isotype Armenian hamster IgG).

4.1.6 Oligonucleotides

All oligonucleotides were purchased from MWG-Biotech AG (Ebersbach, Germany).

target	primer name	sequence	application	probe
IL1a	IL1a qPCR_f	TTGGTTAAATGAC-CTGCAACA	qPCR	52
	IL1a qPCR_r	GAGCGCTCACGA-ACAGTTG		
IL1b	IL1b qPCR_f	TGTAATGAAAGAC-GGCACACC	qPCR	78

Continued on the next page.

target	**primer name**	**sequence**	**application**	**probe**
	IL1b qPCR_r	TCTTCTTTGGGTA-TTGCTTGG		
IL-6	IL-6_f	GAAGGGCACTGCA-GGATAGA	qPCR	12
	IL-6_r	TCCCCAGAGTGTG-GCAGT		
IL-12p35	IL-12 p35_f	CCAGGTGTCTTAG-CCAGTCC	qPCR	62
	IL-12 p35_r	GCAGTGCAGGAAT-AATGTTTCA		
IL-23p19	IL-23_f2	ATAGCCCCATGGA-GCAACTT	qPCR	25
	IL-23_r	GCTGCCACTGCTG-ACTAGAA		
LMP1-CD40	LMP qPCR_f	GGATGTATTACCA-TGGACAACG	qPCR	139
	LMP qPCR_r	TTGATCTCCTGGG-GTTCCT		
HPRT	HPRT1_f	TCCTCCTCAGACC-GCTTTT	qPCR	95

Continued on the next page.

target	primer name	sequence	application	probe
	HPRT1_r	CCTGGTTCATCAT-CGCTAATC		
Cre	RO334	GGACATGTTCAGG-GATCGCCAGGCG	genotyping	
	RO335	GCATAACCAGTGA-AACAGCATTGCTG		
LMP1-CD40	HL15	AAGACCGCGAAGA-GTTTGTCC	genotyping	
	HL54	TAAGCCTGCCCAG-AAGACTCC		
	HL152	AAGGGAGCTGCA-GTGGAGTA		
IL1b	IL1beta2_f	TTTCCTCCTTGCC-TCTGATG	sequencing	
	IL1beta2_r	ATGTGCTGGTGCT-TCATTCA		

4.1.7 Mouse strains

All mouse strains were breed and kept in the Institute for Immunology at the LMU Munich. The following mouse strains have been used in this work.

BALB/c

BALB/c is an inbred mouse strain developed by H.J. Bagg in 1913. Their coat is white (albino) and they have the $H2^d$ MHC haplotype.

C57BL/6

C57BL/6 is an inbred mouse strain developed by C.C. Little in 1921. Their coat is black and they have the $H2^b$ MHC haplotype.

CD11c-Cre

The CD11c-Cre mice strain was produced in the Lab of Boris Reizis. They expresses the Cre recombinase under control of the CD11c promotor [213]. This mouse allows the deletion of floxed allels in DCs and other CD11c-expressing cells. Mice were kept on the C57BL/6 and BALB/c genetic background.

DC-LMP1/CD40

To obtain DC-LMP1/CD40 animals, CD11c-Cre mice were crossed with LMP1/CD40 mice [209]. The latter mouse strain carries the knock-in of the LMP1/CD40 gene which is preceded by a floxed stop-codon into the ROSA26 locus. The cre-mediated excision of the stop codon then leads to the constitutive expression of the fusion-protein between LMP1, derived from the Epstein-Barr-Virus (EBV), and the intracellular signaling domain of human CD40. The LMP1 domain anchors the protein in the plasma membrane and at the same time leads to a multimerization, which in turn leads to signaling by the CD40 molecule.

DC-LMP1/CD40 mice have been breed on different genetic backgrounds. On pure C57BL/6 genetic background, on pure BALB/c genetic background and on mixed genetic background between C57BL/6 and BALB/c mouse strain (named F1 DC-LMP1/CD40). For the purpose of single nucleotide polymorphism (SNP) study F1 DC-LMP1/CD40 mice were intercrossed to obtain a genetically heterogeneous generation named F2 DC-LMP1/CD40.

$Rag1^{-/-}$

$Rag1^{-/-}$ mice are unable to form B and T cells due to a defect in the V(D)J-recombination machinery [216].

4.2 Methods

4.2.1 Immunological and cell biology methods

4.2.1.1 Harvesting of organs and single cell preparation

Animals were euthanized in a CO_2 chamber. Mice were fixed with needles on a styrofoam pad, disinfected with 70 % ethanol and cut open. Using scissors and fine tweezers organs were removed and placed into phosphate buffered saline (PBS). Spleen and lymph nodes were smashed through a 100 μm cell strainer and washed with ice cold PBS. Red blood cells were lyzed using 1 ml of ACK buffer for 5 min at room temperature. Samples were washed once again, counted with the CASY-counter (OMNI life science, Bremen, Germany) and stored on ice for further analysis.

To analyze cells from the lamina propria, the colon was removed, the fecal matter cleaned away, and then the colon was opened longitudinally and cut into ca. 5 mm long pieces. The pieces were then incubated with HBSS-EDTA for 10 min on a shaker at 37°C in order to remove epithelial cells. In the next step, the pieces were digested once for 30 min and then again twice for 20 min with a mixture of Collagenase IV (157 Wuensch units/ml, Worthington), DNAse I (0.2 mg/ml) and Liberase (0.65 Wuensch units/ml, both from Roche). The supernatant was collected after each step of digestion and cells were always washed with PBS. Collected cells were enriched for immune cells with Percoll gradient centrifugation. For this, cells were resuspended in 40 % Percoll and this solution was underlayed with a 80 % Percoll solution using a glass pasteur pipete. This mixture was than centrifuged for 20 min at 1800 rpm at 4°C without break. Cells at the interphase were collected, washed once, counted using the CASY-counter (OMNI life science, OMNI life science, Bremen, Germany) and stored on ice for further analysis.

4.2.1.2 Flow Cytometry staining

For flow cytometric analysis, $2{\cdot}10^6$ cells per staining were plated into a 96 well plate. Cells were stained for 20 min at 4°C in the dark in 50 μl of antibody mix made with a fluorescence-activated cell sorting (FACS) buffer. With titration, optimal dilution for each antibody has been tested. After the incubation, the cells were washed once with 150 μl FACS buffer and than directly acquired at the FACS.

For CCR7, extracellular antibody staining cells were stained for 15 min at 37°C in the dark in 50 μl of antibody mix made with a FACS buffer.

Samples stained with a biotinylated antibody were then stained with fluorescent labeled streptavidin, also in a volume of 50 μl, at 4°C in dark for 20 min.

For intracellular stainings, cells were fixed and permeabilized after they were stained for all extracellular markers. Fixation was 30 min long and performed with a Cytofix/Cytoperm kit (BD, Heidelberg, Germany) according to the protocol provided by manufacturer. After washing, cells were stained for intracellular markers for additional 30 min.

For the intranuclear staining of FoxP3, T-bet and Helios, cells were washed once and then resuspended in 200 μl 1x Fixation/Permeabilization solution (eBioscience, San Diego, CA, USA) for 20 min at 4°C in the dark. Cells were spun down, the supernatant was removed and the cells were washed twice with 1x Permeabilization Buffer (eBioscience, San Diego, CA, USA). Cells were then stained with a specific antibodies in 50 μl Permeabilization Buffer for 30 min at 4°C in the dark. Afterwards, cells were washed once and acquired at the FACS.

Acquisition was performed using a FACSCalibur or FACSCantoII. Data analysis was performed using FlowJo version 8 and 10 (TreeStar, Ashland, OR, USA).

An advanced version of classical flow cytometry is cell sorting, a technique where a user-defined individual cell population can be diverted from a fluidic stream and collected for further analysis. Cell sorts were performed on a FACSAriaIII and a FACSAria Fusion (BD, Heidelberg, Germany).

4.2.1.3 *In vitro* T cell restimulation

To asses the cytokine secretion potential of a polyclonal T cell population, animals were sacrificed and single cell suspensions prepared as described in section 4.2.1.1. $2{\cdot}10^6$ cells were then plated into 96 well plates and stimulated for four hours at 23°C with phorbol-12-myristat-13-acetat (PMA) and ionomycin in a T cell-medium at a final concentration of 40 ng/ml and 1 μg/ml respectively in the presence of 2 μM Glogi-Stop (BD, Heidelberg, Germany), which blocks protein secretion.

Afterwards, cells were washed twice with a FACS-buffer and stained for extracellular markers as described in section 4.2.1.2. To permeabilize cells, they were then resuspended in 150 μl of BD Cytofix/Cytoperm (BD Bioscience) and incubated for 30 min at 4°C in the dark, washed once with Perm/Wash buffer (BD, Heidelberg, Germany) and stained for the cytokines of interest for 30 min at 4°C in the dark. Afterwards, cells were washed once again and resuspended in a FACS buffer in which they were stored, at 4°C, until acquisition.

4.2.1.4 Depletion of commensal microflora

To deplete as many commensal bacteria as possible, animals were provided a mixture of ampicilin sodium salt (1 g/l), vancomycin hydrochloride (500 mg/l), neomycin sulfate (1 g/l) and metronidazole (1 g/l) in the drinking water for 4 to 5 weeks [217].

4.2.1.5 Anti-IL-1β treatment

Five weeks old C57BL/6 DC-LMP1/CD40 animals, which have been treated with ABX to prevent onset of colitis, were moved to cages containing feces from the rest of the mouse facility for commensal bacteria repopulation and ABX treatment was stopped. At the same time treatment of experimental mice with an anti-IL-1β antibody was started. 0.25 mg of anti-IL-1β antibody (clone B122, isotype Armenian hamster IgG, Bio X Cell, West Lebanon, USA) was injected twice per week intraperitoneally. The control mice were treated with an isotype control antibody. Treatment lasted for 7 weeks. Afterwards animals were sacrificed

and analyzed.

4.2.1.6 Magnetic cell sorting

To purify cell populations based on surface marker expression, magnetic cell sorting (MACS, Miltenyi Biotec) was employed. There are two possible methods for magnetic-activated cell sorting (MACS): labeling the population of interest (positive selection) or labeling of all other cells (negative selection). This method is based on the use of monoclonal antibodies that are conjugated to superparamagnetic microbeads. After labeling, cells were applied to a column that has been placed in a paramagnetic field of a MACS separator. There are different columns for different purposes and for different numbers of cells. Labeled cells (the positive fraction) are retained inside the column due to the magnetic field, while the unlabeled ones (the negative fraction) pass through the column. After loading, the sample column was rinsed three times with a MACS buffer and the eluted fraction was collected. After removal of the column from the magnetic field, the cells retained in the column can be rinsed with pressing MACS buffer trough the column. This method enable us purification of cells by depleting all the others cell types or by depleting a certain cell population.

MACS separation was used in order to purify dendritic cells (CD11c microbeads), to deplete B and T cells (anti biotin microbeads after staining the samples with biotin labeled CD3 and CD19 antibody) and for purification of $CD4^+CD62L^+$ T cells for the purpose of adoptive T cell transfer described in section 4.2.1.7. Cells were isolated from either spleen or lymph nodes. All procedures were performed according to the manufacturers instructions.

4.2.1.7 Adoptive T cell transfer for induction of chronic colitis

Chronic colitis was induced by transferring $CD4^+CD62L^+$ T cells from donor wild type C57BL/6 mice to $Rag1^{-/-}$ recipient. The $CD4^+CD62L^+$ transfer model of chronic colitis published by J. Munder in 2003 [198] resembles $CD45RB^{hi}$ model of colitis [199] with the advantage that here is no need for a FACS sorter, but the cells can be prepared with a MACS purification. $CD4^+CD62L^+$ double positive cells have been isolated from the spleen of wild

type C57BL/6 animals. Single cell suspension from the spleen was performed as described in section 4.2.1.1, only red blood cells were not lyzed. Than, the $CD4^+CD62L^+$ T cell isolation kitII (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for MACS purification. In the first step of MACS purification, all non $CD4^+$ T cells were depleted and in the second step, $CD4^+$ T cells were enriched for cells expressing CD62L. The purity was examined by a FACS analysis and it ranged between 81 and 95 %. $3{\cdot}10^5$ isolated $CD4^+CD62L^+$ T cells were intraperitoneally injected into a $Rag1^{-/-}$ recipient. The weight of injected mice was measured regularly and the mice were opened and analysed on day 15 after T cell transfer.

4.2.1.8 Tissue homogenization for protein isolation

FastPrep System from MP Biomedicals (Solon-Ohio, Burlingame-California, USA) consist of the whole repertoire of different Lysing matrixes and can be used to lyse, grind or homogenize tough and resistant samples.

Before homogenization, tissue samples were snap-frozen in liquid nitrogen. For preparation of whole gut protein homogenate samples, snap-frozen intestinal tissue samples, approximately 50 mg, were homogenized in 500 μl PBS containing a cocktail of protease inhibitors (cOmplete ULTRA Tablets, Roche, Sigma-Aldrich, Taufkirchen, Germany). For homogenization, a tissue homogenizer (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA) was used (FastPrep speed: 6.0, FastPrep time: 40 sec). Homogenized samples were centrifugated at 9000 rpm for 5 min in order to pellet debris, supernatant was collected and spun again at maximum speed. Remaining supernatant was stored at -20°C. So prepared samples have been used for measurements with Cytokine bead array (BD, Franklin Lakes, NJ, USA) and for an IL-1 alpha enzyme linked immunosorbent assay (ELISA) Ready-SET-Go! kit or for an IL-1 beta Pro-form ELISA Ready-SET-Go! kit (both purchased from Affymetrix eBioscience, Santa Clara, CA, USA).

4.2.1.9 Measuring of protein concentration

Whole gut protein homogenate samples were prepared as described in section 4.2.1.8. Protein concentration measurement was performed according to the manufacturers instructions (Qubid Protein Assay Kit, Invitrogen, Eugene, Oregon, USA).

4.2.1.10 Cytometric Bead Array

Whole gut protein homogenate samples were prepared as described in section 4.2.1.8 and protein concentration was measured as described in section 4.2.1.9.

BD Cytokine Bead Array mouse inflammation kit was used according to instructions provided by manufacturer. With this method we can measure up to six different cytokines in each sample (IL-6, IL-10, MCP-1, IFN-g, TNF-a, and IL-12p70). Samples were diluted 1:1 with an Assay diluent and later acquired on a FACSCantoII. Results were analyzed using a FCAP Array Software (Soft Flow Inc.). Cytokine levels were normalized on the total protein level present in each sample, which were measured by a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

4.2.1.11 Murine GM-CSF bone marrow culture

Animals were euthanized as described in section 4.2.1.1 disinfected with 70 % ethanol and cut open. GM-CSF bone marrow dendritic cell culture was established in sterile conditions under a laminar airflow cabinet (Heraeus, Hanau, Germany), like it was published by K. Inaba in 1992 [218]. Cells were plated in 100 mm petridishes in GM-CSF medium at a concentration of $1{\cdot}10^6$ cells/ml. Cells were split on concentration $5{\cdot}10^6$ cells per plate on day 3 and 7 and harvested on day 9 for the purpose of an IL-1 alpha and an IL-1 beta Pro-form ELISA.

4.2.1.12 ELISA for IL-1 alpha and IL-1 beta Pro-form

Whole gut protein homogenate samples were prepared as described in section 4.2.1.8 and protein concentration was measured as described in section 4.2.1.9. Samples were diluted

1:10 with an ELISA ELISPOT diluent and measured with an IL-1 alpha ELISA Ready-SET-Go! kit or with an IL-1 beta Pro-form ELISA Ready-SET-Go! kit (both purchased from Affymetrix eBioscience, Santa Clara, CA, USA) according to instructions provided by manufacturer.

An IL-1 alpha ELISA Ready-SET-Go! kit and an IL-1 beta Pro-form ELISA Ready-SET-Go! kit have also been used for measuring the interleukin level in lipopolysaccharide (LPS) stimulated bone marrow derived dendritic cells (BMDCs) prepared as described in section 4.2.1.11. The BMDCs cells were harvested on day 9 and plated into a 96 well plate in concentration $1{\cdot}10^5$ cells per well. Cells were stimulated for three hours at 37°C with a 50 ng/ml LPS or they were left unstimulated. After incubation cells were centrifuged at 1500 rpm for 5 min and supernatant has been discharged. The 96 well plate was cooled to -80°C and thawed three times. Than centrifuged once more at 1500 rpm for 5 min and the supernatant was transferred to a new 96 well plate and stored at -20°C till further usage. This samples have been diluted 1:3 with an ELISA ELISPOT diluent and have been used for measuring of an IL-1 alpha and an IL1 beta Pro-form as described in the text above to obtain whole gut protein homogenate samples.

4.2.1.13 Fecal protein extraction

Fecal samples were prepared according to protocol for fecal immunoglobulin extraction as published previously [219] and adjusted for smaller amount of starting material.

4.2.1.14 Lipocalin-2 ELISA

Fecal samples were prepared as described in section 4.2.1.13 and diluted 1:600 with the Calibrator Diluent RD5-24 (1X). Lipocalin-2 was measured according to manufacture protocol using a Quantikine ELISA kit for mouse Lipocalin-2/NGAL (R&D Systems Europe, Abingdon, UK).

4.2.1.15 CD40 injection

To evaluate the influence of CD40 signal on DCs animals were intraperitoneal (IP) injected with 200 μg of anti-CD40 antibody clone FGK4.5/FGK45 as described previously [142].

4.2.2 Molecular biology

4.2.2.1 Agarose gel electrophoresis

To visualize and separate deoxyribonucleic acid (DNA) fragments according to size, samples were applied to an agarose gel. The gel consisted of 1 - 2 % agarose disolved in a TAE buffer with addition of an ethidium bromide (0.5 μg/mL) for later DNA visualization under ultraviolet (UV) light. Separation of fragments on the gel was achieved with a constant voltage (80 V) applied to an ectrophoresis chamber containing a conductive buffer (TAE). To estimate the size of the fragments, either a 100 bp or a 1 kb ladder was used (New England Biolabs, Ipswich, MA, USA). The polymerase chain reaction (PCR) fragments either already contained the loading buffer or this buffer (10 % glycerol, xylene cyanol FF) was added before applying the sample to the gel. The DNA samples were visualized by examination under UV light (312 nm, Intas, Goettingen, Germany).

4.2.2.2 Isolation of genomic DNA and RNA

In order to isolate genomic DNA for genotyping, 2 - 5 mm of mouse tail tip was cut and put into a 50 μl digestiom mix consisting of Gitocher buffer, Triton, 2-mercaptoethanol and Proteinase K. This mixture was incubated at 55°C for 6 h, followed by a 5 min incubation at 95°C for Proteinase K inactivation.

The isolation of nucleic acids for other purposes, where better DNA quality is needed (SNP genotyping), was performed with a DNeasy Blood & Tissue Kit (Quiagen, Venlo, Netherlands) according to instructions provided by manufacturer.

For ribonucleic acid (RNA) isolation, trizol RNA isolation protocol was used. Samples were resuspended in 1 ml of trizol and snap-freezed with liquid nitrogen. For RNA preparation

samples were melted and mixed with 300 µl of chloroform, centrifuged at full speed, the aqueous phase was then again mixed with two volumes of chlorophorm and separated from the non-aqueous phase after step of centrifugation. The aqueous phase was then transferred to a fresh tube, mixed with one volume of 2-propanol and 1 µl of GlycoBlue (Ambion, Austin, TX, USA) and incubated for 2 h, or overnight, at -20°C. Precipitated RNA was spun down, the pellet was washed with 70 % ethanol and resuspended in H_2O. So prepared RNA have been used for complementary DNA (cDNA) preparation with a QuantiTec Reverse Transcription Kit (Qiagen, Venlo, Netherlands) according to instructions provided by manufacturer.

4.2.2.3 Polymerase chain reaction (PCR)

PCR is technique which enables amplification of a single, or multiple copies of DNA, in a process where sequence specific primers, which flank the region of interest, amplify a focused segment of DNA trough a thermal cycling in the presence of an enzyme DNA polymerase.

4.2.2.4 Quantitative PCR

Real time quantitative PCR (qPCR), is a technique based on traditional PCR reaction with a few advantages: it combines nucleic acid amplification and detection in one step, smaller amounts of starting material can be used and the product can be quantified based on fluorescent detection. Amplified DNA is fluorescently labelled and the amount of the fluorescence released during DNA amplification is directly proportional to the amount of amplified DNA. This enables measuring of fluorescence during the 30 to 45 cycles of a PCR process. The cycle in which fluorescence can be detected is termed the quantitation cycle (Cq) and is the basic result of the qPCR: lower Cq values mean higher initial copy numbers of the target. There are two different methods to detect the amount of PCR product: SYBR green is a fluorescent dye that interpolates with any double stranded DNA, whereas TaqMan probes bind to a specific sequences.

In this thesis qPCR results were obtained with the TaqMan system and performed with the LightCycler TaqMan Master Kit and the Universal ProbeLibrary Set mouse (both purchased

from Roche Diagnostics Deutschland, Mannheim, Germany) according to the manufacturer instructions on a LightCycler 480 System (Roche Diagnostics Deutschland, Mannheim, Germany). Primers and probes used in this thesis are listed in the table of materials in section 4.1.6. Expression levels were normalized to the HPRT house-keeping gene and relative quantification were calculated using the Delta-Delta Ct method [220].

4.2.2.5 SNP genotyping

High-quality SNP genotyping for the F2 DC-LMP1/CD40 genetically heterogenous mouse strain was performed. DNA was prepared according to instructions provided by the manufacturer using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands). Samples were sent to Illumina (San Diego, CA, USA) for genotyping service using the Mouse Linkage LD chip.

Ilumina Mouse LD Linkage Panel has 377 SNP loci chosen to maximize genetic information across the top ten inbred strains routinely used for mouse crosses. This panel was designed to include approximately four SNPs per each 27 Mb interval across the entire mouse genome. It provides approximately 175 - 200 informative markers per cross and covers the entire mouse genome.

Resulting SNPs in genes were identified with the help of the Mouse Linkage LD chip and have been validated with the multiplication of the SNP region with locus specific primers. Amplified genomic sequences were sequenced by MWG-Biotech AG (Ebersbach, Germany).

4.2.2.6 Statistics

For statistical analysis, PRISM software (GraphPad software, La Jolla, CA, USA) was used. Bar graphs represent mean ± standard error of mean (SEM) and P-values were calculated with a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5 Results

5.1 Phenotypic analysis of DC-LMP1/CD40 animals on different genetic backgrounds

In order to study CD40 signaling without systemic effect of anti-CD40 antibody injection, our group generated a mouse model where only DCs receive constant CD40 signaling [208]. As described in section 3.6.3, transgenic mice with constant CD40 signaling, here called DC-LMP1/CD40 mice, on C57BL/6 genetic background develop B and T cell dependent fatal colitis.

5.1.1 Exclusively C57BL/6 DC-LMP1/CD40 mice developed spontaneous colitis

We observed that colitis development in DC-LMP1/CD40 mouse model depended on the background of the animal. Only DC-LMP1/CD40 animals on C57BL/6 genetic background developed colitis, while mice on BALB/c or F1 (mixed between C57BL/6 and BALB/c) genetic backgrounds did not show any visible signs of disease. Histopathologic examination showed a colitis-like phenotype in the lamina propria of C57BL/6 DC-LMP1/CD40 animals, while no noticeable pathological changes were found in the spleen or any other organs (data not shown). Histopathological analysis for C57BL/6 DC-LMP1/CD40 mice showed a thickening of colon mucosa, extensive lamina propria infiltration of mixed inflammatory mononuclear cells (macrophages, lymphocytes and plasma cells), as well as a loss of crypts, focal cryptitis, ulceration and reduction of goblet cells (Fig.5.1 A). In contrast, F1 DC-LMP1/CD40 animals showed only slight histopathologic changes compared to their background control littermates, while BALB/c transgenic animals showed no changes compared to BALB/c wild type (wt) animals (Fig.5.1 A).

Colitis was in C57BL/6 DC-LMP1/CD40 animals also visible on a macroscopic level by

colon shortening and thickening (Fig.5.1 C). In contrast, the colons of F1 DC-LMP1/CD40 and BALB/c DC-LMP1/CD40 did not show any visible inflammation or shortening (Fig.5.1 B).

Fecal lipocalin-2 was published to be a good non-invasive inflammatory marker [221]. Measurement of lipocalin-2 in DC-LMP1/CD40 animals showed high levels only in C57BL/6 DC-LMP1/CD40 animals, while in their control littermates and in transgenic animals on BALB/c genetic backgrounds, no increase in fecal lipocalin-2 could be observed (Fig.5.1 C). F1 DC-LMP1/CD40 animals had slightly increased levels of fecal lipocalin-2, when compared to their control littermates. However, levels of fecal lipocalin-2 measured in F1 transgenic animals were way below the levels which could be measured in C57BL/6 transgenic animals (Fig.5.1 C).

How constant CD40 triggering in DCs affected the levels of pro-inflammatory cytokines was further analyzed with Cytometric Bead Array (CBA). Cytokines were measured in whole gut homogenate of C57BL/6 DC-LMP1/CD40 and F1 DC-LMP1/CD40 animals with suitable controls. A significant increase of pro-inflammatory cytokines monocyte chemoattractant protein-1 (MCP1), TNF-α, IFN-γ and IL-6 was found, while anti-inflammatory IL-10 was significantly reduced in C57BL/6 DC-LMP1/CD40 animals compared to their littermate controls (Fig.5.2 B, upper panel). However, no change in any of the six analyzed cytokines could be observed for F1 DC-LMP1/CD40 animals (Fig.5.2).

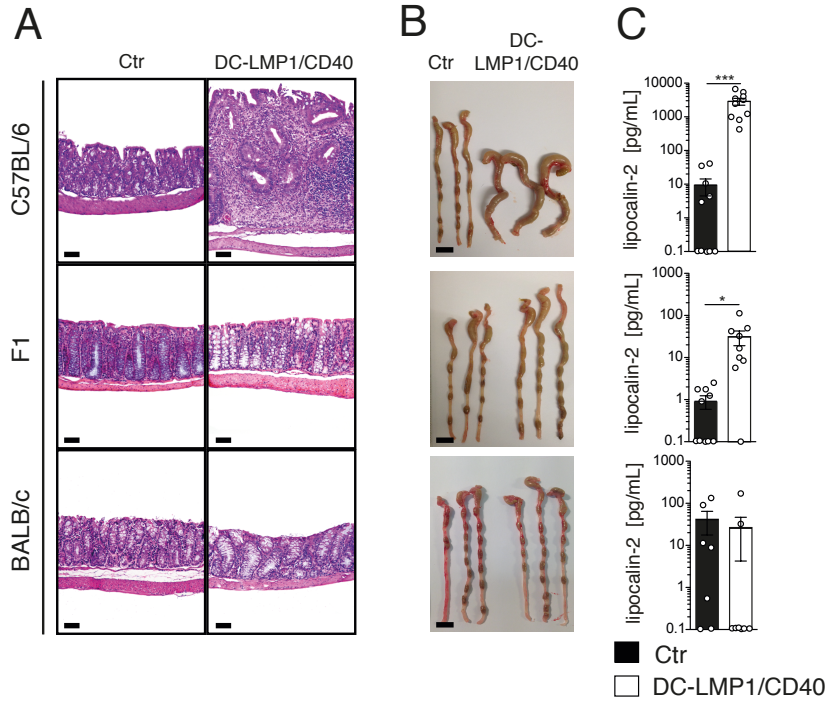


Figure 5.1: DC-LMP1/CD40 animals on C57BL/6 genetic background developed strong intestinal inflammation with colitis. A) DC-LMP1/CD40 mice on C57BL/6 background displayed severe colitis with a thickening of the colon mucosa, extensive proprial infiltration of mixed inflammatory mononuclear cells, loss of crypts and reduction of goblet cells. DC-LMP1/CD40 mice on F1 background showed only slight changes in comparison to controls (Ctr), while transgenic mice on BALB/c background showed no changes in comparison to BALB/c wt mice. Paraffin sections, HE-staining. Bars = 100 μm. **B)** Macroscopic pictures of colons from control and DC-LMP1/CD40 animals on different genetic backgrounds. Bars = 1 cm. **C)** Levels of fecal lipocalin-2 as measured by ELISA in 8-10 week old DC-LMP1/CD40 animals on different genetic backgrounds. Shown are pooled statistics from 3 experiments (n = 6-10). All bar graphs represent mean ± standard error of mean (SEM) where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

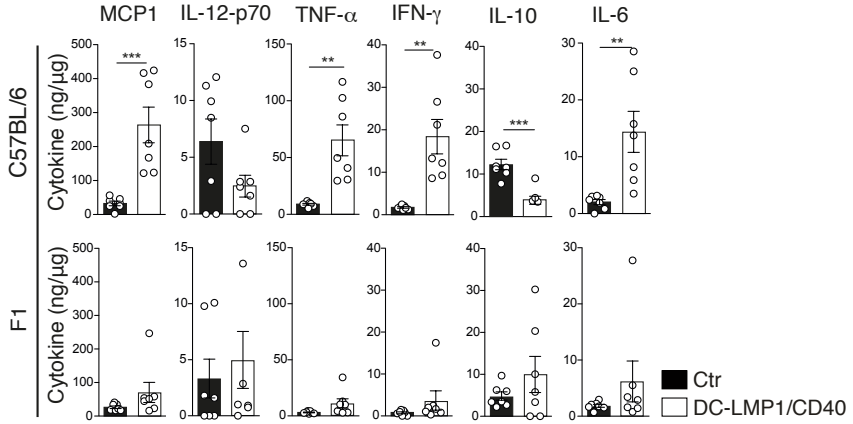


Figure 5.2: Increased proinflammatory cytokines in the intestine of C57BL/6 DC-LMP1/CD40 animals. Levels of pro-inflammatory cytokines were measured in colon homogenates from 8-12 week old DC-LMP1/CD40 mice on C57BL/6 and on a mixed F1 genetic background. Cytokine concentrations were measured with a Cytometric Bead Array (CBA) kit and normalized to the total protein content for each sample. Shown are pooled statistics from 2 experiments (n = 7). All bar graphs represent mean ± standard error of mean (SEM) where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5.1.2 Reduction of tolerogenic CD103$^+$ DCs was independent of the genetic background

From the previous publication it was known that CD103$^+$ DC subsets have been strongly reduced in DC-LMP1/CD40 animals on C57BL/6 genetic background [208]. Therefore, we next analyzed the numbers and frequencies of these DCs also in the other backgrounds.

Analysis of colonic lamina propria (LP) showed strong reduction in the frequencies of CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ tolerogenic DCs in all three transgenic mouse strains (Fig.5.3 A). On the other hand a higher frequencies of CD103$^-$CD11b$^+$ DCs were found in transgenic animals compared to their control littermates. Analysis of DC subsets in mLNs showed similar effect of LMP1/CD40 on CD103$^+$ DCs, which were also strongly reduced in all transgenic animals (Fig.5.3 B).

To find out if the differential effect of the CD40-signaling on CD103$^+$ and CD103$^-$ DCs was due to differential transgene expression, CD103$^+$ and CD103$^-$ DCs were sorted from LP of

C57BL/6 DC-LMP1/CD40 animals and qPCR with primers specific for the LMP1/CD40 gene construct was performed. Surprisingly, we found that the expression of LMP1/CD40 gene was significantly higher in $CD103^-$ DCs when compared to $CD103^+$ counterparts (Fig.5.4). Despite the fact that $CD103^-$ DCs expressed relatively higher levels of the LMP1/CD40 transgene as compared to $CD103^+$ DCs they were not affected by CD40 signaling in the same way like the others DC subsets.

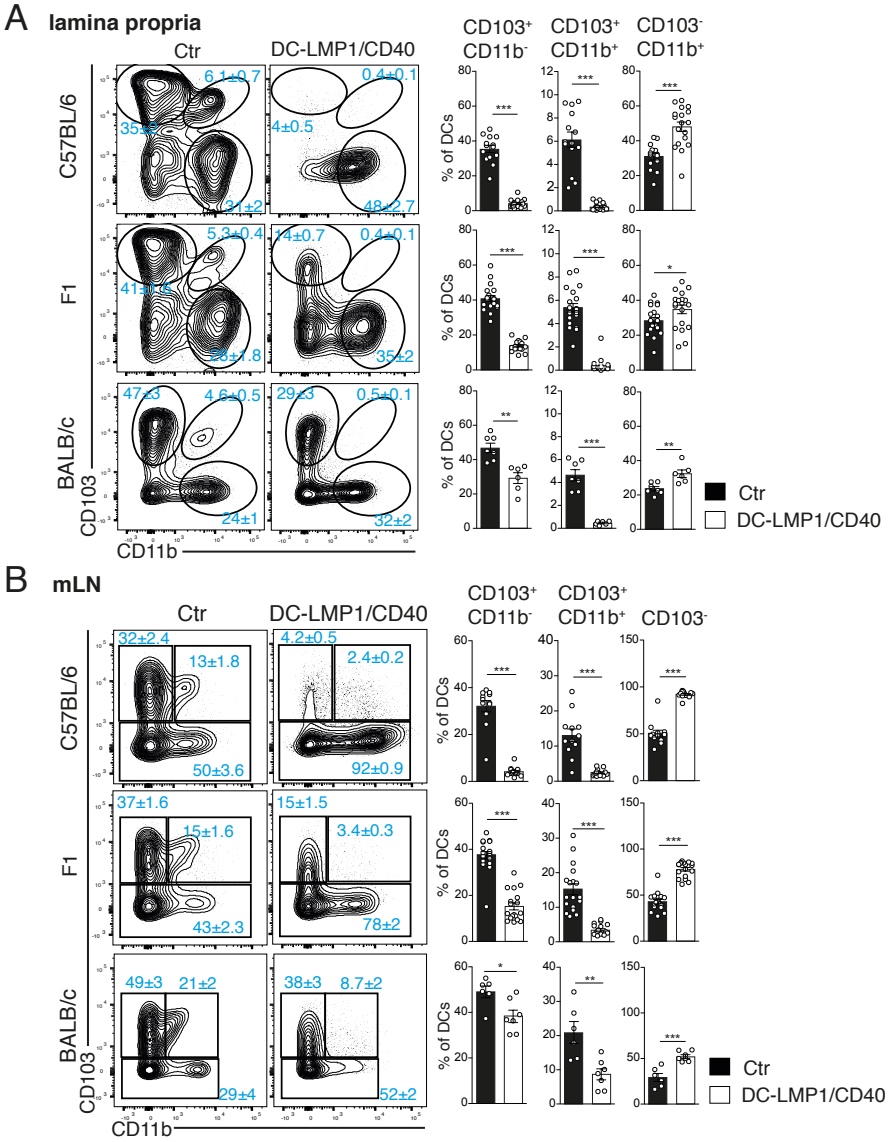


Figure 5.3: CD103$^+$ DCs were strongly reduced in LP and mLN of DC-LMP1/CD40 animals. DC subsets in the LP **(A)** or mLN **(B)** were analyzed in DC-LMP1/CD40 animals on different genetic backgrounds. **A)** LP cells were gated on single cells, live, CD45$^+$MHCII$^+$CD11c$^+$CD64$^-$ cells from control (Ctr) or DC-LMP1/CD40 mice on different genetic backgrounds. Representative FACS-plots are shown, numbers and bar graphs indicate the frequency of DC subsets. Shown are pooled statistics from 5 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 14-18), from 6 experiments for F1 DC-LMP1/CD40 animals (n = 19-20) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 6-7). **B)** mLNs cells were gated on single cells, live, MHCII$^+$CD11c$^+$ cells from control or DC-LMP1/CD40 mice on different genetic backgrounds. Representative FACS-plots are shown, numbers and bar graphs indicate frequency of DC subsets. Shown are pooled statistics from 4 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 12-14), from 5 experiments for F1 DC-LMP1/CD40 animals (n = 18) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 6-7). All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

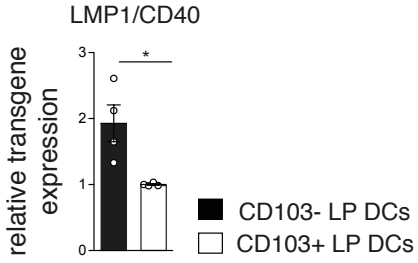


Figure 5.4: CD103$^+$ and CD103$^-$ DCs expressed LMP1/CD40 transgene. CD103$^+$ and CD103$^-$ DCs were sorted from LP (single, live, CD45$^+$MHCII$^+$CD11c$^+$CD64$^-$) of C57BL/6 control and C57BL/6 DC-LMP1/CD40 animals. Trizol RNA isolation protocol was used for RNA isolation, from which cDNA was prepared. Samples were analyzed by qPCR with primers specific for LMP1/CD40 construct. Data was normalized on HPRT expression. Shown is pooled data from 2 experiments (n = 4). All bar graphs represent mean ± SEM where significance was analyzed using a students *t*-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5.1.2.1 CD103$^+$ DCs were stronger reduced in DC-LMP1/CD40 animals on C57BL/6 than on other backgrounds

As shown in Fig.5.3 A and B, CD103$^+$ DCs were strongly reduced in LP and mLNs of DC-LMP1/CD40 transgenic animals of all genetic backgrounds. However, they were more reduced in C57BL/6 DC-LMP1/CD40 animals as compared to BALB/c and F1 DC-LMP1/CD40 animals. In order to compare DC subpopulations in DC-LMP1/CD40 animals on different genetic backgrounds, we calculated the percentage of each DC subset in transgenic mice, where corresponding background wt controls represent 100 % for each respective analyzed DC subset. When we analyzed results in this way, we found that the tolerogenic CD103$^+$CD11b$^-$ DC subset showed the strongest reduction to approximately 10 % of wild type in LP (Fig.5.5 A) and mLNs (Fig.5.5 B) of C57BL/6 DC-LMP1/CD40 animals. F1 transgenic animals showed an intermediate nearly to approximately 40 % of wild type levels reduction of CD103$^+$CD11b$^-$ DC, while frequency of this DC subpopulation was reduced to only 60 % of wild type on BALB/c background. On the other hand CD103$^+$CD11b$^+$ DCs were similarly reduced in LP of all transgenic animals, while they were differentialy affected in mLNs, where C57BL/6 transgenic animals had the lowest frequency, while BALB/c DC-LMP1/CD40 animals had the highest frequencies of this second tolerogenic DC-subpopulation. Additionally, a relative

increase of CD103$^-$CD11b$^+$ DCs was observed in all trangenic animals compared to wt animals, however, the strongest increase could be seen in LP (Fig.5.5 A) and mLNs (Fig.5.5 B) of C57BL/6 DC-LMP1/CD40 animals.

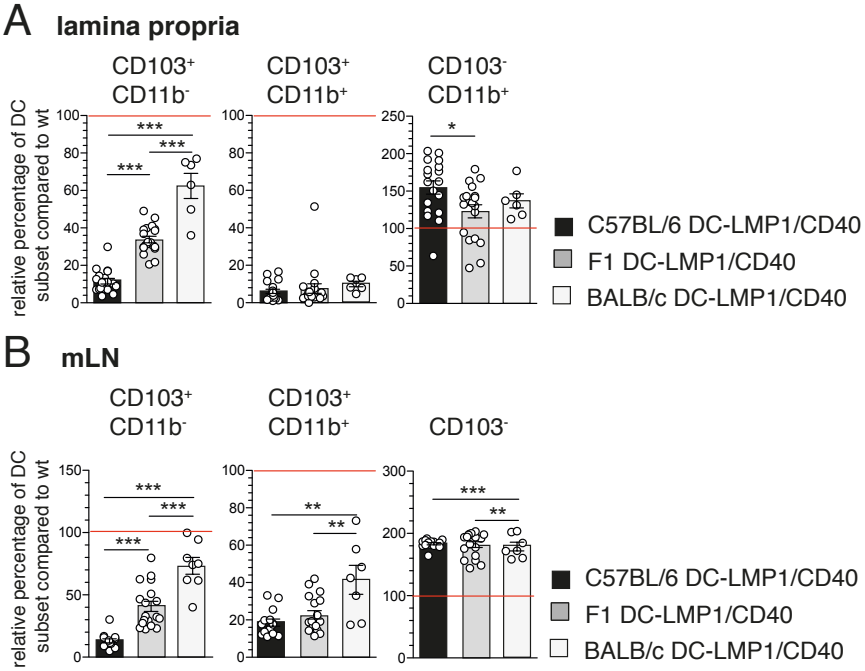


Figure 5.5: Relative changes of DC subpopulations as compared to wild type mice in different genetic backgrounds. DC subsets in the LP **(A)** or mLN **(B)** were analyzed as described in figure 5.3. The frequencies for each DC subset in DC-LMP1/CD40 animals on different genetic backgrounds are shown as relative percentage as compared to corresponding background control, which has been set to 100 % (red line). **A)** Relative frequencies for LP samples shown in figure 5.3 A. **B)** Relative frequencies for mLNs samples shown in figure 5.3 B. All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

CD40 signaling has been previously connected with an improvement of antigen presentation capacity and maturation of DCs [129, 130, 131]. Therefore, surface markers of DCs from DC-LMP1/CD40 animals were analyzed and compared in C57BL/6 DC-LMP1/CD40 and F1 DC-LMP1/CD40 animals. The only strong difference which could be observed between these two transgenic mouse strains was that all DC subsets in C57BL/6 DC-LMP1/CD40 animals consistently showed lower surface expression of MHC class II molecule compared to background control wt animals. This effect could be observed in LP (Fig.5.6 A) and mLNs (Fig.5.6 B). However, significant differential expression of CD86 co-stimulatory molecule in

LP of both transgenic strains could not be observed (Fig.5.6 A), while down-regulation of CD86 on mLNs $CD103^+CD11b^-$ DCs in transgenic animals on both backgrounds was detected (Fig.5.6 B). In contrast, CD86 was upregulated on $CD103^+CD11b^-$ DCs in both transgenic strains and on $CD103^+CD11b^+$ DCs only in F1 DC-LMP1/CD40 animals (Fig.5.6 B). Also CD80 expression on DC subsets was analyzed and a weak up-regulation in LP DCs of both transgenic strains could be detected (Fig.5.6 A), while up-regulation was even stronger on mLN DCs (Fig.5.6 B).

In order to compare activation profiles of DCs in transgenic animals with different genetic backgrounds, the percentage of mean fluorescence intensity (MFI) from co-stimulatory molecules on each DC subset was calculated. The MFI of corresponding wt mice in the same genetic background represented 100 % for analyzed co-stimulatory molecules on each specific DC subset. When analyzed in such a comparative relative way, DCs from C57BL/6 DC-LMP1/CD40 animals showed lower expression of MHC class II molecules on LP DCs, higher expression of CD80 and lower expression of CD86 in mLN DCs as compared to F1 DC-LMP1/CD40 animals (Fig.5.7 A and B).

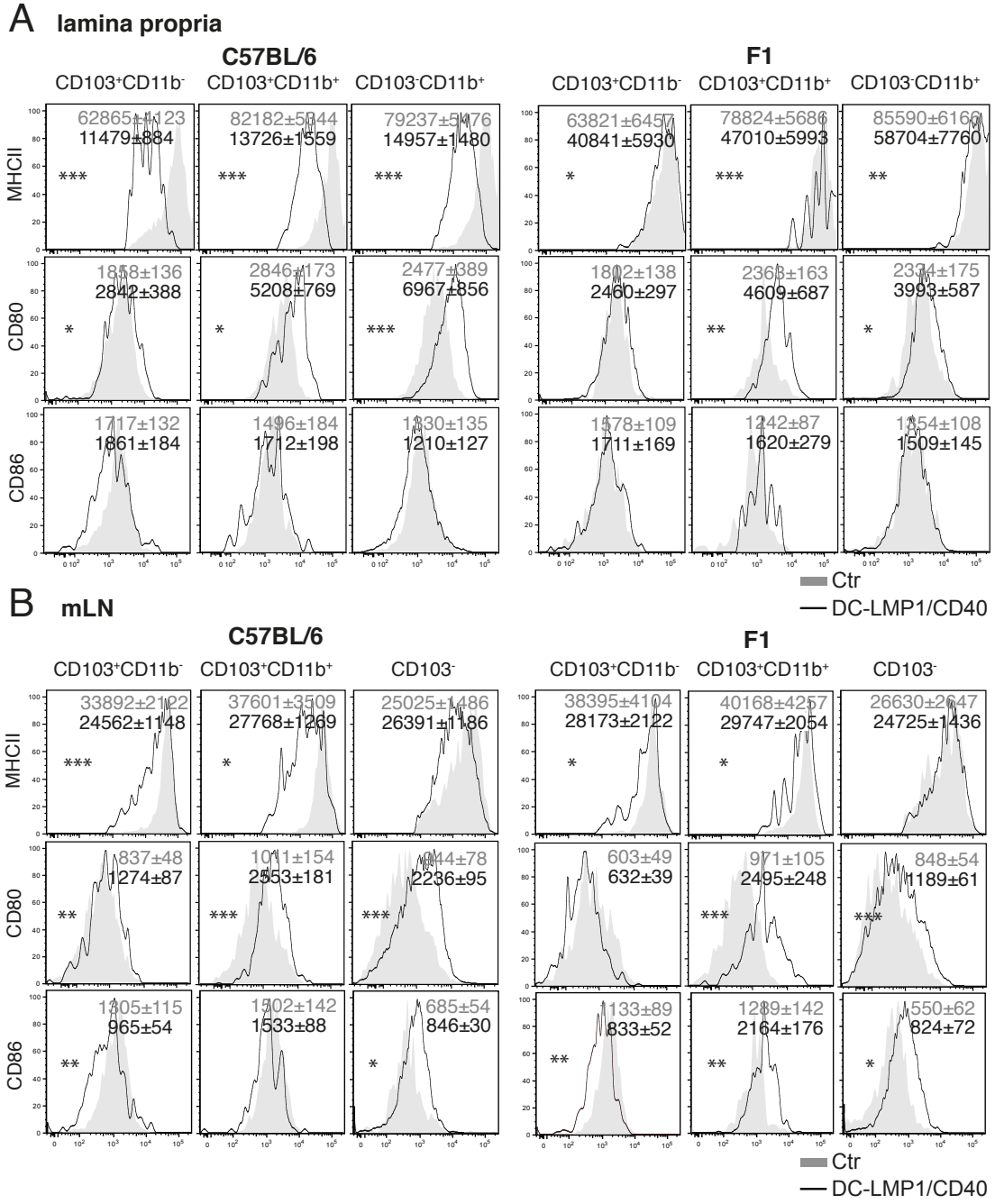


Figure 5.6: DC-LMP1/CD40 DCs from mLN and LP were more activated than their control counterparts. DC subsets of the LP **(A)** or mLN **(B)** were analyzed for the expression of different activation markers. Shown are representative histograms of wt controls (filled grey) and DC-LMP1/CD40 (black line). Numbers represent MFI ± standard error of mean (SEM). **A)** Shown are pooled statistics from 5 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 8-18) and from 6 experiments for F1 DC-LMP1/CD40 animals (n = 12-20). **B)** Shown are pooled statistics from 4 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 6-14) and from 5 experiments for F1 DC/LMP1/CD40 animals (n = 10-18). The significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

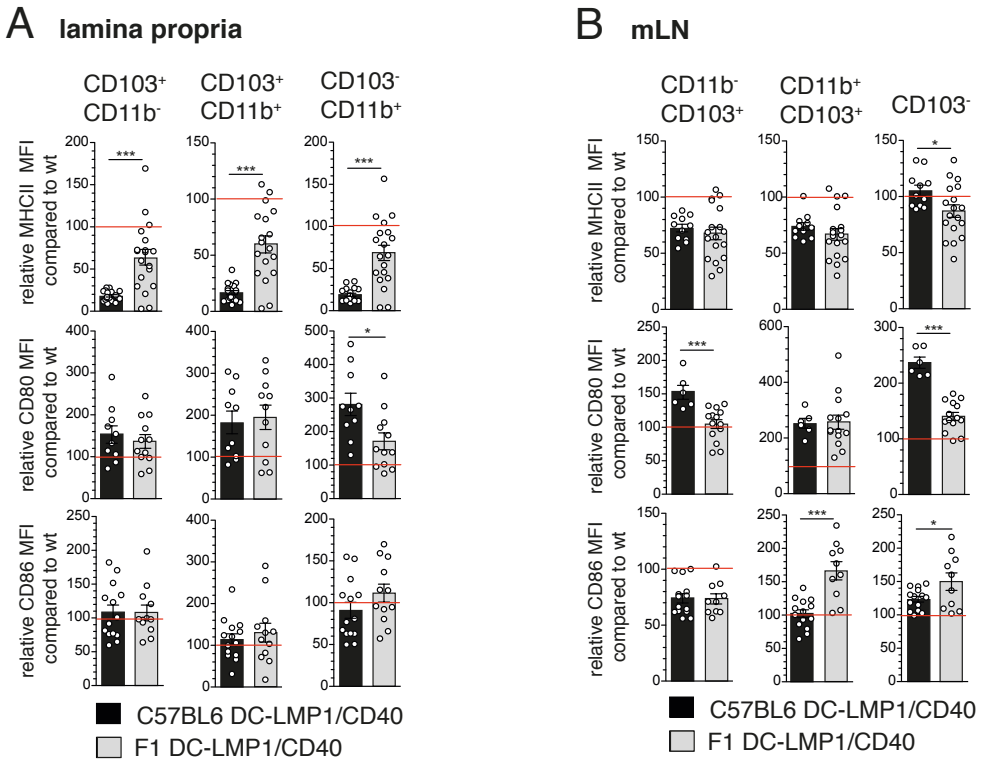


Figure 5.7: Relative changes of surface markers of DC subpopulations as compared to wild type mice in different genetic backgrounds. DC subsets in the LP **(A)** or mLN **(B)** were analyzed as described in figure 5.6. MFI values for activation markers of each DC subset in different DC-LMP1/CD40 animals are shown relative to corresponding background control which was set to 100 % (red line). **A)** Relative calculations for lamina propria samples shown in figure 5.6 A. **B)** Relative calculations were performed for mLNs samples shown in figure 5.6 B. The significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5.1.3 DC-LMP1/CD40 mice lack iTreg cells

Since DC-LMP1/CD40 animals had a strongly reduced frequency of intestinal CD103$^+$ DCs, which were previously shown to be crucial for iTreg cell induction [107, 108], we next investigated the intestinal Treg cell compartment. When comparing total FoxP3$^+$ Treg cells in different organs no differences in Treg frequencies between transgenic and wild type animals could be found [208]. However, when peripherally induced iTreg (Helios$^-$) and thymus induced nTreg cells (Helios$^+$) were analyzed separately, significantly fewer induced iTreg cells was found (Fig.5.8 A). In LP of C57BL/6 DC-LMP1/CD40 animals 13 % of Tregs were iTregs, as compared to 80 % of iTregs in wt animals (Fig.5.8 A). Also in F1 DC-LMP1/CD40

and BALB/c DC-LMP1/CD40 animals a reduction in the iTreg cell compartment could be observed. However, in F1 transgenic animals around 40 % iTregs (F1 controls 80 %) could be detected, while in BALB/c DC-LMP1/CD40 animals 38 % of Tregs were iTregs, as compared to 54 % of iTregs in wt animals (Fig.5.8 A). In LP of all transgenic animals high amounts of nTreg cells could be found which apparently filled the Treg cell compartment.

The separation between iTreg and nTreg cells in mLNs according to Helios expression is not as clear as in LP, but when analyzed the results have shown the same tendency as in LP. However, in mLNs iTreg cells in BALB/c DC-LMP1/CD40 animals were not significantly reduced when compared to wt animals (Fig.5.8 B).

In the next step, the relative amounts of Treg cells according to background control were analyzed analogous to the analysis performed for DC subpopulations. Using this analysis, the amount of iTreg cells between DC-LMP1/CD40 animals of different genetic origins could be compared. The percentage of each Treg subset in transgenic animals, where suitable background wt control represented 100 % for analyzed DC subset, was calculated. This analysis showed that the reduction of iTregs was most prominent in transgenic animals on C57BL/6 background. F1 DC-LMP1/CD40 animals had intermediate levels of iTregs, while transgenic BALB/c animals showed only a very mild reduction of iTregs (Fig.5.9).

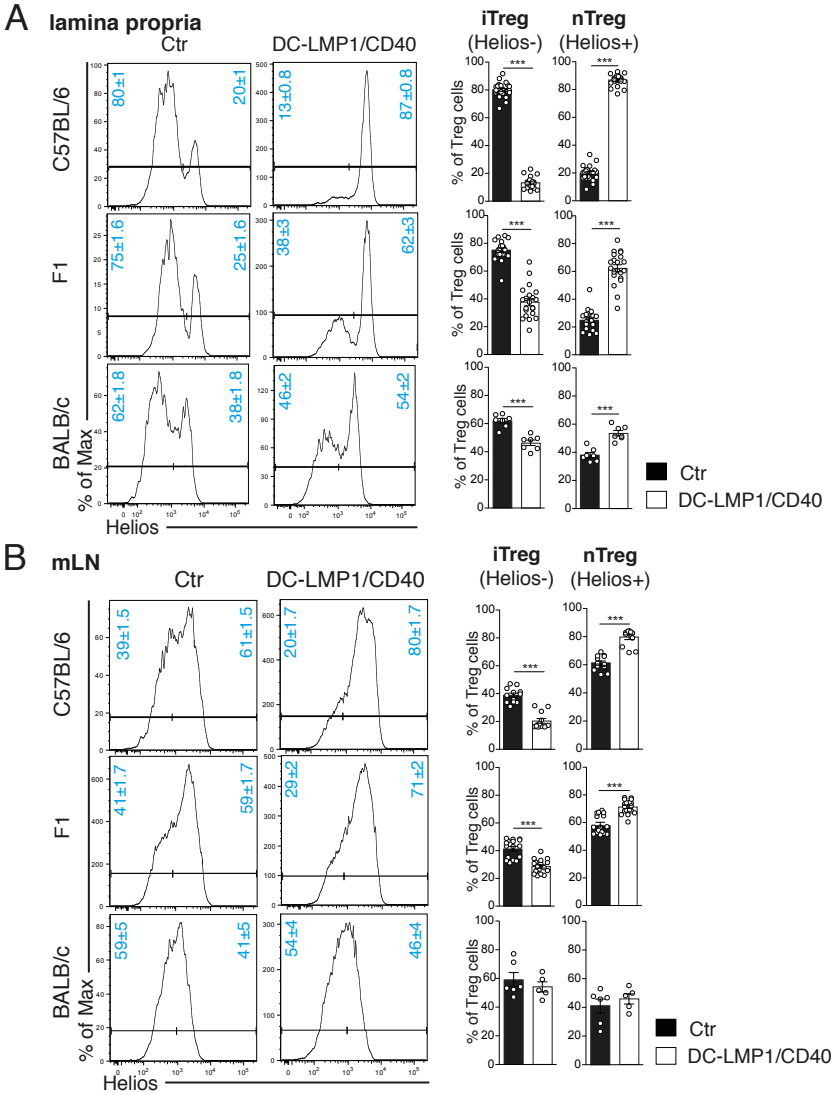


Figure 5.8: iTreg induction was severely impaired in DC-LMP1/CD40 animals. Single cell suspensions of LP **(A)** and mLN **(B)** were analyzed for Treg cells. **A)** Shown are representative FACS-plots of FoxP3$^+$ Tregs found in the LP pre-gated on single, live, CD45$^+$CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ and in the next step subdivided into nTregs and iTregs using Helios as a marker for thymic derived Tregs. Numbers on FACS-plots and bar graphs on their right side indicate the frequency of Treg cell subsets, separately for each analyzed genetic background. Shown are pooled statistics from 7 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 23-26), from 6 experiments for F1 DC-LMP1/CD40 animals (n = 21) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 7). **B)** Shown are representative FACS-plots of FoxP3$^+$ Tregs found in the mLN pregated on single, live, CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ and in the next step subdivided into nTregs and iTregs using Helios. Numbers on FACS-plots and bar graphs on their left side indicate the frequency of Treg cell subsets, separately for each analyzed genetic background. Shown are pooled statistics from 4 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 12), from 4 experiments for F1 DC-LMP1/CD40 animals (n = 16-18) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 5-6). All bar graphs represent mean ± SEM where significance was analyzed using a students *t*-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control.

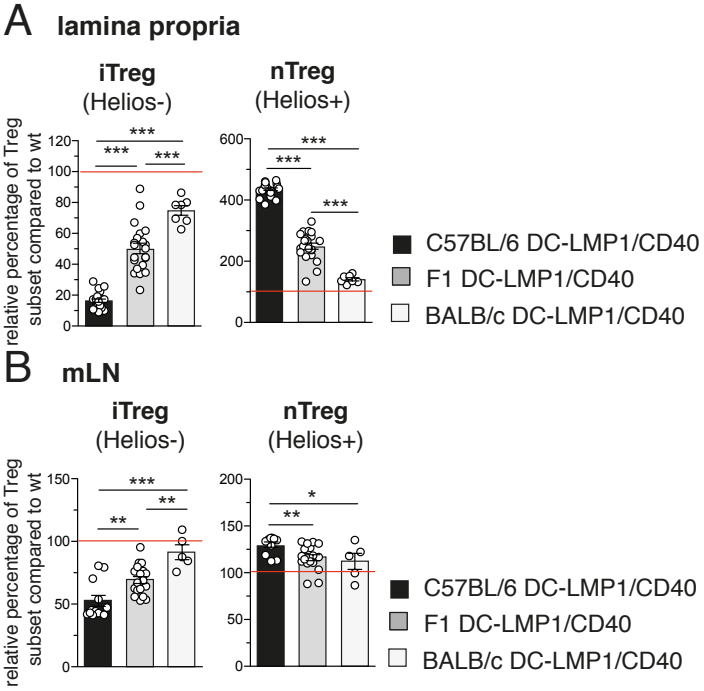


Figure 5.9: Analyses of iTreg and nTreg frequencies relative to wt controls in the corresponding genetic backgrounds. Treg cell subsets in the LP **(A)** or mLN **(B)** were analyzed as described in figure 5.8 A and B. The frequencies for each Treg cell subset in DC-LMP1/CD40 animals on different genetic backgrounds are shown as relative to corresponding background control (red line represents average frequency of background control). **A)** Relative calculations were performed for lamina propria samples shown in figure 5.8 A. **B)** Relative calculations were performed for mLN samples shown in figure 5.8 B. All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5.1.4 More CD103$^+$ DCs resulted in more iTregs

Data of DCs and iTregs in DC-LMP1/CD40 animals on different genetic backgrounds suggested that more CD103$^+$ DCs resulted in more iTregs. Samples from the very same animals were stained for DCs and iTreg cells and plotted on scatter plot, where DC subsets are shown on the y-axis versus iTregs shown on the x-axis. As transgenic animals in different genetic backgrounds were plotted into the same scatter plot, we calculated the percentage of the analyzed DC or iTreg subset in relation to the corresponding wt controls in the very same genetic background. A linear fit was applied to this data and the Pearson coefficient calculated. A strong positive correlation between the presence of CD103$^+$ subsets of DCs and Helios$^-$ iTreg cells in LP (Fig.5.10 A) and in mLNs could be observed (Fig.5.10 B). The cor-

relation was stronger for $CD103^{+}CD11b^{-}$ than for $CD103^{+}CD11b^{+}$ DCs, while analysis of the $CD103^{-}CD11b^{+}$ DCs gave us a negative correlation with iTregs for both analyzed organs (Fig.5.10 A and B). The strength of the co-occurrence between DC subpopulations and iTregs was represented with the Pearson Correlation coefficient (r), whose values can range between -1 (the strongest negative correlation) and +1 (the strongest positive correlation). From the scatter plot could therefore be visible that more $CD103^{+}$ DCs could induce more iTreg cells, where $CD103^{+}CD11b^{-}$ DCs had better tolerogenic properties. On the other hand we could show that higher percentage of $CD103^{-}$ DCs did not lead to higher percentage of iTregs.

Since C57BL/6 DC-LMP1/CD40 animals had the lowest percentage of $CD103^{+}$ DCs, this resulted in the lowest percentage of iTreg cells, while F1 and specially BALB/c transgenic animals had more $CD103^{+}$ DCs and consequently more iTreg cells.

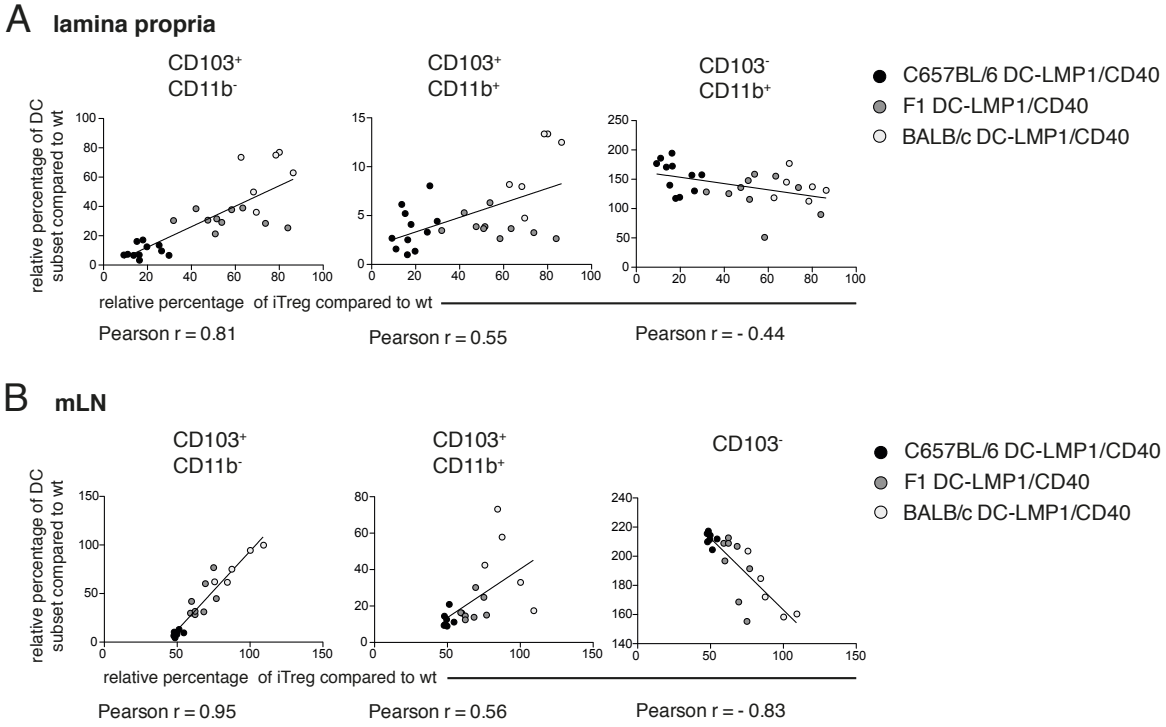


Figure 5.10: Positive correlation between tolerogenic CD103$^+$ DCs and iTreg cells. DC subsets and iTreg cells were analyzed for each mouse at the same time. DCs were prepared and analyzed as described in figure 5.5 (A) for LP and (B) for mLN. iTregs (Helios$^-$) cells were prepared and analyzed as described in figure 5.9 (A) for LP and (B) for mLNs. Each symbol on the scatter plot represents one mouse. Linearly fitted line was added on each scatter plot to visualize the relationship between DC subpopulations and iTreg cells. The strength of co-occurrence between DC subpopulation and iTregs is represented with the Pearson Correlation coefficient (r), whose values range between -1 and +1. **A)** Data for LP was acquired and pooled from 3 separate experiments for C57BL/6 DC-LMP1/CD40 animals (n = 10-12), from 4 experiments for F1 DC-LMP1/CD40 animals (n = 13-14) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 6-7). **B)** Data for mLNs was acquired and pooled from 2 separate experiments for C57BL/6 DC-LMP1/CD40 animals (n = 6-8), from 3 experiments for F1 DC-LMP1/CD40 animals (n = 12) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 5-6).

5.1.5 C57BL/6 DC-LMP1/CD40 mice showed a breakdown of intestinal tolerance

We have previously shown [208] that colitis in C57BL/6 DC-LMP1/CD40 depended on B and T cells, since mice on RAG$^{-/-}$ genetic background did not develop the disease (Fig.3.1 A). Therefore, we further characterized T cells and found a significantly increased amount of IL-17$^+$IFN-γ^+ and IL-17$^-$IFN-γ^+ T cells in LP (Fig.5.11) as well as in mLNs and in the

spleen (Fig.5.12 A and B) of C57BL/6 DC-LMP1/CD40 animals. In contrast to LP, in mLNs and spleen of C57BL/6 DC-LMP1/CD40 animals also IL-17$^+$IFN-γ^- T cells were increased (Fig.5.12 A and B). However, F1 DC-LMP1/CD40 animals showed a different distribution of T cell subsets. There, a high amount of IL-17$^+$IFN-γ^- T cells could be shown in LP (Fig.5.11) and a slightly higher, but also significant amount of these cells in the spleen (5.12 B). In contrast to C57BL/6 DC-LMP1/CD40 animals in mice of the F1 background LP IL-17$^+$IFN-γ^+ T cells were only slightly increased, while IL-17$^-$IFN-γ^+ T cells were not increased (Fig.5.11 and 5.12). Similar like in F1 transgenic animals also in BALB/c DC-LMP1/CD40 animals IFN-γ expressing cells were not increased in any analyzed organ, while the same held true for IL-17 expressing cells (Fig.5.11 and 5.12).

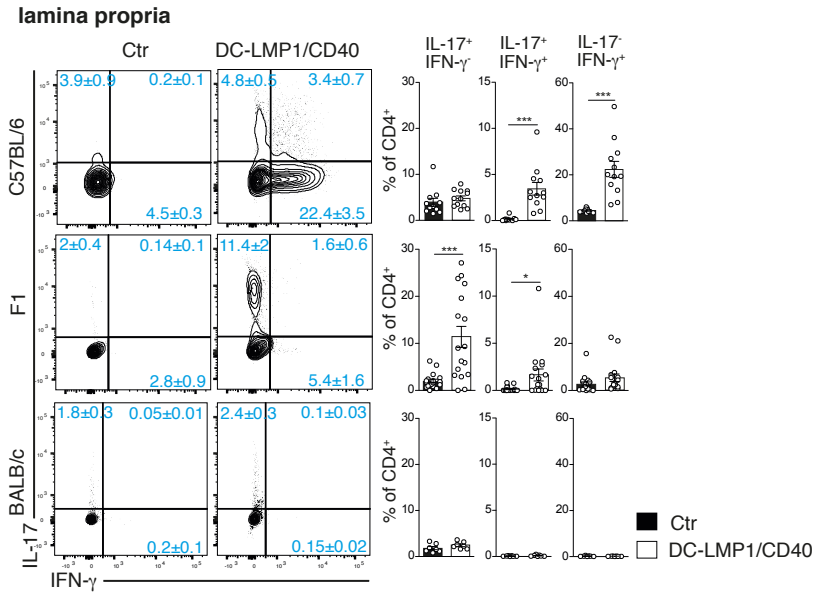


Figure 5.11: Increased number of IFN-γ and IL-17 producing T cells in the LP of DC-LMP1/CD40 animals on C57BL/6 background. T cell functionality was analyzed by stimulating single cell suspensions with PMA/Ionomycin for 4 h, subsequently cells were stained intracelluarly for the production of IL-17 and IFN-γ. Shown are representative FACS-plots with indicated frequencies for LP (gated on single, live, CD45$^+$CD3$^+$CD4$^+$). Bar graphs represent the pooled statistics from 4 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 11-12), from 6 experiments for F1 DC-LMP1/CD40 animals (n = 17-18) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 7). All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control.

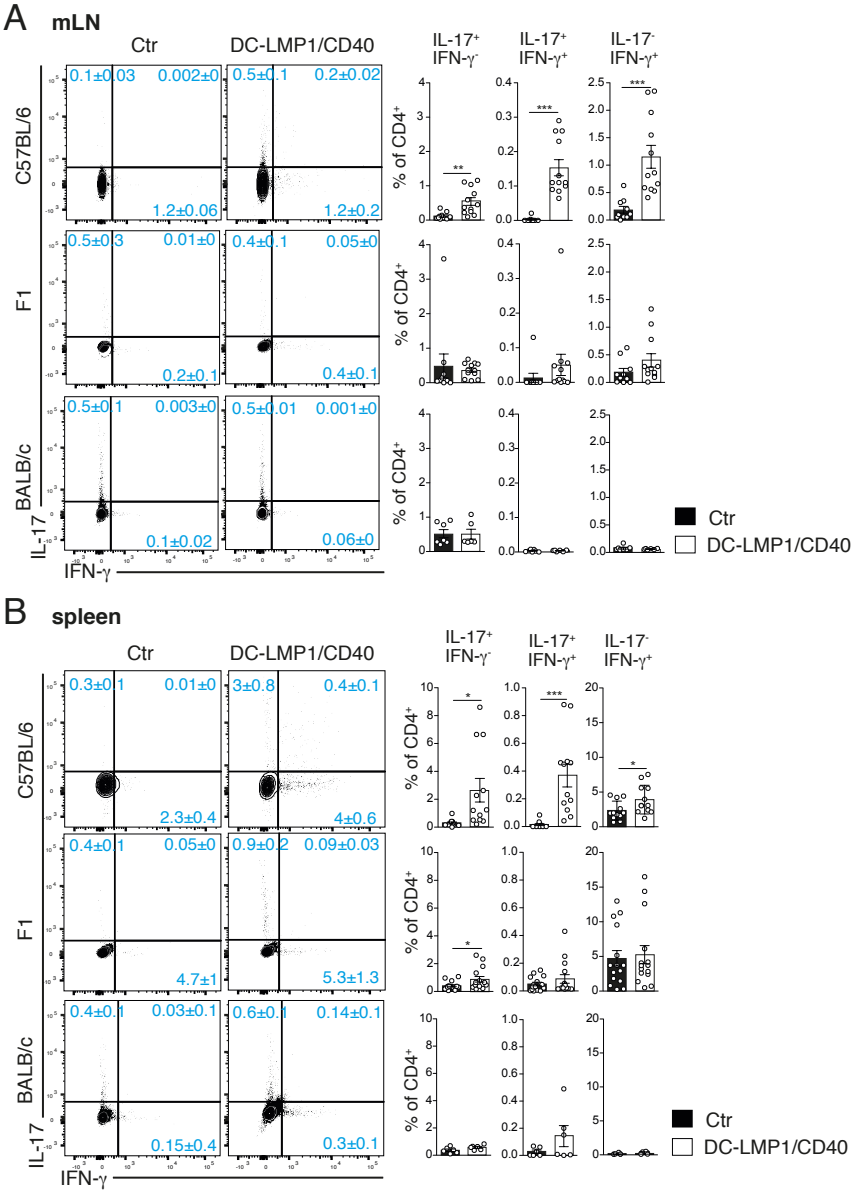


Figure 5.12: Effector T cell subsets in mLN and spleen. T cell functionality was analyzed by stimulating single cell suspensions with PMA/Ionomycin for 4 h, subsequently cells were stained intracelluarly for the production of IL-17 and IFN-γ. Shown are representative FACS-plots with indicated frequencies for mLN **(A)** (gated on single, live, $CD3^+CD4^+$) and for the spleen **(B)** (gated on single, live, $CD3^+CD4^+$). **A)** Bar graphs represent the pooled statistics for frequencies of Th subpopulations from 4 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 10-12), from 4 experiments for F1 DC-LMP1/CD40 animals (n = 10-12) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 6-7). **B)** Bar graphs represent the pooled statistics for frequencies of Th subpopulations from 4 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 12), from 5 experiments for F1 DC-LMP1/CD40 animals (n = 15) and from 2 experiments for BALB/c DC-LMP1/CD40 animals (n = 6). All bar graphs represent mean ± SEM where significance was analyzed using a students *t*-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control.

Similar to the relative calculations for DCs and Tregs shown above, the relative frequencies of effector T cells subsets were calculated for each genetic background of DC-LMP1/CD40 animals separately in relation to their corresponding wild type counterparts. This kind of analysis was performed in order to compare the frequencies of effector T cells between DC-LMP1/CD40 animals of different genetic origins. When results were analyzed like this, we found that C57BL/6 DC-LMP1/CD40 animals had the highest amount of LP IL-17$^-$IFN-γ^+ T cells among all three DC-LMP1/CD40 strains (Fig.5.13 A). In contrast, F1 transgenic animals had the highest relative frequency of IL-17$^+$IFN-γ^- T cells, while the effector T cell compartment of transgenic BALB/c DC-LMP1/CD40 animals was not changed when compared to their BALB/c wt controls (Fig.5.13 A). The situation in mLNs was slightly different, since there C57BL/6 DC-LMP1/CD40 animals had the highest relative increase of all effector T cell subsets (Fig.5.13 B). The same was true for the spleen (Fig.5.13 C).

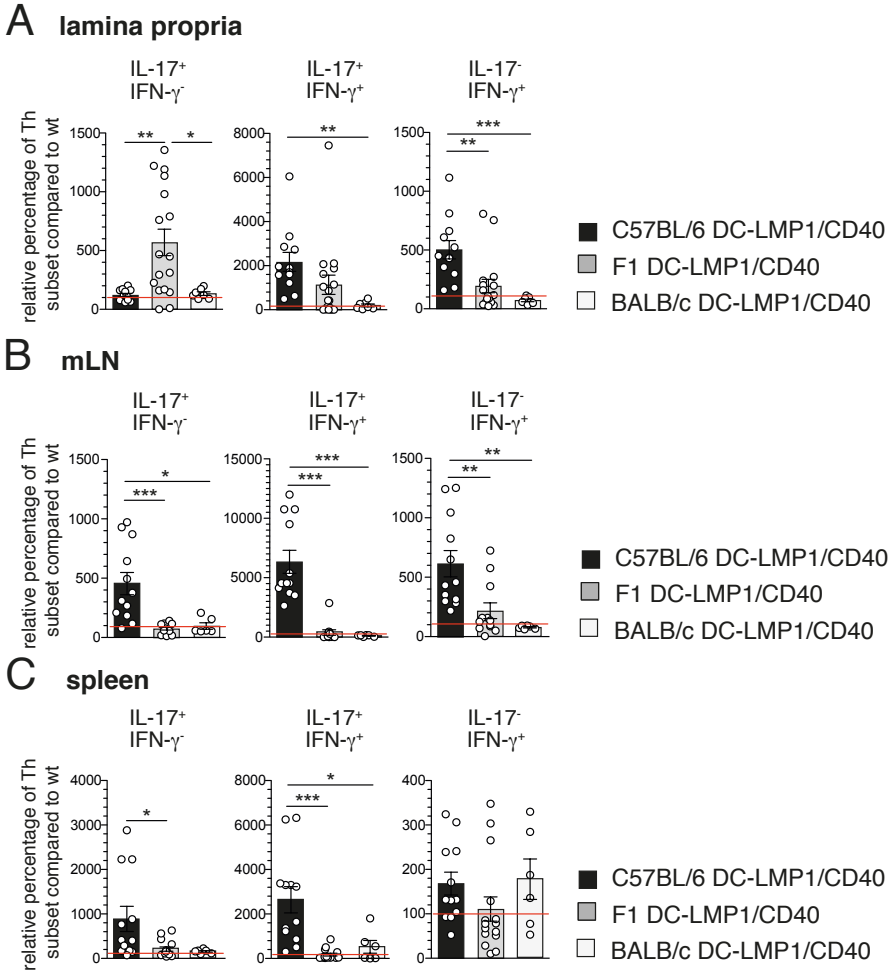


Figure 5.13: Comparative analysis of effector T cells in three different genetic backgrounds. IL-17$^+$IFN-γ^-, IL-17$^+$IFN-γ^+ and IL-17$^-$IFN-γ^+ Th cell subsets in LP **(A)**, mLN **(B)** and spleen **(C)** were analyzed as described in figures 5.11 and 5.12. The frequencies for each Th cell subset in DC-LMP1/CD40 animals on different genetic backgrounds are shown as relative to suitable background control (red line represents average frequency of background control). **A)** Relative calculations were performed for lamina propria samples shown in figure 5.11. **B)** Relative calculations were performed for mLN samples shown in figure 5.12 A. **C)** Relative calculations were performed for spleen samples shown in figure 5.12 B. All bar graphs represent mean ± SEM where significance was analyzed using a students *t*-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5.1.5.1 Th17 cells in C57BL/6 DC-LMP1/CD40 animals showed a more pathogenic phenotype

Th17 cells in the periphery are not a homogeneous population and can confer host protection as well as autoimmune diseases [171, 172]. For their initial commitment to the Th17 lineage, T cells need TGF-β1 and IL-6 in order to differentiate into a more protective Th17 cell type

[173, 175]. However, if they are exposed to IL-23, and according to some publications also IL-1β, they develop a more pathogenic phenotype, which among others includes T-bet expression and IFN-γ production [176, 163, 222]. Since Th17 cells show late developmental plasticity and different degrees of pathogenicity and since we have shown that transgenic animals of different strains differed in the structure of the Th17 repertoire, we performed additional experiments in order to further identify the type of Th17 cells in the different mouse types.

First, T-bet expression in IL-17$^+$IFN-γ^-, IL-17$^+$IFN-γ^+ and IL-17$^-$IFN-γ^+ T cell subsets was compared in C57BL/5 DC-LMP1/CD40 animals. This analysis showed that IL-17$^+$IFN-γ^- cells expressed the lowest level, IL-17$^+$IFN-γ^+ intermediate level and IL-17$^-$IFN-γ^+ the highest level of the transcription factor T-bet (Fig.5.14 A). It is known that IL-17 expression of Th17 cells is transient in some inflammatory settings, which means that cells after IL-23 conversion can stop expressing IL-17 and only express IFN-γ. These cells were therefore named ex-Th17 cells or Th1-like cells [177]. Therefore, this could mean that IL-17$^-$IFN-γ^+ T cells identified in C57BL/6 DC-LMP1/CD40 animals were actually ex-Th17 cells which have lost IL-17 expression. We could show that IL-17$^+$IFN-γ^- T cells from C57BL/6 transgenic animals had significantly higher T-bet levels than their wt controls, suggesting their potential for further differentiation towards a more Th1-like phenotype (Fig.5.14 B). In contrast, this was not found for F1 DC-LMP1/CD40 animals where no elevated levels of T-bet were observed (Fig.5.14 B). The latter suggested that IL-17$^+$IFN-γ^- T cells in F1 DC-LMP1/CD40 animals would not develop further into pathogenic ex-Th17 cells or Th1-like cells. Analysis of T-bet expression in T cells from BALB/c DC-LMP1/CD40 animals was unfortunately not possible since we did not have enough animals.

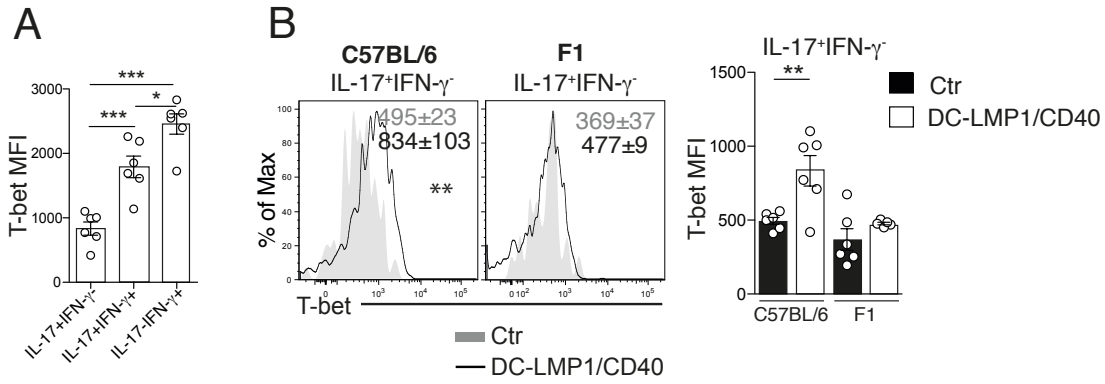


Figure 5.14: IL-17$^+$IFN-γ^- cells from C57BL/6 DC-LMP1/CD40 animals showed a more pathogenic phenotype than those from F1 DC-LMP1/CD40 animals. A) IL-17$^+$IFN-γ^-, IL-17$^+$IFN-γ^+ and IL-17$^-$IFN-γ^+ T cells from C57BL/5 DC-LMP1/CD40 animals were analyzed for T-bet expression. The bar graph represents the pooled statistics for MFI of T-bet expression from 2 experiments (n = 6). **B)** Shown are representative histograms of controls (filled grey) and DC-LMP1/CD40 (black line) for T-bet expression in IL-17$^+$IFN-γ^- T cells from C57BL/6 and from F1 mixed background transgenic animals. The numbers on the histograms represent mean ± SEM, which is additionally displayed on the bar graph on the right side. For statistics, data from 2 experiments was pooled (n = 5-6). Bar graph represents mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

Taken together the extensive phenotypic analysis of DC-LMP1/CD40 mice on different genetic backgrounds showed that only transgenic animals on C57BL/6 genetic background developed colitis-like phenotype, showed high levels of inflammatory marker lipocalin-2 in feces and increased levels of proinflammatory cytokines in the intestine. However, all DC-LMP1/CD40 animals showed strong reduction of tolerogenic CD103$^+$ DC subsets, which was the strongest for C57BL/6 transgenic animals. Additionally, all DC-LMP1/CD40 animals had reduced frequencies of Helios$^-$ iTregs when compared to wt animals. However, mice on C57BL/6 genetic background were the most affected, F1 animals showed the intermediate reduction, while iTregs in BALB/c transgenic animals were only slightly reduced. We could show the existence of positive correlation between CD103$^+$ DC subsets and iTreg cells where CD103$^+$CD11b$^-$ DC subset had stronger tolerogenic properties. The analysis of effector T cell subsets in DC-LMP1/CD40 animals has shown the strong expansion of pathogenic IL-17$^+$IFN-γ^+ T cells only in transgenic animals of C57BL/6 origin, while F1 transgenic animals had more lamina propria IL-17$^+$IFN-γ^- T cells and BALB/c DC-LMP1/CD40 animals did

not have elevated levels of effector T cells when compared to BALB/c wt animals.

5.2 CD40 signaling induced migration of intestinal DCs from LP to mLNs

As described in section 3.4, the alternative way of studying CD40 signaling in DCs is with a simple intra-peritoneal injection of anti-CD40 mAb into the mouse. This is not an ideal method due to the systemic effect of the anti-CD40 antibody on all cells expressing the CD40 molecule, but it offers some advantages over the constitutive DC-LMP1/CD40 model. The biggest disadvantage of the DC-LMP1/CD40 mouse model is that it is always on, meaning that DCs receive a CD40 signal from the moment when they start expressing CD11c. Therefore, it is hard to identify which of the observed effects is a primary effect due to the triggering of the CD40 pathway in DC and which are actually secondary effects.

We published that CD40 signaling induced migration of intestinal DCs from LP to mLNs [208]. To study the influence of CD40 signaling on DCs, anti-CD40 antibody was IP injected in wt C57BL/6 mice and LP and mLNs DC subsets were analyzed 24 h post treatment. A strong reduction of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DC subsets was found in LP (Fig.5.15 A (black)), while they were strongly increased in mLNs of treated animals (Fig.5.15 A (red)). In combination with a measured increase of CCR7 levels, which is a chemokine receptor needed for the migration of cells via the afferent lymph towards mLNs [55], especially on LP $CD103^+$ DCs (Fig.5.15 B), this result suggested that CD40 stimulus induced an increased exit of $CD103^+$ DCs from lamina propria and their further migration towards mLNs. To test if DCs really migrated due to the triggering of the CD40 pathway in them, we injected anti-CD40 in mice which lack the CD40 molecule specifically on $CD11c^+$ DCs. In these mice the treatment did not induce the migration of $CD103^+$ DCs [208], therefore we could argue that the migration of DCs was a direct effect of CD40 crosslinking.

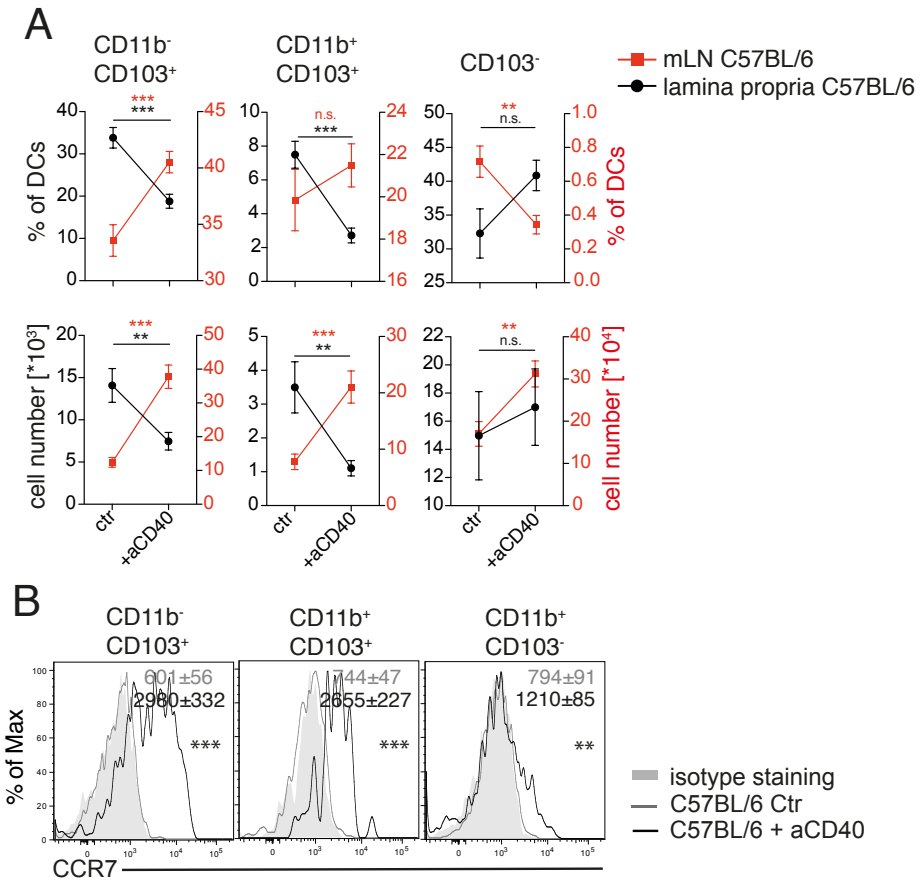


Figure 5.15: Injection of anti-CD40 antibody in wt mouse induced migration of CD103$^+$ DCs from LP to mLNs. A) A percentage of DCs for each DC subset and absolute numbers of cells per DC subset in lamina propria (black) and mLNs (red) are shown. Shown are pooled statistics from 4 separate experiments (n = 11-14). Wild type C57BL/6 animals were IP injected with an anti-CD40 antibody and analyzed 24h post injection. **B)** CCR7 expression was analyzed in different subsets of LP DCs from wt C57BL/6 animals injected with the anti-CD40 antibody (IP injection, 24 hours) or untreated controls. Shown are representative histograms of isotype control staining (filled grey), controls (grey line) and anti-CD40 injected animals (black line) for CCR7 staining in different subsets of LP DCs. Numbers on histograms represent MFI ± SEM from 2 pooled experiments (n = 6). The significance was analyzed using a students *t*-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control; n.s. = not significant. Black stars indicate significance for lamina propria cell subsets, while red stars indicate significance for mLN cell subsets.

5.2.1 Influence of anti-CD40 injection on DCs in C57BL/6 wt and BALB/c wt mice

Since higher frequencies of CD103$^+$ DCs were observed in F1 and BALB/c DC-LMP1/CD40 animals than in transgenic animals on a C57BL/6 genetic background (Fig.5.5), we tested

if differential CD40 signaling between C57BL/6 and BALB/c mice could be the reason for the observed differences. An anti-CD40 mAb was IP injected in each mouse strain and after 24 h DC subsets of LP and mLNs were analyzed. Anti-CD40 treated animals were compared with uninjected controls of the same strain. DCs of both mouse strains responded similarly to the activation of their CD40 pathway. A reduction of $CD103^+$ DCs could be observed in LP, while their frequencies increased in mLNs (Fig.5.16 A and B). However, while many $CD103^+$ DCs migrated from LP to mLNs in BALB/c, this depletion was not as complete as compared to C57BL/6 animals. CCR7 chemokine receptor up-regulation was also found to be stronger expressed on DCs from C57BL/6 animals as compared to these from BALB/c animals, eventually explaining the less efficient migration in the latter (Fig.5.16 C).

After it was observed that BALB/c wt animals had more $CD103^+CD11b^-$ DCs than C57BL/6 wt animals, it was tested if F1 wt animals had an intermediate amount of $CD103^+$ $CD11b^-$ DCs. This hypothesis could be confirmed in LP (Fig.5.17 A) of analyzed animals, while wt animals of different genetic backgrounds did not differ in the frequency of the mLN $CD103^+CD11b^-$ DCs (Fig.5.17 B).

Additionally, the activation status of DCs in C57BL/6 and BALB/c animals after anti-CD40 treatment was compared. Some differences between the two strains could be shown. 24 h after an anti-CD40 injection most of the DC subsets in LP or mLNs showed no change in MHC II expression. Only $CD103^-CD11b^+$ cells in LP of BALB/c animals showed a slight, albeit significant, down-regulation of MHC II expression. In contrast, a strong up-regulation of CD80 and CD86 co-stimulatory molecules on mLN and LP DCs of both mice strains 24 h after triggering the CD40 pathway was observed (Fig.5.18 A and B).

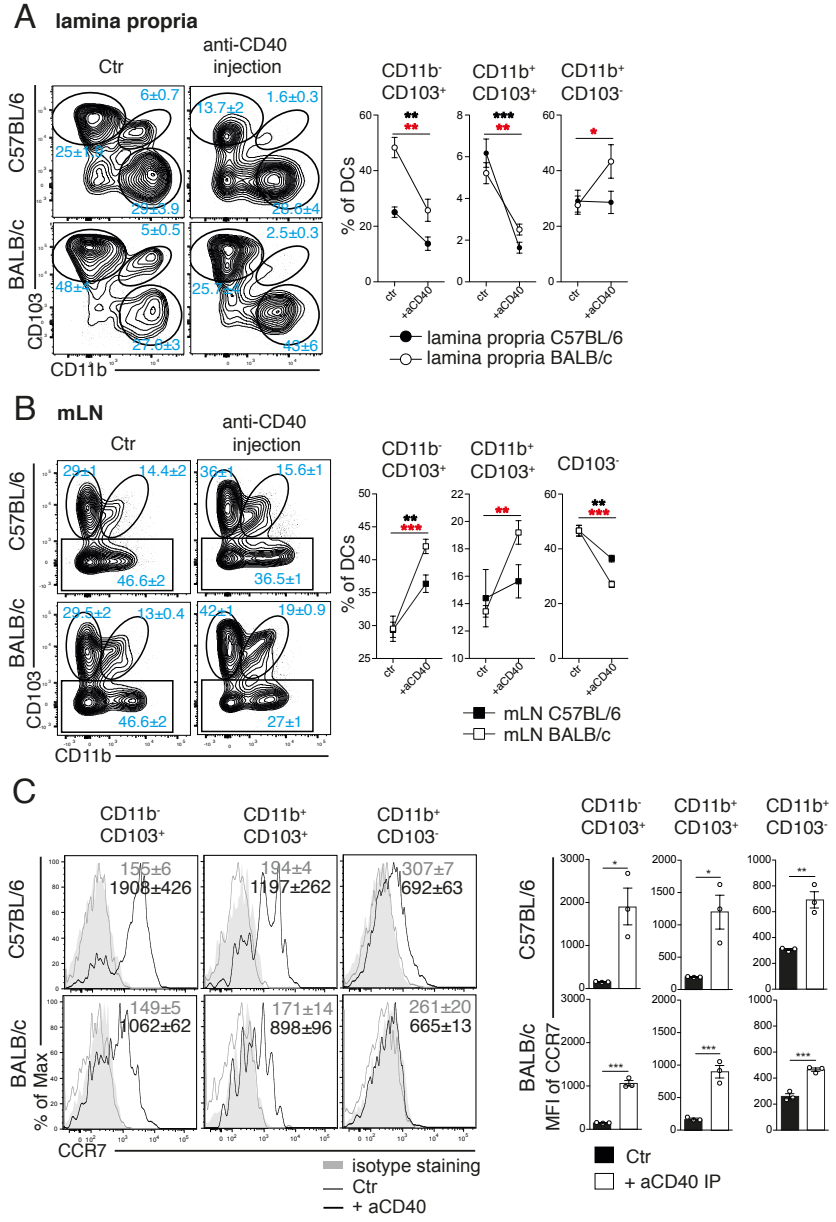


Figure 5.16: Injection of an anti-CD40 antibody had a similar effect on the migration of DCs in C57BL/6 and BALB/c wt animals. DC subsets in the LP **(A)** or mLN **(B)** were analyzed in wt C57BL/6 and wt BALB/c animals injected with an anti-CD40 antibody (24 hours post IP injection) and in un-injected controls of each mouse strain. **A)** Representative lamina propria FACS-plots are shown, numbers and dot graphs indicate the frequency of DC subsets. Shown are pooled statistics from 2 experiments (n = 5-6). **B)** Representative mLN FACS-plots are shown, numbers and dot graphs indicate the frequency of DC subsets. Shown are pooled statistics from 2 experiments (n = 5-6). **C)** CCR7 expression was analyzed in different subsets of lamina propria DCs from wt C57BL/6 and wt BALB/c animals injected with the anti-CD40 antibody (IP injection, 24 hours) or untreated littermates controls. Shown are representative histograms of isotype control staining (grey filled), controls (grey line) and anti-CD40 injected animals (black line) for CCR7 staining in different subsets of LP DCs. Numbers on histograms represent MFI ± SEM (which is additionally displayed in the bar graph on the right side) from 1 representative experiment (n = 3) of two independent experiments. All graphs represent mean ± SEM where significance was analyzed using a students *t*-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control. Black stars indicate significance for C57BL/6 mouse strain, while the red stars indicate significance for BALB/c mouse strain.

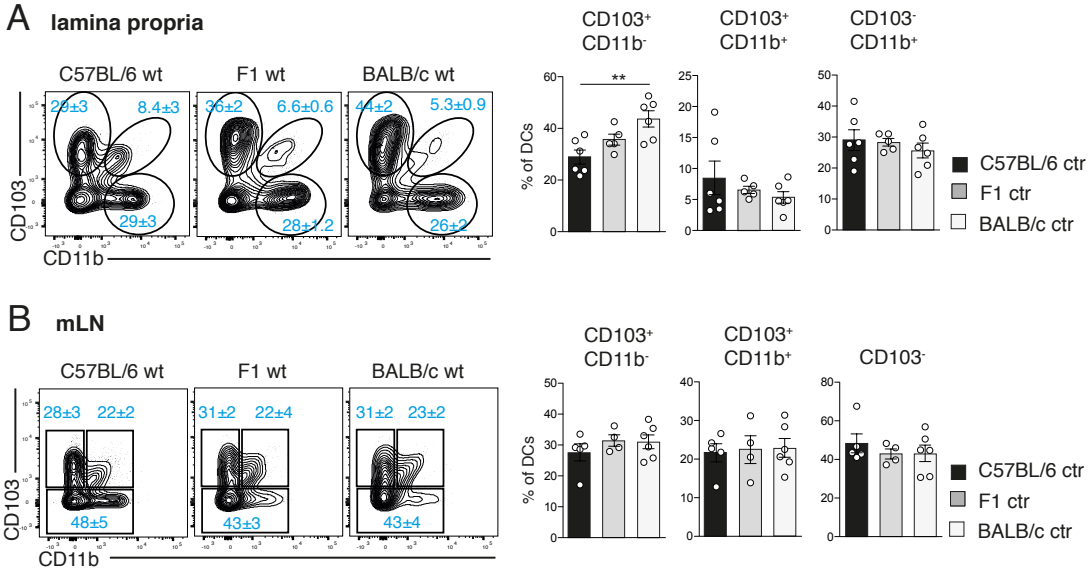


Figure 5.17: BALB/c wt animals had the highest frequency of CD103$^+$CD11b$^-$ DCs. DC subsets in the LP **(A)** or mLN **(B)** were analyzed in wt (wild type) animals on different genetic backgrounds. **A)** LP cells were gated on single cells, live, CD45$^+$MHCII$^+$CD11c$^+$CD64$^-$ cells. Representative FACS-plots are shown, numbers and bar graphs indicate the frequency of DC subsets. Shown are pooled statistics from 2 experiments (n = 5-6). **B)** mLN cells were gated on single cells, live, MHCII$^+$CD11c$^+$ cells. Representative FACS-plots are shown, numbers and bar graphs indicate frequency of DC subsets. Shown are pooled statistics from 2 experiments for C57BL/6 DC-LMP1/CD40 animals (n = 4-6). All bar graphs represent mean ± SEM where significance was analyzed using a students *t*-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

In order to compare the activation profile of anti-CD40 injected animals of different genetic backgrounds, the percentage of MFI from co-stimulatory molecules on each DC subset in anti-CD40 injected animals was calculated. The MFI of the corresponding un-injected genetic background control represented 100 % for the respective analyzed co-stimulatory molecule on each specific DC subset. When the results were analyzed like this, it could be shown that anti-CD40 injection in BALB/c animals activated DCs stronger as compared to C57BL/6 animals. Higher expression of CD80 and CD86 co-stimulatory molecules were observed on CD40-treated DCs of BALB/c genetic background (Fig.5.19 A and B).

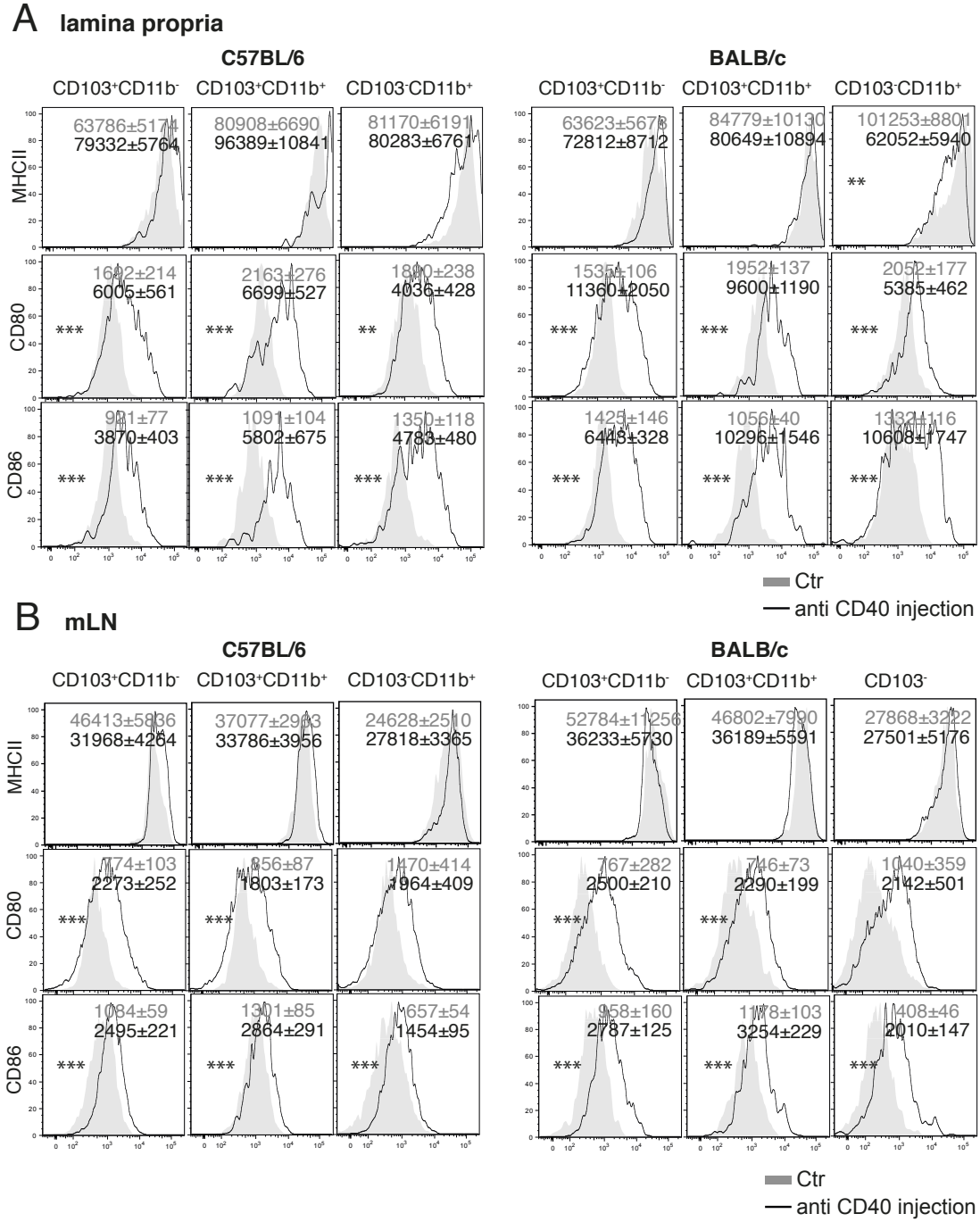


Figure 5.18: DC maturation markers on mLN and LP DCs from anti-CD40 injected C57BL/6 and BALB/c wt animals. DC subsets of the LP **(A)** or mLN **(B)** were analyzed for the expression of different activation markers. Shown are representative histograms of wt controls (filled grey) and anti-CD40 injected animals (black line). The numbers represent MFI ± SEM. **A)** Shown are pooled statistics for LP DCs from 2 experiments for anti-CD40 injected (IP injection, 24h time point) C57BL/6 and BALB/c animals (n = 5-6). **B)** Shown are pooled statistics for mLN DCs from 2 experiments for anti-CD40 injected (IP injection, 24h time point) C57BL/6 and BALB/c animals (n = 5-6). The significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

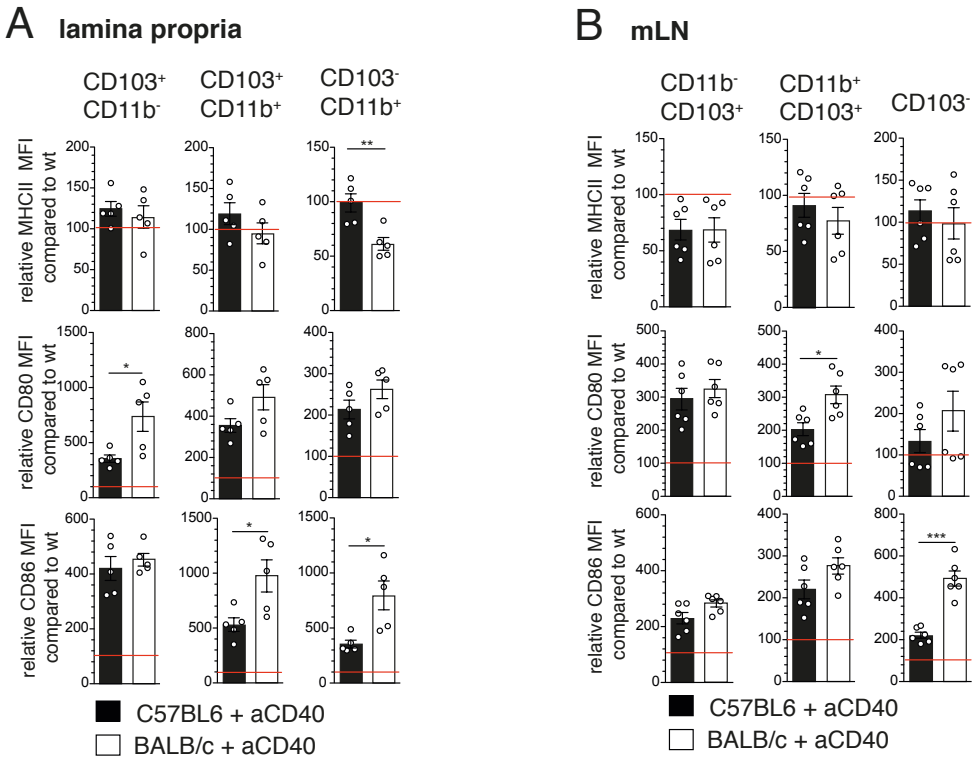


Figure 5.19: Anti-CD40 treated animals on BALB/c genetic background showed higher expression of CD80 and CD86 co-stimulatory molecules. DC subsets in the LP **(A)** or mLN **(B)** were analyzed as described in figure 5.18. MFI values for activation markers of each DC subset in different DC-LMP1/CD40 animals are shown as relative change to suitable un-injected background control (red line represents average frequency of background control). **A)** Relative calculations were performed for lamina propria samples shown in figure 5.18 A. **B)** Relative calculations were performed for mLN samples shown in figure 5.18 B. The significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5.3 Activated CD4 T cells trigger reduction of CD103$^+$ DCs

In order to investigate a potential biological relevance of the observed mechanism we performed the following experiment. CD40 ligand (CD154), the natural ligand for CD40, is expressed on activated T cells *in vivo* [223]. We therefore tested the possibility if activated T cells could induce migration of tolerogenic CD103$^+$ DCs from LP to mLNs, as demonstrated for CD40 antibody triggering or CD40 signaling in DC-LMP1/CD40 mice. Such a mechanism has the potential to shut down tolerance induction in order to make ongoing immune

responses more potent. To this end treg-depleted naïve CD62L$^+$CD4$^+$ T cells were transferred into Rag$^{-/-}$ animals. Since Rag$^{-/-}$ mice have no T cells, transferred T cells started to spontaneously proliferate and became activated. Mice were analyzed 15 days after adoptive T cell transfer and it could be shown that the tolerogenic CD103$^+$CD11b$^-$ DC subset was significantly reduced in LP of experimental animals when compared to Rag$^{-/-}$ control animals (Fig.5.20).

Taken together this experiment suggested that activated T cells have the potential to trigger DCs in order to modulate tolerance versus immunity.

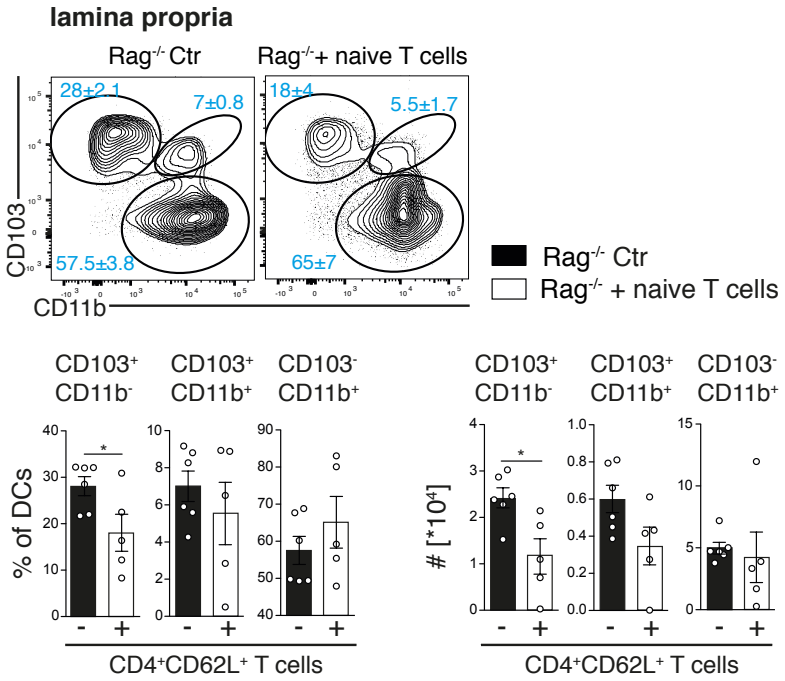
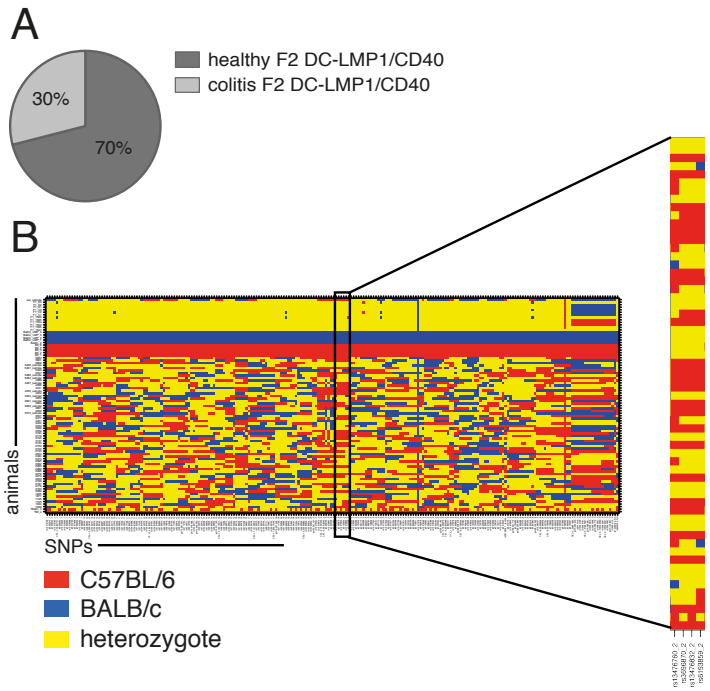


Figure 5.20: Activated CD4 T cells had the capacity to induce migration of DCs from LP. CD4$^+$CD62L$^+$ T cells from donor wild type C57BL/6 mice were transferred into Rag$^{-/-}$ animals and subset distribution of lamina propria DCs was analyzed 15 days after transfer. Shown are representative FACS plots with indicated frequencies of DC subsets. Bar graphs represent the pooled statistics for frequencies and absolute numbers of DC subpopulations from 2 experiments (n = 6). All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control.

5.4 SNP genotyping of F2 DC-LMP1/CD40 mouse strain

F2 DC-LMP1/CD40 animals were a genetically and phenotypically heterogeneous group of mice, where approximately 30 % of animals developed colitis while the rest remained healthy (Fig.5.21 A). This heterogeneity was used for a SNP analysis, with the purpose to identify candidate genes that might be key for different colitis susceptibility between C57BL/6 and BALB/c. DNA samples were collected from F2 DC-LMP1/CD40 animals with visible signs of colitis and a SNP genotyping with the Ilumina Mouse Linkage LD chip (Illumina, San Diego, CA, USA) was performed. The Ilumina Mouse LD Linkage Panel covers 377 SNP loci chosen to maximize genetic information across the top ten inbred strains routinely used for mouse crosses. This genetic information could be used to eventually identify C57BL/6 SNP alleles which contribute to disease in F2 DC-LMP1/CD40 mice with colitis. On the other hand it could also allow the identification of BALB/c SNP alleles which eventually protect from colitis. Only F2 DC-LMP1/CD40 animals with visible signs of colitis (colitis visible on macroscopic level by colon shortening and thickening) were analyzed. This criteria fulfilled approximately 30 % of F2 DC-LMP1/CD40 animals (Fig.5.21 A). Since in addition to these 30 % of F2 DC-LMP1/CD40 animals only C57BL/6 DC-LMP1/CD40 animals developed colitis, it was expected that the SNP study would lead to the identification of a region in the mouse genome where all tested sick F2 DC-LMP1/CD40 animals would be homozygote for C57BL/6 SNP allele. Although, there was no region homogeneous for the C57BL/6 SNP allele, a region on mouse chromosome 2 was identified where all 60 analyzed F2 DC-LMP1/CD40 animals carried at least one C57BL/6 derived allele (Fig.5.21 B). Since we relied on visible signs of colitis and did not perform an extensive histology analysis for every mouse, the study design might not have been precise enough and some F2 transgenic animals with milder colitis might have been included. Therefore, we still took a deeper look in the identified SNP region of chromosome 2, which led to the identification of 16 immunology relevant genes (Fig.5.21 C). qPCR primers for each of these genes were designed to compare expression levels of these

genes between C67BL/6 and BALB/c mice strains. All 11 different defensins have shown to be hard to study with qPCR, because they showed a high degree of homology. The IL1bos, CD93 and Fkbp1a genes were not followed further, since no connection between them and colitis could be found. In contrast IL-1α and especially IL-1β got our attention. Both genes were found to be significantly up-regulated in the inflamed colonic mucosa of patients with ulcerative colitis [224, 225].



C

Gene name	Gene name
Il1a	interleukin 1 alpha
Il1b	interleukin 1 beta
Il1bos	interleukin 1 beta opposite strand
Cd93	CD93 antigen
Fkbp1a	FK506 binding protein 1a
Defb19	defensin beta 19
Defb20	defensin beta 20
Defb21	defensin beta 21
Defb22	defensin beta 22
Defb23	defensin beta 23
Defb25	defensin beta 25
Defb26	defensin beta 26
Defb28	defensin beta 28
Defb29	defensin beta 29
Defb36	defensin beta 36
Defb45	defensin beta 45

Figure 5.21: SNP genotyping for the F2 DC-LMP1/CD40 genetically heterogeneous mouse strain identified 16 candidate genes for different colitis susceptibility between C57BL/6 and BALB/c mouse strain. A) Pie chart representing the percentage of F2 DC-LMP1/CD40 animals (generated with F1 DC-LMP1/CD40 inter-cross) with visible signs of colitis (colitis visible on macroscopic level by colon shortening and thickening) (n = 223). **B)** SNP analysis of F2 DC-LMP1/CD40 animals which developed colitis (n = 60) and control animals to test the accuracy of SNP analysis. Magnified is a region on chromosome 2 that contains at least one copy of the C57BL/6 allele for the analyzed SNP. **C)** List of immunologically relevant genes within this region on the mice chromosome 2.

5.4.1 Testing IL-1α and IL-1β as candidate genes for colitis susceptibility

Due to its high potency to induce inflammation, the activation and secretion of IL-1β is tightly regulated with the formation of inflammasomes [226]. On the other hand, the physiological role in the inflammatory process and biogenesis of IL-1α are less well defined [227].

BMDC cultures from C57BL/6 and BALB/c mouse strains were prepared and stimulated for 3 h with LPS in order to induce intra-cellular production of IL-1 cytokines, which were not secreted in the medium if the inflammasome was not further triggered [228]. In the next step LPS stimulated BMDCs were lysed and intracellular IL-1α and IL-1β cytokine levels were measured with an ELISA assay. Interestingly, it could be shown that BMDCs from C57BL/6 mice produced more IL-1α and IL-1β than BMDCs from BALB/c mice after they were stimulated with LPS (Fig.5.22 A).

Additionally, the IL-1 family cytokine levels in DC-LMP1/CD40 transgenic animals were analyzed. The whole gut homogenate samples from C57BL/6 DC-LMP1/CD40 and F1 DC-LMP1/CD40 animals with suitable wt controls were prepared. The IL-1α and IL-1β cytokine levels were measured with an ELISA assay. A high amount of both cytokines could be detected in the gut samples from C57BL/6 DC-LMP1/CD40 animals, while no signal could be detected in transgenic animals on F1 background or in control wt animals (Fig.5.22 B).

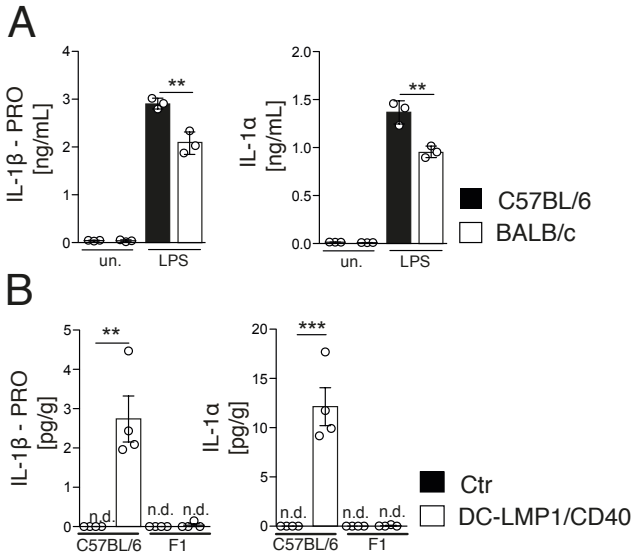


Figure 5.22: IL-1 cytokines were potential candidates for different colitis susceptibility between mouse strains. A) The amount of IL-1α and IL-1β was determined by ELISA in lysates of C57BL/6 and BALB/c BMDCs with and without LPS stimulation. Shown are representative results from one of 2 independent experiments with similar outcome (n = 3). B) The amount of IL-1α and IL-1β was determined by ELISA in whole colon homogenates of C57BL/6 and F1 DC-LMP1/CD40 animals with suitable controls for each background. The measured amount of cytokine was normalized on the starting amount of each sample. Shown are representative results from one of 2 independent experiments with a similar outcome (n = 4). All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. n.d. = not detectable; un. = un-stimulated; LPS = lipopolysaccharides.

Taken together we could show that C57BL/6 derived BMDCs responded on LPS stimulation with higher production of IL-1α and IL-1β cytokines than BALB/c derived BMDCs. Additionally, we could measure high levels of IL-1α and IL-1β cytokines only in whole gut homogenate samples from C57BL/6 DC-LMP1/CD40, while no signal could be detected in F1 DC-LMP1/CD40 or in F1 and C57BL/6 wt animals. However, it is important to be aware that the difference in IL-1 cytokine level between transgenic C57BL/6 and F1 animals is also due to inflammation, which is going on only in transgenic C57BL/6 animals. Therefore, it is hard to say if IL-1 is causative or it is just a consequence of the disease.

Using data-set from the Mouse Genome Database SNPs in IL-1α and IL-1β that were discriminatory between C57BL/6 and BALB/c were identified. Two SNPs were located in the IL-1β gene and one in the IL-1α gene (Fig.5.23 A). All these three SNPs were located in

the non coding three prime untranslated region, which is known to influence polyadenylation, translation efficiency, localization, and stability of mRNA [229, 230]. Therefore, we tested if there was a potential correlation between the SNP in genetically heterogeneous F2 DC-LMP1/CD40 animals with or without disease development. Primers were designed to amplify the SNPs containing regions and these were amplified from F2 DC-LMP1/CD40 animals. The PCR products were run on the agarose gel and the suitable bands representing one of the IL-1 gene segments including SNPs were cut out and sequenced. Sequencing data was analyzed and the SNP in IL-1β gene with the number rs3022903 gave a clear reliable sequence, while the others did not.

The idea was to search for a correlation between C57BL/6 origin of SNP in the IL-1β gene and the development of colitis. As a marker of colitis fecal lipocalin-2 was used. In this part of the study every F2 DC-LMP1/CD40 animal, which was sequenced for IL-1β SNP, was also measured for fecal lipocalin-2. The expectation was that animals with the high levels of lipocalin-2, meaning animals with strong intestinal inflammation, would carry the C57BL/6 allele for SNP in IL-1β locus. However, this was not the case (Fig.5.23 B). Only one F2 DC-LMP1/CD40 animal was found to be a homozygot for allele derived from BALB/c strain, while many animals carried one C57BL/6 and one BALB/c allele. Since those heterozygous mice had the same level of inflammation like the animals carrying two C57BL/6 alleles, we could not confirm our hypothesis.

The reason for the negative result could have been a less than ideal choice of marker for the presence of colitis. Therefore, it was decided to directly measure the level of IL-1β in whole gut homogenate of F2 DC-LMP1/CD40 animals and then correlate these levels with the genetic origin of the SNP in the IL-1β gene locus. This kind of analysis also failed to prove the hypothesis that C57BL/6 origin of IL-1β gene could have been responsible for high levels of IL-1β cytokine in C57BL/6 DC-LMP1/CD40 animals (Fig.5.23 C).

A

gene	SNP	allele in C57BL/6	allele in BALB/c	PCR
Il1a	rs3022902	A	T	not working
Il1b	rs3022904	C	A	not working
Il1b	rs3022903	C	T	working

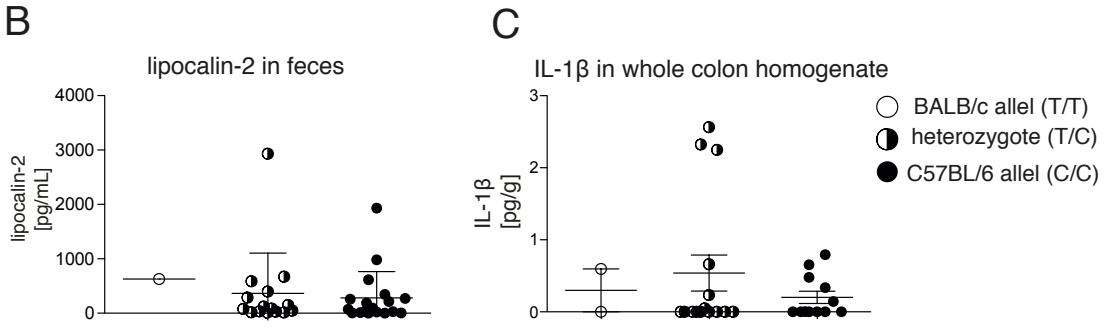


Figure 5.23: Sequencing of IL-1 locus in F2 DC-LMP1/CD40 animals, in order to confirm C57BL/6 derived IL-1β allele as reason that animals develop colitis. A) SNPs located in mouse IL-1 locus on chromosome 2, for which C57BL/6 mouse strain has different allele than BALB/c mouse strain. For all this SNPs sequencing primers were generated, but they worked only for the second SNP in IL-1β gene (rs3022903). **B)** Levels of fecal lipocalin-2 as measured by ELISA in 10-12 weeks old F2 DC-LMP1/CD40 in relation with sequencing data for rs3022903 SNP in IL-1β locus. The dot plot represents data acquired from 38 experimental animals. **C)** Levels of IL-1β as measured by ELISA in whole colon homogenates (measured amount of cytokine was normalized on the starting amount of each sample, measured in grams) of 10-12 weeks old F2 DC-LMP1/CD40 in relation with sequencing data for rs3022903 SNP in IL-1β locus. The dot plot represents data acquired from 29 experimental animals. All graphs represents mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

5.5 Blocking IL-1β prevented colitis development in C57BL/6 transgenic mice

Since high levels of IL-1β cytokine could be measured only in C57BL/6 DC-LMP1/CD40 animals, which developed strong intestinal inflammation, rescue of the animals by blocking the IL-1β cytokine with an anti IL-1β antibody was attempted. The amount of injected antibody was titrated and the length of the experiment was optimized. Five weeks old C57BL/6 DC-LMP1/CD40 animals, which have been treated with ABX to prevent onset of colitis, were moved to cages containing feces from the rest of the mouse facility for commensal bacteria repopulation and ABX treatment was stopped. Doing this we exposed C57BL/6 DC-

LMP1/CD40 animals to bacteria, which caused colitis development in untreated C57BL/6 DC-LMP1/CD40 animals. Simultaneously, we started treatment of experimental mice with an anti-IL-1β antibody, which was injected twice per week intraperitoneally. The control mice were treated with an isotype control antibody. Treatment lasted for 7 weeks (Fig.3.2 A). The weight of treated and control mice was regularly measured (not shown) and at the end of the experiment it could be shown that anti-IL-1β treatment protected C57BL/6 DC-LMP1/CD40 animals from wasting disease. Their average weight was significantly higher than the weight of the isotype control treated mice (Fig.5.24 C). Also macroscopic photos of the colon revealed that the anti-IL-1β treatment protected C57BL/6 DC-LMP1/CD40 from colitis development (Fig.5.24 B). Colons from isotype treated animals were shorter and visibly more inflamed. Additionally, the absence of inflammation by measuring the fecal lipocalin-2 levels [221] could be shown. In contrast to anti-IL-1β treated animals, isotype treated C57BL/6 DC-LMP1/CD40 mice showed high levels of lipocalin-2 (Fig.5.24 D).

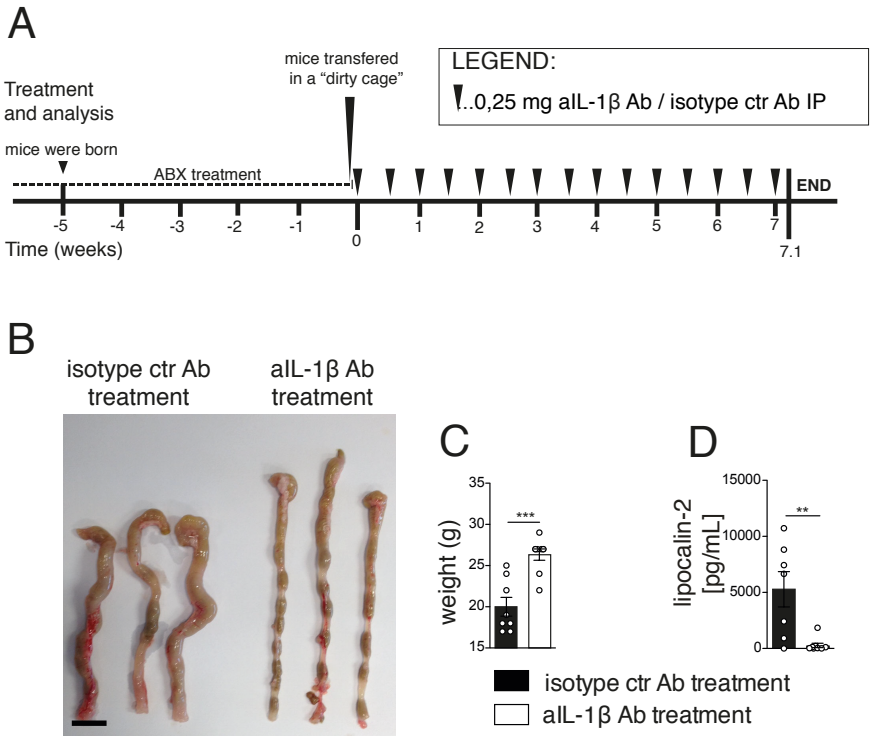


Figure 5.24: Anti-IL-1β treatment could prevent colitis development in C57BL/6 DC-LMP1/CD40 animals. A) Anti-IL-1β treatment experiment design. C57BL/6 DC-LMP1/CD40 mice were born on ABX therapy and were drinking ABX water until the age of 5 weeks. Then the mice were transferred into a dirty cage and the treatment with an anti-IL-1β antibody or isotype ctr antibody was started. 0.25 mg of antibody was intraperitoneally injected twice per week for duration of 7 weeks. The day later mice were sacrificed and analyzed. **B)** Macroscopic pictures of colons from isotype control and anti-IL-1β treated C57BL/6 DC-LMP1/CD40 animals after 7 weeks of therapy. Bar = 1 cm. **C)** Weight of mice measured at the end of experiment. Shown are pooled statistics from 2 experiments (n = 8-10). **D)** Levels of fecal lipocalin-2 as measured by ELISA in the experimental animals at the end of experiment. Shown are pooled statistics from 2 experiments (n = 8-10). All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control, Ab = antibody, IP = intraperitoneal.

5.6 Blocking IL-1β prevented the expansion of IFN-γ^+ T cells

After it was shown that anti-IL-1β treatment prevented colitis development in C57BL/6 DC-LMP1/CD40 mice, it was analyzed if it also prevented the expansion of pathogenic IFN-γ^+ T cells, which probably caused colitis in C57BL/6 DC-LMP1/CD40 animals.

No difference in frequency or absolute number of IL-17⁺IFN-γ⁻ and IL-17⁺IFN-γ⁺ lamina propria effector T cells between C57BL/6 DC-LMP1/CD40 treated with anti-IL-1β mAb or isotype control antibody could be detected. However, a decrease in the frequency and in the number of IL-17⁻IFN-γ⁺ T cells could be observed (Fig.5.25 A). These IL-17⁻IFN-γ⁺ T cells are the effector cell subset which was increased only in C57BL/6 DC-LMP1/CD40 animals while they could not be identified in transgenic animals on F1 or BALB/c genetic backgrounds (Fig.5.11 and Fig.5.12). Therefore, this suggested that they were the ones responsible for colitis development.

Analysis of the mLNs gave a similar result as described for LP, with the only difference being that here only the decrease in the absolute number of IL-17⁻IFN-γ⁺ T cells could be seen, while anti-IL-1β treatment did not affect their frequency (Fig.5.25 B).

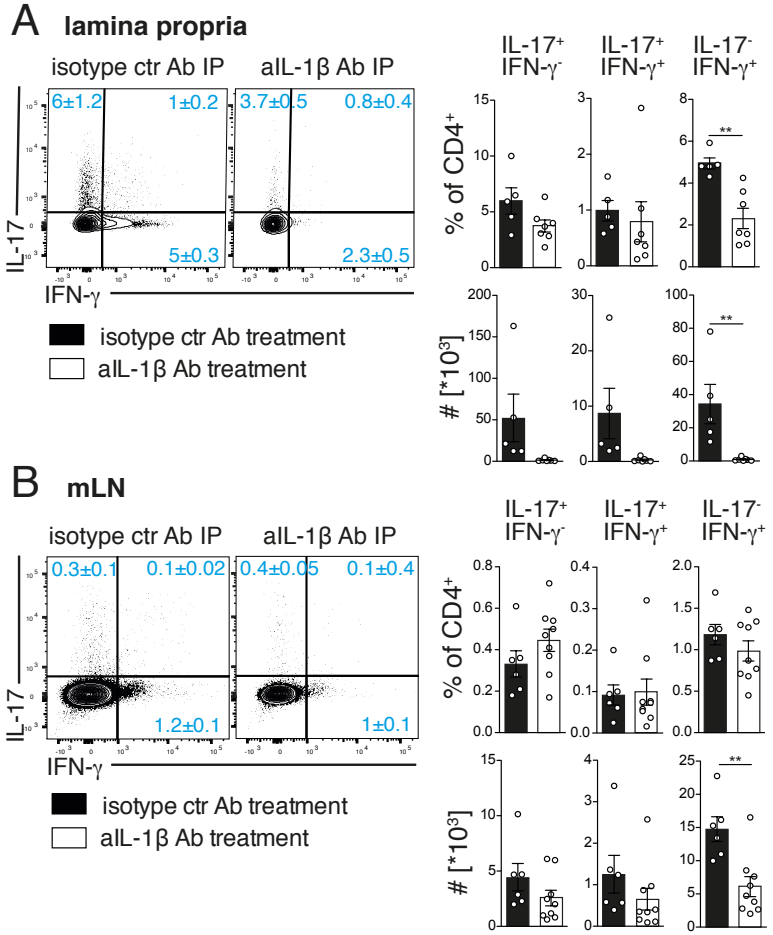


Figure 5.25: Anti-IL-1β treatment strongly reduced the development of IL-17$^-$IFN-γ^+ CD4 T cells. T cell functionality was analyzed by stimulating single cell suspensions of lamina propria cells **(A)** or mLN cells **(B)** from C57BL/6 DC-LMP1/CD40 animals, which were treated with anti-IL-1β or with isotype control, with PMA/Ionomycin for 4 h and subsequently staining cells intracellular for the production of IL-17 and IFN-γ. **A)** Shown are representative FACS-plots with indicated frequencies for LP (gated on single, live, CD45$^+$CD3$^+$CD4$^+$). Bar graphs represent pooled statistics from 2 experiments (n = 5-7). **B)** Shown are representative FACS-plots with indicated frequencies for mLN (gated on single, live, CD3$^+$CD4$^+$). Bar graphs represent pooled statistics from 2 experiments (n = 6-9). All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control, Ab = antibody, IP = intraperitoneal.

5.7 LMP1/CD40 transgene induces IL-12-p35 production in CD64$^+$ macrophages

After showing that high levels of IL-1β could only be detected in colitis C57BL/6 DC-LMP1/CD40 mice (Fig.5.22 B) and that an anti-IL-1β treatment prevented the disease in

these animals (Fig.5.24), we wanted to identify the cell population which produced IL-1β. Since the production of IL-1β could only be detected in transgenic animals, all cell populations expressing the LMP1/CD40 transgene were analyzed. These were CD11c$^+$ cells, which are mostly DCs and macrophages. Surprisingly the expression of IL-1β RNA could not be detected in CD103$^+$ DCs nor in CD103$^-$ DCs (Fig.5.26 A). However, when CD64$^+$ macrophages were sorted from LP of control and transgenic animals, it could be seen that DC-LMP1/CD40 animals on C57BL/6 genetic background produced high amounts of IL-1β (Fig.5.26 B, upper panel). Interestingly, an increase in the amount of IL-1β in CD64$^+$ macrophages derived from F1 and BALB/c transgenic animals could not be observed (Fig.5.26 B). In order to test if observed difference in IL-1β expression was mouse strain background dependent, CD64$^+$ macrophages from ABX treated C57BL/6 DC-LMP1/CD40 animals were analyzed. However, in these animals increased levels of IL-1β could not be detected (Fig.5.26 B, lower panel). Additionally, high levels of IL-23-p19 and IL-12-p35 could be measured in CD64$^+$ macophages from C57BL/6 transgenic animals, while these two cytokines were increased to a smaller extend in CD64$^+$ macophages from F1 transgenic animals. In contrast BALB/c DC-LMP1/CD40 animals and ABX treated C57BL/6 DC-LMP1/CD40 animals only showed elevated levels of IL-12-p35 cytokine, what suggested that elevation of this cytokine was directly caused by LMP1/CD40 transgene, since in these two strains no inflammation could be detected (Fig.5.1 for BALB/c DC-LMP1/CD40 animals and [208] for ABX treated C57BL/6 transgenic animals).

Taken together, cytokine profiles of LP macrophages in transgenic animals of different genetic origins revealed that macrophages in C57BL/6 DC-LMP1/CD40 animals showed the most inflammatory phenotype, while these from F1 DC-LMP1/CD40 animals and especially these from BALB/c DC-LMP1/CD40 animals showed less inflammatory one. High levels of IL-12-p35 cytokine were directly caused by LMP1/CD40 transgene, since this cytokine was measured at elevated levels also in BALB/c transgenic animals and in ABX treated C57BL/6 transgenic animals. Hovewer, elevated levels of IL-23-p19 and IL-1β in C57BL/6

DC-LMP1/CD40 animals were according to these results caused by some secondary inflammatory effect, since ABX treatment reduced their levels on levels present in wt C57BL/6 animals.

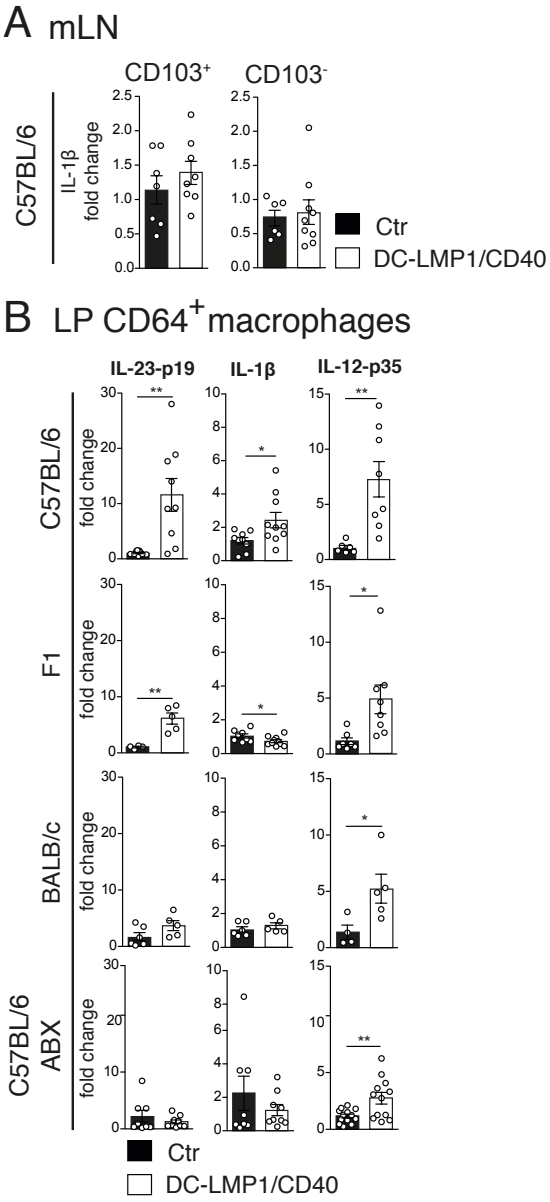


Figure 5.26: CD64$^+$ macrophages produced IL-23p19, IL-1β and IL-12-p35 in C576BL/6 DC-LMP1/CD40 animals upon inflammation, while only IL-12-p35 production was directly caused by LMP1/CD40 transgene. A) CD103$^+$ and CD103$^-$ DCs were sorted from mLNs (single, live, MHCII$^+$CD11c$^+$CD64$^-$) of C57BL/6 control and C57BL/6 DC-LMP1/CD40 animals. Trizol RNA isolation protocol was used for RNA isolation, from which cDNA was prepared. Samples were analyzed by qPCR and data was normalized on HPRT expression. Shown is pooled data (n = 6-9). **B)** CD64$^+$ macrophages were sorted from LP (single, live, CD45$^+$MHCII$^+$CD11c$^+$CD64$^+$) of C57BL/6 DC-LMP1/CD40, F1 DC-LMP1/CD40, BALB/c DC-LMP1/CD40 and ABX treated C57BL/6 DC-LMP1/CD40 animals. Control group for each mice strain background and ABX therapy was included. Trizol RNA isolation protocol was used for RNA isolation, from which cDNA was prepared. Samples were analyzed by qPCR and data was normalized on HPRT expression. Shown is pooled data (n = 4-10). All bar graphs represent mean ± SEM where significance was analyzed using a students t-test, with *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Ctr = control, ABX = antibiotics.

6 Discussion

The fact that genetic background of mouse strains can influences the outcome of experiments has been shown for many different autoimmune diseases before. Different genetic mutations and polymorphisms are the reasons for divergent patterns of immune responses and consequently a different susceptibility to diseases between mouse strains. Conventional transgenic and gene knockout technologies have enabled modeling of human diseases in experimental mice. However, when phenotyping genetically modified mice it is important to consider that targeted genes may not solely contribute to the outcome of the experiments. It is important to be aware of the effect of strain background, microbial milieu, environmental and epigenetic factors [231].

In recent years our group has been studying the constitutive CD40-triggering of DCs, its effects on DCs maturation, on DCs homeostasis and on peripherial tolerance induction [208]. We used the advantage of transgenic mice expressing a LMP1/CD40 transgene [209]. Due to breeding LMP1/CD40 transgenic mice to CD11c Cre animals, only $CD11c^+$ cells such as DCs and macrophages [213] do express the transgene.

Initial experiments have been done with C57BL/6 DC-LMP1/CD40 animals, for which we have shown that the constitutive triggering of the CD40 pathway induced a migration of tolerogenic DC subsets from LP to mLNs. This led to a reduction of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs (Fig.3.1 B), was accompanied by a strong reduction of $Helios^-$ peripherial induced Treg cells (Fig.3.2 A) and resulted in a breakdown of intestinal tolerance with increased amounts of IL-17^+IFN-γ^+ and IFN-γ^+ T cells in lamina propria of transgenic animals (Fig.3.2 B) [208]. Colonic Tregs in these animals could not regulate the inflammatory response against commensal bacteria, which caused a colitis-like phenotype and a drastically shortened life lifespan of C57BL/6 DC-LMP1/CD40 mice (Fig.3.1 A). However, DC-LMP1/CD40 transgenic animals in the BALB/c and in mixed C57BL/6 x BALB/c backgrounds (here called F1 DC-LMP1/CD40 animals) surprisingly did not develop any intestinal pathology (nor any other) (Fig.5.1 A and C). This observation further confirmed the point

of the paper by the Sellers group [231], where they warn that immunological variation between inbred laboratory mouse strains can have a big influence on phenotyping results from genetically modified mice.

BALB/c and C57BL/6 mice are two of the most commonly used inbred mouse strains which are well known to have different immune responses. While differences in disease susceptibility do not come as a surprise, the elucidation of the underlying reasons may help in understanding the molecular cause and thereby help in developing novel treatments.

Several differences between C57BL/6 and BALB/c animals have been described. BALB/c are albino mice and are of the $H2^d$ MHC haplotype, while C57BL/6 have black fur and a $H2^b$ MHC haplotype. A study which compared inbred mice of congenic strains that differ only in their H2 haplotypes has shown the effect of haplotype on the production of several cytokines. Cells from mice with $H2^b$ MHC haplotype responded to *in vitro* mitogen stimulation with lower levels of IL-3, IL-4, IL-5, TNF, IL-6 and IL-10 [232].

Additionally, one big difference between those two strains lies in the Th1/Th2 induction capacity [233]. It was demonstrated that C57BL/6 is a prototypical Th1 mouse strain, whose macrophages upon activation produce more TNF-α, IL-12 and IFN-γ than BALB/c mice, which have a more Th2 directed immune response with a higher production of IL-4 [234, 235]. A different pattern of cytokine production makes BALB/c mice more susceptible to intracellular parasite infections. A good example for that is experimental infection with the intracellular protozoan *Leishmania major*, where the Th1 directed immune response of C57BL/6 mice offers good protection, while the Th2 directed immune response of BALB/c mice is not efficient [236].

On the other hand this type of immune response makes BALB/c mice more resistant to autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) [237, 238], experimental autoimmune myasthenia gravis [239] and experimental autoimmune uveitis [240, 241, 242] when compared to a highly susceptible C57BL/6 mouse strain.

Another study, which compared Treg cells between BALB/c and C57BL/6 mouse strains,

showed that BALB/c mice have more Tregs than C57BL/6 mice and that $CD4^+CD25^-$ (responder) T cells from C57BL/6 mice are in addition notably less susceptible to suppression by Treg cells [243]. However, it is important to mention that this study did not use FoxP3 as a marker to identify Treg cells. In this study all $CD25^+$ cells were assumed to be Tregs, which is according to modern standards not a correct definition anymore, as CD25 is also expressed on all activated T cells [244].

Observations from all these studies highlight the importance of the mouse genetic background in the planning and interpretation of the immunological studies. The above listed differences in immune response between C57BL/6 and BALB/c mouse strain could at least in part contribute to the differences in colitis susceptibility between DC-LMP1/CD40 animals on different genetic backgrounds. However, the purpose of this thesis was to precisely analyze the phenotypical differences between DC-LMP1/CD40 animals on different genetic backgrounds with the goal to identify the exact reason why only C57BL/6 mouse strain develop colitis upon constitutive triggering of the CD40 pathway.

6.1 Phenotypic analysis of DC-LMP1/CD40 animals on different genetic backgrounds

The experiments with DC-LMP1/CD40 animals have shown that transgenic animals on C57BL/6 genetic background developed a colitis-like phenotype, which depended on lymphocytes and commensal bacteria, since $RAG^{-/-}$ C57BL/6 DC-LMP1/CD40 and ABX treated C57BL/6 DC-LMP1/CD40 animals were protected from disease development (Fig.3.1 and 5.1).

From human patients it is known that ulcerative colitis develops in genetically susceptible individuals, due to an inappropriate immune response towards normal gut micro-flora. It was shown that genetic mutations, which increase intestinal barrier permeability lead to a higher risk for disease development [245]. The very same mechanism is used in dextran sulfate

sodium induced model of colitis [246] where mice develop a colitis-like phenotype, which is most likely the result of damage in the intestinal epithelial mono-layer and the penetration of bacteria into gut lamina propria. Similarly, like in our DC-LMP1/CD40 colitis model, also in colitis model of DSS [247] as well as in IL-10 gene-deficient mice [248] ablation of bacteria with ABX significantly improved clinical and histopathological severity of disease.

What is more interesting than the dependency of colitis development on bacteria is that only C57BL/6 animals developed an intestinal pathology as a result of constitutive CD40 stimulation (Fig.5.1). In DC-LMP1/CD40 animals on F1 genetic background no inflammation could be observed on a macroscopic level, while histological analysis has shown only minor changes. Analysis with the non-invasive inflammatory marker lipocalin-2 [221] confirmed that inflammation is highly present in C57BL/6 DC-LMP1/CD40 animals, while F1 transgenic animals showed only slightly increased levels of fecal lipocalin-2 (Fig.5.1 C). In contrast, BALB/c transgenic animals showed no visible signs of inflammation, nor did they have an increased amount of fecal lipocalin-2 (Fig.5.1 C).

When differences between DC-LMP1/CD40 animals on a molecular level were searched for and the colon cytokine profile of transgenic animals on C57BL/6 and F1 genetic backgrounds was analyzed, yet again changes were observed only in C57BL/6 transgenic animals (when compared to controls). Whole gut homogenate samples of C57BL/6 DC-LMP1/CD40 animals have shown a significant increase of pro-inflammatory cytokines MCP1, TNF-α, IFN-γ and IL-6, while anti-inflammatory IL-10 was significantly reduced (Fig.5.2). Increased inflammatory cytokine levels including TNF-α and IL-6 have previously been connected with pathogenesis of colitis [249], where treatment with the TNF-α blockers has proven to be beneficial for patients [250, 251]. For MCP1 is known that it regulates the migration of monocytes from the blood across the vascular epithelium, which is an important factor for the onset of inflammation [252]. Therefore, high levels of MCP1 in C57BL/6 DC-LMP1/CD40 animals might contribute to the extensive lamina propria infiltration of mixed inflammatory mononuclear cells, which could be observed in these animals, while they could not be seen in F1 transgenic animals.

On the other hand IL-12 is known to promote Th1 immune responses and INF-γ production, where its blocking was shown to prevent spontaneous colitis in IL-10 deficient mice [253]. The absence of IL-12-p70 cytokine increase in whole gut homogenate samples of C57BL/6 transgenic animals (Fig.5.2) is in conflict with the qPCR data from CD64$^+$ macrophages in this animals, which showed a significant increase in the expression of IL-12p35 (Fig.5.20), which is a sub-unit of IL-12-p70. Even if high levels of IL-12 in C57BL/6 transgenic animals would help to explain high frequencies of IL-17$^-$IFN-γ^+ T cells present only in transgenic animals of C57BL/6 origin, the data obtained with CBAs are more informative, since it is known that many post-transcriptional mechanisms control the final protein abundance [254].

However, since pro-inflammatory cytokines were not measured in whole gut homogenate samples of ABX treated C57BL/6 DC-LMP1/CD40 animals, the factor of inflammation in C57BL/6 DC-LMP1/CD40 animals can not be excluded. Latter makes it impossible to differentiate if elevated pro-inflammatory cytokine levels were a cause for the disease or were actually its consequence.

6.1.1 Reduction of tolerogenic CD103$^+$ DCs was independent of the genetic background

The effect of CD40 signaling on CD103$^+$ DCs was first shown by the group of Fiona Powrie. In this study CD40 stimulated sorted DCs down regulated CD103 expression and anti-CD40 injection into Rag$^{-/-}$ mice decreased CD103$^+$MHCII$^+$ cells in the spleen and caused wasting disease with colitis [142]. Because of this finding and the constant CD40 signaling in our transgenic mice, CD103$^+$ DC subsets have been analyzed. We recently published that C57BL/6 DC-LMP1/CD40 animals had a strong reduction of CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ DCs, while CD103$^-$ DCs were increased. However, the increase of CD103$^-$ DCs was a secondary inflammatory effect and not a direct effect of the LMP1/CD40 transgene, since it was not the case in ABX treated animals or in Rag$^{-/-}$ animals [208]. The comparison of C57BL/6 DC-LMP1/CD40 animals with transgenic animals on partial or complete BALB/c

genetic backgrounds has shown that a reduction of $CD103^+$ tolerogenic DCs was background independent (Fig.5.3 A and B). The latter made the fact that these animals did not develop the disease even more interesting. However, the percentage of remaining $CD103^+$ DCs was higher in transgenic animals with partial or complete BALB/c genetic backgrounds. Since transgenic animals were on a different genetic backgrounds, the direct comparison between the frequency of DC subsets was not possible. Therefore we calculated the percentage of DCs subset in transgenic animals with the background controls providing a reference point of 100 percent. Relative frequencies calculated in that way allowed us to compare between DC-LMP1/CD40 transgenic animals on different genetic backgrounds and we could show that $CD103^+CD11b^-$ DCs were the most reduced in C57BL/6 transgenic animals (Fig.5.5). From the recently published study of Esterhazy et al. it is known that this $CD103^+CD11b^-$ DC subset is the one with the highest tolerogenic properties as it expresses the highest levels of Aldh1a2, Tgfb2 and Itgb8, enzymes crucial for Treg cells induction and imprinting of gut homing [114]. Differences in the frequency of LP $CD103^+CD11b^+$ DCs between different transgenic animals could not be found. However, it could be demonstrated that BALB/c DC-LMP1/CD40 animals had the highest frequency of these cells in mLNs, while the frequency in C57BL/6 transgenic animals was the lowest. This $CD103^+$ DC subset was shown to be less efficient in Treg induction due to different gene signature with other isoforms of Aldh1a2, Tgfb2 and Itgb8 genes expressed [114]. Additionally, a relative increase of LP $CD103^-$ DCs in C57BL/6 transgenic animals when compared to transgenic animals of other backgrounds could be observed. As already mentioned, we assume that this increase was a secondary effect of the inflammation, since this DC subset was only slightly increased in ABX treated C57BL/6 DC-LMP1/CD40 animals [208]. The latter suggests that the pro-inflammatory milieu in C57BL/6 DC-LMP1/CD40 mice caused changes in the migration and renewal of this heterogeneous and less well described DC subset, while inflammation was significantly weaker in F1 and not detectable in BALB/c transgenic animals and therefore also $CD103^-$ DCs were less increased.

In DC-LMP1/CD40 transgenic animals all CD11c expressing cells received constant CD40 signaling, therefore it was interesting that only $CD103^+$ DCs were reduced. We wanted to show, that the observed difference stems from differences in the biology between DCs subsets and not from a difference in the expression of the LMP1/CD40 transgene between DCs subpopulations. The comparison in the expression of LMP1/CD40 transgene between $CD103^+$ and $CD103^-$ sorted DCs from LP of transgenic animals has shown that the transgene was also expressed in $CD103^-$ DCs. Expression was even higher as compared to the $CD103^+$ DCs (Fig.5.4), which suggests a different sensitivity of DC subsets to CD40 stimulation.

Some studies have reported that CD40 signaling improves the antigen presenting potential of stimulated DCs with an up-regulation of MHC class II and co-stimulatory molecules like CD80 and CD86 [129, 130, 131], whilst others have published that CD40 signaling alone is not sufficient for the complete maturation of DCs, because it only induces the secretion of IL-12-p40 and not its bioactive heterodimer IL-12-p70 [135]. When it was analyzed if the expression of LMP1/CD40 transgene induced DCs maturation and if the degree of maturation differed between C57BL/6 and F1 transgenic animals, a slight background independent up-regulation of the CD80 co-stimulatory molecule on LP DCs could be found. Additionally, a strong down-regulation of MHC II molecule on DCs, which was stronger for C57BL/6 transgenic animals, was observed (Fig.5.6). All studies [129, 130, 131] which reported that CD40 signaling causes up-regulation of MHC class II molecule on DCs, were performed *in vitro*. Therefore, the inconsistency between our observations and results from *in vitro* studies probably arises from distinctive maturation of *in vitro* versus *in vivo* anti-CD40 matured DCs. The phenomen which has previously been reported by the group of Frleta et al. [255]. However, the differences probably also arise from the fact that stimulation with CD40 mAb gives a short strong stimulation, while LMP1/CD40 transgene results in constant stimulation of CD40 pathway. Therefore, the DCs in DC-LMP1/CD40 animals might be in some way adapted to the constant CD40 stimulus, since the intensity of signal stays unchanged.

The next interesting observation was that mLNs $CD103^+CD11b^-$ DCs of both transgenic

strains showed a down-regulation of CD86 co-stimulatory molecule. This was also not consistent with the existing literature about the effects of anti-CD40 mAb on the maturation of DCs. Frleta et al. reported that *in vivo* anti-CD40 matured DCs up-regulated CD86, which peaked around day 2-3 and then declined to baseline level [255]. However, they were only analyzing splenic DCs, while we were interested in mLN and LP DCs, and they stimulated cells with an anti-CD40 antibody, while DCs in our transgenic animals were stimulated due to the genetic construct. The latter is probably the reason for the differences between our observations.

Experiments with C57BL/6 and F1 transgenic animals have shown that DCs in both mouse strains showed similar activation profile. Besides the differential down-regulation of MHC II molecule, no major differences could be observed. This effect was most likely due to inflammation in C57BL/6 but not in F1 transgenic animals.

Lower levels of the CD86 co-stimulatory molecule on the most tolerogenic CD103$^+$CD11b$^-$ DCs subset [114] as well as an overall strong reduction of CD103$^+$ DCs should have an effect on the peripheral immune tolerance, therefore this was our next question to be approached.

6.1.2 DC-LMP1/CD40 mice lack iTreg cells

In our recently published paper we reported that C57BL/6 DC-LMP1/CD40 animals had strongly reduced frequencies of RORγt$^+$Helios$^-$ iTreg cells (Fig.3.2 A). Since F1 and BALB/c DC-LMP1/CD40 transgenic animals did not develop intestinal inflammation, we analyzed if they also lack iTreg cells. We confirmed that Helios$^-$ iTreg cells were reduced in the LP of all DC-LMP1/CD40 animals when compared with controls of the same strain (Fig.5.8). However, as with CD103$^+$ DCs, Helios$^-$ iTreg cells were also the most reduced in C57BL/6 transgenic animals, while in F1 transgenic animals and especially in BALB/c transgenic animals they were present in significantly higher frequencies (Fig. 5.9). This finding would be in line with the lowest frequencies of the most tolerogenic [114] CD103$^+$CD11b$^-$ DCs subset in animals on the C57BL/6 background. CD103$^+$ DCs were shown to have a unique capacity to induce

Foxp3 Treg cells from naïve $CD4^+$ T cells in the periphery, due to their high expression of the enzyme aldehyde dehydrogenase, which converts vitamin A into retinoic acid, and high expression of tissue plasminogen activator, transforming growth factor-beta 2 and latent TGF-β-binding protein 3 [107, 111, 108]. We have shown that in C57BL/6 DC-LMP1/CD40 animals the total amount of $Foxp3^+$ Tregs cells were not changed, when compared with controls [208]. What was changed, was the ratio between nTreg and iTreg cells.

iTreg cells develop in the periphery from naïve $CD4^+$ T cells and have been shown to be crucial for a balance between pro- and anti-inflammatory mechanisms at mucosal interfaces of the gastrointestinal tract, where the immune system comes in close contact with commensal bacteria and food antigens [256]. Their enhanced suppressive capacity and superiority over nTreg cells in gut-specific immune responses has recently been proven in the T-cell transfer colitis model [257]. Therefore, high frequencies of nTreg cells in DC-LMP1/CD40 animals could not compensate for the loss of iTreg cells, since the TCR of iTregs cells are of different specificity and are shaped by local microflora [27, 30].

We were able to show that there was indeed a strong correlation between the frequency of $CD103^+$ tolerogenic DC subsets and the frequency of iTreg cells in the colon lamina propria and in the mLNs (Fig.5.10). Analysis of the $CD103^-CD11b^+$ DCs has shown a negative correlation with iTreg cells in LP and in mLNs (Fig.5.10), what is consistent with literature where $CD103^-$ DCs are described as being more inflammatory and not efficient in inducing iTreg cells [108, 111].

All these observations and reports from other studies suggested that a higher frequency of $CD103^+$ DCs in F1 and BALB/c DC-LMP1/CD40 animals seemed to be sufficient for a partial induction of iTreg cell compartment, which managed to control the immune response against commensal bacteria and has prevented colitis development in these animals. However, the question why constitutive CD40 signaling affected $CD103^+$ DCs of F1 and BALB/c animals to a smaller extent than $CD103^+$ DCs of C57BL/6 animals, remains unanswered.

6.1.3 C57BL/6 DC-LMP1/CD40 mice showed a breakdown of intestinal tolerance

The gastrointestinal tract is the place where the immune system comes in close contact with food antigens and with a diverse microbial ecosystem. It is known that the microflora of a healthy human contains more than 100 trillion microorganisms with a genome which is 100 times larger than the whole human genome [258]. In the gastrointestinal tract, the immune system faces a complex population of beneficial commensal bacteria, which even though they are foreign to the body, do not trigger the immune response in healthy individuals. Therefore, the ability of the immune system to differentiate commensal bacteria from pathogens seems even more remarkable. As far back as in 1995 the group of Duchmann et al. published that normal individuals are tolerant to their own microflora, while they respond to the bacteria of others. However, this is not the case for patients with IBD, who are not tolerant to their own microflora [259].

The breakdown of intestinal tolerance could also be observed in C57BL/6 DC-LMP1/CD40 animals and it resulted in strong intestinal inflammation, which could be prevented by a depletion of bacteria achieved with antibiotics. In transgenic animals with colitis an exacerbated inflammatory Th17/Th1 T cell response could be seen [208]. The latter was most likely a consequence of their lack of iTreg cells, which are known to be crucial for immune cell homeostasis and prevention of uncontrolled expansion of effector T cells against harmless antigens [260]. iTreg and Th17 cell responses in the intestine are supposed to be reciprocally regulated, to enable efficient inflammatory responses during host defense [261]. Both T cell populations require TGF-β for their development, while additional IL-23 and IL-6 signaling promotes differentiation to Th17 cells [262, 263].

In F1 DC-LMP1/CD40 animals a different distribution of T cell subsets has been found. Here, in contrast to C57BL/6 DC-LMP1/CD40 animals IFN-γ^+ T cells were not elevated, while increased amounts of IL-17$^+$ T cells were found in the LP (Fig.5.11). In contrast,

BALB/c DC-LMP1/CD40 animals did not differ from BALB/c wt controls in their T cell subset distribution.

It is known that Th17 cells are not a homogeneous group of cells, since they can be protective [171] or they can be responsible for the development of autoimmune diseases [172]. Th17 cells differentiated only with TGF-β1 and IL-6 are protective [173, 175], whilst their further differentiation with IL-23 [176, 163] results in pathogenic Th17 cells, which express T-bet [176, 163]. Considering this, it might be possible that Th17 cells, which were seen in F1 DC-LMP1/CD40 animals, could be the protective Th17 subset. In order to better characterize them, the T-bet levels in IL-17$^+$IFN-γ^- T cells from C57BL/6 and F1 DC-LMP1/CD40 animals were analyzed. We were able to show that IL-17$^+$IFN-γ^- T cell subset in C57BL/6 DC-LMP1/CD40 animals had a significantly higher expression of T-bet when compared to IL-17$^+$IFN-γ^- T cells from C57BL/6 controls. However, this phenomen could not be observed in IL-17$^+$IFN-γ^- T cells from F1 transgenic animals (Fig.5.14 B). The latter suggested a higher degree of pathogenicity for IL-17$^+$IFN-γ^- T cells of C57BL/6 origin. It has been published that Th17 cells can start expressing IFNγ and in some inflammatory settings even stop expressing IL-17 [177]. In literature these cells are called "ex-Th17" cells or "Th1-like" cells. Studies, as the one from Harbour et al., have reported that the transition from Th17 to Th1-like cells was absolutely required for disease development [264].

Analysis of T-bet in IL-17$^+$IFN-γ^-, IL-17$^+$IFN-γ^+ and IL-17$^-$IFN-γ^+ effector T cells in C57BL/6 DC-LMP1/CD40 animals showed increased levels of T-bet, where IL17 single positive cells had the lowest expression and IFN-γ single positive had the highest T-bet expression (Fig.5.14 A). The latter indicated that IFN-γ^+ T cells, which are present in high frequencies in transgenic animals on C57BL/6 genetic background, might have actually been the ex-Th17 cells, which have lost IL-17 expression to become pathogenic Th17 cells. In order to analyze this possibility, it would be necessary to breed IL-17 and IFN-γ^+ reporter genes into the DC-LMP1/CD40 background to follow the fate of Th17 cells and prove the origin of IFN-γ^+ T cells in C57BL/6 DC-LMP1/CD40 animals. Hirota et al. have published that

IL-1R1 expression on IFN-γ^+ T cells proves their Th17 origin, since real Th1 T cells do not express this receptor [177]. Unfortunately, a good antibody against IL-1R1 does not yet exist. Therefore, the only option would be a qPCR for IL-1R1 gene made with cDNA isolated from sorted IL-17$^-$IFN-γ^+ T cells. Since IL-17 and IFN-γ stainings are intracellular, this requires cell fixation and makes them unsuitable for cell sort and further qPCR analysis. The cross of two reporter genes into our mouse model would be a long process which exceeds the time frame of our study. Therefore, we could not prove that IL-17$^-$IFN-γ^+ T cells, which were increased in C57BL/6 animals, were ex-Th17 cells and not real Th1 cells. However, even if we could not claim this for sure our results strongly suggested that F1 DC-LMP1/CD40 animals had more regulated non pathogenic Th17 cells, while IL-17$^-$IFN-γ^+ T cells in C57BL/6 DC-LMP1/CD40 animals had more alternative phenotype and a potential to develop in pathogenic ex-Th17 cells, which might be responsible for colitis development.

6.2 CD40 signaling induced migration of intestinal DCs from LP to mLNs

As mentioned in the introduction, the effects of CD40 signaling in DCs *in vivo* can be studied in two ways. Additionally to the DC-LMP1/CD40 mouse model, where we have a DC specific triggering of the CD40 pathway, anti-CD40 mAb can be injected intraperitoneally into a mouse and its effects on DCs are analyzed later. Both methods have their advantages and disadvantages. The disadvantage of an anti-CD40 mAb injection and the main reason why we started our study with DC-LMP1/CD40 animals, is that the effect is systemic. All cells which express the CD40 molecule are affected, therefore it is impossible to differentiate between effects of anti-CD40 mAb on DCs and on other cells. Besides DCs CD40 is expressed on follicular DCs, monocytes, epithelial cells, endothelial cells, hematopoietic progenitor cells and carcinomas [118, 119, 120, 121]. A study by Kimura et al. has reported the necroinflammatory response in the liver, as a response to CD40 stimulation of B cells [143]. Therefore the observed

effect on DCs after the anti-CD40 treatment, could be just a secondary effect of a cytokine storm induced by some other cell type.

However, the use of anti-CD40 mAb also has some advantages. The biggest limitation of the DC-LMP1/CD40 mouse model is that the signal can not be induced. This makes it impossible to identify the first event in the cascade of events. Therefore, we used the advantage of anti-CD40 injection to demonstrate that CD40 signaling induces the migration of intestinal DCs from LP to mLNs. After an intraperitoneal injection of anti-CD40 mAb in wt C57BL/6 animals, $CD103^+$ DC subsets were strongly reduced in LP, while their amount increased in mLNs [208]. A similar effect of anti-CD40 antibody has previously been published by the group of Fiona Powrie, who has shown that $Rag^{-/-}$ mice injected with the anti-CD40 antibody had fewer $CD103^+MHCII^+$ cells in the spleen [142].

Kinetics experiments performed by Christian Barthels have shown that the amount of the $CD103^+$ DCs in the mLNs reached its peak 24 h post injection [208]. This finding could be reproduced and in addition CCR7 protein expression on DCs was analyzed. The expression of chemokine receptor CCR7 was increased on LP DCs, where $CD103^+$ DCs had the strongest expression (Fig.5.15).

Since CCR7 is critically important for the migration of LP DCs via afferent lymph to the T cell areas in mLNs [55, 265], this supports our hypothesis that DCs in LP are reduced due to their migration. In addition Christian Barthels could demonstrate that DC migration is induced by direct triggering of DCs by anti-CD40 antibody and not due to some secondary potentially inflammatory effects. To this end he used mice which lack CD40 specifically on DCs (CD11c-Cre x $CD40^{fl/fl}$, (Lutgens et al. unpublished)), where anti-CD40 antibody treatment could not induce DC migration from the LP to the mLNs [208].

In this study the antibody approach was used to compare the effects of anti-CD40 mAb treatment on wt BALB/c and C57BL/6 animals. A purpose of this experiment was to investigate if both mouse strains would react differentially to CD40 triggering. The anti-CD40 antibody treatment of BALB/c and C57BL/6 animals strongly reduced the frequency of

$CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs in the LP of both strains (Fig.5.15 A). In addition, highly increased frequencies of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs in mLNs could be observed (Fig.5.16 B).

However, it could be seen that even if $CD103^+CD11b^-$ DCs in anti-CD40 treated BALB/c animals were strongly reduced, they were still as many as in un-injected C57BL/6 animals. Since mice of the BALB/c strain had more $CD103^+CD11b^-$ DCs as compared to C57BL/6 mice, it was possible that the amount of anti-CD40 antibody used for injection was limiting. However as 200 μg of anti-CD40 antibody were injected, this high amount is hard to imagine to be insufficient to stimulate all DCs in BALB/c mice.

Analysis of CCR7 on LP DCs from anti-CD40 treated C57BL/6 and BALB/c animals has shown that stimulation of CD40 pathway in C57BL/6 animals resulted in stronger up-regulation of CCR7 especially on $CD103^+$ DCs (Fig.5.16 C). The differential up-regulation of CCR7 chemokine receptor on CD103 positive DCs between C57BL/6 and BALB/c animals, as a result of CD40 signaling, could explain why constant CD40 triggering in DC-LMP1/CD40 animals had the strongest effect on C57BL/6 transgenic animals, intermediate on F1 and the smallest ob BALB/c transgenic animals.

Es expected, the analysis of F1 wt animals revealed that they had fewer $CD103^+CD11b^-$ DCs than BALB/c, but more than C57BL/6 animals (Fig.5.17 B). However, different steady state amount of $CD103^+CD11b^-$ DCs between wt animals of C57BL/6, F1 and BALB/c strains probably does not explain the differences which were observed between different strains of DC-LMP1/CD40 animals. While in the anti-CD40 mAb approach it was theoretically possible to inject an insufficient amount of the antibody as a limiting factor, in the LMP1/CD40 transgene setting every single DC should has received identical CD40 stimulation.

Additionally, the activation status of C57BL/6 and BALB/c DC after *in vivo* stimulation with anti-CD40 mAb was compared. We could not observe an effect on expression of MHC II molecule on DC subsets, while CD80 and CD86 co-stimulatory molecules were strongly up-regulated (Fig.5.18). From these results, it could be seen that anti-CD40 mAb (Fig.5.18)

activated DCs more than the LMP1/CD40 transgene (Fig.5.6). A comparison between two mouse strains has shown that BALB/c DCs responded to anti-CD40 mAb stimulation with a higher expression of CD80 and CD86 co-stimulatory molecules (Fig.5.19). Higher expression of co-stimmulatory molecules is a known characteristic of phenotypically mature DCs, which are known to be more prone in inducing immunity than tolerance [12]. This observation was therefore in conflict with data from DC-LMP1/CD40 animals, which showed the highest frequencies of iTregs and the lowest frequencies of Th1/Th17 effector cells in transgenic BALB/c animals. However, in DC-LMP1/CD40 mouse model in contrast to anti-CD40 antibody approach we could not see that DCs of F1 transgenic animals would be phenotypically more mature than those from C57BL/6 transgenic animals (Fig.5.7) (BALB/c DC-LMP1/CD40 animals were due to limitation in experimental animals not analyzed).

6.3 Activated CD4 T cells trigger reduction of CD103$^+$ DCs

Our data from *in vivo* experiments with anti-CD40 mAb and analysis of DC-LMP1/CD40 mice suggested, that CD40 ligand expressing cells in the state of inflammation might be able to temporarily shut down the tolerogenic mechanisms in order to improve the efficiency of the immune response. For example, activated CD4$^+$ T cells, which are known to express CD40 ligand [223], could theoretically triggered the CD40 pathway in DCs and according to our data caused a reduction of tolerogenic CD103$^+$ DCs. This mechanism could reduce the capacity to induce tolerance by iTreg induction and would strengthen the immune responses.

We tried to prove our hypothesis with the CD4$^+$CD62L$^+$ transfer model of colitis [198], which resembles the well known CD45RB^{hi} model [199]. CD4$^+$CD62L$^+$ T cells from donor wt C57BL/6 mice were transferred to a Rag1$^{-/-}$ recipient, which resulted in chronic colitis. When Treg depleted T cells are transferred into "empty" Rag1$^{-/-}$ recipient the transferred

cells strongly proliferate to fill up the empty niche. This activated $CD4^+$ T cells will express CD40 ligand [223] and their effect on DCs of recipient mice was analyzed in our experiments. We could shown that 15 days after the adoptive transfer $CD103^+CD11b^-$ DCs were significantly reduced in the LP (Fig.5.20). This result supported our hypothesis on CD40-CD40 ligand contribution to the balance between tolerance and immunity as tolerogenic DCs were significantly reduced.

6.4 SNP genotyping of F2 DC-LMP1/CD40 mouse strain

An SNP genotyping study using the Ilumina Mouse LD Linkage platform was designed with the purpose of identifying new candidate genes for different colitis susceptibility between C57BL/6 and BALB/c transgenic mice strains. We analyzed only F2 DC-LMP1/CD40 with visible signs of colitis. Since F2 transgenic animals were genetically and phenotypically diverse, we were searching for a region in the mouse genome which would have the C57BL/6 genetic origin in all sick F2 DC-LMP1/CD40 animals. Ilumina Mouse LD Linkage platform included SNP loci spread all over the mouse genome for which the BALB/c and the C57BL/6 mouse strains had different alleles. Since only DC-LMP1/CD40 animals on a C57BL/6 genetic background developed colitis, our hypothesis was that such a region may include a gene which would be responsible for increased colitis susceptibility in transgenic C57BL/6.

However, we could not identify a region in F2 DC-LMP1/CD40 animals which was homozygous for the C57BL/6 background. In contrast we found one SNP which was in every animal heterozygous for C57BL/6 and BALB/c alleles. Although, this was not the outcome we expected, we analyzed this region of chromosome two further and found 16 candidate genes which could be relevant for immunological process (Fig.5.21 C). In order to preform a study with higher resolution we would probably have to include more animals and use another SNP analysis method. In addition it could be important to increase the parameters of

colitis analysis in our mice, as we most likely have included animals with different degrees of disease. This would explain why some of the analyzed F2 DC-LMP1/CD40 mice carried one C57BL/6 and one BALB/c allele for the SNPs in the identified region. Most likely it would have been important to perform precise pathological analysis with histology for every animal included in the SNP study. Nevertheless, among the 16 identified candidate genes we also found IL-1α and IL-1β genes, which are profoundly up-regulated in the inflamed colonic musosa from ulcerative colitis patients [224, 225]. As these genes were of relevance for our colitis model we continued to study their roles.

6.4.1 Testing IL-1α and IL-1β as candidate genes for colitis susceptibility

Interleukins are a sub-type of cytokines, messenger molecules with strong immunomodulatory functions. They can be pro- or anti-inflammatory and have been shown to be important for the differentiation and activation of immune cells [266]. The interleukin one family is known to be composed of strong pyrogens and T cell co-stimulators and contains in addition to IL-1α and IL-1β 9 other members [267]. The regulation of these important inflammatory cytokines especially IL-1β is very strict, as they can cause several autoinflammatory diseases, chronic inflammation, septic shock or even death [267].

Since the SNP study performed with F2 DC-LMP1/CD40 animals suggested IL-1α and IL-1β as potential candidate genes for increased colitis susceptibility of DC-LMP1/CD40 on C67BL/6 genetic background (Fig.5.21 C) and since high levels of these cytokines, which have a high inflammatory potential have been found in ulcerative colitis patients [224, 225], we designed several experiments to study their role in our colitis model.

With the first experiment, where we measured IL-1α and IL-1β production in LPS primed BMDCs of C57BL/6 and BALB/c origin, we wanted to analyze if BMDCs of different strains differ in IL-1α and IL-1β transcription caused by triggering of PRRs with LPS. For induction of IL-1β cytokine transcription, Toll-like receptors have to be activated with LPS or CpG

[268]. The IL-1β precursor is not yet biologically active [269] and can not be secreted. To become mature, the IL-1β precursor has to be cleaved by active caspase-1. However, for the activation of procaspase-1 the activation of the inflammasome is needed. Inflammasomes are multimeric signalling protein complexes, which detect pathogenic microorganisms or sterile stressors and upon activation proteolytically cleave procaspase-1 into active caspase-1 [270]. Due to such a tight regulation less than 20 % of the total synthesized IL-1β precursors leave the cell in the active 17 kDa form [267].

In contrast, IL-1α is constitutively expressed and does not need to be activated to be biologically active [271]. Also, its localization differs from the localization of IL-1β, since it is mostly found on the cell surface as a membrane-bound IL-1α [272].

We measured a pro-IL-1β from the lysed BMDCs and found that C57BL/6 derived BMDCs produced higher amounts of pro-IL-1β and IL-1α as compared to BALB/c derived BMDCs (Fig.5.22 A).

Because of these *in vitro* results we tested IL-1 levels *in vivo* in C57BL/6 and F1 DC-LMP1/CD40 animals. Interestingly, only C57BL/6 DC-LMP1/CD40 animals had high colonic levels of pro-IL-1β and IL-1α cytokines, while F1 transgenic animals and control animals did not have a detectable amounts (Fig.5.22 B). Unfortunately, without analyzing the colonic levels of ABX treated C57BL/6 DC-LMP1/CD40 animals it was impossible to know if high levels of IL-1 cytokines were a cause of the difference which induced the disease in C57BL/6 transgenic mice, or were the consequence of the disease and were induced by some other unknown mechanism.

However, the fact that IL-1β is highly inflammatory, precisely regulated [267], found in patients with ulcerative colitis [224, 225], strongly produced by C57BL/6 BMDCs and at high levels present in colons of inflamed C57BL/6 DC-LMP1/CD40 animals, made it an interesting candidate gene for different colitis susceptibility between BALB/c and C57BL/6 mouse strains. However, experiments which revealed that C57BL/6 allele for IL-1β did not result in a higher level of IL-1β and that the sick F2 DC-LMP1/CD40 animals did not have

the C57BL/6 allele for IL-1β (Fig.5.23 B and C) were disappointing but did not yet disprove the hypothesis that high levels of IL-1β caused colitis in C57BL/6 transgenic animals. Since IL-1β is regulated on so many levels, it would be possible that BALB/c and C57BL/6 mice strains differ in one of the genes important for its regulation. Therefore, we designed some additional experiments, which will be discussed further on, in order to test the role of IL-1β in our experimental model of colitis.

6.5 Blocking IL-1β prevented colitis development in C57BL/6 transgenic mice

Very high levels of IL-1β were measured in humans suffering from IBD [273] and it is known that IL-1β serves to amplify the mucosal inflammation [274, 275]. Animal studies with the *Helicobacter hepaticus* colitis model and T cell transfer model of colitis have identified multiple mechanisms for how IL-1β promotes intestinal pathology and demonstrated that its blocking may be a useful therapeutic approach [276].

Since many years rheumatoid arthritis is being treated with a blockade of IL-1 cytokine activity, particularly IL-1β [277]. Three IL-1 blockers used in patients are available on the market: anakinra [278], canakinumab [279] and rilonacept [280]. So far blocking of IL-1 cytokines is not yet routinely used as a treatment for colitis in humans. However, since IL-1β levels were elevated only in C57BL/6 DC-LMP1/CD40 animals which also develop fatal colitis, we tried to rescue them with anti IL-1β therapy. For this purpose anti-IL-1β mAb was used. Anti-IL-1β mAb was injected intraperitoneally like published previously in an arthritis model [281]. The dose and the duration of treatment had to be optimized for DC-LMP1/CD40 colitis model (Fig.5.24 A).

It could be shown that anti-IL-1β treatment prevented colitis development in C57BL/6 DC-LMP1/CD40 animals, since their colons were visibly less inflamed than the colons of isotype control treated animals (Fig.5.24 B). Mice were noticeably heavier (Fig.5.24 C) and lipocalin-

2, a marker for intestinal inflammation, could not be detected (Fig.5.24 D). This experiment confirmed that IL-1β plays a crucial role in the development of colitis in our C57BL/6 DC-LMP1/CD40 mouse model. However, neutralization of IL-1β with anakinra has been previously used in treatment of two patients with severe infant-onset medical-refractory colitis and fistulizing disease caused by IL-10 receptor deficiency [282]. This treatment successfully reduced intestinal inflammation and led to clinical, endoscopic and histological improvement in both patients.

Study by the group of Shouval [282] in IL-10 receptor deficient patients and our experiments with anti-IL-1β therapy in C57BL/6 DC-LMP1/CD40 animals therefore suggest that similar studies should be performed on other colitis models with a purpose to study potential benefits of IL-1β blockade in treatment strategies of humans with IBD.

6.6 Blocking IL-1β prevented the expansion of IFN-γ^+ T cells

The observation that blocking of IL-1β prevented colitis development in C57BL/6 transgenic animals made us further investigate the potential mechanism behind this effect. One possible explanation connecting IL-1β to the observed differences in C57BL/6 and F1 DC-LMP1/CD40 mice was that IL-1β is necessary for the formation of the IL-17$^-$IFN-γ^+ effector cells, which were elevated only in C57BL/6 DC-LMP1/CD40 animals (Fig.5.11). If the hypothesis that IL-17$^+$ cells in C57BL/6 DC-LMP1/CD40 animals differentiated to IL-17$^+$IFN-γ^+ and IL-17$^-$IFN-γ^+ T cells would hold true, then these were the so called "ex-Th17" or "Th1-like" T cells, which were previously shown to be critical for colitis development [264]. These Th1/Th17 cells have also been measured in high frequency in the intestinal mucosa of IBD patients [283], which further suggests their pathogenicity. The role of IL-1β in this hypothesis would be the induction of IFN-γ in Th17 cells, which has previously been reported by a study in humans with autoimmune disorders [284]. The described mechanism of action

is that IL-1β together with TCR stimulation renders Th17 cells responsive for IL-12 cytokine and stabilizes the T-bet expression which results in pathogenic Th17 cells that express IFN-γ [284]. Given that IL-1β deficient mice do not develop EAE [285], a multiple sclerosis model which was also shown to be dependent on IFN-γ expressing Th17 cells [286], further supports our hypothesis for the role of IL-1β in DC-LMP1/CD40 model of experimental colitis.

Therefore, it was a logical step to analyze the effector T cell compartment in anti-IL-1β treated C57BL/6 DC-LMP1/CD40 animals. Like we predicted from literature about Th17 plasticity and differentiation, we could show that blocking IL-1β blocks the development of IL-17$^-$IFN-γ^+ T cells in treated C57BL/6 DC-LMP1/CD40 animals (Fig.5.25).

6.7 LMP1/CD40 transgene induces IL-12-p35 production in CD64$^+$ macrophages

After it was demonstrated that sick C57BL/6 DC-LMP1/CD40 animals had high levels of intestinal IL-1β and that anti-IL-1β therapy could prevent the disease development in these animals, we wanted to identify which cell subsets produced IL-1β. According to literature, IL-1 cytokines are primarily produced by stimulated monocytes or macrophages [287]. Confirming this, studies with human IBD patients reported that IL-1β was produced by colonic lamina propria monocytes [288, 289]. Since our transgenic animals were bread with CD11c CRE animals which resulted in only CD11c expressing cells receiving constant CD40 stimulation, all these cell subsets were analyzed for the production of IL-1β. No significant upregulation of IL-1β production in CD103$^+$ or CD103$^-$ DCs from C57BL/6 DC-LMP1/CD40 animals, when compared to control littermates, could be found (Fig.5.26 A). However, a high production of IL-1β in CD64$^+$ LP macrophages, which are also known to express CD11c [50], could be detected in C57BL/6 DC-LMP1/CD40 animals (Fig.5.26 B). In contrast no elevated levels of IL-1β in macrophages from F1 and BALB/c transgenic animals could be measured. Although, C57BL/6 DC-LMP1/CD40 transgenic animals had substantially higher level of

IL-1β cytokine, this was reduced back to background level upon removal of commensal bacteria. The latter together with normal levels in F1 and BALB/c transgenic animals showed, that IL-1β elevation was a secondary effect of inflammation and was not directly caused by LMP1/CD40 transgene.

Additionally, we could show that C57BL/6 DC-LMP1/CD40 CD64$^+$ LP macrophages produced high levels of IL-12-p35 and IL-23-p19 cytokines (Fig.5.26 B). IL-12 and IL-23 cytokines are heterodimers which share a common p40 subunit, while p35 subunit is IL-12 specific and p19 is IL-23 specific [290]. However, the analysis of transgenic animals with partial or complete BALB/c genetic background revealed that IL-23-p19 was reduced in CD64$^+$ LP macrophages from F1 DC-LMP1/CD40 animals, while its levels in BALB/c transgenic animals resembled the levels of BALB/c wt controls (Fig.5.26 B). Interestingly, depletion of commensal bacteria and prevention of inflammation with ABX therapy in C57BL/6 transgenic animals reduced IL-23-p19 cytokine level back to C57BL/6 background control level (Fig.5.26 B). The latter suggested that IL-23-p19 was elevated as a consequence of inflammation, which was strong in C57BL/6 transgenic animals and less intense but detectable in F1 transgenic animals (Fig.5.1). In contrast IL-12-p35 was found to be elevated in CD64$^+$ macrophages of all transgenic animals, even in absence of inflammation (Fig.5.26 B). The latter suggested that this was the only cytokine elevated due to direct effect of LMP1/CD40 transgene. However, also IL-12-p35 was the most elevated in C57BL/6 transgenic animals which were not treated with ABX, what suggest that the presence of inflammation even increased IL-12 production. It would be interesting to repeat qPCR analysis for IL-12-p35 gene in the way that the IL-12-p35 levels could be directly compared between transgenic animals of different genetic origins. For this purpose all samples would have to be analyzed in the same qPCR experiment. However, due to small amounts of cDNA which could be obtained out of low numbers of LP CD64$^+$ macrophages, we did not have enough material for this experiment. Therefore, for this purpose an additional sort of CD64$^+$ macrophages for all DC-LMP1/CD40 strains would be needed. However, it is known from the literature that BALB/c and C57BL/6 mouse strains

differ in Th1/Th2 induction capacity [233], since it was shown that C57BL/6 macrophages upon activation produce more IL-12 than BALB/c mice, which have in contrast more Th2 directed immune response [234, 235].

Taken together our results suggested that LMP1/CD40 transgene caused elevated levels of IL-12 cytokine in CD64$^+$ macrophages of DC-LMP1/CD40 animals. As it is known from literature, macrophages from C57BL/6 mouse strain are even in wt animals more prone to produce high levels of IL-12 than those from BALB/c animals. The latter leads to a more Th1 directed immune response in C57BL/6 animals [234, 235] and to a certain degree explains high frequencies of IL-17$^-$IFN-γ^+ T cells present only in C57BL/6 DC-LMP1/CD40 animals (Fig.5.11). However, while IL-12 has been connected with Th1 cell differentiation, IL-23 is supposed to play a role in Th17 cells, where it was shown to be important for the stabilization of genes controlling proinflammatory effector molecules and Th17 cell activation [290]. Since Th17 cells may also express IFNγ and in some inflammatory settings even stop with IL-17 expression [177], without IL-17 and IFNγ reporter genes we could not know if IL-17$^-$IFN-γ^+ T cells present in C57BL/6 DC-LMP1/CD40 animals were ex-Th17/Th1-like cells or were the real Th1 cells. Even if we showed that high levels of IL-1β and IL-23-p19 in C57BL/6 DC-LMP1/CD40 animals were a secondary effect of the inflammation, they still formed a perfect cytokine milieu for the development of the pathogenic Th17 cells [176, 163]. According to literature IL-1β renders Th17 cells responsive for IL-12 and thereby stabilizes T-bet expression in Th17 cells [284]. Our data therefore suggests that C57BL/6 transgenic animals developed strong inflammation caused by IL-17$^-$IFN-γ^+ T cells which were caused by LMP1/CD40 transgene which elevated the IL-12 cytokine levels and were in the next step even more increased by differentiation from IL-17$^+$ T cells in the perfect cytokine milieu of the inflammatory settings with the elevated levels of IL-1β and IL-23-p19.

The expansion of pathogenic Th1/Th17 T cell subsets in C57BL/6 DC-LMP1/CD40 animals could in addition not be regulated since these animals lacked iTreg cells, which were not induced due to the absence of tolerogenic CD103$^+$ DC subsets. We could indirectly show that

CD40 signaling affected BALB/c DCs to a smaller extent than C57BL/6 DCs, since stronger up-regulation of CCR7 chemokine on C67BL/6 derived CD103$^+$ DCs, as a consequence of anti-CD40 mAb injection, could be demonstrated. This finding offered the explanation that due to divergent up-regulation of CCR7 chemokine more CD103$^+$ DCs left LP in C57BL/6 transgenic animals than in BALB/c transgenic animals, while F1 transgenic animals had an intermediate phenotype. Higher frequencies of CD103$^+$ DCs in BALB/c transgenic animals caused higher frequencies of iTregs cells, where F1 transgenic animals had intermediate and C57BL/6 transgenic animals the lowest frequencies.

At the end it seems that all pieces fit together and to a certain degree explain why only C57BL/6 DC-LMP1/CD40 animals developed colitis. However, some additional experiments will be needed in order to get a more precise picture picture.

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