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

Direktor: Prof. Dr. Thomas Brocker

CD40 signaling in dendritic cells modulates tolerance and immunity

Dissertation

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

an der medizinischen Fakultät

der Ludwig-Maximilians-Universität München

vorgelegt von

Christian Barthels

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Gedruckt mit Genehmigung der Medizinischen Fakultät der Ludwig-Maximilians-Universität München

Betreuer	Prof. Dr. Thomas Brocker
Zweitgutachter	Prof. Dr. Ludger Klein
Dekan	Prof. Dr. med. dent. Reinhard Hickel
Tag der mündlichen Prüfung	14.03.2016

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Christian Barthels

This work contains work presented in the following publications:

Barthels, C., Ogrinc, A., Steyer, V., Meier, S., Simon, F.C., Blutke, A., Straub, T., Zimber-Strobl, U., Lutgens, E., Kammel, M., Ohnmacht, C., Garzetti, D., Stecher, B., Brocker, T. CD40-signaling abrogates induction of peripheral RORγt+ regulatory T cells by intestinal CD103+ dendritic cells and causes fatal colitis. Manuscript in preparation

Abbreviations

ABX	antibiotics
AF647-Ova	Alexa Flour 647 conjugated ovalbumin
APC	antigen presenting cell
	allophycocyanin
Batf3	basic leucin zipper transcription factor ATF-like 3
BSA	bovine serum albumin
CBL	cecal bacterial lysate
CD	cluster of differentiation
CDP	common DC precursor
CFSE	carboxyfluorescein succinimidyl ester
CMP	common myeloid progenitor
CNS	conserved non-coding DNA sequence
DC	dendritic cells
DSS	dextran sodium sulfate
ELISA	enzyme-linked immunosorbent assay
FLT3	FMS-like tyrosine kinase
GALT	gut associated lymphoid tissue
GM-SCF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte-macrophage precursor
HSC	hematopoietic stem cell
IFN-γ	interferon-γ
ID2	inhibitor of DNA binding 2
iNOS	inducible nitric oxide synthase
IRF8	IFN regulatory factor 8

LC	langerhans cells
LN	lymph node
mAb	monoclonal antibody
MDP	macropage/DC progenitor
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mLN	mesenteric lymph node
NFIL3	nuclear factor interleukin 3 regulated
Notch2	neurogenic locus notch homolog protein 2
Nrp1	neuropilin-1
Ova	chicken ovalbumin
PAMP	pathogen associated molecular patterns
PMA	phorbol-12-myristat-13-acetat
PRR	pattern recognition receptor
RBP-J	Recombining binding protein suppressor of hairless
RelB	v-rel avian reticuloendotheliosis viral oncogene homolog B
SFB	segmented filamentous bacteria
TCR	T cell receptor
TLR	toll-like receptor
TNBS	trinitrobenzesulfonic acid
TNF-α	tumor necrosis factor-α
Tregs	regulatory T cells
iTregs	(peripheral) induced Tregs
sALT	serum alanine aminotransferase

Contents

1 Summary

Self / non-self discrimination is one of the main features of the immune system of higher animals. While encounter with self-molecules should induce tolerance, the appropriate recognition of non-self antigen triggers immune reactions. A careful balance between induction of immunity and tolerance is especially important at body surfaces such as the intestinal tract, where also foreign, commensal-derived antigens must be tolerated. Dendritic cells (DCs) have key roles in this important equilibrium as they can induce both, immunity and tolerance, depending on their maturation status: immature DCs induce tolerance and mature DCs induce immunity.

To further study signals in DCs that control the decision between immunity and tolerance, we focused on CD40, a signal known to induce incomplete maturation of DCs with regard to expression of costimulatory molecules and cytokines, but to be important for DC licensing.

To investigate the influence of a CD40 signal over a longer period of time and without the effects of the mAb on other cell types, we generated transgenic mice, where DCs receive a tonic CD40-stimulus.

These transgenic animals showed only moderately activated DCs in the spleen in terms of costimulatory molecules and cytokine production. At the same time animals developed a strong colitis, characterized by abundant Th1 and Th17 cell in gut and other organs as well as by highly elevated levels of proinflammatory cytokines like TNF-α and IL-6. Disease development was shown to be dependent on commensal bacteria as well as B and T cells.

In these animals we also observed a strong reduction of $CD103^+$ DC cells in the lamina propria of the gut and additionally in the mesenteric lymph node (mLN). This was accompanied by the absence of induced Tregs in colon and mLN and at the same time the presence of more Th1 and Th17 cells.

These data show that a CD40 stimulus on DC produces incompletely matured DCs in the spleen. But in peripheral organs the same signal induces migration into the draining lymph

nodes and DCs functionally change in a way that prohibits the induction of tolerance. This represents a potential mechanism by which activated $CD40L^+$ T cells can influence immune responses by inhibiting the induction of tolerance.

2 Zusammenfassung

Die Selbst-/Fremdunterscheidung ist eines der Hauptmerkmale des Immunsystems höherer Tiere. Während die Erkennung von Selbstantigen Toleranz induzieren sollte, löst die adäquate Erkennung von Fremdantigen eine Immunreaktion aus. Ein empfindliches Gleichgewicht zwischen der Induktion von Immunität und Toleranz ist besonders wichtig an Körperoberflächen wie dem Darm, wo außerdem fremdes, kommensales Antigen toleriert werden muss. DCs spielen eine Schlüsselrolle in diesem wichtigem Gleichgewicht, da sie in der Lage sind beides, Toleranz und Immunität, in Abhängigkeit von ihrem Reifestatus zu induzieren: unreife DCs induzieren Toleranz und reife DCs induzieren Immunität.

Um die Signale, welche die Entscheidung zwischen Toleranz und Immunität kontrollieren, weitergehend zu studieren, haben wir uns auf CD40 fokussiert, ein Signal von dem man weiß, dass es eine unvollständige Reifung von DCs im Bezug auf kostimulatorische Moleküle und Zytokine induziert, aber dennoch wichtig für die Lizensierung der DCs ist.

Um den Einfluss eines CD40 Signals über einen längeren Zeitraum und ohne die Effekte des mAbs auf anderen Zelltypen zu untersuchen, generierten wir eine transgene Maus, in der DCs einen tonischen CD40 Stimulus erhalten.

Diese transgenen Tiere zeigten nur leicht aktivierte DCs in der Milz was die Expression von kostimulatorischen Molekülen und Zytokinen angeht. Gleichzeitig entwickelten sie aber eine starke Colitis, die sich durch eine hohe Frequenz von Th1 und Th17 Zellen in Darm und anderen Organen sowie ein erhöhtes Niveau von proinflammatorischen Zytokinen wie TNF-α und IFN-γ auszeichnete. Die Entwicklung der Krankheit war abhängig von kommensalen Bakterien sowie B- und T-Zellen.

Außerdem beobachteten wir in diesen Tieren eine Reduktion von $CD103^+$ DCs in der Lamina Propria des Darms und im mLN. Dies wurde begleitet von der Abwesenheit induzierter Tregs in Colon und mLN bei einer gleichzeitigen Erhöhung von Th1 und Th17 Zellen.

Diese Daten zeigen, dass ein CD40 Stimulus unvollständig gereifte DCs in der Milz pro-

duziert. In peripheren Organen aber induziert das gleiche Signal eine Wanderung in den Lymphknoten und ändert die Funktionalität der DCs in einer Art und Weise, welche die Induktion von Toleranz verhindert. Dieses stellt einen potentiellen Mechanismus dar, mit dessen Hilfe aktivierte, $CD40L^+$ T-Zellen eine Immunantwort durch Verhinderung der Toleranzinduktion beeinflussen können.

3 Introduction

3.1 The immune system

A hallmark of all higher animals is the ability to distinguish between self and non-self. By this multicellular organisms protect themselves against different kinds of pathogens (e.g. viruses, bacteria, protozoans).

The immune system of vertebrates is divided into two arms. The first is the so called innate immunity, that consists of different layers such as mechanical barriers, like mucus and skin, antibacterial peptides, such as α-defensins and finally cellular components, like NK cells and granulocytes. The innate immune system is characterized by a very fast reaction rate. But at the same time the receptor repertoire is smaller for the respective antigens and less diverse.

The second arm is the so called adaptive immunity. It consist of B and T cells. Contrary to their innate counterparts the antigen receptors of B and T cells are of very high affinity. This high affinity is accomplished in a process called V(D)J-recombination in which an immense variety of different receptor specificities is generated by a limited amount of genetic segments. These are recombined in a semi-random fashion and allow the adaptive immune system to generate cells that recognize virtually all possible foreign- (and self-) antigens. The cost for this high affinity on the other hand lies in very low precursor frequencies. This low abundance leads to a slow reaction time of these cells when they first encounter their antigen, since they have to undergo several rounds of cell devision, before they are ready to fight pathogens.

In the end the innate immune systems is therefore capable of reacting very fast, but only to a limited number of different structures, while the adaptive immune system reacts much slower, but is able to fight a broad range of antigens.

The second problem arising from the semi-random generation of T and B cell receptors (TCR and BCR) is that a receptor generated in such a way has the potential to react against self-antigens and therefore another step of quality control is necessary. This step is called

tolerance induction and will be discussed in detail later on.

3.2 Dendritic Cells

One key component of the immune system are cells that are capable of taking up antigen and present it in a form that can be recognized by other cells of the immune system. Cells that fulfill this task are so called antigen presenting cells (APCs) and they include macrophages and dendritic cells (DCs). DCs have first been described by Ralph Steinman in 1973 [1], as cells with a tree-like form (gr. dendron = tree). In a series of following papers he showed that this cell type is very potent in inducing mixed lymphocyte reactions [2]. Although the role and special abilities have been subject to controversial debate, now, forty years and one nobel prize later, the important role of DCs in the induction of tolerance and immunity as well as the mere existence of DCs as a separate linage is widely accepted.

3.2.1 Development and heterogeneity of DCs *in vivo*

After Steinman had described DCs in the spleen it was soon discovered that Langerhans cells (LCs), first described by Paul Langerhans in 1868, share a lot of features with DCs [3]. With this finding the idea that DCs may exist in other organs than the spleen and that this type of cell might show a certain degree of heterogeneity *in vivo* was born.

Today two main branches of DCs are distinguished. On the one hand the DC type discovered by Steinman, that has been termed classical DC (cDC) and on the other hand the so called plasmacytoid dendritic cells (pDC). The latter subset is characterized by a plasma cell like shape and the ability to secrete large amounts of type-I interferons in response to viral encounter (reviewed in [4]).

The focus of this work lies on cDCs and the diversity of the different DC subsets in lymphoid and non-lymphoid organs as well as their roles in the induction of tolerance and immunity. All this will be discussed in the following sections.

Very important for the establishment of DCs as an independent lineage was the identi-

fication of distinct precursor cell types with the potential to differentiate into independent DC subsets. This precursor has lost the potential to differentiate into other cell-types of the myeloid or lymphoid lineages.

As all hematopoietic cells, DCs develop from the hematopoietic stem cell (HSC) via several distinct precursor stages into the final DC. One of the first of these precursors is the so called common myeloid progenitor (CMP) that still has the potential to give rise to monocytes, macrophages, granulocytes, erythrocytes and megakaryocytes as well as DCs. This CMP then differentiates via the granulocyte-macrophage precursor (GMP) into the macropage/DC progenitor (MDP) that is able to give rise to many macrophage and DC subsets [5].

As shown in Fig.3.1 the developmental tree branches of into a common DC precursor (CDP) as well as monocytes.

CDPs can still give rise to pDCs and the precursor of cDC (preDC) [7]. These preDCs can be found in the blood circulation and do seed lymphoid as well as non-lymphoid tissue were they finally differentiate into DCs [7].

3.2.2 $CD8^+$ Dendritic cells

cDC can be further divided into $CD11b^+$ DC or $CD8^+/CD103^+$ expressing DCs [8, 9]. These subsets can be found in lymphoid as well as in non-lymphoid tissue. The $CD8^+$ DCs were the first subset of DCs that has been described in the murine system [10]. In non-lymphoid tissue the equivalent subset is not characterized by the expression of CD8 but instead by CD103 expression [11, 12]. It recently became clear that Xcr1, a cytokine receptor, is expressed in lymphoid as well as non-lymphoid $CD8/CD103^+$ DC and thereby is a unifying marker for this DC subset [13, 14].

Cells of this subset share a common transcription profile that sets them apart from other leukocytes [15]. This specific pattern also leads to a unique protein signature for example in the expression of lectins by the $CD8^+$ subset. These include among others Clec9a and DEC205 [16, 17]. The latter is one of the most studied receptors and has been shown to

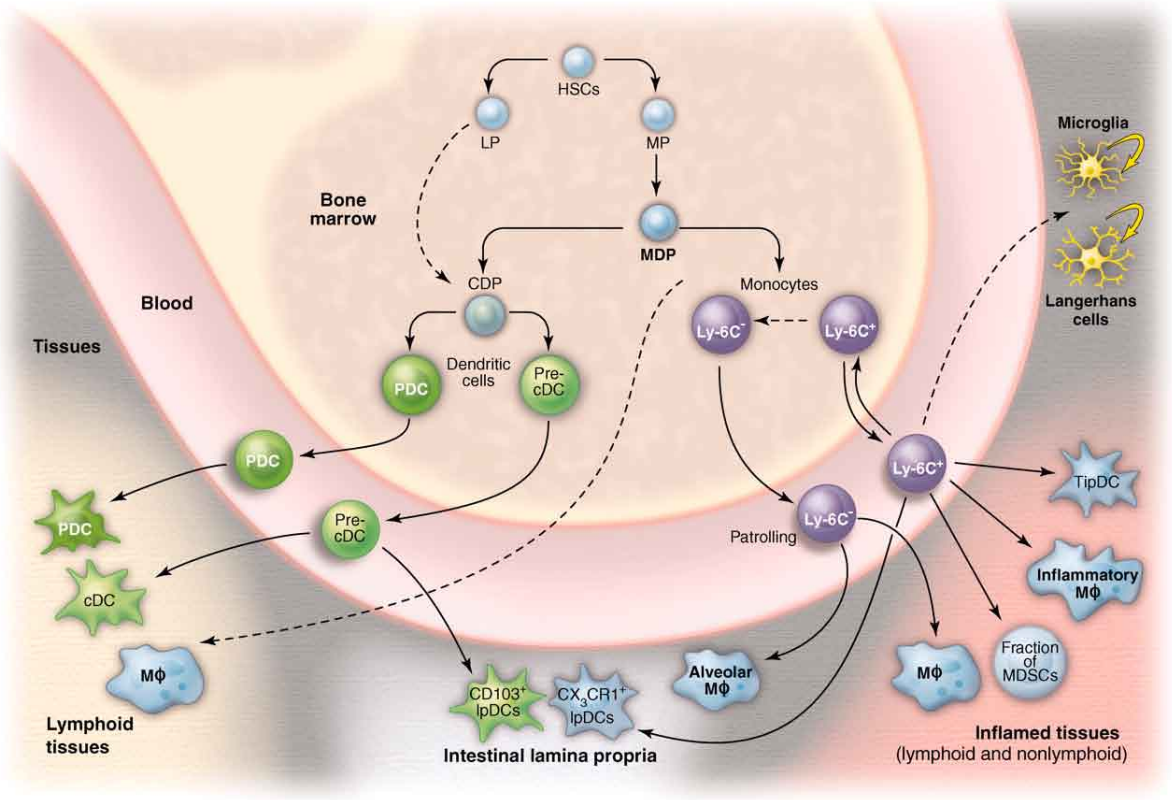

Figure 3.1: Development of different DC subsets and macrophages from the HSC to the mature cell type. Figure is adapted from [6].

mediate antigen uptake and subsequent cross-presentation of antigens [16].

CD8/CD103$^+$ DCs are very effective in presenting exogenous antigen on major histocompatibility complex class I molecules (MHCI), a process termed cross-presentation. This has been described for splenic DCs [18] as well as DCs from non-lymphoid organs [19]. CD8$^+$ DCs also produce high amounts of IL12p70 upon stimulation with TLR-ligands [20].

The development of CD8/CD103$^+$ DCs is dependent on a group of different transcription factors. These include the inhibitor of DNA binding 2 (Id2), the basic leucin zipper transcription factor ATF-like 3 (Batf3) and the IFN regulatory factor 8 (IRF8) as well as the nuclear factor interleukin 3 regulated (NFIL3). The absence of either of these results in a severe impairment in the CD8/CD103$^+$ fraction of the DCs, while at the same time the CD11b$^+$ compartment was unaffected [21, 22, 23, 24, 25]. Interestingly, the absence of BATF3 on the 129S6/SvEv-background leads to the complete absence of CD8$^+$ and CD103$^+$DC in spleen

and periphery, while on the C57BL/6 background CD103$^+$ DC are completely absent, while CD8$^+$ DCs are only reduced in the spleen and normal in lymph nodes (LN) [23, 26].

3.2.3 CD11b$^+$ Dendritic cells

The CD11b expressing DC subset is the predominant one in all lymphoid organs except for the thymus [27]. This subset in most cases does not express CD8 or CD103. In the spleen the CD11b$^+$ subset can be further divided into ESAM^{hi} cells that are of pre-DC origin and require signaling via the neurogenic locus notch homolog protein 2 (Notch2) for their development and ESAM^{low} cells that are probably derived from an early precursor form like the MDP [28]. These different subsets also express other markers such as CD4 to varying degrees and additionally do not have a unifying gene expression signature. In contrast to CD8$^+$ DC, CD11b$^+$ DC are inefficient at cross-presenting antigen but produce more IL-6 and IL-23 and are better equipped to induce CD4 T cell responses [16, 29, 30].

The development of CD11b$^+$ DC is dependent on the transcription factors v-rel avian reticuloendotheliosis viral oncogene homolog B (RelB), Notch2, recombining binding protein suppressor of hairless (RBP-J), IRF2 and IRF4 [28, 31, 32, 33, 34].

3.2.4 The mononuclear phagocytes of the intestine

In recent years the focus of immunological research shifted more towards non-lymphoid tissues of the body and so scientists in the field started to characterize DC subsets found in other tissues, such as the lamina propria of the small and large intestine.

A problem that one faces when looking at mononuclear phagocytic cells in the gut is that tissue macrophages do also express CD11c and in many instances are also positive for MHCII [35, 36]. Hence it is not sufficient to analyze for CD11c- and MHCII-expression to differentiate bona fide DC from macrophages. It has been shown that this problem can be solved by the use of additional markers, such as CD103 as a marker for DCs and CX_3CR1 and CD64 or F4/80 as markers for macrophages [37, 38, 39]

Although CX_3CR1^+ cells have initially been considered DCs, it has been shown recently that they show classical features of macrophages. In addition to the above mentioned markers they show a high phagocytic activity, cytoplasmic vacuoles, abundant cytoplasm and cannot migrate [37, 40]. The strong phagocytic activity is combined with the ability to form protrusions across the epithelial layer of the gut and to sample the gut lumen for antigen [41].

In contrast to DCs in lymphoid organs, which are either positive for CD103/CD8 or CD11b, DCs of the lamina propria can be divided into three subsets on the basis of CD103 and CD11b expression. In the small intestine the majority of DCs express CD11b, while the situation is reversed in the colon [42]. These cells do not only resemble DCs in that they express certain surface markers, but they also have functional properties that are archetypal for DCs. They have been shown to migrate between the lamina propria and the draining mLN at steady state, as well as after administration of toll-like receptor (TLR) ligands [39, 43]. Additionally they are able to prime naïve T cells, induce these T cells to home towards the gut [44] and are derived from classical DC precursor populations [45].

More recently a new population has received much attention. This subset is characterized by an intermediate expression of CX_3CR1 and comprises a mixture of monocytes, macrophages and DCs. The majority are recently extravasated $Ly6C^{hi}$monocytes that stepwise differentiate into macrophages and thereby lose the expression of Ly6C and gain high expression of CX_3CR1 and CD64 [37, 38]. But this CX_3CR1^{int} population also contains bona fide DCs. These DCs express no F4/80 but Zbtb46, a DC-specific transcription factor [46], can migrate in a CCR7-dependent manner and proliferate in response to FLT3L [43, 47].

One of the most prominent features of the $CD103^+$DCs is their ability to induce naïve T cells to become regulatory T cells (Tregs) [48, 49]. This is due to their ability to generate high amounts of retinoic acid (RA) via aldehyde dehydrogenases as well as the ability to convert latent into active TGF-β using $\alpha v\beta 8$ integrin [48, 49, 50]. However, which $CD103^+$ subset plays a role in the induction of Tregs remains to be resolved, since the ablation of either one of those does not lead to a decrease in Treg cell numbers in the lamina propria [23, 29]. A reduction in Tregs in the lamina propria was only observed in a mouse model where both

$CD103^+$ subsets were depleted [51], which would argue for a certain degree of redundancy between the two subsets.

Furthermore $CD103^+$DCs of the gut associated tissue (GALT) are also able to react appropriately towards environmental cues and induce immune reactions if necessary. It has been shown that they lose their tolerogenic properties during intestinal inflammation induced by dextran sodium sulfate (DSS) [52]. In a model of *aspergillus fumigatus* infection $CD103^+CD11b^+$ DCs were shown to be important for Th17 response induction [30] and in another study even a role in steady-state Th17 induction was found [29].

3.3 DC maturation and its role in induction of immunity or tolerance

As already mentioned in the beginning, the semi-random process, in which T and B cell-receptors are generated, makes it necessary that the newly formed receptors are tested for self-reactivity. This is done in a process called tolerance induction. For T cells this happens in the thymus and for B cells in the bone marrow. Cells that pass these tolerance induction then can go on to the periphery and, if primed, can very efficiently fight pathogens.

DCs are uniquely well equipped for both tolerance as well as induction of an immune responses for several reasons. First, they are located in the periphery or in parts of the spleen that are in constant contact with antigens from the circulation, namely the marginal zone. Both locations allow for constant sampling of their surroundings for antigen.

Second, they are very efficiently taking up antigen for processing and presentation (reviewed in [53]).

The third point worth mentioning in this context is the ability of DCs to migrate into the T cell zones, in both, inflammatory and steady state conditions [54].

And the last reason is of course their superior ability to prime naïve T cells, which was already one of the features that Steinman described DCs to have [2].

But the most important point that makes DCs so unique in their ability to induce both tolerance and immunity is their high degree of plasticity. They are either functionally and phenotypically "immature" or, after encounter of pathogens, "mature". DCs sense the presence of so called "pathogen associated molecular patterns" (PAMPs) via different sets of pattern recognition receptors (PRRs) and can then respond very precisely according to the thread. PRRs encompass TLRs, but also receptors like nucleotide oliogomerization domain (NOD) 1 and 2 and proteins of the retinoic acid-inducible gene 1 (Rig1) - like family. In response to the activation via these receptors DCs upregulate MHC (signal 1), costimulatory molecules, such as CD80 and CD86 (signal 2) and start the production of cytokines such as IL-12 (signal 3). Additionally, they shut down antigen uptake and start to migrate into lymphatic organs. T cells that encounter specific antigen on immature DCs receive signal 1 (MHC/peptide-TCR interaction) but not signals 2 and 3, since DCs do neither upregulate costimulatory molecules nor cytokines needed for cell fate decision of T cells.

In recent years immature DCs were defined as MHCII^{low}costim^{low}, although the term was originally intended to include a functional component. This newer definition is somehow problematic since there are instances described in the literature where phenotypically mature DCs were able to induce tolerance [55].

3.3.1 Central tolerance

Central tolerance is induced in primary lymphoid organs, where the respective lineage develops. B cells are tested in the bone marrow, while T cells are selected in the thymus.

T cells that react strongly with MHC-peptide complexes in the thymus can either be converted into Tregs or undergo apoptosis. It has been shown that DCs play vital roles in both of these processes [56, 57]. In a model of constitutive DC depletion it could also be shown that the absence of DCs impairs negative selection in the thymus and leads to a higher output of autoreactive T cells from the thymus of these animals and finally to the development of autoimmunity [58]. The latter study on the other hand is controversial, since a different group

independently generated the same mouse model and could not reproduce the findings of autoimmunity but instead claimed that the observed pathologies are not due to autoimmunity but due to a myleoproliferative disease as a result of abundant FLT3L [59]. The differences between this two studies have not yet been completely worked out.

3.3.2 Peripheral tolerance

While negative selection in the thymus deletes most of the autoreactive T cells, it is still an incomplete process. To keep up tolerance towards antigens that have not been selected against in the thymus, peripheral mechanisms have to be in place, to fill this gap. For the reasons mentioned above, DCs are especially well equipped for this task and there are several studies that show a role of DCs in the induction of peripheral tolerance [60]. T cells that encounter their antigen on immature DCs are then either driven into anergy, a state of T cell-unresponsiveness, or can be turned into Tregs. These, peripherally induced Tregs, will be called iTregs in this thesis.

iTregs

The importance of iTregs was underscored in experiments using *in vitro* generated Tregs. It is well documented that mice without the ability to form Tregs die shortly after birth [61]. And while thymus-derived "natural" Tregs (nTregs) are indispensable to prevent lethality of FoxP3-deficient mice, iTregs are important to avoid the development of autoimmunity and chronic inflammation for example in the gut [62]. This nicely shows, that nTregs are more important to prevent the fatal outcome, but iTregs are needed to preclude certain, special aspects of chronic inflammation. This is in line with findings showing a role of certain bacterial strains in the induction of iTregs [63] and big differences between TCR repertoires of nTregs and iTregs [64], reflecting the different antigens recognized.

The development of FoxP3$^+$ iTregs has been shown to critically depend on a stretch of conserved DNA in the FoxP3-promotor region, called "conserved non-coding DNA sequence-

1" (CNS1) [65]. Animals with a deletion of this region showed a strong reduction of iTreg generation in GALT and other parts of the periphery. Although these animals lack most of the iTregs, they did not develop any autoimmunity and were also not more susceptible to DSS-induced colitis. While this is surprising, it might be explained by an increase in the development of other regulatory cells in the periphery, like Tr1 cells, that might compensate for the loss of FoxP3$^+$ Treg-induction [65].

The phenotypic differentiation of nTregs and iTregs *in vivo* has been a topic of great controversy in the literature. Early on Helios, a member of the Ikaros-transcription factor family, has been proposed as a marker exclusively expressed on nTregs [66]. This expression of Helios might also be influenced by the way that the cells are activated, when the Treg-cell program is induced and by the type of APCs involved [67, 68].

Another marker that has been shown to be expressed preferentially on nTregs, is the molecule Neuropilin-1 (Nrp1) [69, 70]. This is a very attractive marker for nTreg vs iTreg discrimination, since it is an extracellular molecule, which would allow easy purification of iTregs. However it has also been shown, that under inflammatory conditions iTregs can upregulate Nrp1 [70].

Very recently the lab of Gerad Eberl developed a new way to distinguish between iTregs and nTregs. They showed that a subset of FoxP3$^+$ cells does also express the transcription factor RORγt and is negative for Helios [71]. These cells can be found in small numbers throughout the body, but are strongly increased in mLN and the lamina propria of small and large intestine. They can not be found in young animals before weaning, but increase over time, and their development is also blocked in germ-free animals. Interestingly they are also not found in animals that lack DCs, which again highlights the importance of DCs in the induction of peripheral tolerance.

DCs in peripheral tolerance induction

To test the influence of DCs on the induction and the upkeep of Treg cells several DC depletion models have been employed. A complete and constitutive deletion of DCs did not change the

thymic Treg output and also in the periphery no differences were detected [58, 59]. Contrary to this, another study found a correlation between the numbers of DCs and Tregs per animals [72]. In opposition to the first two studies the latter used no chronic depletion, but on the one hand an induced reduction and on the other hand an induced increase in DC numbers. In these induced models they were able to observe a positive correlation between DC and Treg numbers. A chronic depletion might lead to compensatory mechanisms, rescuing a reduced Treg induction, while the use of CD11c-Cre on the other side might have side effects in other cell populations.

A series of studies addressed the role of DCs in tolerance induction by targeting antigen to certain DC subsets using different mAbs [16, 73, 74]. Adoptively transfered T cells were very well tolerized in this animals, when mAb conjugates were injected at steady state, without further adjuvants. One question addressed by this targeting strategy was whether different subsets of DCs show varying potential to induce tolerance and if there may even exist a subset that exclusively induces tolerance. Initial experiments to solve this question were carried out by the Nussenzweig lab, where the above described targeting approach was used to target antigen to $CD8^+$ and $CD11b^+$DCs and both subsets could induce tolerance [16].

In order to avoid problems arising from potential signaling functions of the targeted receptor, genetic models have been generated. The antigen is here expressed using a loxP-flanked stop-cassette in front of the antigens, which is excised using a tamoxifen-inducible CD11c-Cre molecule [75]. This study also confirmed the potential of DCs to induce peripheral T cell tolerance *in vivo*, although it does not allow to differentiate DC subsets, since all DCs and most macrophages are positive for CD11c.

The first study claiming a special role for a certain subset was published in 2007 by the group of Fiona Powrie, who showed that $CD103^+$DCs from the small intestinal lamina propria and the mLN were superior in inducing Tregs when compared to $CD103^-$DCc [48]. This study showed that Treg induction is dependent on the ability of $CD103^+$DCs to metabolize vitamin A to produce RA and on the presence of TGF-β. Other studies following the initial one showed, that one of the key features of $CD103^+$DCs, that enables them to induce Tregs, is

the expression of the integrin αv, that can convert latent into active TGF-β [76].

DCs of the lamina propria comprise two different CD103$^+$ subsets, one of them coexpressing CD11b, while the other one does not. The initial studies however did not address if these are different in their potential to induce Tregs. The CD103$^+$CD11b$^-$ subset is developmentally dependent on the transcription factor Batf3 and can therefore be depleted by the knock-out of Batf3 [23]. Using this knock out mice, no differences in the Treg-compartment of the lamina propria were observed. Additionally, the animals did not show any signs of intestinal inflammation or higher susceptibility to dextran sodium sulfate (DSS)-induced colitis in contrast to animals having all DC subsets.

The CD103$^+$CD11b$^+$ subset on the other hand has been shown to express the human langerin molecule in a transgenic setting and can therefore be depleted using a human langerin-DTA mouse model [51]. When these animals were crossed to a Batf3$^{-/-}$ background, thereby deleting both CD103$^+$DC subsets in the lamina propria, a moderate reduction in the Treg number of the lamina propria was observed. This was accompanied by a reduction in the frequency of CCR9$^+$Tregs, a marker which cells have to upregulate to enter the lamina propria. Taken together, it seems as if both CD103$^+$ DC subsets in the GALT can induce iTreg development. This would fit to a model, in which the environment, a DC is derived from, is more important for its tolerogenic potential, than the mere subset it belongs to.

The idea that the environment is very important for the potential of DCs to induce tolerance is further strengthened by the observation, that CD103$^+$ DCs also lose their ability to induce iTregs during colitis [52]. This of course goes back to the idea, that immature DCs excel at inducing tolerance, while mature DCs would induce an immune response.

In line with the idea of the environment being important for tolerance induction is the fact, that oral administration of antigen is superior to any other way that an antigen could be administered, when it comes to tolerance induction [77, 78]. This phenomenon is called oral tolerance. It can also be seen, when transgenic T cells are transfered into animals that receive their respective antigen orally. The T cells respond to this by converting to FoxP3 expressing Tregs [49]. For this process the antigen has to be taken up, processed and then

presented to the naïve T cells within the mLN or other lymphoid structures. Here DCs are essential, because even though macrophages are able to sample luminal content of the gut and take up such antigen [41], they are unable to migrate [39] and therefore do not reach lymphoid structures to interact with T cells and induce FoxP3 expression. The transport of this antigen is actually achieved by DCs, that either can take up the antigen themselves [79] or can take over antigen from macrophages via gap-junction proteins [80].

This, together with the above mentioned ability to generate molecules like RA and TGF-β, makes DCs absolutely essential in the induction of oral tolerance.

3.4 The influence of CD40 on DCs

CD40 is a transmembrane glycoprotein with a molecular mass of 48 kDA. Its role has initially been investigated predominantly in B cells, where CD40 has a proliferative effect when bound by its ligand CD154 (CD40L). However, CD40 is not just expressed by B cells, but instead also on monocytes, epithelial and endothelial cells as well as on DCs.

The role of CD40 signaling in DCs is less well understood then its role in B cells. It has been shown by different groups that the ligation of CD40 on DCs matures them and therefore makes them more effective at antigen presentation via upregulation of MHCII and costimulatory molecules [81, 82, 83]. This means that signal 1 and 2 can be delivered by DCs that have been been activated via CD40.

It is less clear whether DCs, activated this way, are able to deliver a signal 3 to T cells. It has been shown that CD40 ligation on DCs leads to production of proinflammatory cytokines like IL-12 [84] *in vitro* and that the injection of anti-CD40 mAb has immunostimulatory effects *in vivo*, when antigen is delivered directly to certain DC subsets [73, 74]. In contrast, several studies show that CD40 ligation on DC alone is not sufficient, but it always needs a combination of CD40-signal and TLR-ligand to get a full activation of DCs [85, 86, 87].

The discrepancy between these studies may have several reasons. The first reason is that when anti-CD40 mAb is used *in vivo*, it does not only have an effect on DCs but also on

other cells expressing CD40, most notably B cells. And it has been shown that this leads, among other effects, to liver inflammatory disease, that is supposed to be dependent on B cells being present [88]. Therefore it is unclear whether the observed DC maturation is only due to anti-CD40 treatment or if a mixture of this and proinflammatory cytokines / danger signals released from necrotic liver cells is responsible for the maturation of the DCs. The second reason concerns studies that have been carried out *in vitro*. Here the cells are under the influence of growth-factors as well as serum and plastic, so that again the results of mixed influences on DCs are observed. Because of the above mentioned reasons *in vivo* data gathered using mAb-injection is of limited use.

Another very interesting effect of anti-CD40 *in vivo* is limited to the $CD103^+$ subset of DCs. The group of Fiona Powrie could show that the injection of anti-CD40 mAbs leads to the disappearance of $CD103^+MHCII^+$ cells from the spleen after injection into $Rag^{-/-}$ animals. Additionally the expression of CD103 on sorted $CD103^+$ DCs was lost after they were incubated with anti-CD40 *in vitro* [89]. $Rag^{-/-}$ animals injected with anti-CD40 mAb suffered from wasting disease that could not be prevented by the transfer of regulatory T cells. Because of the role of $CD103^+$ DCs on the peripheral induction of tolerance, the depletion of these cells by the application of a mAb is a possible valuable tool to investigate their role *in vivo*. At the same time it is limited due to the described side effects of the mAb application.

To study the influence of CD40 without these interfering influences, we developed a mouse model in which selectively DCs receive a constitutive CD40 signal.

3.4.1 The DC-LMP1/CD40 mouse model

To generate a model in which only DCs receive a CD40 signal, we made use of the LMP1/CD40 model developed in the laboratory of Ursula Zimber-Strobl [90]. In this mouse a Stop-cassette flanked by two loxP sites and followed by the gene for the fusion protein LMP1/CD40 was knocked into the rosa26 locus of the mouse (fig. 3.2). Since the rosa26 locus is transcriptionally very active one can be certain that the fusion protein is expressed optimally.

In this study the LMP1/CD40 mouse was crossed with a mouse strain expressing the Cre recombinase under the control of the CD11c promotor. CD11c is almost exclusively expressed by DCs and macrophages [31]. Because of that we termed this mouse strain DC-LMP1/CD40.

The fusion protein LMP1/CD40 consists of the transmembrane domain of the Ebstein-Barr virus (EBV) protein LMP1 (amino acids 1-187) coupled to the intracellular part of the human CD40 domain (aa 223-280). EBV infects B cells and can establish a live long persistence in these cells and can also potentially cause malignant lymphoproliferative disorders in individuals that are immune compromised. To achieve this, EBV expresses six nuclear proteins (EBV nuclear antigens, EBNA) and two latent membrane proteins (LMP1 and LMP2a). LMP1 has many similarities to CD40 when it comes to its function in B cell proliferation and survival. The protein consists of a short N-terminal cytoplasmic tail, six transmembrane domains and a long C-terminal cytoplasmic tail and is the necessary factor in the immortalization of B cells *in vitro*. The transmembrane part allows LMP1 to aggregate and thereby facilitates ligand free signaling via recruitment of TRAFs [91]. LMP1 and CD40 signal transduction leads to the activation of NF-κB, JNK as well as p38/MAPK [92, 93].

The second part of the fusion protein is the intracellular part of the human CD40 molecule. It has previously been shown that a human CD40 molecule can rescue the phenotype caused

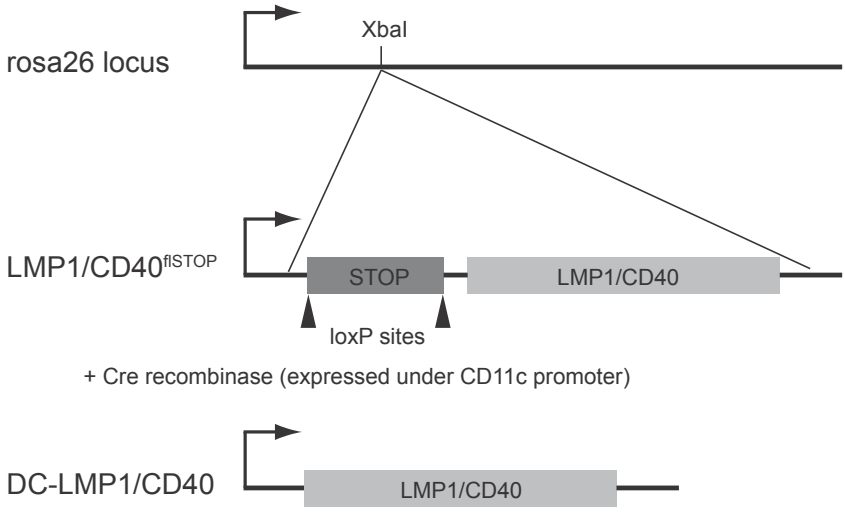

Figure 3.2: Schematic representation of the rosa26 locus in LMP1/CD40 mouse (middle panel) as well as in DC-LMP1/CD40 mice (bottom panel).

by the deletion of the mouse CD40 molecule [94].

Chimeric molecules of LMP1 and CD40 have been shown to be able to deliver ligand-free signals very similar to normal CD40 signals *in vitro* [95].

The first *in vivo* use of the chimeric molecule was reported in a model were the LMP1/CD40 molecule was activated using a CD19-Cre [90]. As expected, the chronic CD40 stimulus lead to B cell lymphoma development in mice older then 12 weeks, further underscroring the role of CD40 signaling in B cell proliferation and survival.

3.5 Aim of the thesis

DCs are uniquely well equipped to induce both tolerance and immunity. The decision which of the two is induced is thought be made dependent on the maturation status of a DC. This in turn can be influenced by a broad variety of different stimuli. One of them is CD40 which is discussed in the literature as both, immune stimulatory, but also showing potential to generate tolerogenic DCs.

The goal of this study was to circumvent disturbing factors of anti-CD40 mAb treatment and instead to investigate the effects of a CD40 signal delivered to DCs directly and how this influences their potential to induce tolerance or immunity. To this end we generated a transgenic mouse model in which DCs receive a constant, ligand-free CD40 signal under the control of the CD11c promotor. This allowed us to investigate *in vivo* the influence of a CD40 signal on the tolerogenic potential of DCs in general, but also gave us the opportunity to analyze the potential at different sites of the body.

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, BD), incubator (Hera cell, Heraeus Kendro Laboratory Products, Hanau, Germany), laminar airflow cabinet (Heraeus), 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), real-time PCR machine (CFX96 Real Time System, BIO-RAD, Hercules, CA, USA), vacuum pump (KNF Neuberger, Munzingen, Germany), vortex-Genie2 (Scientific Industries, Bohemia, NY, USA), water bath (Grant Instruments Ltd., Barrington Cambridge, UK).

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4.1.2 Consumables

disposable syringe filter (0.2 + 0.45 μm)	Nalgene Nunc Int., Rochester, NJ, USA
disposable injection needle (26 G x 1/2")	Terumo Medical Corporation, Tokyo, Japan
disposable syringe (1+5 ml) reaction	Braun, Melsungen, Germany
reaction container 5 ml (FACS)	BD, Franklin Lakes, NJ, USA

reaction container 15 ml and 50 ml	Greiner, Frickenhausen, Germany
BD Microtainer	BD, Franklin Lakes, NJ, USA

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
PBS:	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	2 mM KH_2PO_4
	pH 7.4
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 % (v/v) FCS
	10 mM EDTA
	10 mM HEPES
FACS buffer:	PBS
	2 % (v/v) FCS
	0.01 % (v/v) NaN_3
T cell-medium:	RPMI
	10 % (v/v) FCS
	100 U/ml Penicillin
	100 g/ml Streptomycin
	500 mM β-mercaptoethanol
CFSE-staining buffer:	PBS
	0.03 % FCS
50x TAE buffer:	242 g Tris HCl
	57.1 mL 100 % (v/v) acetic acid
	100 mL 0.5 M EDTA (pH 8.0)

	H_2O ad 1 L
10x Gitocher:	670 mM Tris HCl pH 8.8
	166 mM $(NH_4)_2SO_4$
	65 mM $MgCl_2$
	0.1% (v/v) gelatin
1x Gitocher buffer:	5 μL 10x Gitocher buffer
	2.5 μL 10 % (v/v) Triton X-100
	0.5 μL β-mercaptoethanol
	3 μL proteinase K (10 mg/ml)
	39 μL H_2O

4.1.5 Antibodies

Table 4.3: Antibodies used in flow cytometry

epitope	clone	conjugate	manufacturer
B220	RA3-6B2	PerCP	BD Bioscience
CD3	145-2C11	PE-Cy7	ebioscience
CD4	GK1.5	PE, PE-Cy7, APC	BD Bioscience
CD8	53-6.7	BV 421, APC-eFlour780	ebioscience
CD11b	M1/70	APC-eFlour780	BD Bioscience
CD11c	N418	PE-Cy7, APC	BD Bioscience
CD25	PC61.5	PerCP-Cy5.5	ebioscience

Continued on next page

Table 4.3 – continued from previous page

epitope	clone	conjugate	manufacturer
CD44	IM7	FITC	BD Bioscience
CD45	30.F11	PerCP, BV 421	biolegend
CD45.1	A20	eFlour450	biolegend
CD45.2	104	APC	biolegend
CD45RA	14.8	PE	BD Bioscience
CD62L	MEL-14	APC	BD Bioscience
CD64	X54-517.1	APC	biolegend
CD70	FR70	biotin	ebioscience
CD80	16-10A1	biotin	ebioscience
CD86	GL-1	biotin	BD Bioscience
CD90.1	OX-7	PerCP	BD Bioscience
CD103	M290	PE	BD Bioscience
CD172a	P84	APC	BD Bioscience
FoxP3	FJK-16s	AlexaFlour647 or eFlour660	ebioscience
MHCII	M5/114.15.2	FITC, PerCP	ebioscience
Ly6C	AL-21	FITC	BD Bioscience
Helios	22F6	FITC	ebioscience
RORγt	AFKJS-9	PE	ebioscience
NK1.1	PK136	PE, PE-Cy7	BD Bioscience
TCR-b	H57-597	FITC	ebioscience
V-a2	B20.1	PE	biolegend
F4/80	BM8	PE-Cy7	biolegend
IL-17A	TC11-18H10	PE	BD Bioscience
IFN-γ	XMG1.2	APC	ebioscience

Continued on next page

Table 4.3 – continued from previous page

epitope	clone	conjugate	manufacturer
Streptavidin		BV421	biolegend

4.1.6 Oligonucleotides, peptides and proteins

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

OTI- ($Ova_{257-264}$, SIINFEKL) and OTII-peptide ($Ova_{323-339}$, ISQAVHAAHAEINEAGR) were purchase from PolyPeptide Group (Strasbourg, France).

Ovalbumin protein grade II and VII were bought from Sigma-Aldrich (St. Louis, MO, USA).

4.1.7 Mouse strains

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

Table 4.4: Genotyping primers

knock-out/transgene	primer name	primer sequence
Cre	RO334	GGACATGTTCAGGGATCGCCA-GGCG
	RO335	GCATAACCAGTGAAACAGCAT-TGCTG
LMP1/CD40	HL15	AAGACCGCGAAGAGTTTGTCC
	HL54	TAAGCCTGCCCAGAAGACTCC
	HL152	AAGGGAGCTGCAGTGGAGTA
CD40fl	CD40flox_wt_fw	TCTTTGGGAGCACTGAAGAG
	CD40flox_wt_rv_1	TACCAAAGCAAGAACGCAGA
	CD40flox_flox_rev	GATCGTTGAAGAAGGAGGTG
CX3CR1	CX3CR1_wt	GTCTTCACGTTCGGTCTGGT
	CX3CR1_com	CCCAGACACTCGTTGTCCTT
	CX3CR1_mut	CTCCCCCTGAACCTGAAAC

CD11c-Cre

The CD11c-Cre mouse was produced in the Lab of Boris Reizis and expresses the Cre recombinase under control of the CD11c promotor [31]. This mouse allows the deletion of floxed allels in DCs and other CD11c-expressing cells.

DC-LMP1/CD40

To obtain DC-LMP1/CD40 animals, CD11c-Cre mice were crossed to LMP1/CD40 mice [90]. 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 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 x Rag1$^{-/-}$

DC-LMP1/CD40 mice have been crossed onto a Rag1$^{-/-}$ background. Rag1$^{-/-}$ mice are unable to form B and T cells due to a defect in the V(D)J-recombination machinery [96].

OTI

This mouse strain carries a transgenic T cell receptor specific for the ovalbumin-derived peptide SIINFEKL that is presented to CD8^{+} T cells in the context of H-2K^{b} [97]. The T cell receptor of these mice uses the Vα2 and the Vβ5 segments and T cells can be visiualized using mAbs directed against these two segments.

OTII

OTII is a TCR-transgenic mouse line that carries a TCR specific for the peptide residues 323-339 of ovalbumin that are presented in the context of I-A^{b} [98]. At the same time these cell show crossreactivity to retroviral superantigens expressed in the thymus, which leads to a strong negative selection.

DC-CD40 KO

DC-CD40 KO mice were generate by crossing CD11c-Cre mice with $CD40^{fl/fl}$ mice, thereby generating a DC-specific knock-out of the CD40 molecule. These mice were generate by Prof. Esther Lutgens and Dr. Norbert Gerdes.

4.2 Methods

4.2.1 Immunological and cell biology methods

4.2.1.1 Harvesting of blood and organs and single cell preparation

Animals were sacrificed by cervical dislocation after they had been sedated using Isoflurane. Organs were removed using scissors and fine tweezers and put into RPMI medium. Spleen, thymus and lymph nodes were then digested with DNAse I (0.2 mg/ml) and Liberase (0.65 Wünsch units/ml, both Roche) for 30 min at 37°C. Afterwards the organs were passed through a 100 μm cell strainer, washed once with cold PBS and red blood cells were lyzed using ACK buffer for 5 min at room temperature. Cells were washed once again and counted using CASY-counter (OMNI life science) and used for further analysis or experiments.

To analyze cells from the lamina propria, colon was taken from a mouse, fecal content removed, the colon opened longitudinally and cut into ca. 5 mm big pieces. The pieces were then incubated with HBSS-EDTA for 10 min on a shaker at 37°C, the supernatant containing epithelial cells was discarded and gut parts were washed twice with icecold PBS. Afterwards the colon was digested once for 30 min and then twice for 20 min with a mixture of Collagenase IV (157 Wuensch units/ml, Worthington), DNAse I (0.2 mg/ml dissolved in PBS) and Liberase (0.65 Wuensch units/ml, both Roche, dissolved in HBSS with FCS), the supernatant was collected after each digestion and the cells were washed once with PBS.
Cells from all three digestions were combined and immune cells enriched using gradient centrifugation. For this cells were resuspended in 40 % Percoll and this solution was overlayed onto a 80 % Percoll solution. Centrifugation was carried out for 20 min at 1800 rpm and 4°C without break. Cells at the interphase were collected, washed once and used for further

analysis.

4.2.1.2 Flow Cytometry staining

For flow cytometric analysis $2{\cdot}10^6$ cells were used per staining in a 96 well plate. Cells were washed once with 100 μl FACS buffer and then stained for 20 min at 4°C in the dark in 50 μl of antibody mix in FACS buffer. Each antibody has been titrated for optimal use. After the incubation cells were washed once with 150 μl FACS buffer and then either directly acquired by FACS or fixed for overnight storage using FACS buffer containing 2 % (v/v) paraformaldehyde.
Cell that have been stained with a biotinylated mAb were stained in a second step with a fluorescently labeled streptavidin also in a volume of 50 μl at 4°C in dark for additional 20 min.

For intracellular stainings cell were fixed and permeabilized after they have been stained for all extracellular markers. For the staining of FoxP3 cells were washed once and then resuspended in 200 μl 1x Fixation/Permeabilization solution (eBioscience) for at least 30 min at 4°C in the dark. Cells were spun down, the supernatant removed and the cells washed twice with 1x Permeabilization Buffer (eBioscience). Cells were then stained with FoxP3-specific antibody in 50 μl Permeabilization Buffer for 30 min at 4°C in the dark. Afterwards cells were washed once and acquired by FACS.
Acquisition was either performed using a FACSCalibur or FACSCanto II. Cell sorting was performed at FACSAria (all BD). Data analysis was performed using FlowJo version 8 and 9 (TreeStar, Ashland, OR, USA).

4.2.1.3 Depletion of commensal bacteria

To deplete as many commensal bacteria as possible animals were provided with 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 at least 3 weeks [99].

4.2.1.4 ELISA for commensal- and food-reactive antibodies

The cecum of C57BL/6 mice was removed, opened longitudinally, transfered into a 2 mL eppendorf cup, containing 1.5 ml PBS and cecal content was expelled by vigorously vortexing. Remaining cecal tissue was removed and PBS and cecal content was transfered into tubes with Lysing Matrix E (MP Biomedicals) and then homogenized using the FastPrep system (MP Biomedicals) for 45 s at maximum speed.

Samples were spun down and supernatant was collected, filtered and spun again at maximum speed. The protein concentration was determined and the cecal bacterial lysate (CBL) was stored at -20°C until used.

CBL was diluted in carbonate buffer to a final concentration of 50 ng/ml and 100 μl of this was coated per well over night at 4°C. Wells were washed five times with PBS 0.05 % (v/v) Tween20. Afterwards unspecific binding was blocked using 200 μl PBS with 0.5 % (v/v) MMP for two hours at room temperature and wells were then again washed five times with PBS 0.05 % (v/v) Tween20.

Serum of mice was diluted either 1:300 or 1:600 and 100 μl of this was added to a well, incubated for two hours at room temperature and washed again for five times with PBS 0.05 % (v/v) Tween20. For detection isotype specific antibodies coupled to horseradish peroxidase were used at a dilution of 1:4000 in blocking buffer for 2 hours at room temperature.

After another round of washing the ELISA was developed using 100 μl of 3,3',5,5'-tetramethylbenzidin solution. The reaction was stopped by adding 50 μl 2 N H_2SO_4. Optical density was measured at a wavelength of 450 nm with 630 nm as a reference wavelength.

4.2.1.5 *In vivo* antigen uptake assays

To investigate uptake and processing of complete protein animals were injected with a mixture of 100 μg AlexaFlour647-Ova and 100 μg DQ-Ova. Animals were sacrificed 2 hours later, spleen taken and processed as described before for flow cytometry staining.

4.2.1.6 Generation of bone marrow chimeras

To generate bone marrow chimera recipient mice were irradiate with two separate doses of 550 rad using a Cesium source (Gammacell 40, AECl, Mississauga, Canada). Irradiated animals were reconstituted with $5{\cdot}10^6$ bone marrow cells, 1:1 mixed from Ly5.1$^+$ and Ly5.2$^+$ bone marrow. To prevent infection, animals received 1.2 g/l neomycin in water ad libitum for 4 weeks. Animals were analyzed 8 to 10 weeks after reconstitution.

4.2.1.7 Magnetic cell sorting

To purify cell populations based on surface marker expression, magnetic cell sorting (MACS, Miltenyi Biotec) was employed. This technique uses antibodies reactive to certain surface antigens coupled to magnetic beads. After cells have been incubated with these antibodies for an appropriate amount of time, cells can be applied to a column placed in a paramagnetic field. Labeled cells are retained on the column, while unlabeled cells are washed away. Thereafter, columns are rinsed three times and the eluted fraction can be collected. This opens the interesting possibility of purifying cells by depleting all other cell types and not touching the cell of interest.

This technique was actually employed when CD8$^+$ and CD4$^+$ T cells were purified for adoptive transfer experiment (CD8$^+$ T cell Isolation Kit, negative selection and CD4$^+$ T cell Isolation Kit, negative selection). All procedures were performed according to manufacturer instructions.

4.2.1.8 Adoptive T cell transfer

To transfer transgenic T cells into experimental animals spleens from transgenic animals were taken and the respective T cells purified using either CD4 or CD8 T cell enrichment kits. Purity of the cell suspension was checked using anti-CD4 or anti-CD8 antibody in combination with an appropriate Vβ antibody specific for the respective TCR.

If not stated otherwise $0.5{\cdot}10^6$ CD8$^+$ or $2{\cdot}10^6$ CD4$^+$ T cells were transfered intravenously

into syngenic and sex-matched mice. Behavior of the transfered cells could be followed by analyzing the congenic markers CD45.1 or CD90.1.

4.2.1.9 CFSE labeling and *in vivo* cytotoxicity assay

Cells were resuspended at a concentration of $10{\cdot}10^6$ in CFSE-staining buffer. Then CFSE was added to a final concentration of 2.5 μM, mixed immediately and incubated for 10 min at 37°C. Afterwards, 2 ml of FCS was added, the mixture incubated for 2 min at RT to stop the labeling reaction and the cells were washed three times with PBS. The cells were filtered after this and used in proliferation assays.

If the CFSE labeled cells were supposed to be used in an *in vivo* cytotoxicity assay, whole spleen was divided into three equal parts, subsequently loaded with different concentrations of peptide and then stained with different concentrations of CFSE. Cells with high peptide concentration (2 μg/ml) were labeled with 2.5 μM CFSE, cells that did not receive any peptide were labeled with 0.33 μM CFSE and cells with low peptide concentration (2 ng/ml) were labeled with 70 nM CFSE. The three groups were mixed in a 1:1:1 ratio, separation of the peaks was verified by FACS and injected into animals that previously received cytotoxic T cells.

After 16 h animals were sacrificed and spleens analyzed for the presence of CFSE-labeled cells. The calculation of specific lysis was performed on the basis of two of those peaks, while the other peak served as backup, in case of a fast killing.

Specific lysis was calculated on the basis of the ratio R, with

$$R = \frac{\%\ unloaded\ cells}{\%\ peptide\ loaded\ cells}$$

This ratio was then used to calculate the specific lysis as

$$specific\ lysis = \left(\frac{R_{unimmunized\ animals}}{R_{immunized\ animals}}\right) \cdot 100$$

4.2.1.10 *In vitro* T cell restimulation

To asses 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 put into 96 well plates and stimulated with Phorbol-12-myristat-13-acetat (PMA) and ionomycin in T cell-medium at a final concentration of 40 ng/ml and 1 μg/ml respectively for four hours in the presence of 2 μM Golgi-Stop (BD).

Afterwards, cells were washed twice with FACS-buffer and then stained extracellularly as described in section 4.2.1.2. To permeabilize cells, they were resuspended in 150 μl of BD Cytofix/Cytoperm (BD Bioscience) and incubated for 20 min at 4°C in the dark, washed once with Perm/Wash buffer (BD) and stained for cytokines of interest for 30 min at 4°C in the dark. Then cells were washed once again and resuspended in FACS buffer until they were acquired.

4.2.1.11 Immunofluorescence staining

Organs were directly embedded in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands) and snap frozen in liquid nitrogen. Sections of 5-7 μm were cut using a cryostat instrument (Leica Microsystems, Wetzlar, Germany), fixed for 20 min at -20°C using aceton and subsequently dried over night in the dark.

Before staining the slides were adjusted to room temperature and rehydrated for 15 min with PBS containing 0.25 % (w/v) bovine serum albumin (BSA). To minimized unspecific binding, slides were incubated with PBS, 0.25 % BSA containing 10% mouse serum.

Staining was performed in a moist chamber in the dark for 30 min at room temperature. After staining the slides were washed and cover slides put on. The slides were analyzed using a BX41 microscope equipped with a F-view II camera and cellF software (all from Olympus, Hamburg, Germany).

4.2.1.12 Cytokine bead array

Blood of animals was taken via puncturing the heart. The blood was transfered into a microtainer tube (BD, Franklin Lakes, NJ, USA) and incubated at room temperature for at least three hours, so that the blood could coagulate. Afterwards the tube was centrifuged at 8000 rpm for 5 min at room temperature and the serum was frozen at -20°C until use.

To measure up to six different cytokines in the same amount of serum the BD Cytokine Bead Array mouse inflammation kit was employed. Serum samples were titered in the beginning to ensure an optimal concentration for the assay. The assay procedure was then performed according to manufacturers instructions.

Acquisition of the samples was performed using a FACSCantoII. Results were analyzed using FCAP Array Software (Soft Flow Inc.)

4.2.2 Molecular biology

4.2.2.1 Agarose gel electrophoresis

To visualize DNA fragments and to separate them according to size, they were applied to gel-electrophoresis on an agarose gel. The agarose gel was prepared by dissolving 0.8 - 2 % (w/v) agarose in TAE buffer, depending of the fragmentsize that was supposed to be visualized. To estimate the size of the fragments, either a 100 bp or a 1 kb ladder was used (New England Biolabs, Ipswich, MA, USA). PCR fragments either already contained loading buffer (10 % glycerol, xylene cyanol FF) or it was added directly before applying the sample to the gel. DNA samples were visualized using ethidium bromide (0.5 μg/mL) that was added to the gel followed by examination with UV light (312 nm, Intas, Goettingen, Germany).

4.2.2.2 Isolation of genomic DNA and RNA

To isolate genomic DNA for genotyping, 2-5 mm of mouse tail tip was cut, put into 50 μl 1x Gitocher buffer and incubated at 55°C for 6 h. Proteinase K was inactivated at 95°C for 5 min.

The isolation of nucleic acids for other purposes was done using the following kit according to manufacturers instructions:

- DNeasy Blood & Tissue Kit (Quiagen)
- RNeasy Mini Kit (Quiagen)
- RNeasy Micro Kit (Quiagen)

4.2.2.3 Transcriptional profiling of DCs

To analyze the gene expression profile of DCs under different conditions, DCs were ex-vivo sorted using the markers indicated in the results section. Purity was assessed by FACS, cells were washed once and then resuspended in buffer RLT (Quiagen) to disrupt the cells and unfold all proteins. This was immediately snap-frozen and then kept at -80°C. The gene expression was analyzed using the mouse immunology panel for the nanostring platform (NanoString Technologies, Seattle, WA, USA). Processing of the samples was done by a collaboration partner.

Data analysis was performed using R and nSolver analysis software (NanoString Technologies).

4.2.2.4 Statistics

Statistical analysis was performed using PRISM software (GraphPad software, La Jolla, CA, USA). Unless stated otherwise all bar graphs represent mean ± standard error of mean (SEM) and significance was analyzed using a students t-test, with *: $P = 0.01$ to 0.05, **: $P = 0.001$ to 0.01 and ***: $P < 0.001$.

5 Results

5.1 Influence of anti-CD40 injection on DCs in different organs

To investigate the influence of CD40 signaling on DCs, anti-CD40 mAb was injected into $Rag^{-/-}$ animals, since an influence on splenic DC was already published in $Rag^{-/-}$ animals [89]. Three days after injection $CD103^+$DCs were completely absent from the spleens of animals treated with an anti-CD40 mAb (Fig. 5.1 A). Since $CD103^+$DCs play an important role in peripheral tissues we also analyzed different DC subsets in the lamina propria of the gut. Here we also observed a strong reduction of the relative amount of both $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs while the other subset was relatively increased (Fig. 5.1 B). To test if this effect holds true in animals that have B and T cells, anti-CD40 mAb was injected into C57BL/6 animals and the three intestinal DC subsets were analyzed. Also in lamina propria of T and B cell sufficient animals the two $CD103^+$ subsets were strongly reduced, although we found a substantial amount of remaining $CD103^+CD11b^-$ cells (Fig. 5.1 B lower panel).

To look for other effects caused by the injection of anti-CD40 mAb, serum was taken and livers were analyzed macroscopically. Again this was performed in $Rag^{-/-}$ animals, where we were able to compare our results to published data and in C57BL/6 animals to see if there are differences when B and T cells are present. We could observe the formation of white spots in the liver of $Rag^{-/-}$ animals 72 h after injection, indicating an ongoing inflammation (Fig. 5.2 A). This finding was further supported by elevated serum levels of alanin-amino-transferase (sALT), a marker for liver damage [88]. This could be observed in $Rag^{-/-}$ and C57BL/6 animals. The sALT levels were strongly increased 72 hours after injection of the anti-CD40 mAb (Fig. 5.2 B) indicating liver damage and strong side effects.

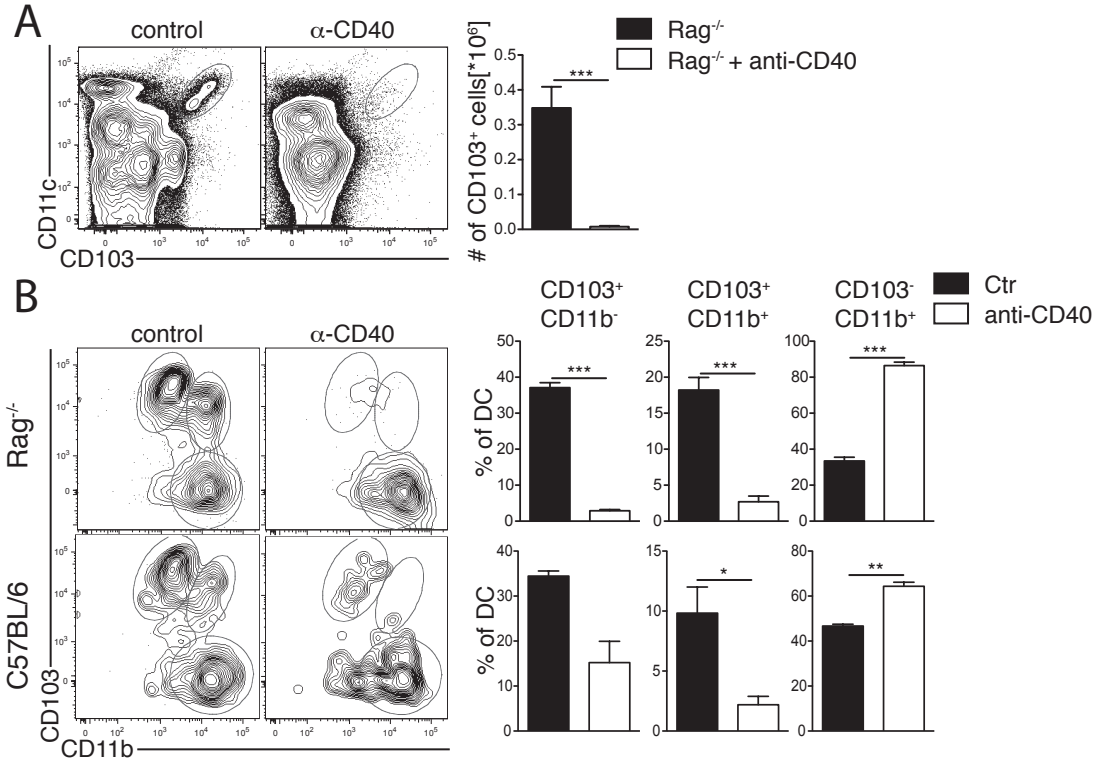

Figure 5.1: Injection of anti-CD40 mAb leads to a reduction of CD103$^+$ DCs in different organs C57BL/6 and Rag$^{-/-}$ animals were injected with anti-CD40 mAb, sacrificed after 72h and analyzed for the presence of CD103$^+$ DCs. **A**) Live cells of Rag$^{-/-}$ spleens were analyzed for the expression of CD103. Shown are representative FACS-plots of one of three experiments (n = 3 animals per group). Statistics represents pooled results from these three experiments. **B**) Expression of CD103 and CD11b on CD11c$^+$MHCII$^+$CD64$^-$ cells in the lamina propria after anti-CD40 injection in C57BL/6 and Rag$^{-/-}$ animals. Representative results of 3 and 2 repetitions with n= 3 respectively.

Additionally we did find a rapid increase in serum levels of proinflammatory cytokines such as MCP-1, TNF-α, IFN-γ and IL-6 (Fig. 5.2 C). Serum levels of these cytokines peaked between 16 and 24 h after injection and were back to baseline after 72 hours.

Taken together these data showed a selective reduction of CD103$^+$ DCs after receiving a CD40 signal. On the other hand our data also showed strong side effects caused by the injection of the anti-CD40 mAb. This made it impossible to asses the influence that a CD40 signal has directly on DCs.

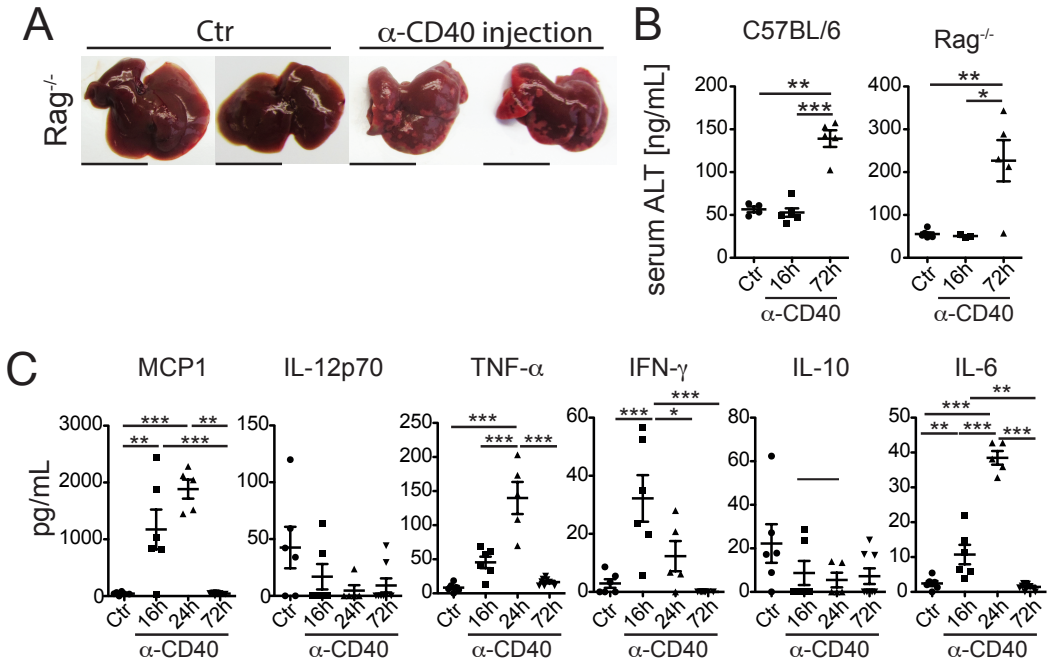

Figure 5.2: anti-CD40 mAb induces strong liver inflammation and leads to increased proinflammatory cytokines in serum. **A**) Macroscopic pictures of livers from control animals and animals that have been injected with anti-CD40 72h before. **B**) Serum levels of the liver specific enzyme ALT as measured by ELISA. **C**) Serum levels of the proinflammatory cytokines. Serum was taken from C57BL/6 animals at the indicated timepoints after anti-CD40 injection and analyzed using cytometric bead array. Statistic significance was analyzed using a One-Way-ANOVA test.

5.2 Characterization of spleens from DC-LMP1/CD40 animals

To analyze the effects of a CD40 signal on DCs we generated DC-LMP1/CD40 mice in which a CD40 signal is delivered specifically to cells which express CD11c (see also section 4.1.7). These mice are born in mendelian ratios but are smaller then their littermates.

Since we wanted to analyze the influence of CD40 signaling in DCs in the context of induction of tolerance and immunity we started our analysis by looking at the spleen as a major site of induction of systemic immune reactions.

The architecture of the spleen of DC-LMP1/CD40 mice was largely intact (Fig. 5.3 A). B cell zones and DC localization were unchanged in DC-LMP1/CD40 animals when compared

to controls. No increase in extramedullary hematopoiesis, as indicated by Ter119 staining, was detected. The anti-Ter119 mAb reacts with mature erythrocytes and some progenitor stages of erythrocytes [100]. The number of lymphocytes per spleen on the other hand was strongly reduced in all subsets analyzed, while at the same time a strong increase in granulocytes was detected. Monocyte and pDC numbers remained unchanged (Fig. 5.3 B).

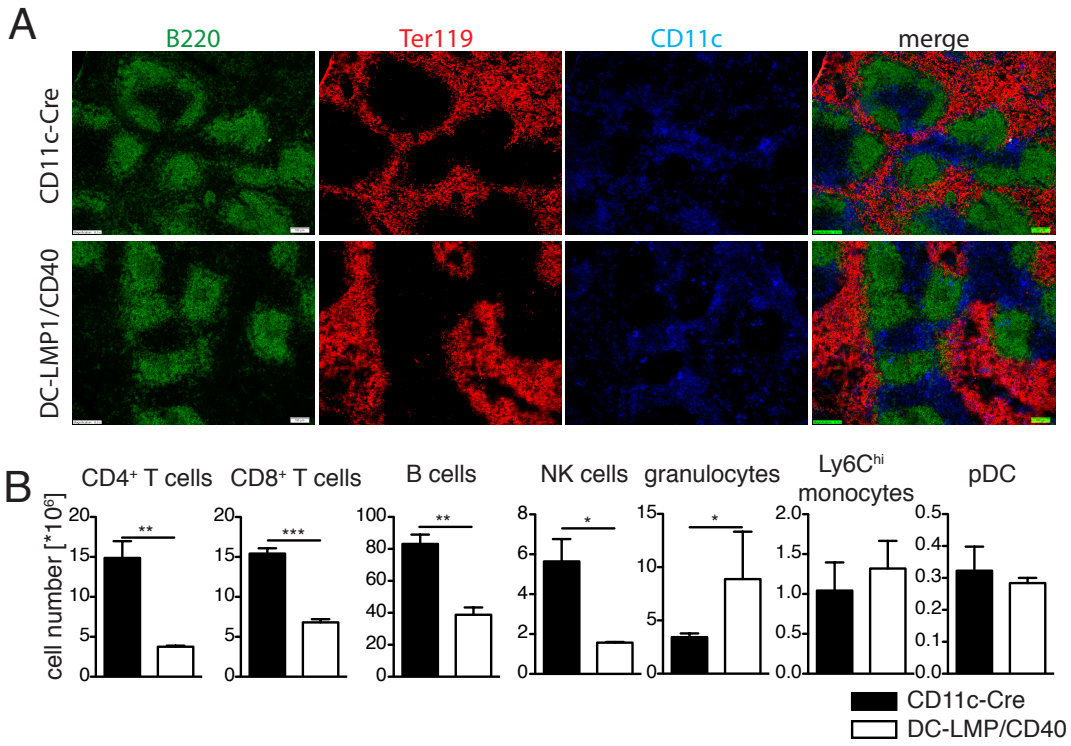

Figure 5.3: Structure of the spleen is unchanged although the lymphocyte numbers are reduced. A) Frozen sections of spleens from control as well as DC-LPMP1/CD40 animals were stained for B cells (B220), DCs (CD11c) and erythrocytes (Ter119). Bars respresent 100 μm. **B**) Cell numbers of different cell types in spleen.

To further investigate the influence of a CD40 signal specifically on splenic DCs, single cell suspensions of spleens from control and transgenic animals were prepared and presence of DCs as well as the subset distribution of $CD8^+$, $CD11b^+$ and double negative (DN) DCs was analyzed (Fig. 5.4 B, C and D). We found that absolute number of DCs reduced in spleen. This reduction was due to a decrease in the number of $CD8^+$ DCs.

Next, we analyzed whether the CD40 signal leads to phenotypical and functional maturation of DCs. To this end we analyzed the expression of MHCII as well as costimulatory molecules,

both of which are upregulated on activated DCs. The expression of CD70, CD80 and CD86 was analyzed on the entirety of splenic DCs. There was no upregulation of CD70 and CD86 while CD80 was considerably upregulated compared to control animals (Fig. 5.4 E). Another sign of DC maturation is the upregualtion of MHCII on the surface. However, the CD40 signal induces a significant downregulation of MHCII on the surface (Fig. 5.4 A).

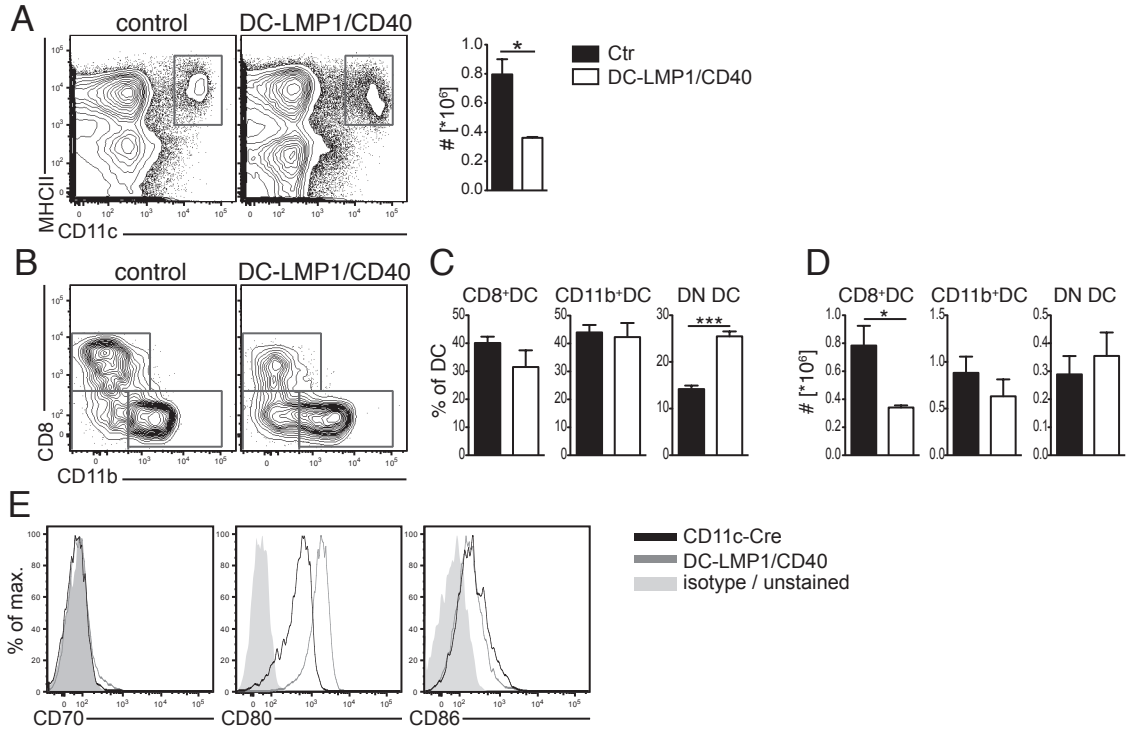

Figure 5.4: Splenic DC subsets and activation status. A) Representative FACS plots of DCs in DC-LMP1/CD40 animals and statistics of cell numbers as representative results of one of 4 experiments (n = 3 per experiment). **B**) Representative FACS-plots of splenic DC subsets as differentiated by the expression of CD8 and CD11b. Plots are gated on live $CD11c^{+}MHCII^{+}$ cells. **C**) Relative amount of cells in the respective subset. Data pooled from two independent experiments (n = 6). **D**) Number of cells in the respective subset. Data pooled from two independent experiments (n = 6). **E**) Expression of costimulatory markers by splenic DCs, representative results of 3 experiments (n = 3).

In order to functionally asses the capacity of DCs in DC-LMP1/CD40 animals to induce tolerance, we made use of a T cell transfer-model. For this, transgenic T cells of known antigen-specificity are transfered into recipient animals. They can then easily be followed *in vivo* by looking at a congenic marker and can be functionally analyzed using the known peptide to which the T cells react.

To this end we purified and transfered OTI T cells into either control or DC-LMP1/CD40 animals. Control animals were left untreated or were injected with ovalbumin protein (Ova) with or without additional anti-CD40 mAb. DC-LMP1/CD40 animals did only receive Ova. If the CD40 signal would lead to a full functional maturation of DCs we would be able to find proliferation of OTI cells without any further stimulus in DC-LMP1/CD40 animals and these cells would also be able to kill target cells loaded with their cognate peptide.

Such an expansion of OTI cells was only observed in the case of control animals immunized with Ova and anti-CD40 mAb. To a much lesser extend this was also seen in control animals that only received Ova, but not in DC-LMP1/CD40 animals (Fig. 5.5 A). To test the functionality of the transfered OTI T cells, splenocytes loaded with the peptide recognized by OTI and unloaded control splenocytes were transfered into the immunized animals at day 15 after immunization. After overnight incubation the specific lysis of these target cells was analyzed. Killing was only observed for the animals that have received additional anti-CD40 mAb (Fig. 5.5 B). These findings indicated that DCs in DC-LMP1/CD40 animals were not able to prime the transgenic OTI T cells. This might either be because the CD40 signaling in DCs did not lead to a maturation status of DC sufficient to induce T cell responses or because the CD40 signal induced changes that did not allow the DCs to take up or process the protein antigen.

To make sure that the differences observed were not due to changes in the antigen-uptake and -processing machinery in DC-LMP1/CD40 animals, we tested these capacities experimentally. For this we injected a mixture of fluorescent AlexaFlour647-Ova and DQ-Ova into transgenic and control animals. DQ-Ova is a fluorescent derivative of the Ova-protein, in which the fluorescence is quenched and this quenching is relieved under acidic conditions e.g. in lysosomes. It can therefore serve as a marker for antigen-processing. Two hours after injecting this mixture, DCs in the spleen were analyzed. As shown in Fig. 5.5 C DC-LMP1/CD40 animals are able to take up and process antigen equally well or even better then wild type control DCs.

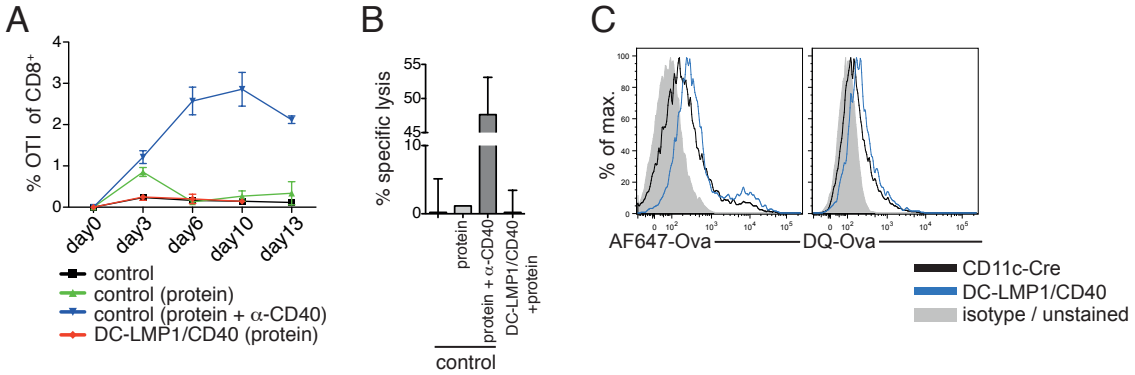

Figure 5.5: DC-LMP1/CD40 mice induce tolerance in CD8 T cells. OTI T cells were transfered and their proliferation was analyzed after Ova protein injection. **A**) Kinetic of OTI cell proliferation after injection of Ova. Cells were gated on live lymphocytes. **B**) At day 15 after immunization CFSE labeled target cells loaded with peptide or control cells were injected in animals of the different groups and specific lysis was analyzed 16 h later. **C**) Antigen uptake and processing in live $CD11c^{+}MHCII^{+}$ cells was analyzed after intravenous injection of AlexaFlour647-Ova and DQ-Ova. Animals were sacrificed two hours later and DCs analyzed for fluorescence of AlexaFlour647-Ova (uptake) and DQ-Ova (processing). Representative plot n = 6.

Taken together, our results indicate that CD40 signaling in DCs does not lead to phenotypical or functional maturation. Instead we find immature DCs that are unable to induce immune reactions.

In order to take a closer look at the changes, that the CD40 signal causes in DCs, we performed gene expression analysis of more than 500 genes. To avoid effects caused by T cell/DC interaction the analysis was performed in animals on the $Rag^{-/-}$-background. We compared DC-LMP1/CD40 animals to $Rag^{-/-}$ and in addition $Rag^{-/-}$ animals that had been injected with anti-CD40 mAb 16 hours before DCs were sorted. This gave us the possibility to compare the influence that a CD40 signal delivered by the transgene to the effects that are observed when cells are targeted by anti-CD40 mAbs. For this, live $CD11c^{+}MHCII^{+}$ cells were sorted from spleen, lysed and the mRNA copy number was analyzed using the nanostring platform.

A principal component analysis was performed, to asses how similar the samples within one group are to one another and to see how similar the different groups were. This analysis clearly shows that all three experimental groups are distinct in their expression pattern from

each other (Fig. 5.6 A).

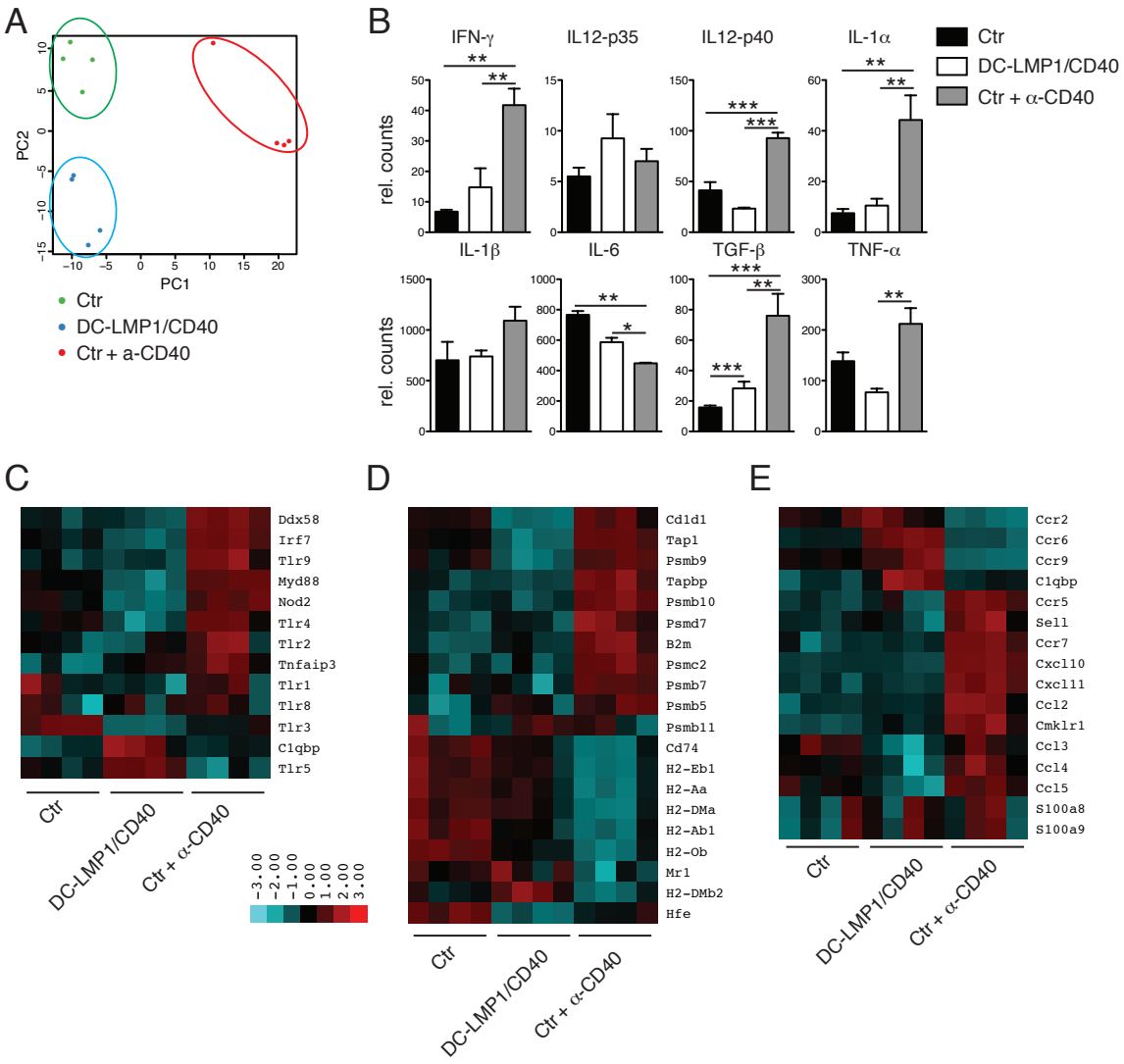

Figure 5.6: Gene expression of DCs from DC-LMP1/CD40 animals is more similar to those from controls than DCs from animals injected with anti-CD40 mAb. DCs of animals on the Rag$^{-/-}$ background were sorted based on high expression of MHCII and CD11c and used for a nanostring gene expression analysis. **A**) Principal component analysis of the different DC-groups. **B**) Expression of proinflammatory cytokines. Heatmap of genes that are implicated in **C**) pattern recognition and associated signal transduction, **D**) antigen-uptake and -presentation for MHCI as well as for MHCII and **E**) chemokines and chemokine receptors.

We further analyzed how the different CD40 signals influence the expression of mRNA for proinflammatory cytokines. As shown in Fig. 5.6 B there was no induction of cytokine transcription observed when transgenic DCs are compared to their wild type counterparts. The only exception was a small increase in the levels of the anti-inflammatory TGF-β. In contrast, the injection of anti-CD40 antibody leads to a strong upregulation of IFN-γ, IL12-

p40, IL1-α, TNF-α and TGF-β. The increase in TGF-β was also much stronger than in transgenic DCs. IL12-p35 was hardly expressed at all under steady state conditions and was also not induced by either way of CD40 signal delivery.

Next we looked at expression levels of genes that play a role in the detection of pathogens and the corresponding signal transduction (Fig. 5.6 C). Here again the DCs treated with anti-CD40 antibody showed a very different expression-pattern from the wild-type and transgenic DCs. Most of the RNA were strongly upregulated after anti-CD40 injection with the exception of mRNAs for toll-like-receptor (TLR)3, 5 and C1q binding protein (C1qbp). Transgenic DCs on the other hand showed a remarkably similar expression pattern as compared to wild-type except for C1qbp and TLR5, responsible for detecting the bacterial protein flagellin [101], which are upregulated and TLR3, specific for double stranded DNA, which is downregulated compared to wild-type.

Another important function of DCs is the processing of antigen and the subsequent loading of these antigenic fragments onto MHC molecules. We also performed a cluster analysis with genes playing roles in these processes and received similar results as with the previous two groups of genes, namely more similarity between wild-type and transgenic DCs and clearly a very different profile for DCs after stimulation with anti-CD40 antibody (Fig. 5.6 D). One striking change was a strong upregulation of genes with a role in processing and loading onto MHC class I molecules (Tap, Tapbp, β2m, proteasomal subunits) after antibody-treatment. In contrast, the genes important for MHCII loading (e.g. CD74, H2-DMa, H2-DMb2) were strongly downregulated after antibody treatment and were also slightly downregulated in transgenic DC when compared to the wild-type. This is also mirrored in the MHCII protein-expression levels (Fig. 5.4 A).

The last group of genes that we analyzed in more detail are chemokines and chemokine-receptors expressed by DCs. Similar to the other genes, we also find DCs from DC-LMP1/CD40 animals being closer related to controls and less similar to anti-CD40 injected animals.

5.3 DC-LMP1/CD40 animals develop spontaneous colitis

As described above, DC-LMP1/CD40 animals were born in mendelian ratios but were visibly smaller than their littermates. This was due to spontaneous development of colitis as shown macroscopically by colon thickening and shortening (Fig. 5.7 A) or using fecal lipocalin-2 as a non invasive marker for intestinal inflammation (Fig. 5.7 B) [102]. Disease could be detected in four to five week old mice and its development could be prevented, when animals were supplied ad libitum with a mixture of four different antibiotics (ABX), to deplete as much of the commensal bacteria as possible. The same was true if the DC-LMP1/CD40 animals were crossed onto a T and B cell-deficient background. This clearly showed that disease development was dependent on the presence of bacteria as well as on cells of the adaptive immune system.

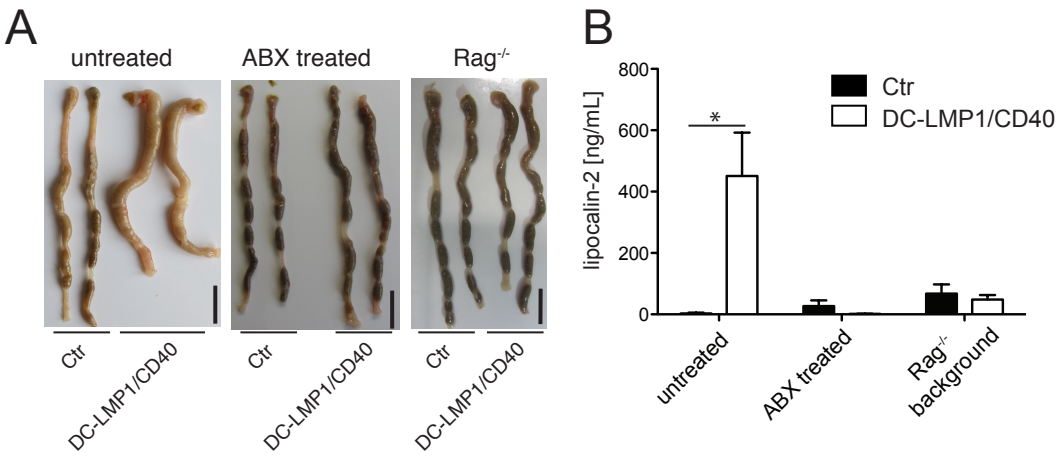

Figure 5.7: DC-LMP1/CD40 animals develop strong colitis, that is dependent on B and T cells and commensal bacteria. A) Macroscopic pictures of colons from control and DC-LMP1/CD40 animals on different genetic backgrounds or after administration of a mixture of ampicilin, neomycin, vancomycin and metronidazol. **B**) Levels of fecal lipocalin-2.

5.4 Influence of CD40-signaling on phagocytic cells in the lamina propria of the colon

5.4.1 Mononuclear phagocytes in the lamina propria of DC-LMP1/CD40 animals

Because of the described colitis, we went ahead and characterized the phagocytic cells of the colonic lamina propria. In contrast to the spleen, macrophages of the gut are also positive for CD11c and MHCII. Therefore, a more complex panel of markers is needed to investigate and differentiate macrophages and DCs.

Single cell suspensions of lamina propria were stained for CD11c and MHCII and cells highly positive for those molecules and negative for F4/80 or CD64, both markers for macrophages, were analyzed for expression of CD11b and CD103. Using this panel one can distinguish three DC subsets (Fig. 5.8 A). Of those, the frequency of the two $CD103^+$ subsets is strongly reduced in the DC-LMP1/CD40 animals on the wild-type background (Fig. 5.8 A). The $CD103^+CD11b^-$ cells are unchanged in numbers, while $CD103^+CD11b^+$ are reduced and $CD103^-CD11b^+$ showed an increase in cellularity (Fig. 5.8 C).

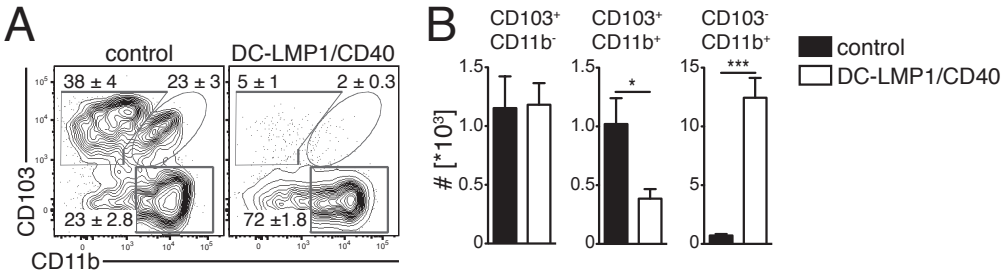

Figure 5.8: $CD103^+CD11b^+$ DCs are strongly reduced in the lamina propria of DC-LMP1/CD40 animals. **A**) Representative FACS plots of DCs of the colonic lamina propria from DC-LMP1/CD40 animals under steady state conditions. Cells were gated on $CD11c^+MHCII^+F4/80^-$. Numbers in FACS plots indicate percentage of DC subsets as mean ± SEM (n = 9) **B**) Cell numbers show pooled results from two experiments (n = 6).

In order to find out if this reduction in $CD103^+$ DCs is a secondary effect of the colitic inflammation or a direct effect of the transgene expression, we repeated this analysis in animals that have been treated for three weeks with ABX or that were on the $Rag^{-/-}$-

background and therefore lacked inflammation. In both groups we found a decreased frequency of CD11b^{-}CD103^{+} and CD11b^{+}CD103^{+} populations (Fig. 5.9 A). Also the absolute numbers of CD103^{+} DCs were strongly reduced in DC-LMP1/CD40 animals on the Rag$^{-/-}$ background and this tendency was also observed for CD11b^{+}CD103^{+} DC in DC-LMP1/CD40 animals treated with ABX, although it did not reach statistical significance (Fig. 5.9 B). The number of CD103^{-} DCs was also increased in animals treated with ABX but to a much lesser degree than in untreated animals. In animals on the Rag$^{-/-}$-background we did not find an increase in CD103^{-} DCs.

Macrophages of the gut play an essential role in the homeostasis of the gut and at the same time might express the LMP1/CD40 transgene due to some CD11c expression [103]. To further characterize if macrophages are changed in the transgenic animals, we used a panel of markers developed in the lab of Bernard Malissen [38], allowing to differentiate distinct developmental stages of Ly6C^{hi} monocytes from the moment they leave the circulation (population P1 in Fig. 5.10 A) and enter the tissue until they finally become anti-inflammatory macrophages (population P3/P4 in Fig. 5.10 A). Using this marker-set we could show that

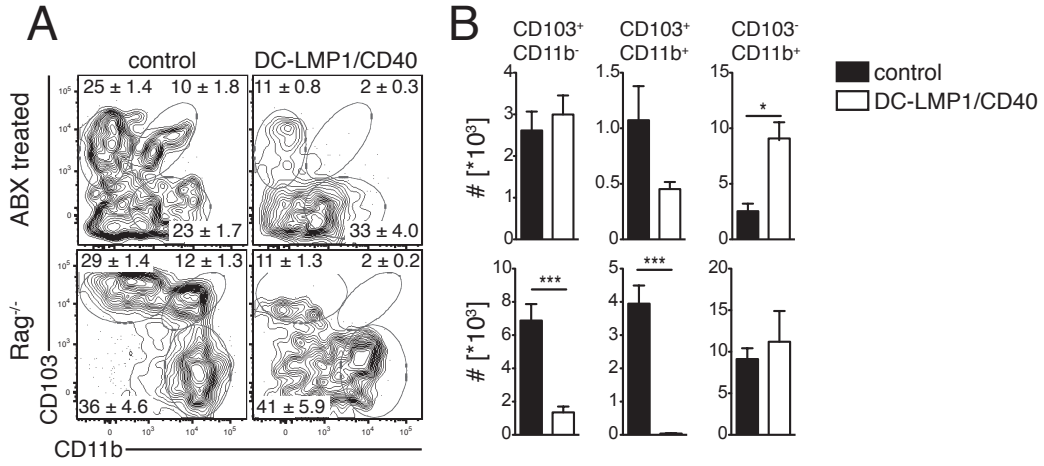

Figure 5.9: CD103^{+} DC reduction is independent of inflammation in DC-LMP1/CD40 animals. A) Representative FACS plots of DCs of the colonic lamina propria from DC-LMP1/CD40 animals on the Rag$^{-/-}$ background or after ABX-treatment. Cells were gated on CD11c^{+}MHCII^{+}F4/80^{-}. Numbers in FACS plots indicate percentage of DC subsets as mean ± SEM from one of two independent experiments (n = 3). **B**) Representative cell numbers of one of two experiments.

in DC-LMP1/CD40 animals the percentage of cells in the "waterfall"-gate that fall into the P3/P4 gate was significantly reduced compared to the wild-type controls. The overall cell number on the other hand was strongly increased in all three subsets differentiated here. This is due to the strong inflammation observed in DC-LMP1/CD40 animals which leads to an overall increase in cellularity of the colon. To investigate the influence of the inflammatory milieu we analyzed macrophages in ABX-treated animals as well as in $Rag^{-/-}$-mice. After ABX treatment we found the distribution of the three subsets unchanged as compared to the wild type. In contrast, we also saw a reduction in P3/P4 in DC-LMP1/CD40 animals on the $Rag^{-/-}$-background. Taken together, this indicated that changes in macrophages observed in untreated animals are due to the inflammatory milieu and not caused by the transgene expression.

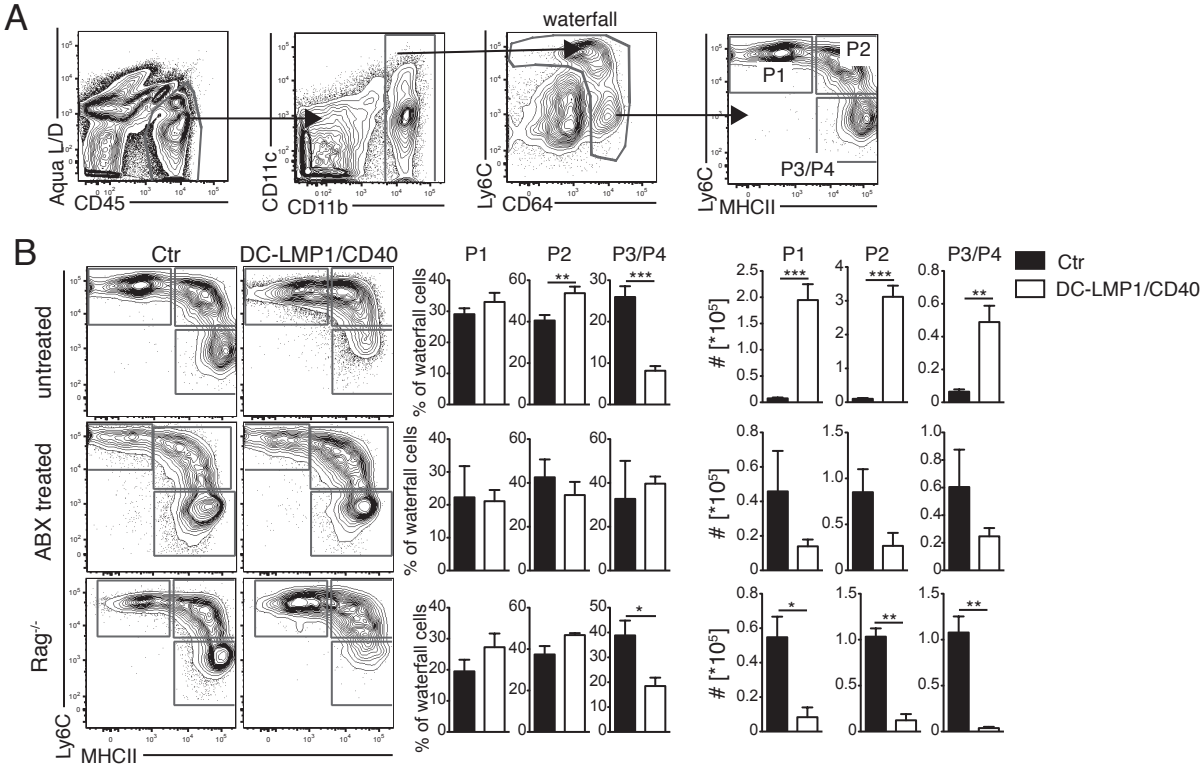

Figure 5.10: Development of macrophages in DC-LMP1/CD40 animals. **A**) Gating strategy to analyze the development from $Ly6C^{hi}$ monocytes towards tissue-resident macrophages. **B**) Distribution of cells inside the "waterfall"-gate into the gates P1, P2 and P3/P4 in untreated C57BL/6, ABX treated C57BL/6 and $Rag^{-/-}$-animals (n = 3)

The absolute number of macrophages on the other hand was strongly reduced in both

groups and in all subsets analyzed, although it did not reach statistical significance after ABX treatment. This finding implicates that the reduction in the relative amount of P3/P4 might be dependent on a bacterial signal in addition to the CD40 stimulus and also hints at a possible dependence of macrophages in the lamina propria on cells of the adaptive immune system and commensal bacteria to properly differentiate.

Since we found a strong reduction of CD103$^+$ DCs in the lamina propria of the colon and these cells are known to migrate towards the draining lymph node, we analyzed DCs in the mesenteric lymph node for the presence of the four DC subsets that can be differentiated in this organ by CD103 and CD11b. We found a small reduction of the percentage of DCs inside the live cell gate but this was compensated for by a larger number of living cells, so that the final number of DCs per mLN was the same (Fig. 5.11 A). Again this was independent of the proinflammatory milieu since we found the same in animals that were treated with ABX.

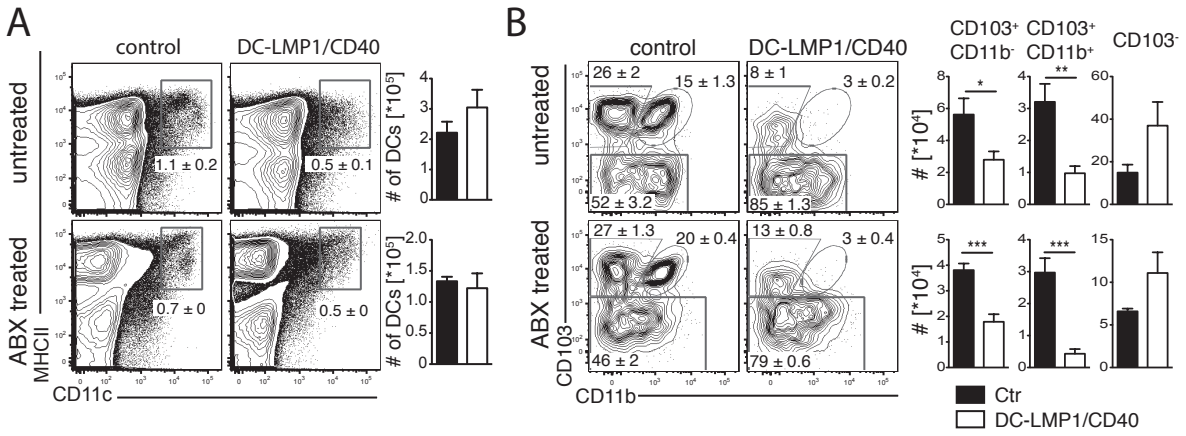

Figure 5.11: CD103$^+$ DCs are strongly reduced in mLN of DC-LMP1/CD40 animals. **A**) Representative FACS plots of DC gate in mLN of control and DC-LMP1/CD40 animals with and without ABX treatment and the statistics for these CD11c$^+$MHCII$^+$ cells. **B**) DCs from A were analyzed using CD103 and CD11b. Number in representative FACS plots are mean ± SEM (statistics for n = 10 untreated animals and n = 5 ABX treated ones).

Next we analyzed the DC subsets that can be found in the mLN and could indeed confirm the results of the lamina propria. The CD103$^+$CD11b$^-$ subset was reduced by about 60 % when looked at the relative amount and 50 % when absolute cell numbers were considered (Fig. 5.11 B). This reduction was even stronger in the CD103$^+$CD11b$^+$ compartment. Here we found reductions between 70 and 80 %.

All these results were again independent of an inflammation, since we found the same in animals treated with ABX. Because we found the same numbers of DCs, defined as $CD11c^+MHCII^+$ cells, but a reduction in both $CD103^+$ populations, it is also to be expected that an increase of another subset would be detected. Indeed, this was the case for the $CD103^-$ compartment, although it did not reach statistical significance.

Using the transgenic model, we were unable to differentiate if this reduction is due to migration or cell death or both. To address this we made again use of the anti-CD40 injection system.

5.4.2 Mononuclear phagocytes in the lamina propria after injection of anti-CD40 mAb

Although the DC-LMP1/CD40 system has the advantage that one can directly deliver a CD40 signal to DCs without targeting other cells, at the same time it has the disadvantage, that it is an "always-on"-sytem in which DCs receive the CD40 signal from the moment they express CD11c. Therefore, it is difficult to define the first event in a cascade of events. To circumvent this problem we utilized a system where the CD40 signal is delivered using anti-CD40 mAb injection.

A kinetic was performed, where DCs in lamina propria and mLN were analyzed 16, 24 and 72 hours after antibody injection. We stained for the presence of all three different DC populations and followed them over time.

In the lamina propria we could detect a decrease of all DC subsets as soon as 16 h after injection of the antibody. This decrease progressed until the end of the experiment and no recovery in cell numbers was observed. At 72 h after injection there were hardly any $CD103^+$ cells left in the lamina propria and also the number of $CD103^-$ DCs was strongly reduced(Fig. 5.12 A and B). This decrease was accompanied by an increase in CCR7 mRNA-expression by $CD11c^+MHCII^+$ cells of the lamina propria already 16 h after anti-CD40 injection as determined by nanostring expression analysis (Fig. 5.12 C).

CCR7 expression could result in migration of DC from tissues to draining LNs [104]. Accordingly we could detect an increase in the the absolute number of all three DCs subsets in the mLN (Fig. 5.12 A and B). This increase was transient, peaked at 24 h after anti-CD40 mAb injection and fell back to initial levels 72 h after injection.

This data suggest that CD40 stimulus of DCs induced an increased exit of DCs from the lamina propria and a subsequent migration towards the draining lymph node.

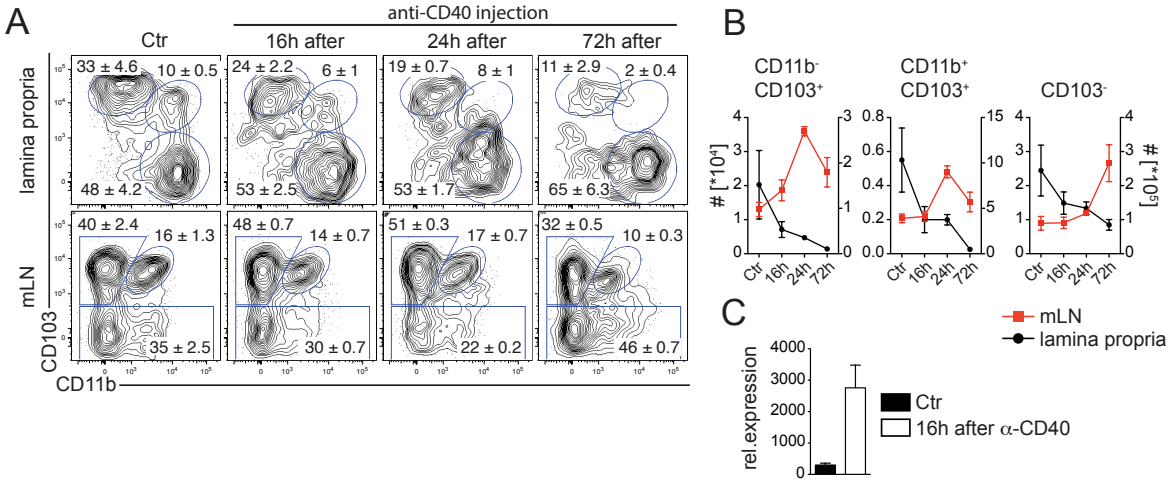

Figure 5.12: DCs decrease in lamina propria after anti-CD40 injection while they transiently increase in mLN. A) $CD11c^{+}MHCII^{+}CD64^{-}$ cells were analyzed for the expression of CD103 and CD11b in lamina propria and mLN over 72 h after anti-CD40 injection. Representative FACS-plots of one of two independent experiments. Numbers indicate percentage of cells within a subset as mean ± SEM (n = 6 per group). **B**) Cell numbers of cells within the subsets defined in A) (n = 6 per group). **C**) Relative expression of CCR7 mRNA in $CD11c^{+}MHCII^{+}$ cells of the colonic lamina propria from control and anti-CD40 treated animals.

To exclude that this effect was mediated by secondary effects of the mAb injection on other $CD40^{+}$ cells, we made use of a mouse-model in which CD11c-Cre cuts the floxed CD40-allel (DC-CD40 KO mice), thereby rendering DCs $CD40^{-}$ and insensitive towards an anti-CD40 antibody. These animals were injected with anti-CD40 antibody and 72 h after injection DCs of the lamina propria were analyzed for CD103 and CD11b expression. As shown in Fig. 5.13 A $CD103^{+}$ DCs were completely absent from the lamina propria of control animals injected with the antibody, while the levels of $CD103^{+}$ cells were basically unchanged in DC-CD40 KO animals. This data shows that anti-CD40 mAb has to target DCs directly in order to induce their exit from the lamina propria and is not mediated by secondary effects.

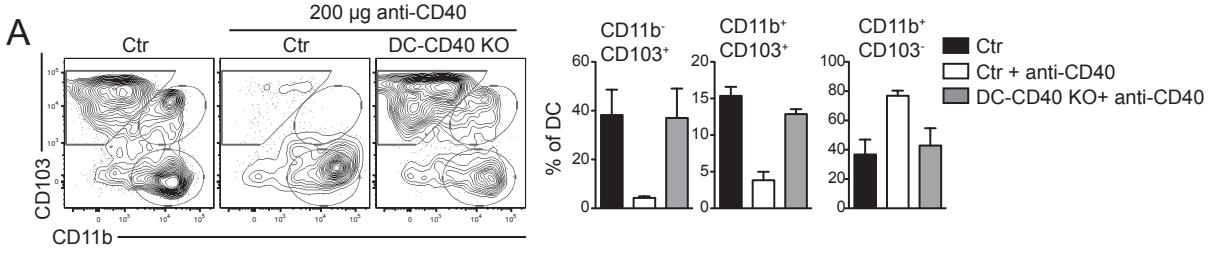

Figure 5.13: CD103$^+$DC reduction is dependent on CD40 expressed by DCs. A) DC-CD40 KO animals and controls were injected with anti-CD40 mAb and three days later lamina propria was stained for the presence of the different DC subsets (n = 5, pooled results from two independent experiments).

Subsequently, we investigated the fate of DCs after they reached the mLN. To this end, we performed flow cytometry based apoptosis assays in DCs isolated from the mLN of wild type animals injected with anti-CD40 mAb either 16 h or 24 h before the analysis. Cells were stained intracellular for active caspase 3, a late effector caspase essential for many apoptotic pathways, and cells positive for this marker were considered to be apoptotic. The frequency of apoptotic cells within the different DC subsets was largely unchanged (as shown in Fig. 5.14 A and B). Contrary to this, the number of cells in a given subset for caspase3$^+$ cells was strongly increased 16 h after injection.

Since we found a shift in macrophage subset distribution in untreated DC-LMP1/CD40 mice (see Fig. 5.10), we also analyzed the distribution of cells within the waterfall gate,

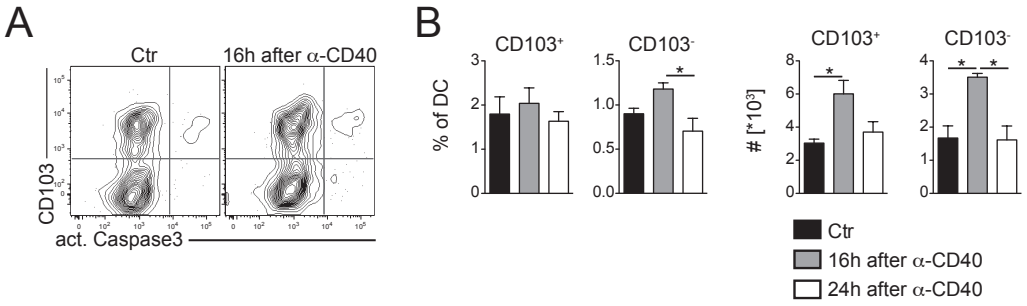

Figure 5.14: The number of apoptotic cells is increased in mLN after anti-CD40 injection Animals were injected with anti-CD40 antibody and CD103$^+$ and CD103$^-$ DCs in the mLN were analyzed at the indicated time points for the presence of apoptotic cells. **A**) Representative FACS plots of active caspase 3 expression. **B**) Frequency of active caspase 3$^+$ cells within the different subsets. **C**) Number of cells positive for active caspase 3. Data represents one experiment n = 3

after we injected anti-CD40 antibody. We saw a transient increase in populations P1 and P2, in percentage as well as in cells numbers. Parallel to that cells in the P3/P4 gate did decrease transiently (Fig. 5.15). All population reached the initial level again at day three after injection. This finding suggest that CD40 injection also influences macrophages and changes the composition of macrophage-precursors and mature macrophages. If this influence is a direct one or mediated by secondary effects remains unclear. But since macrophages have been shown to be unable to migrate it is highly likely that they die in this setting [39].

In order to get an idea how the signal delivered by anti-CD40 antibody and the LMP1/CD40 transgene influences the gene expression in colonic mononuclear phagocytes, we performed transcriptome analysis of $CD11c^{+}MHCII^{+}$ cells. For this, $CD11c^{+}MHCII^{+}$ cells were sorted from the lamina propria of the colon. To avoid secondary effects due to the strong inflammation on the normal C57BL/6 background, experiments were performed on the $Rag^{-/-}$ background. Cells routinely had a purity of 85-95 % after sorting. In analogy to the DCs from spleen, these cells were analyzed using the mouse immunology panel from NanoString.

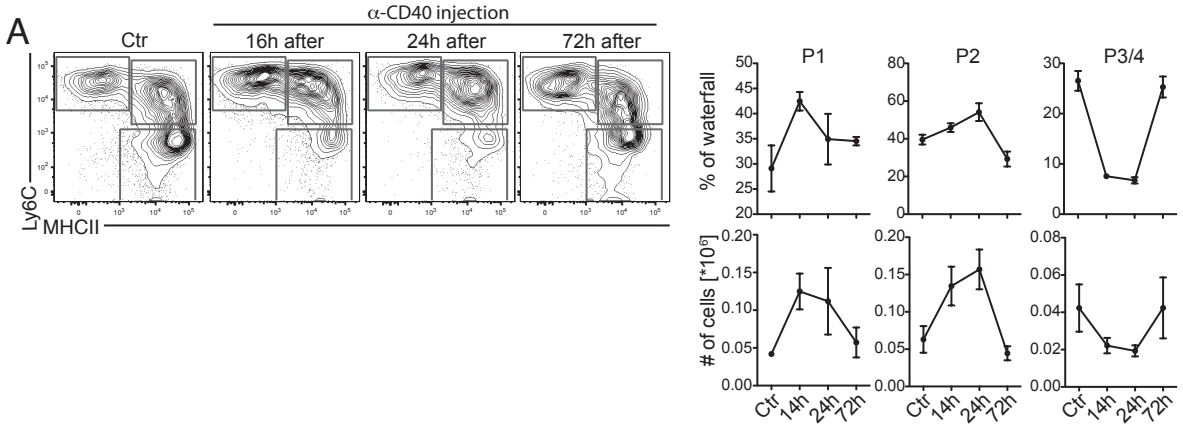

Figure 5.15: Number of mature macrophages decreases in lamina propria after anti-CD40 mAb injection. Animals were injected with anti-CD40 antibody and the distribution of cells in the three waterfall subsets was analyzed. **A**) Representative FACS plots of waterfall cells (gated on live, $CD45^{+}$, $CD11b^{+}$ cells). **B**) Statistics of subset distribution. Data represents one experiment (n = 3)

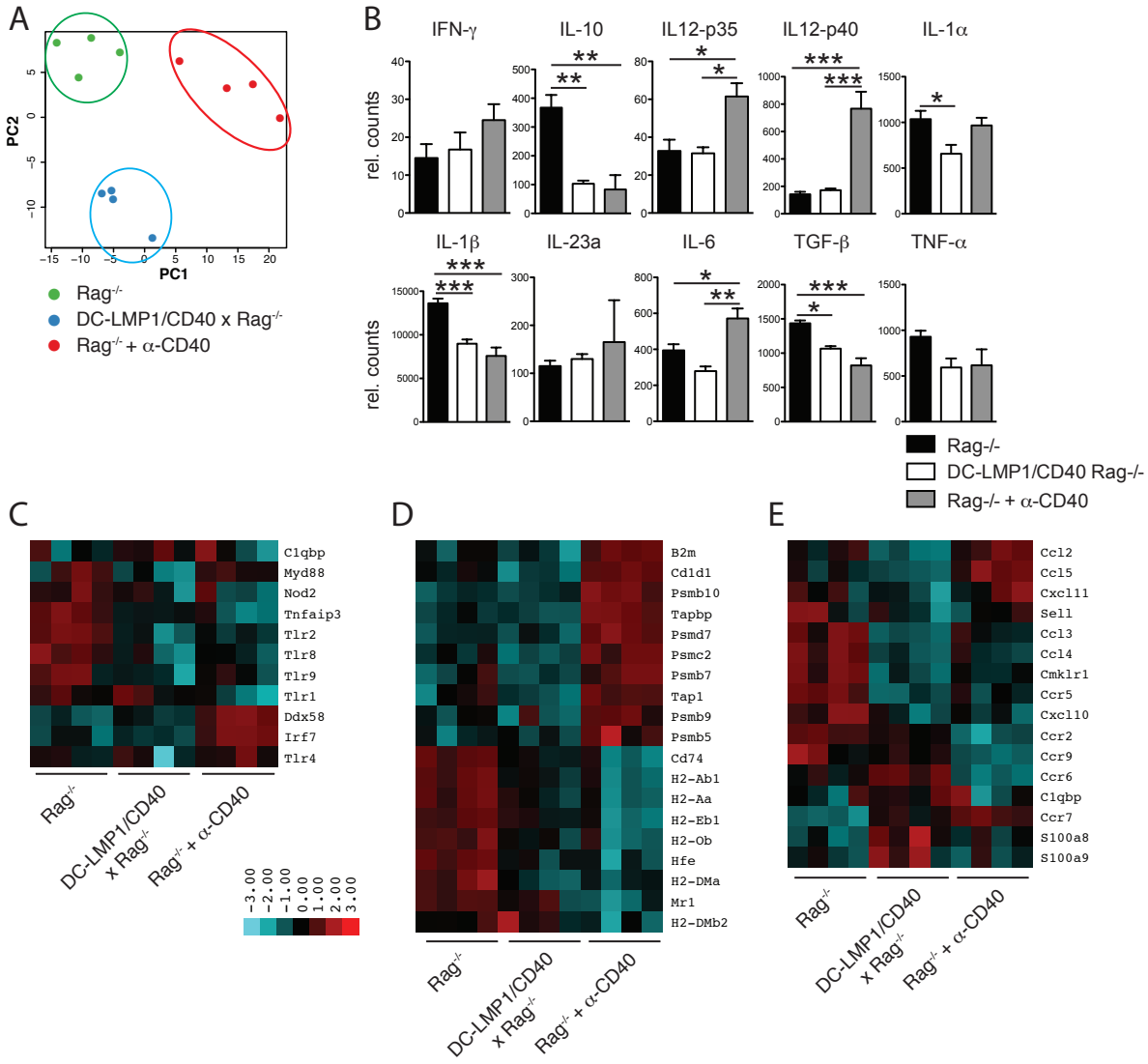

Figure 5.16: Gene expression of mononuclear phagocytes in DC-LMP1/CD40 animals are different from those in control animals and animals injected with anti-CD40 mAb. Mononuclear phagocytes were sorted based on high expression of MHCII and CD11c and used for a nanostring gene expression analysis. **A**) Principal component analysis of the different groups. **B**) Expression of proinflammatory cytokines. **C**) Heatmap of genes that are implicated in pattern recognition and the associated signal transduction. **D**) Heatmap of gene implicated in antigen-uptake and -presentation for MHCI as well as for MHCII. **E**) Heatmap of chemokine and chemokine-receptor gene expression.

A principal component analysis showed, that the $CD11c^{+}MHCII^{+}$ cells of the gut of DC-LMP1/CD40 animals are highly different from those of control animals injected with anti-CD40 mAb. The variance inside the antibody treated group is considerably higher than in the spleen or in the other groups from the lamina propria, but the expression profiles of the

samples still clusters together (Fig 5.16 A).

The cytokine expression profile for the lamina propria again showed no upregulation of any proinflammatory cytokine for DC-LMP1/CD40 animals, but instead a downregulation of IL1-α and IL1-β and additionally a substantial downregulation of the anti-inflammatory molecules IL-10 and TGF-β. The latter was also observed after antibody injection, where on the other hand also an increase of IL6, IL12-p35 and -p40 was observed (Fig 5.16 B).

The expression of genes implicated in pattern-recognition is way more variable in phagocytes from the lamina propria than in those from spleen. In general we could observe an upregulation of Tlr4, Ddx58 and Irf7 and a downregulation of Tlr1, -2, -8, -9 and Nod2 in animals treated with anti-CD40 antibody, when compared to the wild-type. A downregulation of Tlr2, -8, -9 and Nod2 could also be observed for phagocytes from transgenic animals. Interestingly Tnfaip3, the gene coding for the protein A20 which is involved in regulating NF-κB signaling, was strongly downregulated in both transgenic and antibody-treated animals, when compared to the controls (Fig 5.16 C).

In complete agreement with the results from the splenic DCs we found that the treatment with anti-CD40 mAb led to the upregulation of the MHC class I machinery and the downregulation of the class II machinery. The latter is a feature shared between DCs from transgenic and antibody treated animals (Fig 5.16 D).

The chemokine and chemokine-receptor expression differs from the other groups of genes shown here in that we find more genes that are regulated the same way in transgenic animals and wild type controls injected with anti-CD40 antibody. Most of these genes are downregulated (Ccl3, -4, Ccr5, Cxcl10 and Cmklr1). The notable exception from this rule is the expression of CCR7 that is induced in both groups of CD40 stimulated phagocytes. In addition to this, anti-CD40 stimulated phagocytes also show upregulated expression of Ccl2, -5 and Cxcl11 (Fig 5.16 E).

In summary, gene expression of phagocytic cells from the GALT of DC-LMP1/CD40 animals differ considerably from DCs in control animals with or without anti-CD40 injection.

Interestingly though, we were unable to observe production of proinflammatory cytokines in DC-LMP1/CD40 animals in the population of cells analyzed here.

5.5 Effector mechanism of colitis in DC-LMP1/CD40 animals

Experiments in which DC-LMP1/CD40 animals were crossed onto the $Rag^{-/-}$-background and thereby eliminating the influence of B and T cells showed that the development of colitis was dependent on either one or both of these cell types. To further study the role of adaptive immunity in disease development we looked at antibody and T cell responses.

5.5.1 Bacterial reactive antibodies

Serum was taken from DC-LMP1/CD40 animals of different ages and used as a primary antibody in an ELISA. The ELISA plate was coated with a homogenate of cecal contents from healthy C57BL/6 mice, therefore containing bacterial- as well as food-antigens. Isotype-specific antibodies were used as secondary antibodies, to be able to differentiate the isotype reacting to the antigens.

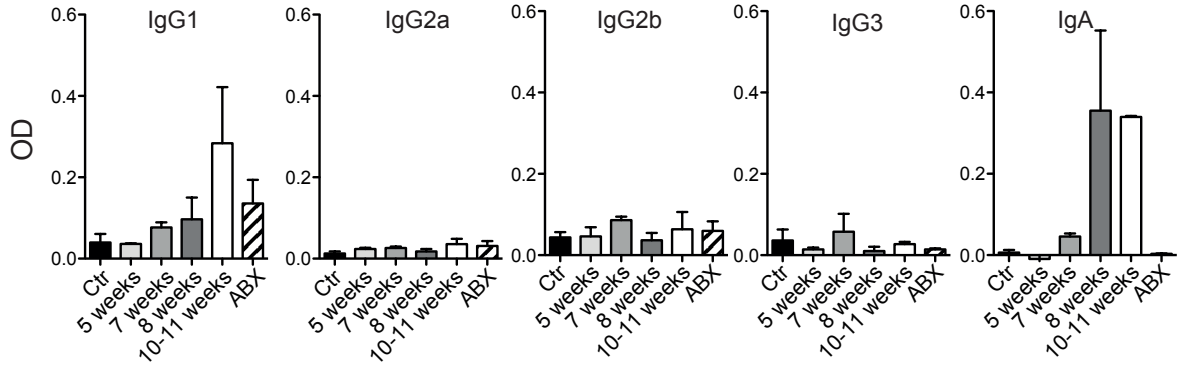

Figure 5.17: Serum of DC-LMP1/CD40 animals shows reactivity towards cecal contents. Cecal bacterial lysates were coated onto an ELISA-plate and serum from mice of different ages was used as primary antibody in an ELISA (n =3-5).

As shown in Fig. 5.17 we found an increase in reactivity against commensal antigens in animals older then 7 weeks. This reactivity was confined to antibodies of the IgG1 and

IgA isotype. Additionally, we showed that the treatment of these animals with ABX led to the absence of reactivity towards the commensal antigens in the serum of these animals, suggesting that rather bacterial than food-antigen was recognized by these mAbs.

5.5.2 Regulatory T cells

Because of the well established connection between $CD103^+$DCs and Treg generation [48, 49], we decided to look in detail into the Treg compartment of DC-LMP1/CD40 mice. Tregs were defined as CD4 T cells with high expression of FoxP3 as well as a varying expression of CD25. Using this gating strategy we found no differences between control and DC-LMP1/CD40 animals in the frequency of Tregs in the CD4 T cell compartment in spleen, mLN or the lamina propria (Fig. 5.18 A).

We next employed a staining scheme that has been developed by Caspar Ohnmacht in the lab of Gerad Eberl [71], that allowed us to differentiate thymus-derived Tregs (nTregs) from Tregs that have been induced in the periphery (iTregs). As shown in Fig. 5.18 B one can distinguish two populations within the $FoxP3^+$ population when using the markers Helios and RORγt. nTregs are $Helios^+ROR\gamma t^-$ and iTregs are $Helios^-ROR\gamma t^+$. In wild type animals between 60 and 80% of Tregs in the lamina propria of the colon are iTregs. In DC-LMP1/CD40 animals this population is virtually absent and all Tregs are thymus-derived nTregs.

This finding was recapitulated in a model were OTII cells were transfered into control and DC-LMP1/CD40 animals and these were fed with ovalbumin in drinking water for five days. Antigen delivered by the oral route usually leads to the induction of tolerance. In fact, this can be observed in the wild type control were we find a substantial induction of FoxP3 expression in the OTII population in the mLN. In DC-LMP1/CD40 animals on the other hand, this population of $FoxP3^+$ OTII cells was almost absent, again showing a strong defect in Treg induction (Fig. 5.18 C).

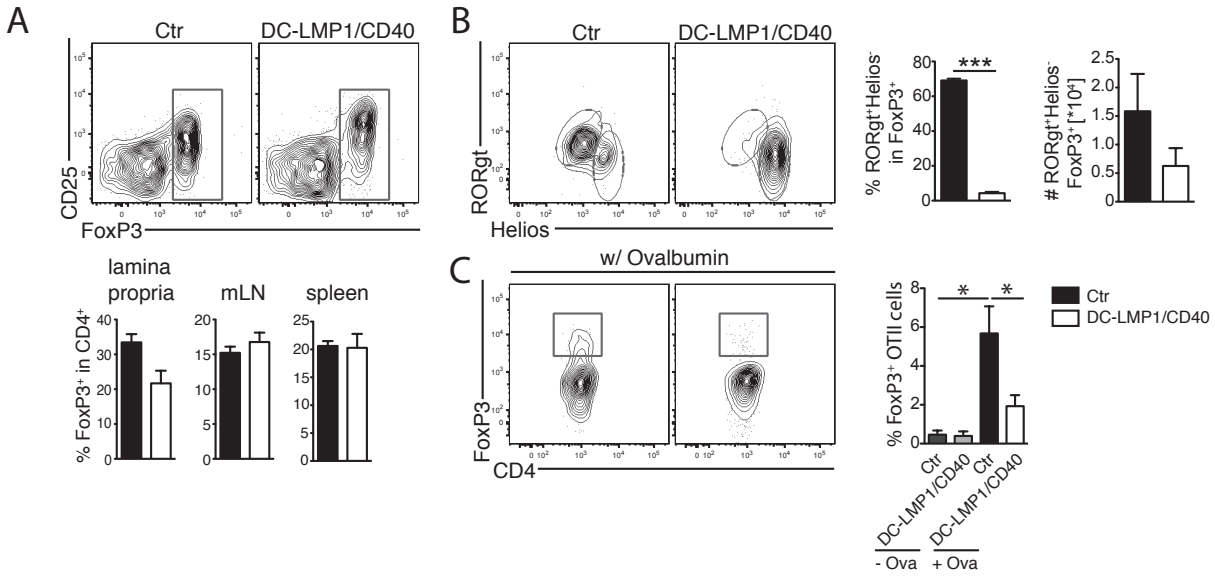

Figure 5.18: Tregs are present at normal frequencies in all organs, while iTregs are missing. A) Representative FACS-plots of Treg stainings in the lamina propria of control and DC-LMP1/CD40 mice. Cells are gated on live $CD3^+CD4^+$ cells (top row). Bottom row shows representative statistics of one of at least two experiments for Tregs in the indicated organs (n = 3). **B**) Tregs found in the lamina propria of the colon were further subdivided into nTregs ($Helios^+ROR\gamma t^-$) and iTregs ($Helios^-ROR\gamma t^+$). Representative result of one of three experiments (n = 3) **C**) OTII cells have been transfered into control and DC-LMP1/CD40 animals that have been treated with ABX for 4 weeks and these animals were given ovalbumin in drinking water for 5 days. The expression of FoxP3 was analyzed in OTII cell recovered from mLN. Representative result of one of three experiments (n = 3)

To test if the observed lack of Treg induction was dominant, we generated mixed bone marrow chimeras of wild type and DC-LMP1/CD40 bone marrow that could be differentiated using CD45.1 and CD45.2 respectively. To make sure that we did not transfer cells that have been primed in a colitogenic environment and thereby transfer cells that in turn might have an influence on the DCs or disease development, we transfered DC-LMP1/CD40 bone marrow of animals on the $Rag^{-/-}$-background.

We did not observe any visual signs of colitis in any of the chimeras. To look at a cellular level we characterized DCs in lamina propria and mLN and also stained for Tregs in the gut.

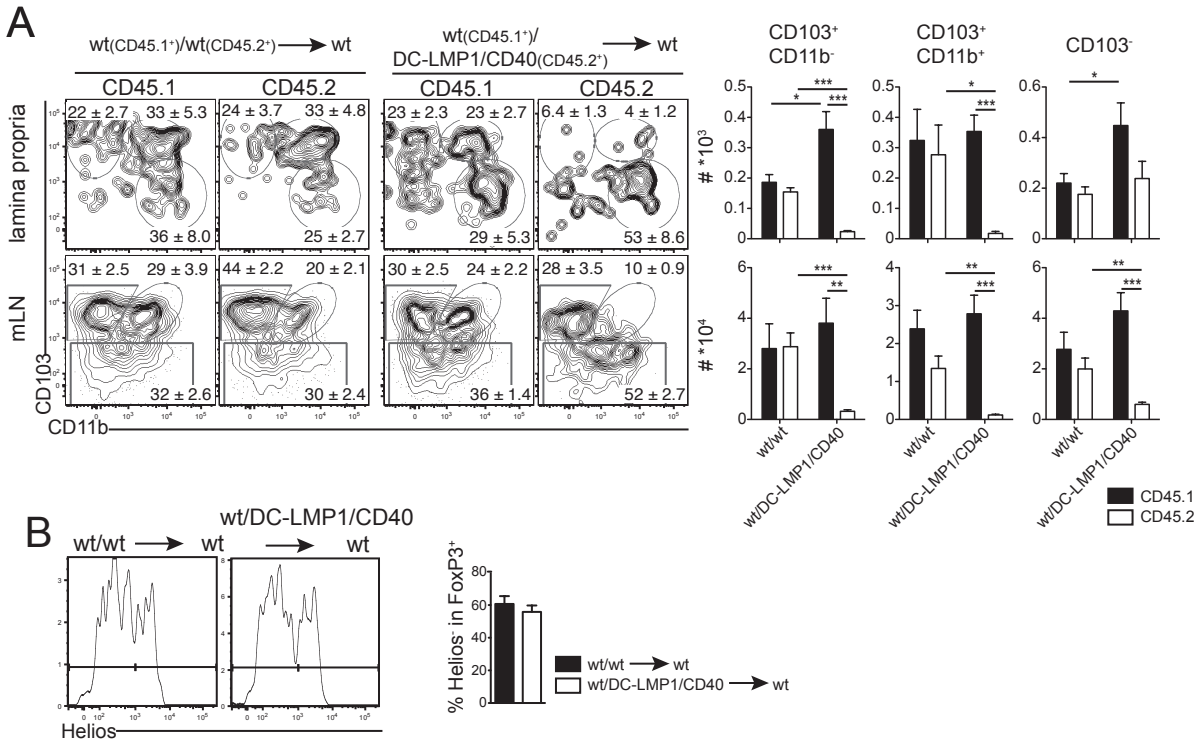

Figure 5.19: iTregs can be induced in mixed bone marrow chimera. A) Representative FACS-plots of DC subsets from lamina propria and mLN of both types of chimera. Cells are gated on live CD11c$^+$MHCII$^+$F4/80$^-$ cells (top row). Statistics are shown for n = 6, number in FACS plots indicate mean ± SEM. **B**) Representative FACS plots of FoxP3$^+$ Tregs found in the lamina propria of the colon further subdivided into nTregs(Helios$^+$) and iTregs (Helios$^-$). Statistics for n = 6.

In the chimeric setting the DC-LMP1/CD40 bone marrow gave rise to reduced levels of CD103$^+$ DCs in the lamina propria, while CD103$^-$ DCs were present at normal frequencies and numbers. DCs derived from the wild type bone marrow, co-transfered into the same animals, reached comparable levels to those from the control chimera (Fig. 5.19 A upper row).

Interestingly, in mLN all DCs derived from DC-LMP1/CD40 bone marrow are strongly reduced when compared to DCs derived from the co-transfered wt bone marrow or to the numbers observed in the wt/wt chimera. Although within the DC-LMP1/CD40-derived DCs we find frequencies of CD103$^+$CD11b$^-$ and CD103$^-$ DC comparable to those from wt-derived DCs, the overall number of DCs derived from DC-LMP1/CD40 bone marrow in mLN is lower. This indicates a strong disadvantage of DCs derived from DC-LMP1/CD40 animals compared

to the wt-derived DCs.

Nevertheless, we could use this model, in which we had CD103$^+$ DCs derived from normal and transgenic bone marrow side by side in one animal, to investigate if the suppressive influence of transgenic DCs on the development of iTregs is dominant. For this the lamina propria of both mixed chimera was stained for the presence of Tregs and these were then classified as iTregs (Helios$^-$) and nTregs (Helios$^+$). Using this scheme we could show that there was normal induction of Tregs in the periphery of mixed bone marrow chimera also in animals that received a mixture of wt and DC-LMP1/CD40 animals.

Therefore we concluded that the lack of iTregs in DC-LMP1/CD40 mice was not a dominant effect caused by the transgenic DCs, but rather caused by lack of appropriate DCs, needed for iTreg induction.

5.5.3 Th1 and Th17 cells

In order to investigate the fate of T cells in DC-LMP1/CD40 we performed *ex-vivo* T cell polyclonal restimulations of T cells from the lamina propria and mLN with phorbol-12-myristat-13-acetat (PMA) and ionomycin. The cells were then stained intracellularly for the presence of Il-17 and IFN-γ.

In lamina propria of DC-LMP1/CD40 animals higher frequencies of IFN-γ^+ and IFN-γ^+IL17$^+$ producing T cells were found. At the same time the frequency of IL-17$^+$ T cells was unchanged (Fig. 5.20 A). The absolute cell numbers of all these subsets were increased in DC-LMP/CD40 animals (Fig. 5.20 B). This increase was due to the strong increase in overall cellularity due to the inflammation.

In mLN only the IFN-γ^+IL17$^+$ T cells were increased while both other subsets remained unchanged in frequency and cell numbers (Fig. 5.20 A and B).

This clearly shows that T cells in the lamina propria are more activated than their control counterparts and also indicates that the response takes place in the lamina propria itself rather then in the draining lymph nodes. This is another indication that the antigens recognized

by these T cells can be found, similar to the antigens recognized by the mAbs described in section 5.5.1, rather in the gut than elsewhere.

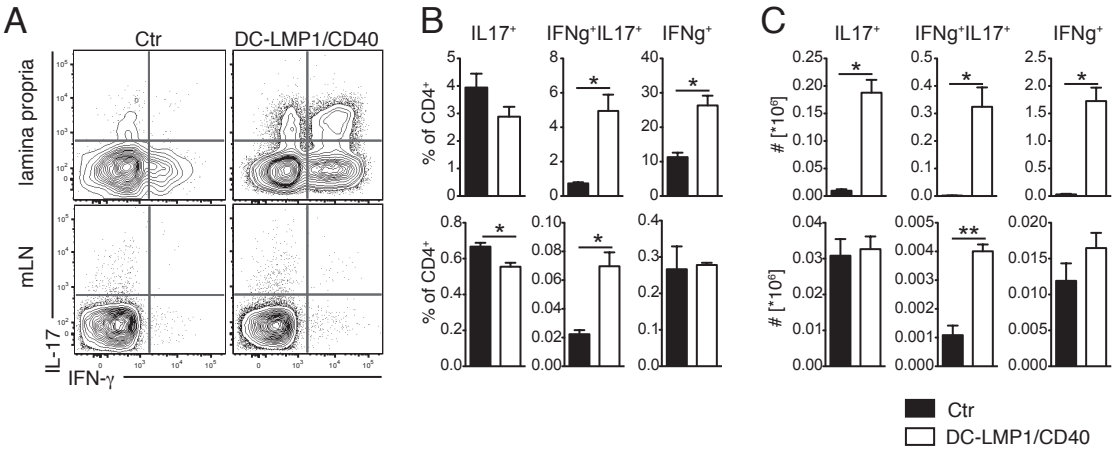

Figure 5.20: DC-LMP1/CD40 animals show increased levels of proinflammatory T cells in the lamina propria. A) Representative FACS-plots of IL-17 and IFN-γ production by T cells of lamina propria and mLN. Cells are gated on live $CD3^+CD4^+$ cells. **B**) Frequency of T cells secreting IL-17 or IFN-γ or both. **C**) Absolute numbers of T cells secreting IL-17 or IFN-γ or both. (always n = 3)

6 Discussion

The influence of CD40-signaling on DCs is still not fully understood and literature is controversial on the question to what degree a CD40 signal leads to phenotypical and functional maturation of DCs (see page 17). Here we were able to investigate the role of a CD40 signal delivered by an antagonistic antibody in DC maturation and function. Additionally, we also developed a system in which DCs receive a ligand-free CD40 stimulus, which let us analyze the effect of CD40 in DCs without the effects, that an antibody has on other cell types.

Using this system we were able to show very different effects of such a signal on DCs in various locations. DCs in spleen largely remained unchanged in phenotype and function, while DCs of the GALT showed major changes in subset distribution and function. These shifts, in the end, also led to the development of severe colitis in animals expressing the transgene.

6.1 Anti-CD40 mAb injection causes liver inflammation independent of B and T cells

The injection of anti-CD40 antibody led to strong liver inflammation in B and T cell sufficient wild type animals as can be seen by increased serum level of alanine amino transferase (Fig. 5.2). This inflammation, with its secondary effects, very much limits the value of the information regarding the influence of CD40 signaling on DCs. These findings, on the other hand, are in line with previous publications concerning anti-CD40 injection into wild type animals by the group of Francis Chisari [88].

Contrary to published results, though, we could also observe increased sALT levels accompanied by macroscopically visible inflammation in the liver even in $Rag^{-/-}$ animals (Fig. 5.2 A and B). Previously, the damage in the liver has been attributed to the presence of B cells in the liver that get activated by the anti-CD40 antibody because this inflammation was not observed in SCID animals, but could be found after B cells from wild type were transfered

into SCID animals [88].

Our results strongly argue against a role for B cells in CD40 mAb-induced liver inflammation, contrary to previous studies [88]. On a cellular level there is no difference observable between $Rag^{-/-}$ and SCID animals, both are lacking all mature B and T cells. It is possible that other influences, that have not been controlled for might play a role. In the end it is unclear how these differences can be explained but our data suggests that the effects leading to liver inflammation are independent of adaptive immunity. The inflammation is more likely mediated by other cells expressing CD40 such as epithelial or endothelial cells.

The injection of anti-CD40 mAb also causes a cytokine storm (Fig. 5.1 C), characterized by rapidly increasing serum levels of MCP-1, TNF-α, IFN-γ and Il-6. The increase is transient and peaks already between 16 and 24 h after injection. And while DCs are in principle able to produce TNF-α, IFN-γ and IL-6 [105, 106] and they have also been shown to be produced after CD40 ligation [82], it is not possible to differentiate the source of this cytokine storm after antibody injection. IL-6 for example can also be produced by B cells after CD40 ligation [107].

Another problem complicating the interpretation of the data gathered using antibody injection is, that many of the cytokines have a direct role in DC biology. For example MCP-1 is a chemoatractant and TNF-α can change expression of costimulatory molecules [55]. This makes it impossible to distinguish between primary effects, caused by the mAb acting on DCs and secondary effects, caused by cytokines secreted by other cells and thereby limits the use of anti-CD40 mAb injection *in vivo* to investigate its role on DCs.

6.2 DC-LMP1/CD40 animals develop colitis

In order to circumvent the off target problems when using anti-CD40 antibody, we generated a mouse that expresses a constitutive active CD40 molecule under the control of the CD11c promotor.

These DC-LMP1/CD40 animals developed severe colitis early on in life as indicated by

swelling and shortening of the colon and by increased levels of lipocalin-2. Lipocalin-2 was shown to be a reliable, non invasive marker for intestinal inflammation, which in addition, was also a quantitative indicator, as shown in a model of dextran-sodium sulfate-induced (DSS) colitis [102]. Using this marker, we could show that disease development was dependent on the presence of T and B cells as well as on commensal bacteria (Fig. 5.7).

The commensal dependency of disease development found in our study is in line with the role of bacteria in other colitis models. It has independently been shown for many of the commonly used colitis mouse models, like the DSS-induced colitis [108], the T cell transfer colitis [109] and for colitis caused by IL-$10^{-/-}$ [110], in all of which disease could not be induced in the absence of bacteria in the intestine.

This protection from colitis by bacterial depletion was mostly attributed to the absence of bacterial antigen that T cells can react to [109].

There are also examples of commensal bacterial strains which are capable of inducing intestinal pathology under certain conditions, like changes in the environment or the presence of genetic factors. One example of this is *segmented filamentous bacteria* (SFB), a bacterium that has to be present in SCID-animals to develop colitis after transfer of $CD4^{+}CD45RB^{+}$ cells [111]. Another example of a bacterial strain causing colitis is *helicobacter hepaticus* which is essential for disease development in a T cell transfer colitis model [112].

A role for T cells in intestinal pathology has been demonstrated in a model of induced colitis in which T and B cell deficient animals receive naïve effector T cells and subsequently develop wasting disease and show histological signs of colitis [113].

Therefore, the dependence of colitis development on bacteria and adaptive immunity observed in DC-LMP1/CD40 animals is well in line with results from other colitis models. Additionally, it fits well to a model where loss of tolerance and concomitant priming of adaptive immunity to commensal derived antigens caused colitis.

6.3 In a sterile environment LMP1/CD40 signaling produces tolerogenic DCs

We started the characterization of the DC-LMP1/CD40 animals by looking at cells in the spleen. Because of previous work showing that a CD40 signal, without additional stimuli does not cause full maturation of DCs [85, 86, 87], we expected to find DCs that are largely unchanged in their phenotype and function in the sterile environment of the spleen, where DCs, in contrast to the colon usually have no contact with bacteria.

The overall architecture of the organ was still largely unchanged in DC-LMP1/CD40 animals, with intact T and B cell zones, DCs located in the marginal zone and in the T cell zones. Interestingly, in DC-LMP1/CD40 animals we found, in spite of the normal spleen architecture, a strong decrease in all lymphocyte cell types and on the other hand an increase in granulocytes. This is an indication that the inflammation, observed in the gut (see discussion below) might also spread to other organs. This effect is probably mediated by changes in serum cytokine levels, as described by Stefanie Meier [115]. Proinflammatory cytokines were found to be upregulated in this study, which might be part of the reason for an increased granulocyte recruitment or granulopoiesis in spleen.

A detailed DC subset analysis in the spleen revealed a relative increase of DN DCs in DC-LMP1/CD40 animals (Fig. 5.4). However, this did not hold true for absolute number. The analysis of numbers instead revealed a decrease in $CD8^+$ DCs.

Of all costimulatory molecules tested we only observed a moderate upregulation of CD80 on DCs of transgenic animals (Fig. 5.4 E). In addition to this being the only upregulated costimulatory molecule, CD80 has recently been implicated not just in costimulatory activities, via CD28 interaction, but also shows coinhibitory potential by interacting with B7-H1 [116]. This complex interaction of CD80 makes it difficult to asses the outcome of such a slight upregulation and does not rule out the potential of DC-LMP1/CD40 DCs to still induce tolerance.

The fact that we were also unable to find any indication of upregulated signal 3 by DCs from DC-LMP1/CD40 spleen, which is needed for a productive T cell activation further raised the possibility that DCs are capable of tolerance induction. CD70 was unchanged in comparison to the controls and so were the typical proinflammatory cytokines like IL12 and IL6 (Fig. 5.4 E and Fig. 5.6 B). The lack of IL-12 secretion is in agreement with the work of Caetano Reis e Sousas lab [87]. He showed that a CD40 stimulus, delivered via antagonistic mAb, alone fails to induce IL-12 production but that a bacterial stimulus has to be present, which is missing in spleens.

Most importantly, we were able to show, in a T cell transfer model, that DCs in DC-LMP1/CD40 animals are not activated in a way that would allow them to prime T cells without any further stimulus (Fig. 5.5). DC-LMP1/CD40 animals were unable to induce any expansion of the transfered $CD8^+$ T cells and did also not induce any cytotoxic activity in these T cells.

Taken together this clearly shows that DCs in the spleen of DC-LMP1/CD40 animals do not reach an activation state that would allow them to prime and propagate a T cell response without any additional stimulus. The injection of anti-CD40 antibody on the other hand shows the expected, strong adjuvant effect [73], that allows for T cell expansion and cytotoxic activity.

Interestingly the expansion of T cells in the DC-LMP1/CD40 animals is even significantly reduced compared to control animals, when both groups only received Ova. To rule out that this was due to a reduced capacity in antigen-uptake or -processing, we compared the two parameters by injecting different flourescent variants of Ova. Using these we could clearly show that the uptake, as indicated by MFI of AF647-Ova in DCs, and processing, indicated by MFI of DQ-Ova, was not different between the two genotypes. One drawback of the DQ-Ova system is, however, that it only indicates degradation of the Ova in an acidic compartment [117]. This of course leaves open the question of the subsequent fate of the antigen. And one has to consider that the number of $CD8^+$ DCs in the spleen was strongly reduced and

this subset is the one, that is able present peptides from extracellular protein especially well onto MHCI molecules (cross presentation) [118]. If one takes into account that the amount of T cell expansion is higher in DC-LMP1/CD40 animals, when immunized with the peptide recognized by OTI [115], it is very likely that the weaker expansion after protein immunization is due to the changed subset distribution and the resulting reduction in cross-presentation capacity.

Overall we were able to show that a sterile CD40 signal, selectively delivered to DCs, does not cause full maturation of these cells. This is not just shown by phenotypic markers but also by the retained capability to induce tolerance in a transfer model.

6.4 Influence of CD40 on phagocytic cells in the GALT

Because of the strong intestinal inflammation in DC-LMP1/CD40 animals we characterized the phagocytic cells of the GALT. For this we differentiated DCs into three subsets on the basis of CD103- and CD11b-expression. Using this set of markers one can distinguish two $CD103^+$DC subsets in the lamina propria itself, of which one expresses CD11b and the other one does not. Additionally, there is a subset expressing CD11b but not CD103. In DC-LMP1/CD40 animals the frequency of $CD103^+$ cells was strongly reduced, while the $CD11b^+CD103^-$ cell were relatively increased.

6.4.1 Anti-inflammatory Macrophages

The $CD103^-CD11b^+$ cells found inside the $CD11c^+MHCII^+$ population, represent mostly macrophages and their precursors. To further analyze them we used a staining panel that allowed us to follow the development from monocytes, that recently extravasated from blood into the tissue, to mature macrophages [38]. We could show a reduction in the frequency of mature macrophages in the gate P3/P4 in DC-LMP1/CD40 animals (Fig. 5.10 B). This reduction was also observed in transgenic animals on the $Rag^{-/-}$ background but not after

depletion of bacteria by ABX. This indicates that the decrease is independent of proinflammatory cytokines and driven by the CD40 signal itself with an additional bacterial-derived signal. This idea is further strengthened by the notion that also after anti-CD40 mAb injection we see a strong, but transient, decrease in populations P3/P4.

Again, because of the strong increase in cellularity, we find higher numbers of all three subsets in untreated DC-LMP1/CD40 animals, although the relative increase is lowest for the subsets P3/P4. Interestingly, the numbers of macrophages are strongly reduced in all three population in ABX and $Rag^{-/-}$ mice, although it did not reach statistical significance in ABX treated animals. Since this reduction is observed under both non inflamed conditions, one conceivable explanation is a necessary cross talk with DC subsets which are reduced under both these conditions. Another possibility would be a direct influence of the transgene, that might be expressed at least in some subsets.

P3/P4 is a heterogeneous population, which could be further divided into a CX_3CR1^{med}- and a CX_3CR1^{high}-fraction [37, 38]. This differentiation can only be made using reporter animals for CX_3CR1 gene expression, because the available antibodies do not have an affinity high enough to distinguish between those two populations.

Populations P3 and P4 are not just phenotypically different but they also differ in their functional profile. Cells within P4 are macrophages that are insensitive towards TLR stimulation, even under colitogenic conditions. In comparison to that one sees an increase in responsiveness towards LPS in P3 cells, when colitis is induced [37].

The reason for the relative reduction of cells in P3/P4 is unclear, but since the P4 cells are partially positive for CD11c and also a subfraction of P3 cell shows CD11c expression, a direct influence of LMP1/CD40 signaling on these cells can not be completely ruled out. In fact in CD11c-Cre x ROSA-RFP animals one can detect a RFP signal in cells that are $Ly6C^{-}$ within the waterfall (data not show). These cells would correspond to P3 and P4 cells in the waterfall staining. Since RFP and LMP1/CD40 are knocked into the same locus and the stop-cassette is removed by the same Cre, it is likely that we also see some transgene expression in P3 and P4. Although it is unclear whether this in turn leads to the decrease in

P3 and P4 cells or if this is mediated by other mechanisms.

In favor of a direct influence of CD40 signal on these populations is also the data gained by using the anti-CD40 antibody. Here also the numbers of P3/P4 cells drops shortly after injection, while cell number for P1 and P2 increase transiently. Since these cells follow an ordered sequence of differentiation steps, it is likely that cells in P3 and P4 disappear and this in turn leads to an increased influx of Ly6C^{hi} monocytes that in the end fill up the niche left open. Because macrophages in the lamina propria are derived from monocytes this niche is filled up quickly. Monocytes are readily available in the circulation and are able to replace the macrophages within 72 hours as show by adoptive transfer experiments [38].

In addition to the reduction in P3/P4 we did also find significantly more cells in P1 and P2 in DC-LMP1/CD40 animals during colitis and the increase in cell number is relatively higher than the increase in P3/P4. This finding is completely in line with a reported break in the development from Ly6C^{hi} monocytes towards the anti-inflammatory macrophages during intestinal inflammation [37]. Accordingly, this increase in cell numbers for P1 and P2 macrophages was not observed in animals after ABX treatment or on the Rag$^{-/-}$. Cells in the P2 gate are not yet fully adapted to an anti-inflammatory role in the gut and do still respond to TLR-ligands and produce inducible nitrite oxide synthase (iNOS), TNF-α and IL-23 [37, 38, 119]. Via these mechanisms cells in P2 might help support colitis development. A role in the initiation of disease on the other hand seems unlikely, since the cells are not able to express the transgene, due to a lack of CD11c expression.

6.4.2 Influence of CD40 signaling in CD103$^+$ DCs

Injection of anti-CD40 antibody in both C57BL/6 and Rag$^{-/-}$ animals led to a strong decrease of all DC subsets in the lamina propria of these animals (Fig. 5.12). Contrary to that, in DC-LMP1/CD40 animals we found a selective reduction of CD103$^+$ DC subsets (Fig. 5.10). In contrast to that, we found an increase in the frequency of the CD103$^-$CD11b$^+$ DCs subset. If colon of non-inflammed animals was analyzed, we could still find a reduction in frequency

of $CD103^+$ DCs, which then was also mirrored in reduced numbers of $CD103^+$ DCs, with the exception of the $CD103^+CD11b^-$ subset in the ABX treated animals. In untreated and ABX-treated animals these reduction was also seen in the gut draining mLN. Thus we observed an overall decrease of $CD103^+$ DC in the GALT of animals that received a CD40 stimulus. $CD103^-$ DCs show the same pattern after anti-CD40 injection but not in the transgenic animal, where the constant pro-inflammatory milieu probably leads to changes in recruitment and replenishment of this more heterogeneous subset.

Furthermore, using the DC-CD40 KO mouse model we were able to show that the absence of $CD103^+$ DCs after anti-CD40 mAb injection was directly caused by the influence of the antibody on the DCs and not by any side effects caused by the cytokine storm or the liver inflammation.

Hence, we were able to use the antibody injection model to further elucidate what happens to DCs after CD40 stimulation. We could show that already 16 h after injection the frequency and number of all DC subsets were decreasing in the lamina propria, while at the same time frequency and numbers were increasing in the mLN. This was accompanied by an increase in CCR7 expression in $CD11c^+MHCII^+$ cells in the lamina propria 16 h after injection. And although this population also contains a big proportion of macrophages, these cells are highly unlikely to be responsible for the increase in CCR7 message, since they have been shown to be sessile and unable to migrate under physiological conditions [39].

Taken together this data strongly suggest that a CD40 signal in DCs of the lamina propria induces them to migrate towards the draining lymph node. This phenomenon has been described before, although in the work of Persson et al. [29] this migration was only observed for $CD103^+CD11b^+$ subset and only after injection of anti-CD40 and LPS. Contrary, we observed a migration of all DC subsets after anti-CD40 injection alone. These differences might be explained by a differential colonization of these animals with microbiota. On the other hand we also observed this migration also in animals after ABX treatment. But a role of bacterial-derived signal still can not be ruled out completely, since the depletion of the

microbiom is, although very efficient, still incomplete.

The described migration of DCs to the mLN on the other hand is just transient and apoptosis assays showed that in parallel to the increase in number of DCs also the number of cells undergoing apoptosis was increased (Fig. 5.14). In contrast, we did not find any indication of an increase in the rate of apoptosis after anti-CD40 antibody injection. However, there have been reports, that the opposite is the case and that CD40 in the immunological synapse can inhibit apoptosis via Akt1 activation [120]. The process of apoptosis is normal for DCs and helps to limit the time that an antigen is presented by activated DCs and has even been implicated in tolerance induction [121].

The situation is of course different in DC-LMP1/CD40 animals. Here the CD40 signal is given to the DCs as soon as they upregulate CD11c and thereby also activate Cre-recombinase. Because of this we were unable to find an early migration followed by cell death, but instead we are looking at a changed equilibrium between cells in gut and mLN. We still found a strong reduction of $CD103^+$ DCs in the lamina propria, but in addition also $CD103^+$ subsets in the mLN are reduced, with the $CD103^+CD11b^+$ being stronger reduced then the $CD103^+CD11b^-$ subset. In addition we also observed a strong increase in $CD103^-CD11b^+$ DCs in the lamina propria of untreated and ABX-treated animals. These cells, although $CD11b^+$, are probably not inflammatory DCs, because those have been reported to be also positive for F4/80 and CD64 and all cells considered to be lamina propria DCs in this work, have been defined as negative for F4/80 and CD64. The increase might instead be explained by compensatory mechanisms that step in due to the long lasting change in DC function and the resulting inflammation.

All this clearly points out that CD40 signaling induces migration of $CD103^+$ DCs into the draining lymph node were the cells die not long after they arrive. And while DCs constantly migrate from tissue into draining lymph nodes, the application of anti-CD40 mAb greatly increases migration and DCs migrate earlier then they would have under normal steady-state conditions.

6.5 Comparative transcriptome analysis of DCs after CD40 stimulation

In order to analyze the maturation status of DCs after CD40 signaling and to compare the different influences of a CD40 signal derived from the transgene with those from the anti-CD40 antibody, a gene expression analysis was performed. Animals on the $Rag^{-/-}$ background were used since the inflammatory environment in animals on the wild type background induced strong secondary effects that confounded the analysis (Ferdinand Simon, unpublished results). The study focused on $CD11c^{+}MHCII^{+}$ cells from spleen and lamina propria.

A principle component analysis indicated on a general level that the expression of DC-LMP1/CD40 DCs is very different from the pattern found after anti-CD40 antibody injection.

This is even more pronounced when the profile of DC-LMP1/CD40 DCs in the spleen was analyzed for transcripts especially important for DC biology, such as antigen uptake and processing or pattern recognition, which are largely unchanged compared to the wild type (Fig. 5.6). At the same time this also indicates that many changes observed after anti-CD40 mAb injection are either indirect effects of the antibody on DCs or that the strength of the signal received by DCs might differ. The outcome in any case is a much more proinflammatory gene expression profile, characterized by high levels of IFN-γ, IL-1α and TNF-α in DC from animals treated with anti-CD40 mAb when compared to DC-LMP1/CD40 DCs.

For the interpretation of the gene expression data from the lamina propria one has to keep in mind that a cell isolation on the basis of CD11c and MHCII expression leads to distinct mixtures of DC and macrophage subsets from different mice. In the case of wild type animals this yielded a mixture of DCs and macrophages, while after CD40 stimulation we did not find a lot of DCs but were left almost exclusively with macrophages. Consequently we were comparing not just expression of DCs under different conditions to one another but different mixtures of heterogeneous cell populations.

But even under this conditions the expression pattern found in cells from DC-LMP1/CD40

animals clearly differed from the cells sorted after anti-CD40 mAb injection (Fig. 5.16).

Phagocytes from the lamina propria also showed an increase in certain proinflammatory cytokines, such as IL-12 and IL-6, after anti-CD40 antibody injection, but more importantly the amount of transcripts for anti-inflammatory cytokines was dramatically decreased. The main source of IL-10 in the $CD11c^{+}MHCII^{+}$ cell fraction from lamina propria are macrophages of the P4 population [37], which were strongly reduced in both CD40 models. TGF-β is also downregulated in both CD40 treated groups, although not as strongly as IL-10. TGF-β has been shown to be produced by macrophages [122], as well as by $CD103^{+}$DC, a cell type which is absent from these samples [48].

Interestingly we were not able to find any signs of DC activation in cytokine gene expression levels for the transgenic animals, neither in spleen nor in lamina propria. For splenic DCs this is completely in line with our finding of a more tolerogenic DC phenotype in this organ. DCs from the GALT on the other hand might be expected to show a more activated phenotype. Although we were unable to find clear indication of DC activation in the expression analysis of DCs from the lamina propria of DC-LMP1/CD40 animals, this does not necessarily mean that the transgene does not lead to such an activation. The sort of such a heterogeneous population only captures a certain timepoint and of course also only the cells that remain in the organ. When we consider the fast kinetics of $CD103^{+}$ DCs leaving the lamina propria after anti-CD40 mAb injection, we can assume that cells which received the signal will already have left the lamina propria and consequently do not have an influence on the expression profile. In order to circumvent this one would have to analyze DCs arriving at the mLN, which is difficult in $Rag^{-/-}$ animals where secondary lymphoid structures do not form correctly [96].

An especially interesting cluster of genes is the one containing molecules implicated in processing and loading of MHC class I and II molecules. Anti-CD40 mAb injection causes a strong upregulation of molecules of the MHCI loading and processing machinery in DCs from spleen and lamina propria, while the LMP1/CD40 transgene has no detectable effect on this cluster of genes. Interestingly, molecules with a role in MHCII antigen processing, loading and

presentation show lower expression in both CD40-groups. This effect was more pronounced in the lamina propria but could also be observed in splenic DCs. In the end these changes could also be observed on protein levels with a lower MHCII expression per cell detectable in all tissues. In contrast mature DCs show higher expression of MHCII molecules on the surface [123], a finding arguing for a more immature state of DCs after CD40 stimulation.

In spleen, the gene Tnfaip3 (A20) was strongly upregulated after anti-CD40 mAb injection and still showed an substantial upregulation in transgenic DCs. DC-specific A20 knock-out animals showed a spontaneous maturation of DCs that finally led to fatal autoimmunity [114, 124]. These studies underlined an important role of A20 in the downregulation of receptor signaling pathways from molecules like TLRs, Nod2 and also CD40. A20 shuts off NF-κB signaling by removing activating ubiquitinations and at the same time adds ubiquitins to sites that mark proteins for degradation [125]. It seems plausible to assume that an increase in the expression of Tnfaip3 is caused by increased NF-κB signaling, caused by CD40 signaling, especially since in B cells the transgene LMP1/CD40 has already been shown to signal in part via NF-κB [90]. The observed increase in Tnfaip3 expression would pose a possibility to counteract the increased signal a DC receives via CD40.

Interestingly, a set of genes that are upregulated by splenic DCs in transgenic animals but not after anti-CD40 mAb injection comprises the chemokine receptors CCR6 and CCR9. Both receptors are important for DCs to home towards the gut [126]. This might be indicative of a cytokine milieu inducing migration of DCs towards the site of inflammation.

Overall the gene expression profile of DC-LMP1/CD40 DCs is in many key aspects of DC biology unchanged when compared to wild type DCs. This is in stark contrast to DCs after CD40 mAb injection, causing a massive change in the transcriptome. Additionally, we were unable to show a higher degree of DC maturation on mRNA level in the spleen, which confirms the phenotypical data.

6.6 Mechanisms leading to colitis in DC-LMP1/CD40 animals

The permanent reduction of CD103$^+$ DCs at steady state which was found in DC-LMP1/CD40 animals allowed us to investigate the influence of CD40 signaling on DCs on the induction of tolerance and immunity in the GALT.

We did find an almost complete absence of induced Tregs while at the same time Th1 and T cells producing IL17 and IFN-γ cells were increased. It has been shown previously that CD103$^+$ DCs have a superior capacity to induce Tregs and these cells were reduced in frequency in our animals [48, 49]. Induced Tregs in turn are crucial to maintain tolerance in Treg depleted mice, even after they have been reconstituted with nTregs [62]. One reason for this might be a dramatic shift in the TCR repertoire found in iTregs induced in the GALT when compared to nTregs. This distinct TCR specificity is shaped by interactions of Tregs with commensal and food antigens [64], and may explain why even though we found normal frequencies of nTregs in lamina propria of DC-LMP1/CD40 animals, these were unable to prevent intestinal inflammation.

In another model, in which both CD103$^+$ DC populations have been genetically deleted [51], reduced numbers of Tregs in the lamina propria of the gut were found. The reduction of colonic Tregs in this model was attributed to a reduced induction of CCR9$^+$ Tregs in the mLN. Contrary to the DC-LMP1/CD40 animals there was no development of autoimmunity or a higher susceptibility towards DSS-induced colitis observed. This is probably due to two reasons. First, the absence of Tregs induced in the mLN is not sufficient to cause colitis without further stimulation and generation of effector T cells. Second, since both DC subsets, that induce a Th1 or Th17 response [29, 30] are missing and these cells function as drivers of intestinal inflammation, it is not surprising that the animals are not prone to T cell mediated colitis. And although we found a reduction of CD103$^+$ DCs, we were also able to show that these cell do develop but they prematurely leave the lamina propria. Therefor, we do still have DCs that are able to prime in DC-LMP1/CD40 animals.

In contrast to the decrease in iTregs we found a strong increase in levels of IFN-γ- and IFN-γ IL17 double producing T cells in lamina propria while the frequency of IL17$^+$ T cells was unchanged. The role of Th17 cells has been shown to be a more protective one in most models. For example, in a model of T cell transfer colitis animals actually developed more severe disease when IL-17$^{-/-}$ T cells were injected [127]. And while this anti-inflammatory role of Th17 cells holds also true in a DSS model [128], their role in a model of trinitrobenzesulfonic acid(TNBS)-induced colitis is pro-inflammatory [128]. This varying role makes it difficult to assess the role that the Th17 cells play in our model, but it seems more likely that IL-17 producing T cells are no drivers of the intestinal inflammation, since their frequency within the CD4 compartment is unchanged.

The role of Th1 cells in intestinal inflammation is much clearer. They have been shown to be one of the driving forces in intestinal inflammation [129]. This fits very well with the strong increase of these cells that we see in the DC-LMP1/CD40 animals. In addition also the IL17 IFN-γ double producing cells might play a role in pathology, since they are also increased in all organs analyzed in this study as well as in the work of Ferdinand Simon (unpublished results).

One open question is which cell type actually primes the T cell responses, when DCs of the GALT are as strongly reduced. In contrast to the Batf3$^{-/-}$ x huLang-DTA mice, where both CD103$^+$ DCs subsets never develop [51], we can still find CD103$^+$ DC in DC-LMP1/CD40 in considerable numbers and the CD103$^-$ subsets was even increased in cell numbers. Our data supports a model where after receiving the CD40-signal migration is induced to LN were they finally die. This leads to a changed equilibrium which causes a reduction of CD103$^+$ DCs but not to their complete absence.

In addition, in DC-LMP1/CD40 animals CD103$^+$ DCs also lost the potential to induce peripheral Tregs. Whether the T cells in the end are primed by these functional changed CD103$^+$ DCs or whether other DC subsets perform the priming is not completely clear. But Th1 and Th17 cells in the end play a central part in the development of intestinal inflammation.

6.7 Conclusion and outlook

In this study we analyzed the influence of CD40 signaling on DCs in different tissues and its influence on tolerance and immunity. To this end, we employed a novel mouse model, in which DCs directly receive a CD40 signal via expression of the LMP1/CD40 transgene expressed under the control of CD11c-Cre.

We were able to show that CD40 stimulus alone is unable to completely mature DCs of the spleen. Such maturation was neither observed on a phenotypical level, considering the expression of costimulatory molecules and proinflammatory cytokines, nor on a functional level, as shown by tolerance induction in a model of CD8 T cell transfer. This finding is in line with previous works showing that CD40 signaling alone is not sufficient to fully mature DCs.

DCs of GALT on the other hand show dramatical changes. We found that the CD40 signal causes migration of $CD103^+$ DCs from the lamina propria towards the mLN. DCs that arrived at the mLN die there soon after. In DC-LMP1/CD40 animals the continuous CD40 signal leads to constant migration of DCs and their subsequent death, which finally results in a shift of the equilibrium leading to a strong reduction of $CD103^+$ DCs in lamina propria and mLN.

However, not just the subset distribution but also the function of the DCs is changed by the CD40 signal. DC-LMP1/CD40 animals completely lose their potential to induce peripheral tolerance by iTreg generation, but instead may prime naïve T cells to become IFN-γ- and IFNγ IL-17-producing T cells. This breakdown of tolerance leads to the development of fatal colitis.

This model nicely underlines the importance of a tight regulation of DC maturation to keep up tolerance induction.

The functional change observed in DCs after CD40 stimulation actually represents an attractive mechanism by which the activation of a T cell, and its subsequent upregulation of CD40L, can transiently shut down the ability of DCs to induce tolerance and instead make them mount an immune response. This would allow T cells, that already have been primed,

to help amplify the immune response and thereby induce a quick, strong reaction against a pathogen. This would also be self limiting, since DCs undergo apoptosis after antigen is no longer found in the system and T cells would not be activated any more.

At this point it is unclear whether the activation and generation of $CD40L^+$ T cells would have a similar effect on DCs in vivo, but this theory can be proven by the transfer of either preactivated T cells or by activating them *in vivo*. If CD40L on T cells shows similar effects on DCs, it would also be of importance to test if this interaction is antigen dependent or if it is facilitated just by CD40L-CD40 interaction.

Another important question that might be answered using the DC-LMP1/CD40 animals is, if the cell that would have become an iTreg is the same cell which start to secrete IL-17 and IFN-γ. To address this we will transfer OTII cells into DC-LMP1/CD40 animals and instead of analyzing FoxP3 expression we will investigate if the cells are able to produce IFN-γ or any other proinflammatory cytokines, all of which should not be induced, when antigen is administered orally. This would help elucidate the question of how plastic T cells are and also show the influence of DCs on T cell fate decision.

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Acknowledgements

Herrn Prof. Dr. Brocker danke ich für die Möglichkeit meine Dissertation in seiner Gruppe anzufertigen. Desweiteren möchte ich mich für die vielen, ausführlichen Diskussion danken, die offene Tür wusste ich immer zu schätzen. Nicht zu letzt bedanke ich mich sehr herzlich für die vielen Möglichkeiten diese Arbeit auch im internationalem Rahmen vorzustellen.

Dr. Tobias Straub möchte ich für seine Hilfe bei der Auswertung der Geneexpressionanalysen sowie bei allen anderen Fragen der Bioinformatik und Statistik danken.

Prof Esther Lutgens und Dr Norbert Gerdes danke ich für die Überlassung der DC-CD40 KO Mäuse.

Vielen Dank an Dr. Caspar Ohnmacht, ohne dessen Ideen wir vielleicht immer noch über Tregs nachdenken würden.

Andrea Bol danke ich für die exzellente Pflege der Mäuse und auch für die vielen "Extrawürste", die sie mir gebraten hat.

Ana Orginc, Ferdinand Simon, Juliane Maurer und Stefanie Meier danke ich für die tolle Zusammenarbeit am LMP-Projekt, ohne die wir sicherlich noch nicht da wären, wo wir jetzt sind.

Allen Kollegen der AG Brocker danke ich außerdem für die tolle Atmosphäre, die viele lange Tage deutlich erträglicher gemacht hat.

Ein besonderer Dank gilt Christine Ried, ohne die unser Labor sofort im Chaos versinken würde. Vielen Dank aber vor allem für die viele Hilfe bei unzähligen Versuchen!

Caroline Bernhard danke ich für die Korrektur dieser Arbeit, aber natürlich auch für all die netten Mittagessen.

Meinen Eltern danke ich dafür, dass sie mir immer alle Freiheiten gelassen haben und trotzdem immer da waren, wenn es darauf ankam. Dank dafür, dass ihr immer ein offenes Ohr habt!

Mein ganz besonderer Dank gilt Claudi ohne die ich sicherlich die Arbeit nicht so schnell

hätte verfassen können. Ich freue mich schon auf eine Zeit nach der Doktorarbeit mit dir und der Lütten.