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Maturation status of dendritic cells controls induction of tolerance versus immunity

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Author's declaration

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation „Maturation status of dendritic cells controls induction of tolerance versus immunity" von mir selbständig und ohne unerlaubte Hilfe angefertigt wurde. Ich habe mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Hilfen und Quellen bedient. Die Dissertation wurde in der jetzigen oder ähnlichen Form bei keiner anderen Hochschule eingereicht und hat auch noch keinen anderen Prüfungszwecken gedient.

Stefanie Meier

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List of abbreviations

Ag	Antigen
APC	1. Antigen presenting cell
	2. Allophycocyanin
Batf3	Basic leucine zipper transcription factor, ATF-like 3
BM	Bone marrow
cDC	Conventional DC
CD	Cluster of differentiation
cDNA	Complementary DNA
CDP	Common DC progenitor
CFSE	Carboxyfluorescein-diacetate-succinimidylester
CHS	Contact hypersensitivity
CLP	Common lymphoid progenitor
CLR	C-type lectin receptors
CMP	Common myeloid progenitor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dDC	Dermal DC
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
Flt3(L)	FMS-like-tyrosine-kinase 3 (ligand)
For	Forward
GM-CSF	Granulocyte macrophage colony-stimulating factor
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP	Horseradish peroxidase
Id2	Inhibitor of DNA binding 2
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
JAK	Janus kinase
ko	Knockout
LMP	Latent membrane protein
LN	Lymph node
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MDP	Macrophage-DC progenitor
MHC	Major histocompatibility complex
min	Minute
mLN	Mesenteric lymph node
mRNA	Messenger RNA
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
NOI	Nitric oxygen intermediates
nt	Nucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PE	Phycoerythrin
PerCP	Peridinin chlorphyll protein
PRR	Pattern-recognition receptor

qPCR	Quantitative PCR
Rev	Reverse
RNA	Ribonucleic acid
RT	1. Room temperature
	2. Reverse transcription
SA	Streptavidin
sec	Second
SHIP1	Src homology 2 domain-containing inositol-5'-phosphatase 1
SIINFEKL	$OVA_{257-264}$
siRNA	Small interfering RNA
sLN	Skin-draining lymph nodes
SOCS1	Suppressor of cytokine signaling 1
SP	Spleen
STAT	Signal transducer and activator of transcription
Ta	Annealing temperature
TCR	T cell receptor
TGF	Transforming growth factor
T_H cell	T helper cell
TiP DC	TNF/iNOS-producing DC
TLR	Toll-like receptor
Tm	Melting temperature
TNF	Tumor necrosis factor
TRAF6	TNFR-associated factor 6
Treg cell	Regulatory T cell
UBC	Ubiquitin C
UTR	Untranslated region
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

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1 Summary

DCs control T cell tolerance and immunity in peripheral lymphoid organs. A critical hallmark of DC biology is their capacity to change their maturation status in response to inflammatory signals. Current immunological concepts suggest that the decision to tolerize T cells or to induce T cell immunity directly depends on the maturation status of DCs. However, it has been suggested that mature DCs have both, tolerogenic and immunogenic potential. To test this model, we sought to generate transgenic mice in which DCs receive a chronic stimulation signal and are induced to become mature, but not immunogenic. To this end, we designed a transgenic mouse model with a DC-specific expression of the fusion protein LMP1/CD40, which mimics chronic CD40 stimulation. We used this model to study how DC maturation influences tolerance induction *in vivo*.

The resulting DC-LMP1/CD40 mice developed colitis, wasting disease, splenomegaly, increased levels of serum pro-inflammatory cytokines and increased levels of serum IgA and IgM. Fully matured DCs were detected in the intestines but not in the spleens of these mice, suggesting that additional triggers in the gut may lead to complete maturation of the DCs and be the cause of intestinal pathology. However, tolerance was induced in transfer experiments where CD8 T cells were administered systemically.

We therefore conclude that the constitutive CD40 signal lowers the activation threshold of DCs. This signal is itself not sufficient to induce autoimmunity in most organs and tissues, but additional stimuli probably by microbes at border-surfaces of the body, such as gut and skin, strongly activate DCs and trigger inflammatory autoreactive immune responses. The work presented in this thesis indicates that CD40 crosslinking is an important mediator to amplify activation signals towards T cells *in vivo*, but that this is only the case when microbial stimuli are additionally present.

These findings demonstrate that DC maturation needs tight and organ-specific regulation in order to avoid autoimmune disorders.

2 Zusammenfassung

Dendritische Zellen (*dendritic cells*, DCs) regulieren T-Zell-Toleranz und -Immunität in den peripheren Lymphorganen. Ein besonderes Kennzeichen der DC-Biologie ist die Fähigkeit, ihren Reifezustand als Antwort auf Entzündungssignale zu ändern. Aktuelle immunologische Konzepte weisen darauf hin, dass die Entscheidung T-Zell-Toleranz oder T-Zell-Immunität zu induzieren direkt mit dem Reifezustand der DCs zusammenhängt. Trotzdem wird vermutet, dass reife DCs sowohl tolerogenes als auch immunogenes Potential haben können. Um dieses Modell zu testen, wollten wir eine transgene Maus entwickeln, in der DCs ein dauerhaftes Stimulationssignal erhalten und angeregt werden, reif aber nicht immunogen zu werden. Zu diesem Zweck haben wir ein transgenes Mausmodell mit einer DC-spezifischen Expression des Fusionsproteins LMP1/CD40 entworfen, das chronische CD40-Stimulation imitiert. Wir haben dieses Modell verwendet, um herauszufinden, wie die Reifung von DCs die Toleranzinduktion *in vivo* beeinflusst.

Die transgene DC-LMP1/CD40-Maus entwickelte eine Dickdarmentzündung, das Wasting-Syndrom und Splenomegalie, außerdem waren erhöhte Level von inflammatorischen Zytokinen sowie von IgA und IgM im Serum messbar. Voll ausgereifte DCs wurden im Darm dieser Mäuse entdeckt, jedoch nicht in der Milz. Dies deutet darauf hin, dass zusätzliche Stimuli im Darm zur vollständigen Reifung der DCs führen und somit der Grund für die Pathologie des Darms sein können. Dennoch wurde in Transferexperimenten, in denen CD8 T-Zellen systemisch appliziert wurden, Toleranz induziert.

Deshalb kommen wir zu dem Schluss, dass das konstitutive CD40-Signal die Aktivierungsschwelle von DCs erniedrigt. Dieses Signal als solches reicht aber in den meisten Organen und Geweben nicht aus um Autoimmunität zu induzieren. Allerdings können zusätzliche Stimuli, wahrscheinlich Mikroorganismen, an den Körpergrenzen wie im Darm und in der Haut DCs aktivieren und autoreaktive Immunantworten hervorrufen. Die Arbeit, die in dieser Dissertation präsentiert wird, zeigt, dass die CD40-Vernetzung ein wichtiger Mediator ist, um die an T-Zellen gerichtete Aktivierungssignale *in vivo* zu vergrößern. Allerdings ist dies nur der Fall, wenn zusätzlich mikrobielle Stimuli vorhanden sind.

Diese Erkenntnisse zeigen, dass die strikte und Organ-spezifische Regulierung der DC-Reifung äußerst wichtig ist um autoimmune Funktionsstörungen zu vermeiden.

3 Introduction

3.1 The immune system

All multicellular organisms possess an immune system to defend themselves from pathogens such as viruses, bacteria, or parasites. The vertebrate immune system can be divided into two main arms: the innate and the adaptive system. The innate immune system consists of physical and chemical barriers, such as skin, gastric acid, mucus or tears as well as cells, such as phagocytes and natural killer cells, and physiological systems, such as complement. The innate immune system has limited specificity but provides a quick first defense after infection and plays a key role in stimulating adaptive immune responses.

The adaptive immune system is composed of specific antibody production by B cells (humoral immunity) and antigen-specific T-cell activity (cell-mediated immunity). Common lymphoid progenitor cells develop in the bone marrow and migrate to the thymus where they mature into T-cells. They express an antigen-binding receptor, the T-cell receptor, on their surface. The T-cell receptor recognizes only antigen that is bound in peptide form to major histocompatibility complex (MHC) molecules, cell-membrane proteins expressed on antigen-presenting cells (APCs).

B cells develop in the bone marrow and migrate to the spleen for their final maturation. They act as APCs and produce highly specific antibodies against antigens.

3.2 Dendritic cells

The most effective APCs are the dendritic cells (DCs). DCs were first described by Steinman and Cohn in 1973 (Steinman and Cohn, 1973). Their high efficiency as APC is partly due to their constitutive expression of MHC class II (MHC-II) molecules and ability to express a number of costimulatory molecules, such as CD80/86 and CD40. Their positioning *in vivo*, namely tissues in contact with the external environment, allows them to capture antigens before migrating to lymphoid tissues, where they can induce potent T-cell clonal expansion and effector function.

The ability of DCs to regulate immunity is dependent on DC maturation. A variety of factors can induce maturation following antigen uptake and processing within DCs,

including whole bacteria or bacterial-derived antigens, inflammatory cytokines, ligation of specific cell surface receptors, and viral products. Immature DCs are recruited to sites of inflammation in peripheral tissues following pathogen invasion. After internalization of foreign antigens and processing, DCs migrate to T-cell-rich areas within lymphoid organs via blood or lymph, present the antigen and induce immune responses.

3.2.1 DC development

DC differentiation is dependent on a combination of cytokines and transcription factors. DCs develop from bone marrow derived hematopoietic stem cells. These differentiate into a common myeloid progenitor (CMP) that gives rise to DCs, monocytes, macrophages, granulocytes, megakaryocytes and erythrocytes (Liu and Nussenzweig, 2010). CMPs differentiate into granulocytes/macrophage progenitors (GMP), which give rise to macrophage-dendritic cell progenitors (MDP). MDPs have the potential to differentiate into common DC precursors (CDP) or monocytes (Fig. 3-1). CDPs can give rise to either plasmacytoid DCs (pDCs) or the direct precursors of most of the DC subsets that are discussed in detail in the next chapter. The transcription factors Batf3, IRF8 and Id2 play an important role in the final differentiation to CD8+ lymphoid tissue DCs, whereas the transcription factors RelB, IRF2, IRF4 and RBP-J are important for the differentiation to CD8- lymphoid tissue DCs. The development of non-lymphoid tissue DCs is predominantly dependent on Flt3 and M-CSF recognition.

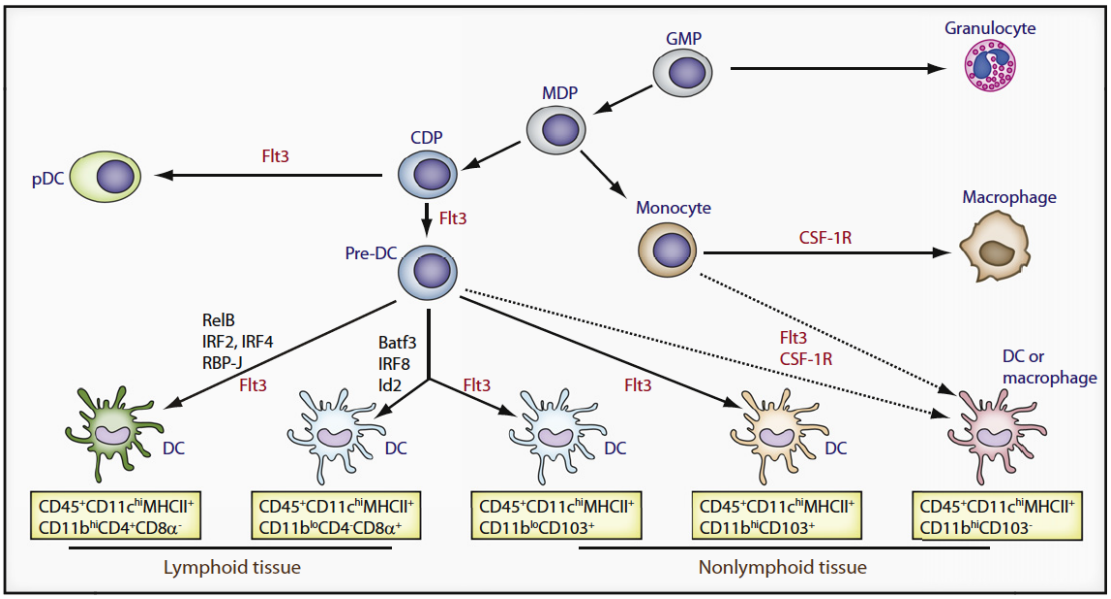

Figure 3-1: Origin of Batf3-IRF8-Id2-dependent and -independent tissue-resident DCs in mice.

This figure illustrates the precursors, transcription factors (black), and cytokine receptors (red) required for the development of each population. Adapted from (Hashimoto et al., 2011).

3.2.2 DC subsets

There are several ways to distinguish the different DC subsets (Steinman and Idoyaga, 2010). One is to differentiate between monocyte-independent DCs, which require Flt-3L for their development and are mostly found in lymphoid tissue, and monocyte-derived DCs, which are dependent on M-CSF (Schmid et al., 2010). Other nomenclatures distinguish conventional or classical DCs from plasmacytoid DCs, which do not originate from the same precursor, or resident from migratory DCs. Here, I focus on the differences between lymphoid and non-lymphoid tissue DCs. However, lymphoid and non-lymphoid tissues can contain similar DC subsets (Steinman and Idoyaga, 2010).

3.2.2.1 Lymphoid tissue DC

Lymphoid tissue DCs reside in the spleen, lymph nodes, thymus and mucosal associated tissues. DCs are classified into two main subsets, namely the classical and the plasmacytoid DCs. The classical DC (cDC) subset consists of CD8+ and CD8- DCs (Naik, 2008). CD8+ DCs are mainly found in the T cell areas of the spleen and lymph nodes in the steady state and are specialized for the uptake of dying cells and

the presentation of endogenous antigens, such as viral antigens. In addition to presentation via MHC-II, CD8+ DCs are also capable of crosspresentation of exogenous antigens via the MHC class I (MHC-I) pathway to CD8+ T cells (den Haan et al., 2000). In contrast to their CD8+ counterparts, CD8- DCs localize predominantly in the marginal zone (Leenen et al., 1998). They are specialized in presenting exogenous antigens via MHC-II (Dudziak et al., 2007). The lymph nodes also contain migratory DCs. These cells derive from the periphery where they act as sentinels for pathogen and peripheral self-antigen. Then they migrate towards draining lymph nodes accompanied by a kind of maturation (Naik, 2008). These migratory DCs include Langerhans cells and dermal DCs that drain to the skin-draining lymph nodes (Merad and Manz, 2009).

Plasmacytoid DCs, also called natural interferon-producing cells, accumulate mainly in the lymphoid organs and express TLR7 and TLR9, which recognize single stranded RNA and unmethylated CpG sequences, respectively. pDCs are therefore good responders to viral antigens and produce large amounts of type I interferons (IFN) upon exposure to live and inactivated virus. However, the role of pDCs *in vivo* is still matter of debate, as in pDC-depleted mice it was shown that, pDCs provide an immediate but limited source of type I IFN that restricts viral burden only in the very early phase of infection (Swiecki et al., 2010). In other studies, it was demonstrated that pDC-knockout mice live into old age without obvious signs of autoimmunity or inflammation, suggesting that pDCs are not important mediators of tolerance (Cisse et al., 2008; Reizis et al., 2011).

Thymic DCs are mostly found in the thymic medulla and play an important role in the negative selection of thymocytes (Brocker et al., 1997). Here, the CD8+ DCs seem to derive from an early thymocyte progenitor while the thymic CD8- Sirp-α+ DCs are thought to have an extra-thymic origin (Wu and Shortman, 2005) and home to the thymus. It has been speculated that these migratory thymic DCs carry antigen to the thymus contributing to the establishment and maintenance of central tolerance (Bonasio et al., 2006).

The mucosal-associated tissues comprise lymphoid tissue in the nasopharynx, Peyers patches and isolated lymphoid follicles in the small intestine, and isolated follicles and the appendix in the large intestine. The DCs found in these tissues resemble splenic DC (Iwasaki, 2007).

3.2.2.2 Non-lymphoid tissue DC

Non-lymphoid tissue DCs can be found in the skin, lung, liver, kidney, pancreatic islets and the intestine (Fig. 3-2). They populate stratified squamous tissue like the epidermis or connective tissue. These cells are called interstitial DCs (Helft et al., 2010).

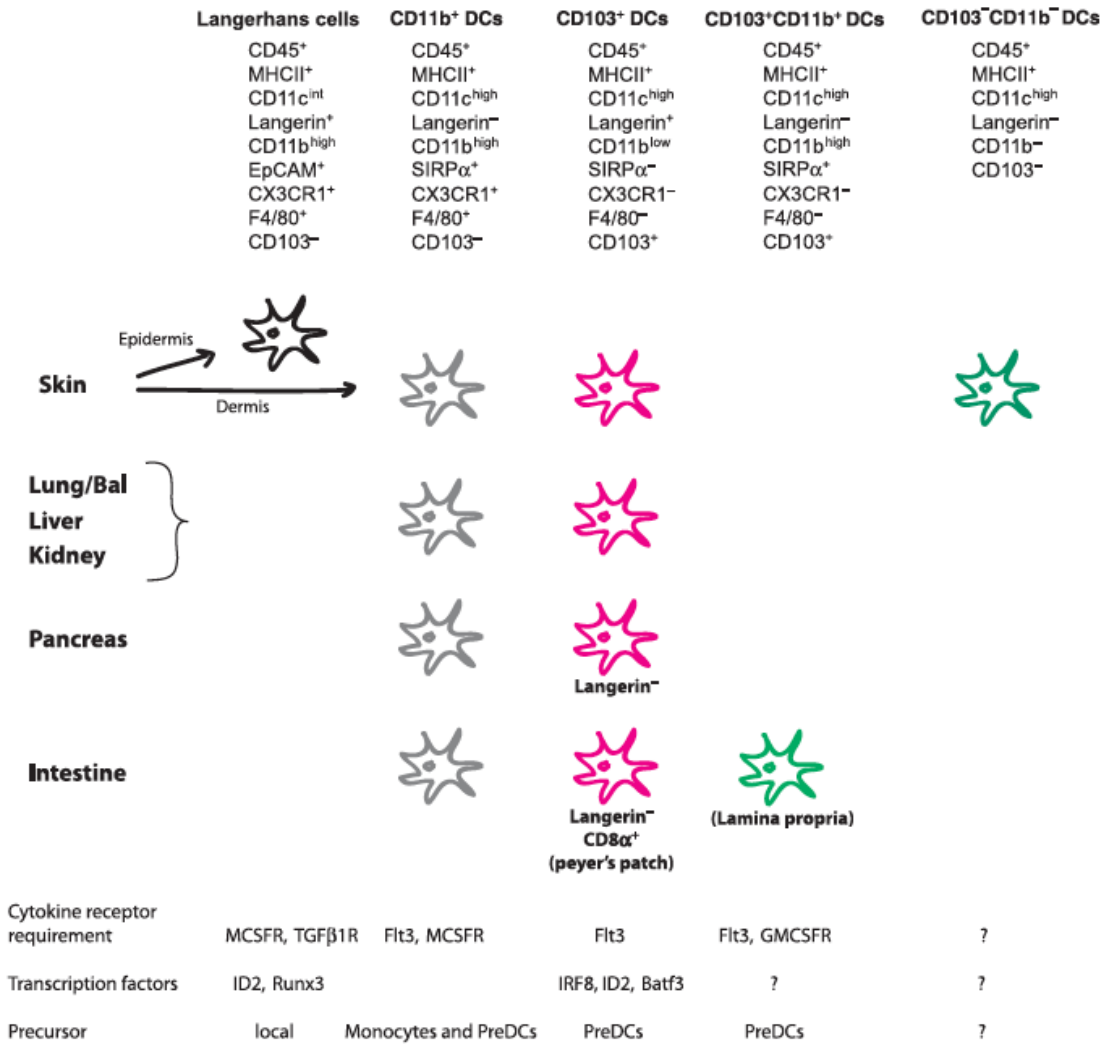

Figure 3-2: Characterization of tissue-resident DCs.

Two DC compartments with distinct cell surface phenotype and immune function have been identified in most non-lymphoid tissues. The nature of the bone marrow precursor that gives rise to each DC subset, the growth factor receptor requirements, and the transcription factors that control their development are summarized here. Additional DC populations can be found in the skin and intestine. In the skin, embryonically derived DCs also called Langerhans cells, populate the epidermis. In the intestine, a population of CD103+ DCs with a phenotype and regulatory program that is distinct to that of most non-lymphoid tissue CD103+ DCs populates the lamina propria, Adapted from (Helft et al., 2010).

Both types are found in the skin. Langerhans cells are M-CSF dependent (Ginhoux et al., 2006) and are found within the epidermis and other stratified squamous tissue, while dermal DCs belong to the interstitial DC subset (Merad et al., 2008).

DCs in the lung parenchyma resemble interstitial cutaneous DCs, as there are two main populations, the CD103+ and the CD11b+ DCs (Sung et al., 2006).

Liver, kidney and pancreatic islet DCs express similar markers to dermal and lung DCs, including CD103 and CD11b (Ginhoux et al., 2009).

Intestinal DCs accumulate mainly in the intestinal lymphoid tissue, such as Peyer's patches, lymphoid follicles and the mesenteric lymph nodes. However, they are also found in the lamina propria, the muscular layers and the serosa. Dendritic cells in gut-associated tissue must have a highly tolerogenic phenotype as they come into contact with a large number of commensal bacteria and food antigens. However, these cells are also able to fight enteric pathogens. CD103+ DCs promote the differentiation of T regulatory (Treg) cells via a mechanism that involves retinoic acid (RA) and TGF-β (Coombes et al., 2007; Sun et al., 2007), and CD103- DCs can drive the differentiation of T_H17 and T_H1 cells (Coombes and Powrie, 2008). In the following section I will further describe the several DC subsets in the lamina propria DCs (Hashimoto et al., 2011).

CD103- CD11b+ DCs arise from circulating monocyte precursors and are M-CSF dependent (Bogunovic et al., 2009; Schulz et al., 2009; Varol et al., 2009). They are poor T cell stimulators (Schulz et al., 2009) and do not migrate effectively to the draining lymph nodes during oral infection (Bogunovic et al., 2009; Ginhoux et al., 2009; Varol et al., 2009).

The development of CD103+ CD11b+ DCs depends on Flt3L and GM-CSF and they derive from DC-restricted precursors (Bogunovic et al., 2009; Varol et al., 2009). Contrary to CD103- CD11b+ DCs, CD103+ CD11b+ DCs migrate to the draining lymph node upon oral microbial stimuli (Bogunovic et al., 2009; Schulz et al., 2009).

A third subset of DCs is found in the lamina propria. These CD103+ CD11b- DCs also originate from DC-restricted precursors and depend on the same transcription factors as lymphoid tissue CD8+ DCs, including Batf3 (Hildner et al., 2008), IRF8 (Aliberti et al., 2003; Schiavoni et al., 2002; Tailor et al., 2008) and the inhibitor of DNA protein Id2 (Hacker et al., 2003) (Fig. 3-1). CD8+ and CD103+ CD11b- DC

subsets also share common functions, as they can express high amounts of IL-12, cross-present antigens to CD8+ T cells and drive the differentiation of CD8+ effector T cells (Hashimoto et al., 2011).

3.2.3 DC maturation

In response to environmental stimuli, immature DCs start a process called maturation, during which they undergo multiple phenotypical and functional changes. The process of DC maturation involves a redistribution of MHC molecules from intracellular endocytic compartments to the DC surface, down-regulation of antigen internalization, an increase in the surface expression of costimulatory molecules, morphological changes, cytoskeleton reorganisation, secretion of chemokines, cytokines and proteases, and surface expression of adhesion molecules and chemokine receptors (Steinman and Idoyaga, 2010).

3.2.4 Induction of Immunity by DC

DCs possess the ability to stimulate naïve T cells. Because of their ability to strongly up-regulate costimulatory molecules, they are more potent T cell stimulators than B cells and macrophages (Steinman and Witmer, 1978). When DCs mature, the T cells not only get the antigen-specific signal 1 that is mediated through the T cell receptor (TCR), but also are stimulated via CD28 (signal 2) because of the up-regulation of B7 molecules on DCs (Kapsenberg, 2003). This process leads to T cell proliferation.

After having received signal 2, CD4+ T cells up-regulate their expression of CD40L, which binds to CD40 receptors on the DCs, stimulating the DCs to produce cytokines (signal 3). Depending on the type of pathogen-associated molecular pattern (PAMP) that was recognized by the DC, appropriate cytokines are produced for the induction of specific types of T helper (T_H) cells, in a process known as T helper polarization. There are three main T_H cell subtypes, which differ in their cytokine profiles and their functions in the immune response. T_H1 cells mainly secrete the pro-inflammatory cytokine IFN-γ and are responsible for killing intracellular pathogens and for perpetuating autoimmune responses. T_H2 cells produce IL-4, -5 and -13, which are associated with the promotion of IgE production and eosinophilic responses, and

therefore play an important role in fighting extracellular pathogens and allergies. A third subset of T_H cells, the T_H17 cells, are potent inflammatory mediators and contribute to several autoimmune disorders, such as asthma, rheumatoid arthritis and inflammatory bowel disease (IBD). They are also important for immune responses against fungi and extracellular bacteria. T_H17 cells secrete the cytokines IL-17A and IL-17F, which activate and recruit neutrophils, IL-21, which activates the T_H17 cells in an autologous manner and activates B cells, and IL-22, which has pro-inflammatory and tissue-protective functions (Hemdan et al., 2010).

The priming of CD8+ T cells must be tightly regulated, as cytotoxic T lymphocytes (CTL) can induce potent immune responses and therefore, if not controlled, could cause severe autoimmunity. To avoid this, CD8+ T cells lack CD40L, rendering them incapable of directly stimulating DCs. Thus, they are dependent on help provided by CD4+ T_H cells that recognize the same antigen on the DC and 'license' it via CD40 in order to prime CD8+ T cells (Smith et al., 2004).

3.2.5 DC in tolerance induction

An important feature of the immune system is the capacity for recognizing and fighting foreign antigens, while remaining tolerant to self-antigens. If this process becomes imbalanced and self-antigen is recognized as foreign, autoimmune diseases may result. There are at least two important mechanisms that are responsible for tolerance maintenance: central and peripheral tolerance, during which deletion, anergy or induction of Treg cells occurs.

3.2.5.1 Central tolerance

The induction of central tolerance takes place in the primary lymphoid organs. B cells are selected in the bone marrow and T cells in the thymus, where autoreactive clones are negatively selected and undergo apoptosis. DCs play a pivotal role during this process. It has been shown that targeted expression of MHC-II molecules on thymic DCs, but not on cortical or medullary epithelial cells, B cells or macrophages, is sufficient to negatively select I-E reactive CD4+ T cells, and to a less complete extent, CD8+ T cells (Brocker et al., 1997). Furthermore, it has been demonstrated that $CD8^{lo}$

Sirp-α+ DCs in the thymus efficiently induce the generation of Treg cells and negative selection (Proietto et al., 2008). The important role of DCs in maintaining central tolerance was underscored by a study showing that constitutive ablation of DCs in mice breaks self-tolerance of CD4+ T cells and results in spontaneous fatal autoimmunity (Ohnmacht et al., 2009).

3.2.5.2 Peripheral tolerance

Although negative selection in the thymus is a highly effective mechanism, some autoreactive T cells still escape to the periphery. It is therefore important that peripheral tolerance mechanisms exist that can inactivate these T cells. DCs play an important role in maintaining peripheral tolerance, as they are capable of inducing deletion, anergy and the induction of Treg cells. In the steady state, DC pick up dead cells, process them and present them in the context of MHC to T cells, which are thereby inactivated (Steinman et al., 2000). It has been shown that a DC-specific deficiency in uptake of apoptotic material inhibits crosspresentation *in vivo* leading to an accumulation of self-reactive T cells in the periphery (Luckashenak et al., 2008).

DCs must be immature during the presentation of self-antigen as autoimmunity would arise if they were entirely immunocompetent (Reis e Sousa, 2006). This reasoning led to the general understanding that immature DCs induce tolerance and mature DCs induce immunity. But this paradigm of immature vs. mature DC is currently under debate (Joffre et al., 2009; Lutz and Schuler, 2002; Reis e Sousa, 2006). Our group and others have shown that tolerogenic DC express substantial amounts of costimulatory molecules (Albert et al., 2001; Kleindienst et al., 2005; Menges et al., 2002), that the expression of costimulatory molecules is necessary for tolerance induction (Perez et al., 1997; Probst et al., 2005) and that immature DC are ignored by T cells (Albert et al., 2001).

3.3 The CD40 receptor

CD40, a member of the tumor necrosis factor (TNF) receptor superfamily, is a 48 kDa transmembrane glycoprotein receptor that was first discovered on B lymphocytes but is also expressed by dendritic cells, monocytes, activated macrophages, endothelial,

epithelial cells and some tumor cells. The murine CD40 receptor consists of 305 amino acids (AA) with an extracellular domain of 193 AA containing the characteristic four cysteine-rich repetitive motives of the TNF-receptor family. In addition, the receptor contains a leader sequence of 21 AA, a transmembrane domain of 22 AA and an intracellular signaling domain with a length of 90 AA.

The signaling cascade downstream of CD40 is initiated once the trimeric ligand CD40L is bound to the receptor. CD40L is primarily expressed on activated CD4+ T cells, but has also been found on platelets, mast cells, DCs, macrophages, basophils, eosinophils, NK cells and B cells, as well as non-haematopoietic cells (smooth muscle cells, endothelial cells, and epithelial cells) (Schonbeck and Libby, 2001).

The signaling domain of CD40 receptor lacks intrinsic catalytic activity, but contains two PxQxT-motives that bind to TNF receptor associated factor (TRAF) 1, 2, 3, 5 and 6 upon CD40L binding (Harnett, 2004). TRAF binding to the cytoplasmic tail of CD40 subsequently leads to the activation of the canonical and non-canonical nuclear factor-κB (NF-κB) pathways (Berberich et al., 1994; Coope et al., 2002) and the mitogen activated protein kinases (MAPKs) extracellular signal regulated kinase (Erk), c-Jun N-terminal kinase (Jnk) and p38 (Harnett, 2004; van Kooten and Banchereau, 2000).

The AA-sequence of the human CD40 receptor is 62% identical to that of the murine receptor. They share 78% identity in the intracellular domains. The last 32 carboxylterminal AA of the human CD40 are completely conserved in the mouse sequence. Furthermore, it had been shown that the human CD40 can rescue the phenotype of CD40-deficient mice (Yasui et al., 2002), suggesting that the findings of studies performed in one species are likely to also be applicable to the other (van Kooten and Banchereau, 2000).

3.3.1 CD40 on dendritic cells

Following the discovery of CD40 on DCs (Freudenthal and Steinman, 1990; Romani et al., 1989), it was also shown that CD40 signaling induces the maintenance of high levels of MHC-II and up-regulation of costimulatory molecules such CD80 and CD86 (Caux et al., 1994; Pinchuk et al., 1994; Sallusto and Lanzavecchia, 1994). Therefore, DCs become more effective APCs after CD40 crosslinking, a process which had

already been shown for B cells (Clark and Ledbetter, 1994). However, DCs and B cells respond differently to CD40 stimulation, with DCs producing higher levels of certain inflammatory cytokines and chemokines (Cella et al., 1996; Kiener et al., 1995).

Several studies show that DCs acquire immunostimulatory capacities, such as the secretion of pro-inflammatory cytokines like IL-12, upon CD40 ligation *in vitro* (Cella et al., 1996) as well as *in vivo* during immunizations (Bonifaz et al., 2002; Hawiger et al., 2001; Liu et al., 2002). However, there are several limitations to these systems. *In vitro*, the DCs are also stimulated by growth factors, serum and plastic, which can have synergistic effects with CD40 ligation. The *in vivo*-stimulations were performed by the injection of an agonistic anti-CD40 antibody that has been shown to induce a B cell-dependent inflammatory liver disease (Kimura et al., 2006), not excluding indirect and DC-unspecific effects. Furthermore, the outcome of such experiments is dependent on the purity of the antibody, which could be contaminated by other inflammatory stimuli.

Other groups have shown that CD40-signaling induces incomplete DC maturation *in vivo* (Ahonen et al., 2004; Sanchez et al., 2007; Schulz et al., 2000). In these studies, only combined CD40/TLR signals completely matured DCs, including IL-12 and type I IFN secretion and CD70 up-regulation.

As the anti-CD40 antibody is not a good choice for *in vivo* studies because of the side effects it possibly causes, a modified constitutively active CD40 molecule that can be expressed specifically on DCs was considered for the project.

3.4 LMP1 and CD40

The Epstein-Barr virus belongs to the family of γ-herpes viruses, infects mainly human B cells and can establish latency in long-lived memory B cells. During the first expansion phase of the virus, called latency phase III, nine viral proteins are expressed. These so-called latent proteins are located either in the nucleus, EBV nuclear antigen (EBNA)-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP, or in the plasma membrane, latent membrane proteins (LMP)1, LMP2A and LMP2B, of the infected cell. In recent years it has been shown that some viral proteins bear functional similarities to cellular proteins that are involved in the

differentiation, proliferation and survival of B cells. LMP1 imitates an active CD40 receptor contributing to B cell immortalization and tumor development. LMP1 consists of 386 AAs containing 186 hydrophobic AAs of the transmembrane domain and 200 AAs of the cytoplasmic signaling domain at the carboxyl-terminus. The transmembrane domain is composed of six membrane-spanning segments that are connected through loops with each other and that mediate aggregation with other LMP1 proteins. These aggregations in the plasma membrane act as multimeric signaling complexes producing a ligand-independent constitutively active signal. As already mentioned, LMP1 resembles CD40 in function (Graham et al., 2010; Lam and Sugden, 2003) and both proteins can be partly replaced by each other (Kilger et al., 1998; Rastelli et al., 2008; Zimber-Strobl et al., 1996). To further compare the two molecules, fusion proteins of the transmembrane domain of LMP1 and the cytoplasmic domain of CD40 and vice versa were generated. These studies provided further evidence that the proteins have similar functions and show equal activation of NF-κB (Busch and Bishop, 1999), equal induction of growth factor expression (Hatzivassiliou et al., 1998), and equal transformation functions (Hatzivassiliou et al., 2007) *in vitro*. *In vivo* studies also revealed functional similarities (Rastelli et al., 2008; Uchida, 1999).

3.5 The fusion protein LMP1/CD40

The fusion protein LMP1/CD40 consists of the transmembrane domain of LMP1 (AA 1-187) and the intracellular part of the human CD40 receptor (AA 223-280) (Fig. 3-3). *In vitro* characterization of the fusion protein demonstrated that LMP1/CD40 acts as a constitutive CD40 receptor (Gires et al., 1997). *In vivo*, it was shown that B cell-specific expression of LMP1/CD40 led to an activated phenotype, prolonged survival and increased proliferation of B cells (Homig-Holzel et al., 2008). Furthermore, constitutive CD40 signaling in B cells induced selective and constitutive activation of the non-canonical NF-κB pathway and a high incidence of lymphoma development between the age of 12 and 19 months.

Little is known about LMP1/CD40 expression on dendritic cells. In a recent *in vitro* study with human DCs, LMP1 and LMP1/CD40 were introduced into an HIV-1 construct to produce virions encoding these proteins in order to improve HIV vaccines

(Gupta et al., 2011). Transduction of DCs and macrophages with these viruses induced up-regulated costimulatory molecules and cytokine production by these cells.

CD40 crosslinking activates NF-κB. The canonical NF-κB pathway in DCs leads to an early production of pro-inflammatory cytokines, whereas it was shown that the non-canonical NF-κB pathway leads to the expression of the tolerance-inducing and immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) (Tas et al., 2007). As already mentioned, it was demonstrated that LMP1/CD40 in B cells constitutively activated the non-canonical NF-κB pathway. If this is also true for DCs, this would be another indication that a certain activation level is beneficial for tolerance induction.

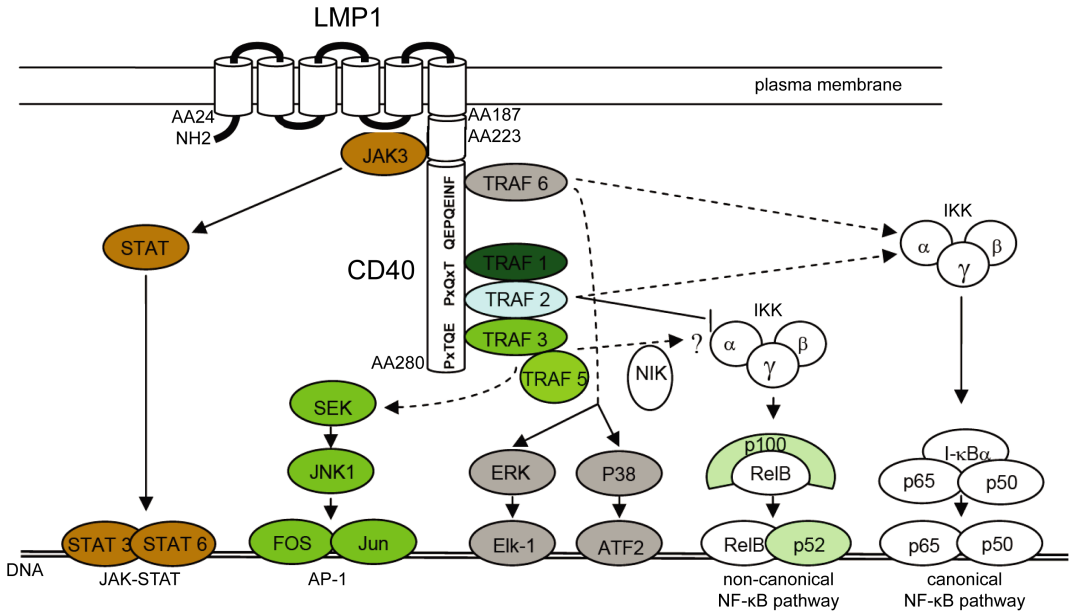

Figure 3-3: Schematic representation of the LMP1/CD40 fusion protein.

The fusion protein consists of the transmembrane domain of LMP1 (AA 1-187) and the intracellular part of the human CD40 receptor (AA 223-280). Adapted from (Hömig, 2005).

4 Aims of the thesis

DCs control T cell tolerance and immunity in peripheral lymphoid organs. A critical hallmark of DC biology is their capacity to change their maturation status in response to inflammatory signals. Current immunological concepts suggest that the decision to tolerize T cells or to induce T cell immunity directly depends on the maturation status of DCs. Furthermore, DC maturation needs to be strictly controlled to avoid 'spontaneous' maturation of DCs. However, mature DCs are not necessarily licensed DCs that produce signal 3. Therefore, although matured in respect to surface markers, mature DCs can possibly induce tolerance (Reis e Sousa, 2006). To test this model, we sought to generate transgenic mice in which DCs receive a chronic stimulation signal and are induced to become mature, but not licensed. To this end, we designed a transgenic mouse model with a DC-specific expression of the fusion protein LMP1/CD40, which mimics chronic CD40 stimulation. We used this model to study how DC maturation influences tolerance induction *in vivo*.

5 Materials and Methods

5.1 Materials

5.1.1 Antibodies

Specificity (anti-mouse)	Conjugate	Clone	Supplier
B220	PerCP	RA3-6B2	Becton, Dickinson & Co. (BD), Franklin Lakes, NJ, USA
CD3	PerCP	145-2C11	BD
CD4	PE PerCP PE-Cy7	GK1.5	BD
CD8	PE PerCP APC-Cy7	53-6.7	BD
CD11b	PE	M1/70	BD
CD11c	APC	HL3	BD
CD19	PerCP PE-Cy7	1D3	BD
CD25	FITC	PC61	BD
CD44	PE	Pgp-1, Ly-24	BD
	Alexa Fluor 405	IM7.8.1	Life Technologies, Carlsbad, CA, USA
CD45.1 (Ly5.1)	APC eFluor 450	A20	eBioscience, San Diego, CA, USA

CD45.2 (Ly5.2)	APC	104	eBioscience
CD62L	PE	Mel-14	BD
CD70	Biotin	FR70	eBioscience
CD80	PE	16-10A1	BD
CD86	PerCP	GL-1	Biolegend
CD90.1	FITC PerCP	OX-7	BD
IL-12 (p40/p70)	PE	p40/p70	BD
IL-17A	PE	TC11-18H10	BD
IFN-γ	PE APC	XMG1.2	BD
F4/80	PE	BM8	BD
Foxp3	Alexa Fluor 647	FJK-16s	eBioscience
MHC-II (I-A/I-E)	FITC Biotin	M5/114.15.2	eBioscience
Ly6C	FITC	AL-21	BD
Ly6C/G (Gr-1)	FITC	RB6-8C5	Biolegend
NK1.1	PE PE-Cy7	PK136	BD
Vα2 TCR	FITC APC	B20.1	BD
Vβ5.1/5.2 TCR	FITC PE	MR9-4	BD

Table 5-1: Antibodies used in flow cytometry

For western blotting, LMP1/CD40 protein was detected by rabbit anti-human CD40 antibody (sc975) (Santa Cruz Biotechnology). A donkey anti-rabbit HRP-conjugated secondary antibody (Jackson Immuno Research) was used. β-actin was detected using a rabbit anti-mouse β-actin antibody (13E5) (Cell Signaling Technology).

Streptavidin-APC was purchased from Life Technologies, Streptavidin-PE from Southern Biotec. Streptavidin-PerCP and Streptavidin-APC-Cy7 from BD.

The agonistic anti-CD40 antibody (clone FGK45.5, isotype rat IgG2a) for immunizations was purchased from Miltenyi Biotec.

5.1.2 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.

5.1.3 Consumables

Centricon filter	Millipore Corporation, Billerica, MA, USA
Disposable syringe filter (0.2 + 0.45 μm)	Nalgene Nunc Int., Rochester, NJ, USA
Bottle filter	Nalgene Nunc Int. Rochester, NJ, USA
Disposable injection needle (26 G x 1/2“)	Terumo Medical Corporation, Tokyo, Japan
Disposable syringe (1+5 ml)	Braun, Melsungen, Germany
Reaction container 0.2 ml	Nunc, Wiesbaden, Germany
Reaction container 1.5 ml und 2 ml	Eppendorf, Hamburg, Germany
Reaction tube 5 ml	BD, Franklin Lakes, NJ, USA
Reaction tube 15 ml und 50 ml	Greiner, Frickenhausen, Germany

Other materials and plasticwares were purchased from BD, Nunc (Wiesbaden, Germany) and Greiner.

5.1.4 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 (Coulter Counter Z2, Beckman Coulter, Krefeld, Germany), centrifuge (Rotixa RP, Hettich, Tuttlingen, Germany), chemical scale (Kern, Albstadt, Germany), 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 (Lightcycler, Roche, Basel, Switzerland or 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). All other devices are mentioned in the methods section.

5.1.5 Media and soilutions

ACK buffer	8.29 g NH_4Cl
	1 g $KHCO_3$
	37.2 mg Na_2EDTA
	H_2O ad 1 l
	pH 7.2-7.4 adjusted with 1 N HCl
	sterilized by 0.2 μm filtration
PBS	150 mM NaCl
	10 mM Na_2HPO_4
	2 mM KH_2PO_4
	pH 7.4 adjusted with 5 N NaOH
PBS-FBS	Dulbecco's PBS without Ca^{2+}/Mg^{2+}
	2% FBS (v/v)

FACS buffer	PBS
	2% FBS (v/v)
	0.01% NaN_3 (v/v)
MACS buffer	Dulbecco's PBS without Ca^{2+}/Mg^{2+}
	0.5% FBS (v/v)
	2 mM EDTA
50x TAE buffer	242 g Tris
	57.1 ml 100% acetic acid (v/v)
	100 ml 0.5 M EDTA (pH 8.0)
	H_2O ad 1 l
10x Gitocher buffer	670 mM Tris pH 8.8
	166 mM Ammonium sulfate
	65 mM $MgCl_2$
	0.1% Gelatin
1x Gitocher buffer (amounts per sample, 50 µl total)	5 µl 10x Gitocher buffer
	2.5 µl 10% Triton-X
	0.5 µl β-mercaptoethanol
	3 µl proteinase K (10 mg/ml)
	39 µl H_2O

Solutions for Western blotting:

Lysis buffer (1x)	PBS, pH 7.4-8 or
	mammalian lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl)
	1% NP-40 (or Taurocholat, Triton-X100)
	1 mM PMSF

	2 mM Benzamidine HCl
Sample buffer (5x)	0.5 M Tris-HCl, pH 6.8
	0.3% Glycerin (99%)
	0.1% SDS
	0.093% DTT (for reducing buffer)
	few grains bromphenol blue
Running buffer (5x)	150 g (0.4 M) Tris
	750 g (2 M)Glycine
	ddH_2O to 5 l
	To use: dilute 1:5, add 5 ml 20% SDS
Blotting buffer (1x)	10 mM $NaHCO_3$
	3 mM Na_2CO_3
	20% Methanol
Washing buffer (1x)	0.05% Tween in PBS (PBST)
Blocking buffer (1x)	5% Nonfat dried milk in PBS
Stripping buffer (1x)	7.5 g (100 mM) glycine, pH 2.9
	ddH_2O to 1 l

Cell culture media

All culture media and solutions were purchased from Gibco (Invitrogen, Carlsbad, CA, USA) unless otherwise stated.

DC medium	RPMI 1640 + glutamine, (PAA, Pasching, Austria)
	5% FBS (inactivated, v/v)
	500 mM β-mercaptoethanol
	100 U/ml penicillin

	100 µg/ml streptomycin
	25 ng/ml GM-CSF
Freezing medium	90% FBS
	10% DMSO

5.1.6 Mouse strains

All mice were bred and maintained in the mouse facility of the Institute for Immunology (LMU, Munich, Germany).

CD11c-Cre

These mice express the Cre recombinase under the CD11c promoter (Caton et al., 2007) and were provided by Boris Reizis, Columbia University, New York. Mice were kept on the B57BL/6 background.

DC-LMP1/CD40

The fusion gene LMP1/CD40 was inserted into the rosa26-locus in LMP1/CD40^{flSTOP} mice (Hömig-Hölzel et al. 2008). The LMP1/CD40 gene was cloned downstream of a loxP-flanked stop cassette. The LMP1/CD40^{flSTOP} mice were provided by Ursula Zimber-Strobl, Institute of Clinical Molecular Biology and Tumor Genetics, Helmholtz Center Munich.

When breeding LMP1/CD40^{flSTOP} mice to CD11c-Cre mice, the expression of LMP1/CD40 takes place in CD11c+ cells. In the following sections these double transgenic mice are referred to as DC-LMP1/CD40. Mice were kept on the B57BL/6 background.

Ly5.1

Ly5.1 mice express the Ly5.1 allele and were kept on the B57BL/6 background.

OT-I

CD8 T cells from OT-I mice express the transgenic Vα2/Vβ5 TCR specific for OVA $(ovalbumin)_{257–264}$ in the context of MHC-I H-2K^b (Hogquist et al., 1994). These mice were kept on the B57BL/6 background expressing the Ly5.1 allele.

5.1.7 Peptides, proteins and oligonucleotides

The peptide $OVA_{257-264}$ and was purchased from PolyPeptide Group (Strasbourg, France).

Oligonucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany) and are indicated in the relevant context in the methods section. Sequencing reactions were carried out by Sequiserve (Vaterstetten, Germany) or MWG-Biotech AG.

5.2 Methods

5.2.1 Cellular and immunological methods

5.2.1.1 Adoptive transfer

This method involves transfer of T cells from a donor mouse into a recipient mouse. T cells were isolated from lymph nodes of donor mice using negative selection (MACS, see 5.2.1.11). The purity of T cells was determined by flow cytometry (see 4.2.1.5) before i.v. transfer into syngenic recipients of the same sex. The congenic markers Ly5.1 allowed subsequent detection of transferred T cells in the recipient.

5.2.1.2 Cell culture

Culture of dendritic cells

Addition of the cytokine GM-CSF leads to *in vitro* differentiation of DCs from bone marrow over the course of several days. A modified version of Inaba's protocol (Inaba et al., 1992) was used. Bone marrow cells were depleted of erythrocytes (Mouse Erythrocyte Lysing Kit, R&D Systems) and $1x10^6$ cells/ml were cultured in DC-medium in a total amount of 10 ml per 100 mm plate at 37°C and 5% CO_2. Fresh medium was added every 2-3 days.

5.2.1.3 CFSE staining and in vivo cytotoxicity assay

Labeling of cells with CFSE (carboxyfluorescein-diacetate-succinimidylester, Life Technologies) allows tracking of cells both *in vitro* and *in vivo*. CFSE diffuses into the cell, where it binds to amino groups of proteins. By cleavage with intracellular esterases, CFSE becomes a fluorescent dye. For *in vivo* cytotoxicity assays, single cell suspensions of whole splenocytes were incubated with $OVA_{257-264}$-peptide (2 ng/ml or

2 μg/ml) for 30 min at 37°C. For CFSE labeling, cells were resuspended in prewarmed PBS containing 0.1% FBS and 0.1 μM (peptide low cells), 0.5 μM (unloaded control cells) or 2.5 μM CFSE (peptide high cells) was added while vortexing to ensure homogeneous staining. Cells were incubated for 10 min at 37°C and protected from light. The reaction was stopped by adding an equal volume of pure FBS. The cells were washed two times with PBS and resuspended in the desired amount of PBS. $20x10^6$ $OVA_{257-264}$-loaded, CFSE-labeled target cells (33% peptide low, 33% control, 33% peptide high cells) were injected i.v. and 21 h later killing of target cells was analyzed in the spleens by flow cytometry.

5.2.1.4 Determination of cell numbers

A Coulter counter Z2 instrument (Beckman Coulter) was used to determine cell numbers. Cell count and size is measured by the change of electrical resistance that a cell causes by passing through a small whole in an electrode. For analysis 10 μl of cell suspension was diluted in 10 ml conductive solution (Isoton II, Beckman Coulter) and 2 drops of a lytic reagent (ZAP-OGLOBIN II, Beckman Coulter) were added to remove residual erythrocytes.

5.2.1.5 Flow cytometry - Fluorescence-Activated Cell Sorting (FACS)

Flow cytometry can detect various characteristics of single cells such as size, granularity and molecular marker expression. Cells are stained with fluorochrome-coupled antibodies against surface or intracellular antigens. Cells in a fluid stream pass a laser beam and several detectors. The resulting information is collected and can be used for identification of distinct cell populations within a heterogeneous mixture of cells.

An advanced development of classical flow cytometry is cell sorting. On a specialized instrument (FACSAria, BD) the cell population of interest can be defined by the user and then be collected by electrostatic droplet deflection.

In a 5 ml reaction tube 50 μl of a single cell suspension ($1-5x10^6$ cells) were mixed with 50 μl of a 2x concentrated antibody solution at an appropriate dilution (antibodies were titrated before use). The tubes were incubated in the dark at 4°C for 20 min. The cells were then washed with 2-3 ml FACS buffer to remove excess of unbound antibodies (300 x g, 4°C). If biotinylated antibodies were used, a second

staining step with fluorochrome-conjugated streptavidin followed. For intracellular cytokine staining splenocytes ($2x10^6$) were stimulated for 4 h in 1 ml with 100 ng/ml LPS for DCs, 1 μg/ml ionomycin and 40 ng/ml PMA for T cells in the presence of 2 μM GolgiStop (BD), which blocks protein secretion. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD) according to the manufacturer's protocol.

Prior to acquisition all samples were filtered (41 μm mesh; Reichelt Chemietechnik, Heidelberg, Germany) to remove cell aggregates. Data were aquired on a FACSCalibur with two lasers (488 and 633 nm) or on a FACSCanto II instrument with three lasers (488, 633 and 405 nm) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

5.2.1.6 Harvesting of blood and organs from mice

Harvesting peripheral blood

Before blood extraction, mice were placed under an infrared lamp for few minutes to generate vasodilatation. Mice were put in a trap and a small cut was made in the tail vein with a scalpel blade. In an eppendorf tube 3-5 drops (100-150 μl) of blood were collected and mixed with 50 μl heparin-sodium (25000 I.E./5 ml, Ratiopharm, Ulm, Germany).

Harvesting organs and preparation of single cell suspensions

Mice were sacrificed by cervical dislocation, fixed with needles on a styrofoam pad, disinfected with 70 % ethanol and cut open. Thymus, lymph nodes and spleen were harvested with fine tweezers and kept on ice in RPMI medium. For generation of single cell suspensions organs were placed in a petri dish (Ø 5 cm) between two 150 μm meshes (Reichelt Chemietechnik) and mashed with a 1 ml syringe plunger (Omnifix, Braun, Melsungen, Germany). For optimal recovery of dendritic cells (DC) organs were treated by enzymatic digestion: injection with a solution containing Liberase DL (0.65 Wünsch units/ml) and DNase I (0.2 mg/ml, both from Roche) and incubation for 25 min at 37°C, followed by mechanical dispersion using a cell strainer (100 μm, BD).

For preparation of bone marrow the hind legs were removed. The bones were cleaned from muscles, separated into tibia and femur and quickly disinfected with 70% ethanol. The terminal parts of the bones were cut open and the bone marrow was flushed out with needle and syringe. For large-scale isolation bones were placed in medium and carefully fragmented with a mortar and pestle. Bone marrow was harvested from the supernatant and filtered through a cell strainer.

For preparation of lamina propria cell suspensions, colons were removed, opened longitudinally, and washed of fecal contents. Intestines were then cut into 0.5 cm pieces, transferred into 15 ml conical tubes, and washed once with 10 ml PBS containing 0.5 % BSA. The colons were shaken at 200 rpm for 15 min at 37°C in 10 ml RPMI, supplemented with 5% FBS and containing 5 mM EDTA to remove the epithelial layer. This process was repeated once. The remaining tissue was then incubated for 10 min at RT in 10 ml RPMI/5% FCS/15mM HEPES followed by shaking at 200 rpm for 1 h at 37°C in 3 ml RPMI/5% FCS/15mM HEPES supplemented with 0.65 Wünsch units/ml Liberase DL (Roche). After incubation the cell suspension was filtered, pelleted and resuspended in ice-cold RPMI/5% FCS/5 mM EDTA. The incubation with Liberase was repeated once with the remaining tissue.

Erythrocyte lysis

Erythrocytes from peripheral blood were lyzed using Pharm Lyse reagent (BD) according to the manufacturer's instructions.

Cell pellets from organs were resuspended in 4 ml ACK buffer and left for 5 min at RT. 10 ml FACS buffer was then added and the cells were centrifuged (5 min at 4°C, 300 x g) prior to resuspension in culture medium or FACS buffer. A more gentle treatment (Mouse Erythrocyte Lysing Kit, R&D Systems) was used according to the manufacturer's instructions for bone marrow preparations or if cells were used for intracellular cytokine staining.

After erythrocyte lysis cell pellets were resuspended in the desired amount of medium or FACS buffer.

5.2.1.7 Generation of bone marrow chimeras

Recipient mice were lethally irradiated with two separate doses at 550 rad using a Cesium source (Gammacell 40, AECL, Mississauga, Canada) and supplied with neomycin (1.2 g/l, Sigma-Aldrich) containing drinking water for five weeks. Chimeras were analyzed 8-10 weeks after bone marrow transfer. For mixed chimeras, Ly5.1 mice were irradiated and reconstituted with 50 % Ly5.1 and 50 % Ly5.2 bone marrow from DC-LMP1/CD40 mice.

5.2.1.8 Detection of cytokines via a cytometric bead array system

25 ml undiluted mouse serum samples were used for the parallel detection of mouse IL-6, IL-10, MCP-1, IFN-g, TNF-a, and IL-12p70 in mouse serum. The mouse inflammation bead array system (BD) was used according to the manufacturer's instructions and analyzed with a FACSCanto (Becton Dickinson). Data were analyzed with the FCAP Array Software (Soft Flow).

Frozen intestinal or splenic tissue samples were homogenized in PBS containing a cocktail of protease inhibitors (Sigma-Aldrich) via a FastPrep-24 (MP Biomedicals) homogenizer. After centrifugation at 10,000 x g to pellet debris, concentrations of cytokines in supernatants were measured with the cytometric bead array (BD). Cytokine levels were normalized to the total protein levels present in each sample, which were measured by Qubit fluorometer (Invitrogen).

5.2.1.9 Ig Isotype ELISAs

Immunoglobulin isotypes were determined using a commercial ELISA kit (SouthernBiotech).

5.2.1.10 Histology

Organs were embedded in O.C.T. compound (Sakura Finetek, Zoeterwoude, The Netherlands), snap frozen and cut in 5 μm sections on a cryostat instrument (Jung Frigocut 2800 E, Leica Microsystems, Wetzlar, Germany). Sections were air-dried for at least 1 h, fixed with acetone (-20°C for 10 min) and stained by standard protocol with anti-CD4-FITC (clone GK1.5, eBioscience), anti-CD11c-PE (clone N418, eBioscience), anti-B220-AF647 (RA3-6B2, eBioscience), anti-MOMA-Bio (MOMA-1, BMA Biomedicals) and SA-Cy5 (Life Technologies). Analysis was performed on a

BX41 microscope equipped with a F-view II camera and cell^F software (all from Olympus, Hamburg, Germany).

For histopathological examination, organs were fixed in 10 % formalin and embedded in paraffin or glycolmethacrylate and methylmethacrylate (GMA/MMA) (Hermanns et al., 1981). Sections of 3.0 µm (paraffin) or 1.5 µm (glycolmethacrylate and methylmethacrylate) thickness were stained with haematoxylin and eosin (HE), and with Giemsa.

For the staining of Langerhans cells, ears were removed close to the head and split into dorsal and ventral halves. The latter were put dermal side down floating on 1 ml of 0.5 M ammonium thiocyanate in a 12-well plate for 20 min at 37°C. Ear halves were placed on a flat surface and the epidermis was carefully removed and floated on cold PBS. Epidermal sheets were then placed on labeled glass slides and fixed in acetone at RT for 5 min. After fixing, the epidermal sheets were washed with PBS and circled with a hydrophobic pen. Afterwards the slides were placed in a humidified box and 100 µl blocking solution (0.25% BSA in PBS with 10% mouse serum) was added. After 30 min of blocking at RT, the blocking solution was removed and 60 µl staining solution (Fc block 1:400, biotinylated IA/IE 1:100 in blocking solution) was added and incubated for 30 min at RT. Epidermal sheets were then washed three times with PBS and incubated with 60 µl secondary antibody (SA-Cy3 1:400 in blocking solution) for 30 min at RT. After staining, sheets were washed three times with PBS and embedded in 50-100 µl Fluoromount-G (Southern Biotech) under a cover slip.

For the detection of anti-nuclear antibodies in the serum, the Mosaik Basisprofil (Euroimmun, Lübeck, Germany) was used according to the manufacturer's instructions. Sera were diluted 1:20 and the anti-mouse Cy-3 antibody was diluted 1:400 in PBS-Tween (included in the kit).

5.2.1.11 Magnetic cell sorting (MACS)

Magnetic cell sorting (MACS, Miltenyi Biotec) is a technique that allows isolation of various cell subpopulations based on their expression of different antigens on the cell surface. In general there are two possible methods for cell sorting: labeling the population of interest (positive selection) or labeling all other cells (negative selection). The MACS principle is based on the use of monoclonal antibodies that are

conjugated to superparamagnetic microbeads. After labeling, the cells are applied to a column that is placed in a magnetic field of a MACS separator. There are different columns for different purposes and for different numbers of cells. Labeled cells (the positive fraction) are retained inside the column by the magnetic field, while the unlabeled ones (the negative fraction) pass through. The column is washed three times with MACS buffer to remove excess unlabeled cells. After removal of the column from the magnetic field, the cells retained in column can be eluted. MACS separation was applied to purify dendritic cells (CD11c microbeads, positive selection) and $CD8^+$ T cells ($CD8^+$ T cell Isolation Kit, negative selection) from cells isolated from spleen and lymph nodes. All procedures were performed according to the manufacturer's instructions.

5.2.1.12 Depletion of gut commensal microflora

Animals were provided ampicillin sodium salt (1 g/L; Roth), vancomycin hydrochloride (500 mg/L; Roth), neomycin sulfate (1 g/L; Roth), and metronidazole (1 g/L; Sigma-Aldrich) in drinking water (Rakoff-Nahoum et al., 2004) from week 3 to 10.

5.2.2 Molecular biology methods

5.2.2.1 Agarose gel electrophoresis

This technique was used to separate DNA fragments according to their length. By comparison to a 100 bp or 1 kb ladder (New England Biolabs (NEB), Ipswich, MA, USA) the actual size of the fragments was estimated. Before gel loading the DNA samples were mixed with gel loading dye (10% glycerol, xylene cyanol FF). Separation was carried out by application of constant voltage (80 V) to an electrophoresis chamber containing conductive buffer (TAE). Depending on the size of the DNA fragment of interest different amounts of agarose were used (0.5-2% w/v). DNA was visualized by addition of ethidium bromide to the gel (0.5 μg/ml) and subsequent examination under UV light (312 nm, Intas, Goettingen, Germany).

5.2.2.2 DNA and RNA isolation and purification

The following kits were used according to the manufacturer's protocols. During RNA isolation residual amounts of DNA were removed by on-column DNase I treatment. All kits were purchased from Qiagen (Hilden, Germany):

Isolation of genomic DNA	DNeasy Blood & Tissue Kit
Isolation of total RNA	RNeasy Mini Kit RNeasy Micro Kit (for small cell numbers)

Isolation of genomic DNA without DNA precipitation for genotyping PCR:

2-5 mm tips from mouse tails were incubated in 50 µl of 1x Gitocher buffer for 6 hours at 55°C followed by 5 min at 95°C.

5.2.2.3 Measurement of nucleic acid concentration

Nucleic acid concentrations were determined by UV absorbance measurement at 260 nm. For this purpose samples were either used undiluted and measured directly with a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA, USA) or diluted in plastic cuvettes (Brand, Wertheim, Germany) and measured with a Biophotometer (Eppendorf, Hamburg, Germany). The 260/280 ratio is an indicator of nucleic acid purity: values between 1.8-2 are desirable, as this means a low amount of protein contamination.

5.2.2.4 Polymerase chain reaction (PCR)

Using this method, DNA sequences in CD11c-Cre and DC-LMP1/CD40^{flSTOP} mice were amplified from a small amount of template tail DNA. For this process specific primers that flank the region of interest are used. A cycle of alternating temperatures allows DNA denaturation, primer annealing and DNA synthesis.

Primers:

CD11c-Cre for: ACTTGGCAGCTGTCTCCAAG

Cre-ORF rev: GCGAACATCTTCAGGTTCTG

LMP1 for: AGGAGCCCTCCTTGTCCTCTA

CD40 rev: CTGAGATGCGACTCTCTTTGCCAT

Reaction composition:

1 µl digested tailpiece

2.5 µl Primer for (10 pmol/µl)

2.5 µl Primer rev (10 pmol/µl)

12.5 µl ReddyMix PCR Master Mix (Abgene, Epsom, UK)

6.5 µl H_20

PCR reactions were performed with a T3 Thermocycler (Biometra) using the following program:

CD11c-Cre:

Step 1:	95°C	5 min	
Step 2:	95°C	30 sec	
Step 3:	63°C	30 sec	
Step 4:	72°C	30 sec	back to step 2 (30 cycles)
Step 5:	72°C	10 min	
Step 6:	4°C	∞	

LMP1/CD40:

Step 1:	94°C	5 min	
Step 2:	94°C	45 sec	
Step 3:	55°C	45 sec	
Step 4:	72°C	1 min 15 sec	back to step 2 (31 cycles)
Step 5:	72°C	10 min	
Step 6:	4°C	∞	

5.2.2.5 Quantitative PCR (qPCR)

Quantitative PCR is used to determine the exact amount of a particular DNA sequence within a sample. There are two different methods to detect the amount of PCR product in 'real time' during the PCR reaction: SYBR green is a fluorescent dye that intercalates with any double stranded DNA, whereas TaqMan probes bind specific sequences. These probes are oligonucleotides that are labeled with fluorescent dyes, which only give a signal when the probe is bound to DNA. The cycle number (crossing point, CP) when fluorescence intensity exceeds a certain threshold is correlated with the initial amount of the relevant template DNA.

Detection with TaqMan probes

Equal amounts of RNA were used for cDNA synthesis with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The TaqMan assay was performed with the LightCycler TaqMan Master Kit (Roche) and the Universal ProbeLibrary Set mouse (Roche) according to the manufacturer's instructions on a CFX96 Real Time System (BIO-RAD) using the primers and probes listed in Table 3. Expression levels were normalized to Ubiquitin C and relative quantification was calculated using the ΔΔCT-method (Fleige et al., 2006).

Gene	Forward Primer	Reverse Primer	Probe #
UBC	5’-GACCAGCAGAGGCTGATCTT-3’	5’-CCTCTGAGGCGAAGGACTAA-3’	11
IL-1β	5’-TGTAATGAAAGACGGCACACC-3’	5’-TCTTCTTTGGGTATTGCTTGG-3’	78
IL-4	5’-GAGAGATCATCGGCATTTTGA-3’	5’-AGCCCTACAGACGAGCTCAC-3’	2
IL-6	5’-GAAGGGCACTGCAGGATAGA-3’	5’-TCCCCAGAGTGTGGCAGT-3’	12
IL-10	5’-CAGAGCCACATGCTCCTAGA-3’	5’-GTCCAGCTGGTCCTTTGTTT-3’	41
IL-12p35	5’-CCAGGTGTCTTAGCCAGTCC-3’	5’-GCAGTGCAGGAATAATGTTTCA-3’	62
IL-12p40	5’-GCGCAAGAAAGAAAAGATGAA-3’	5’-TTGCATTGGACTTCGGTAGA-3’	82
IL-17A	5’-CAGGGAGAGCTTCATCTGTGT-3’	5’-GCTGAGCTTTGAGGGATGAT-3’	74
IL-17F	5’-CAAGAAATCCTGGTCCTTCG-3’	5’-GAGCATCTTCTCCAACCTGAA-3’	45
IL-21	5’-TCAGCTCCACAAGATGTAAAGG-3’	5’-GCCTTCTGAAAACAGGCAAA-3’	100
IL-22	5’-TTTCCTGACCAAACTCAGCA-3’	5’-TCTGGATGTTCTGGTCGTCA-3’	17
IL-23p19	5’-ATAGCCCCATGGAGCAACTT-3’	5’-GCTGCCACTGCTGACTAGAA-3’	25
IFN-γ	5’-GGAGGAACTGGCAAAAGGAT-3’	5’-TTCAAGACTTCAAAGAGTCTGAGG-3’	21
Foxp3	5’-TCAGGAGCCCACCAGTACA-3’	5’-TCTGAAGGCAGAGTCAGGAGA-3’	78
TNF-α	5’-CTGTAGCCCACGTCGTAGC-3’	5’-GGTTGTCTTTGAGATCCATGC-3’	79
IDO	5’-GGGCTTTGCTCTACCACATC-3’	5’-AAGGACCCAGGGGCTGTAT-3’	22
TGF-β	5’-TGGAGCAACATGTGGAACTC-3’	5’-CAGCAGCCGGTTACCAAG-3’	72

Table 5-2: Primers and probes for quantitative TaqMan PCR.

5.2.2.6 Western blotting

Lysates were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. Signals were visualized with Western Lightning-ECL (PerkinElmer Inc., MA, USA).

5.2.3 Statistical analysis

P-values were calculated with Student's t test using PRISM software (GraphPad software, La Jolla, CA, USA) and are defined as: ***: $P < 0.001$, **: $P = 0.001$ to 0.01, *: $P = 0.01$ to 0.05, n.s.: $P > 0.05$. Error bars represent standard error of the mean (SEM).

6 Results

To study the role of constitutive CD40 signaling in DCs, we used mice expressing a fusion protein comprising the signaling domain of the human CD40 and the transmembrane domain of LMP1 (LMP1/CD40). The mice were generated by crossing mice containing a loxP-flanked transcription and translation termination sequence (STOP-cassette) upstream of the LMP1/CD40 coding sequence (Homig-Holzel et al., 2008) with CD11c-Cre transgenic mice expressing Cre recombinase in CD11c+ DCs (Caton et al., 2007). When Cre is expressed it excises the STOP-cassette and the LMP1/CD40 transgene is placed under the control of the ubiquitously active rosa26 promoter (Fig. 6-1). Mice carrying one LMP1/CD40 knock-in allele and one CD11c-Cre allele will be referred to as DC-LMP1/CD40 mice. As controls, either LMP1/CD40^{flSTOP} or CD11c-Cre mice were used.

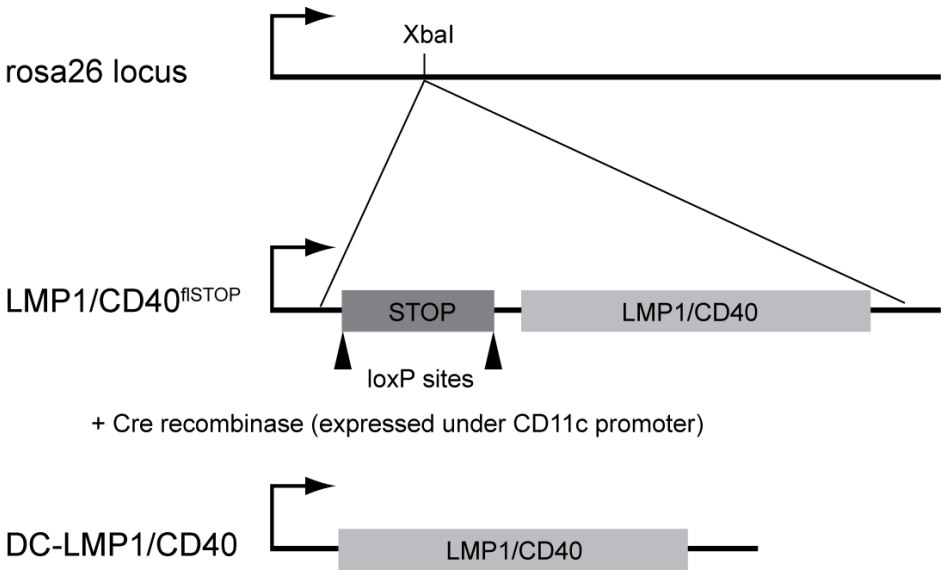

Figure 6-1: Conditional expression of LMP1/CD40.

Schematic representation of the rosa26 locus with the knock-in of the loxP-flanked STOP-cassette and the LMP1/CD40 transgene. After Cre recombinase is specifically expressed in CD11c+ cells, the STOP-cassette is excised and the expression of the transgene LMP1/CD40 takes place under the control of the ubiquitously active rosa26 promoter.

6.1 LMP1/CD40 expression in DC of DC-LMP1/CD40 mice

We first determined whether LMP1/CD40 is expressed in bone marrow derived dendritic cells (BM-DCs). For this purpose we generated BM-DCs from CD11c-Cre and LMP1/CD40^{flSTOP} control mice and the double transgenic DC-LMP1/CD40 mice. Only the double transgenic mice expressed the transgene (Fig. 6-2, A). Furthermore, LMP1/CD40 expression was detected in protein lysate of BM-DCs by Western blot analysis (Fig. 6-2, B).

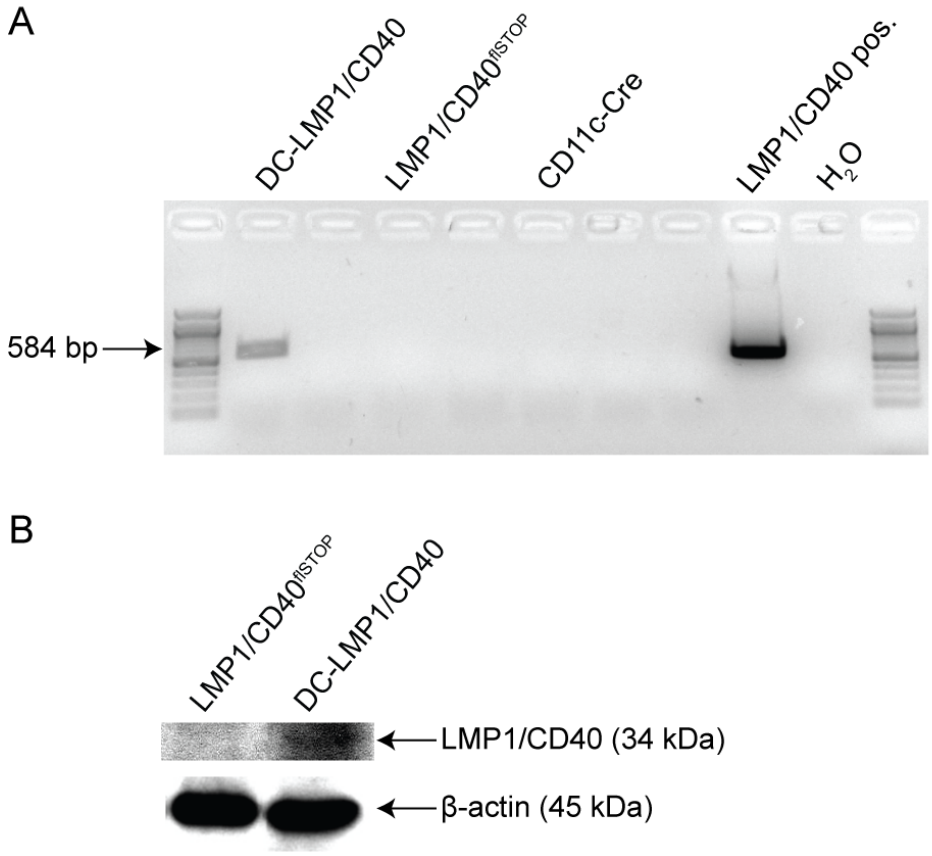

Figure 6-2: Expression of LMP1/CD40.

(A) RT-PCR for LMP1/CD40 (Primers: LMP1 forward and CD40 reverse) on RNA isolated from bone marrow derived DC from control and DC-LMP1/CD40 mice. As a positive (pos.) control, 293-F cells were transfected with a vector containing LMP1/CD40. (B) Detection of human CD40 protein by Western blotting of cell lysates from bone marrow derived DC from control and DC-LMP1/CD40 mice. As a loading control, β-actin was detected.

6.2 Low activation of DC in spleen and lymph nodes

As it is known that CD40 triggering on DCs leads to up-regulation of activation markers (O'Sullivan and Thomas, 2003), we analyzed the phenotype of DCs isolated from spleen and skin-draining lymph nodes from DC-LMP1/CD40. The splenic DCs showed lower MHC-II expression and no mature phenotype compared to LPS-activated DCs and control mice (Fig. 6-3, A). As there might be differences in the maturation status between DC subsets, we also distinguished between CD8+ and CD8- DCs. The splenic DCs contained fewer CD8+ DCs (Fig. 6-3, B). Again, no up-regulation of CD86 was observed, and only a weak up-regulation of CD80 in both subsets. In the skin-draining lymph nodes we observed a reduction of migratory DCs (MHC-II++CD11c+) (Fig. 6-3, C). Notably, neither migratory nor resident DCs showed strong up-regulation of CD80 and CD86. Only a small shift of CD80 was detected. These results indicate that constitutive CD40 signaling in *ex vivo* DCs does not lead to a significant increase in expression of costimulatory molecules.

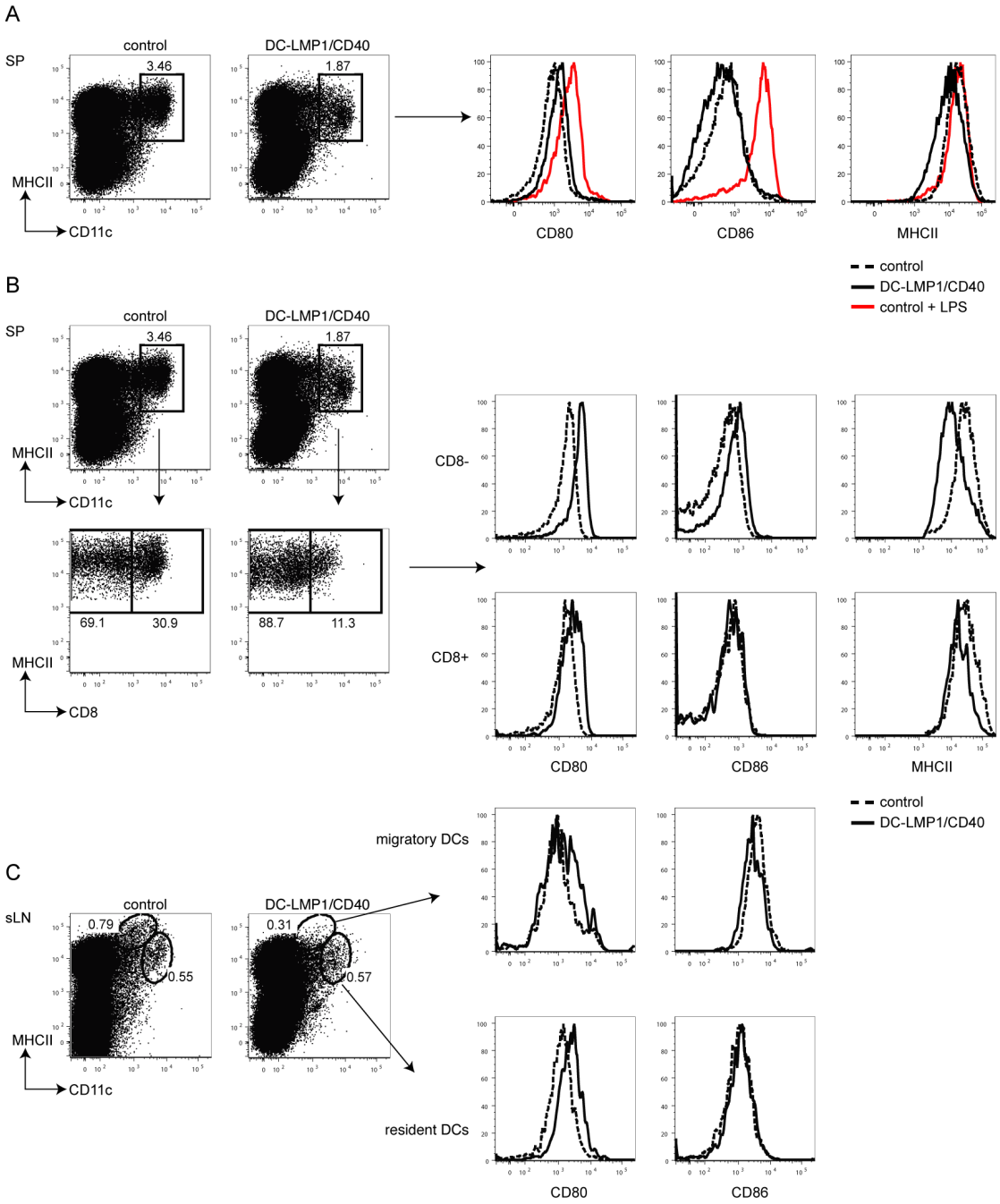

Figure 6-3: Low up-regulation of CD80.

Single cell suspensions of spleens (SP, A+B) and skin-draining lymph nodes (sLN, C) from control mice, control mice injected with 20 µg LPS 16 hours before analysis and DC-LMP1/CD40 mice were stained for CD11c, MHC-II (I-A/I-E), CD80 and CD86. The results are representative of two independent experiments with similar results, n=3.

DCs secrete the pro-inflammatory cytokine IL-12 after CD40 stimulation *in vitro* (Cella et al., 1996), but IL-12 secretion is thought to require additional signals *in vivo* (Schulz et al., 2000). To test if our mice spontaneously secrete IL-12 and if they can be triggered to secrete it in the steady state, we stimulated whole splenocytes with or

without LPS for 3 hours and analyzed IL-12 production by intracellular cytokine staining. Splenic DCs of DC-LMP1/CD40 mice did not secrete IL-12 spontenously and were able to produce IL-12 after LPS stimulation with no significant difference to control DCs (Fig. 6-4) These results indicate that CD40 stimulation alone is not sufficient for the induction of IL-12 production.

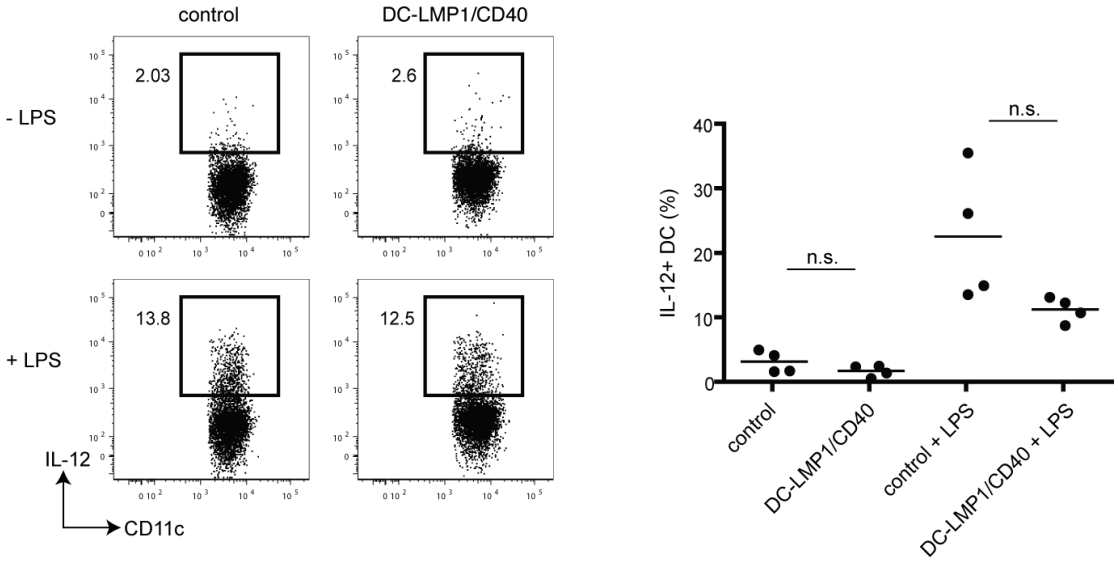

Figure 6-4: No increase in IL-12 production.

Single cell suspensions of spleens were stimulated with 100 ng/ml LPS for 3 h *in vitro* followed by staining of CD11c and MHC-II and intracellular cytokine staining for IL-12. Data pooled from two independent experiments, n=4.

6.3 OT-I T cell proliferation and killer assays

To test whether CD40 stimulated DCs are able to induce proliferation of T cells and convert them into effector cells or rather would tolerize them, we transferred $5x10^4$ OT-I T cells expressing the congenic marker Ly5.1 into control and DC-LMP1/CD40 mice by i.v. injection. After 24 h mice were immunized with 20 µg of the MHC-I K^b-restricted $OVA_{257-264}$ peptide and one control group received in addition 50 µg agonistic CD40 antibody (anti-CD40). Blood was analyzed by flow cytometry on the following days. In mice additionally immunized with anti-CD40, OT-I T cells expanded substantially in response to presented antigen, whereas OT-I T cells in DC-LMP1/CD40 mice did not proliferate to the same extent, but rather resembled the control mice that also received only the peptide (Fig. 6-5, A). To test the function of

these T cells we performed an *in vivo* cytotoxicity assay. On day 16, $OVA_{257-264}$ peptide-loaded, CFSE-labeled target cells were injected i.v. and 21 h later killing of the target cells was analyzed in the spleens by flow cytometry. Target cells with the higher amount of peptide (CFSE high) are the first to be killed by cytotoxic T lymphocytes (CTLs), whereas the cells loaded with the lower amount (CFSE low) are only killed when a higher number of CTLs or CTL with high antigen avidity are present. The CFSE intermediate cells are unloaded and serve as control cells. Killing of the target cells was observed only in the anti-CD40 antibody treated control (Fig. 6-5, B). Therefore DCs activated by LMP1/CD40 are not able to induce effector functions of CD8+ T cells, but rather convert them into tolerant or anergic cells.

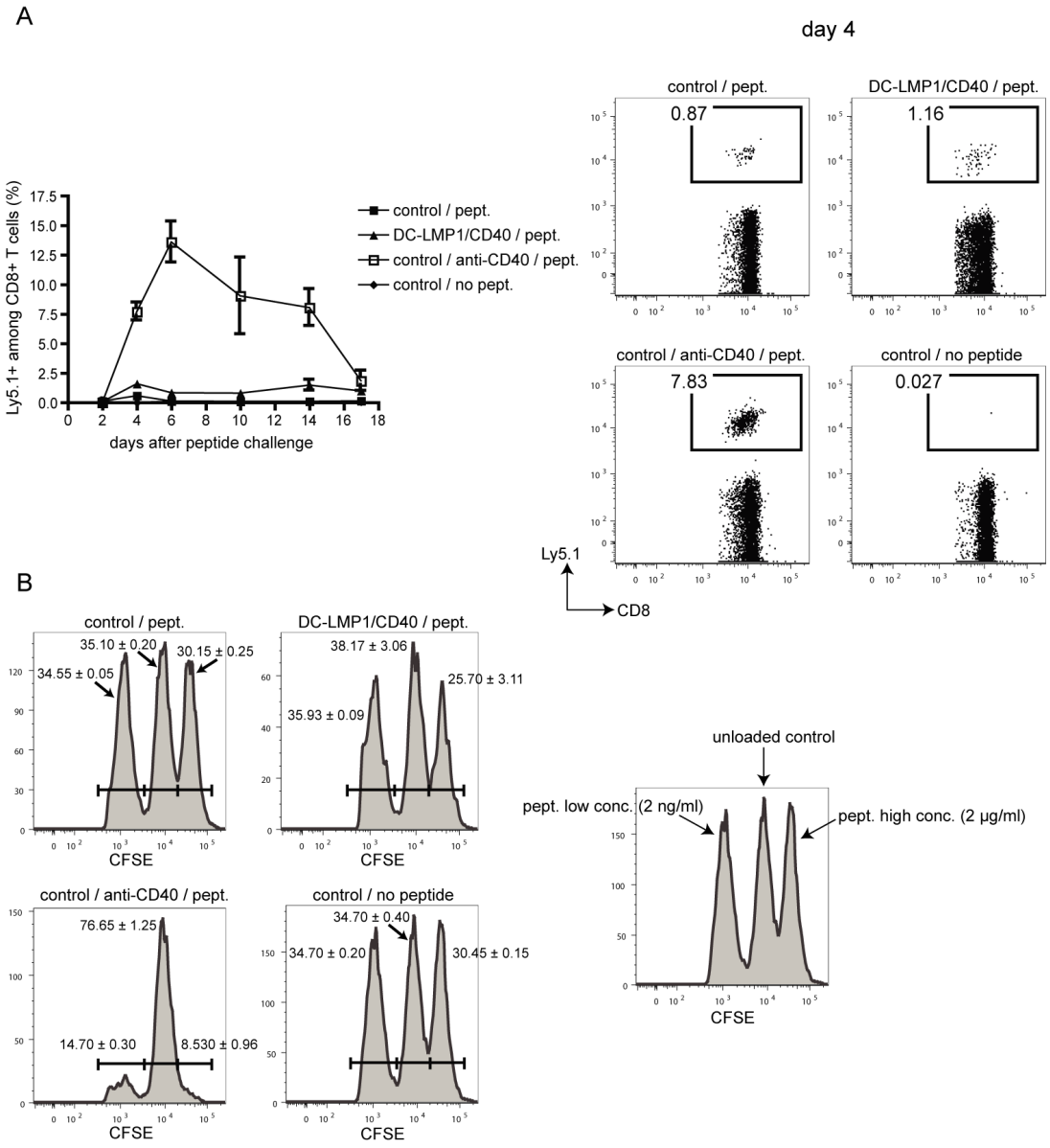

Figure 6-5: No T cell expansion and effector function after immunization with OVA-peptide.

$5x10^4$ OT-I T cells (Ly5.1-positiv) were transferred i.v. into control and DC-LMP1/CD40 mice. After 24 h mice were immunized with 20 µg $OVA_{257-264}$ and one group additionally with 50 µg anti-CD40 Ab. (A) Expansion of T cells was measured in the blood by flow cytometry. (B) *In vivo* cytotoxicity assay. On day 16, $OVA_{257-264}$-loaded, CFSE-labeled target cells were injected i.v. and 21 h later killing of the target cells was analyzed in the spleens by flow cytometry. Data pooled from two independent experiments, n=5-7.

6.4 DC-LMP1/CD40 mice develop severe pathology

6.4.1 Weight loss and reduced survival

DC-LMP1/CD40 mice were born at the expected Mendelian ratio (data not shown) but they appeared smaller in size (Fig. 6-6, picture) and developed wasting disease.

Their body weight was reduced by approximately 35% as compared to controls at 8 weeks of age (Fig. 6-6). We concluded that the weight loss of DC-LMP1/CD40 mice could be an indication for an immune disorder and therefore we also monitored the survival of the mice.

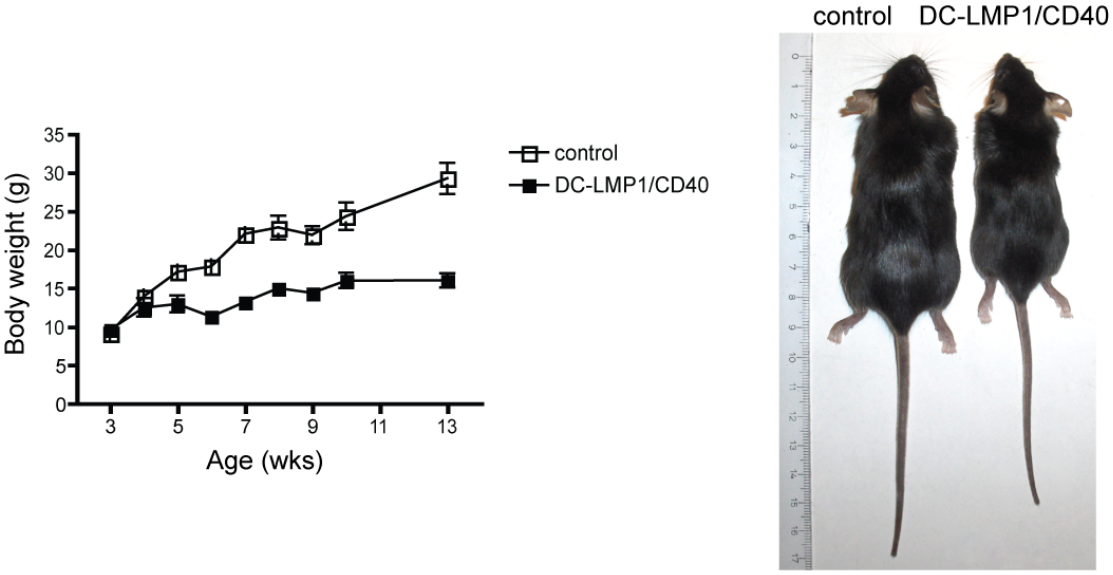

Figure 6-6: Reduced body weight.

Left: Body weight of controls (open squares) and DC-LMP1/CD40 (filled squares) between 3 and 13 weeks of age. n=3-12 per time point. Right: Picture of an 8-wk-old DC-LMP1/CD40 mouse and a negative littermate.

50 % of DC-LMP1/CD40 mice died within the first 12 weeks of age and 100 % of the mice died within 18 weeks (Fig. 6-7) indicating a severe pathology.

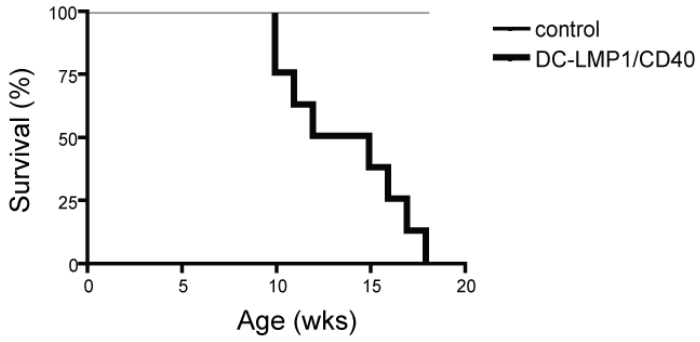

Figure 6-7: Reduced survival.

Kaplan-Meier plot of survival study of controls (thin line; n=8) and DC-LMP1/CD40 mice (thick line; n=8), p<0.0001.

6.4.2 Splenomegaly and colon shortening

As the weight loss and the reduced survival of DC-LMP1/CD40 mice were pointing to an immune disorder, we macroscopically examined the organs of the mice. Analysis of peripheral lymphoid organs of 8-12 week old DC-LMP1/CD40 mice showed that the spleen was enlarged and the colon was thickened and shortened (Fig. 6-8), indicative of intestinal inflammation. These results were detected in every mouse that was sacrificed during the study.

Figure 6-8: Colon shortening and splenomegaly.

Size of colon and spleen of indicated mice on a metric scale. Pictures are representative of at least n=20 mice per group.

6.4.3 Expansion of granulocytes in the spleen and blood

To determine the possible causes of splenomegaly, we analyzed the different cell types in the spleen. The total number of splenocytes was not significantly different between DC-LMP1/CD40 and control mice (Fig. 6-9). Also the amount of CD8- DCs, macrophages and NK cells was not altered in transgenic mice. DCs, T and B cells were significantly reduced. In contrast, granulocyte numbers were significantly increased in the DC-LMP1/CD40 mice and seemed to displace DCs, T and B cells.

An increase in granulocyte number is generally associated with inflammation (Nathan, 2006).

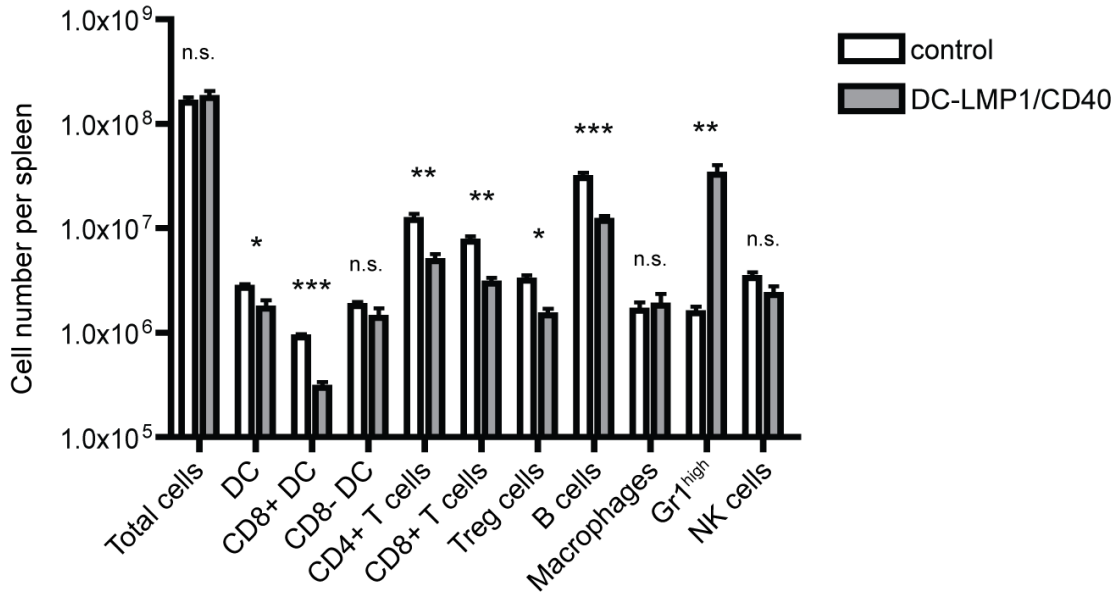

Figure 6-9: Expansion of granulocytes in the spleen.

Cell counts of indicated populations in the spleen of 8–12 week old controls (open bars) or DC-LMP1/CD40 mice (filled bars). Pooled results are from three independent experiments. n=6.

Next, we wanted to know how granulocytes developed over time in DC-LMP1/CD40 mice. Therefore mice were bled weekly from week 3 to 8 and blood was analyzed by flow cytometry for granulocytes, T, B and NK cells. At week 3, similar proportions of all cell types were found in DC-LMP1/CD40 and control mice (Fig. 6-10). The increase in granulocytes and the decrease in T, B and NK cell numbers started at week 4. After 8 weeks of age, granulocytes were increased more than 3-fold and other cell types were reduced to approximately 10% of that observed in wildtype control mice. Therefore we concluded that the splenomegaly might be the result of strong granulocytic infiltrations.

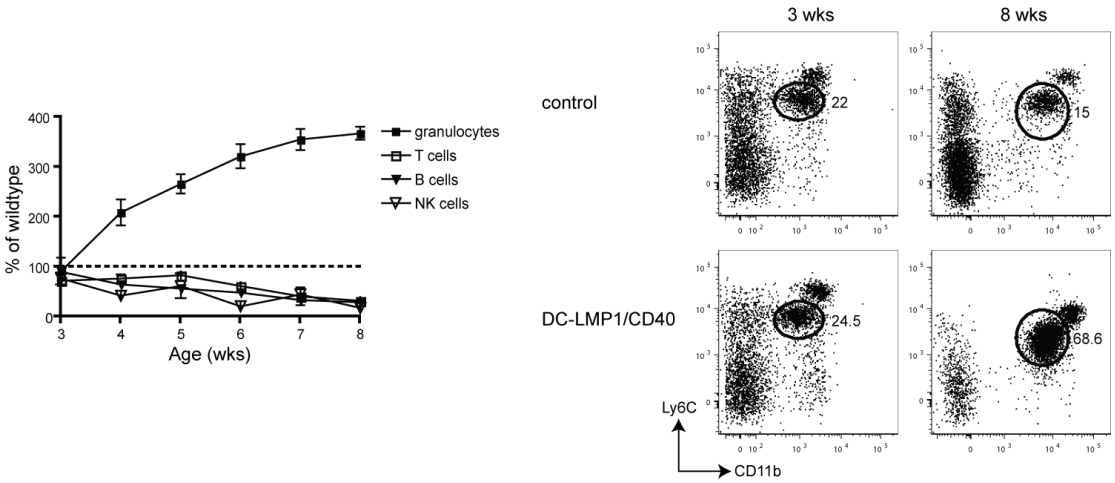

Figure 6-10: Expansion of granulocytes in the blood.

Mice were bled from week 3 to week 8 and blood was stained for T, B, NK cells and granulocytes and analyzed by FACS, n=3-6 per time point.

6.4.4 Infiltrations of granulocytes in the colon

Having observed colon thickening macroscopically, we next analyzed colon tissue by histology. DC-LMP1/CD40 mice displayed colitis with severely thickened colon walls, marked hyperplasia of stromal cells and extensive infiltration of granulocytes and lymphocytes within the lamina propria, reduction of goblet cells, and focal ulceration of the mucosa (Figure 6-11). These results confirmed the macroscopical observation of colon thickening and indicated an immunopathological effect in transgenic mice.

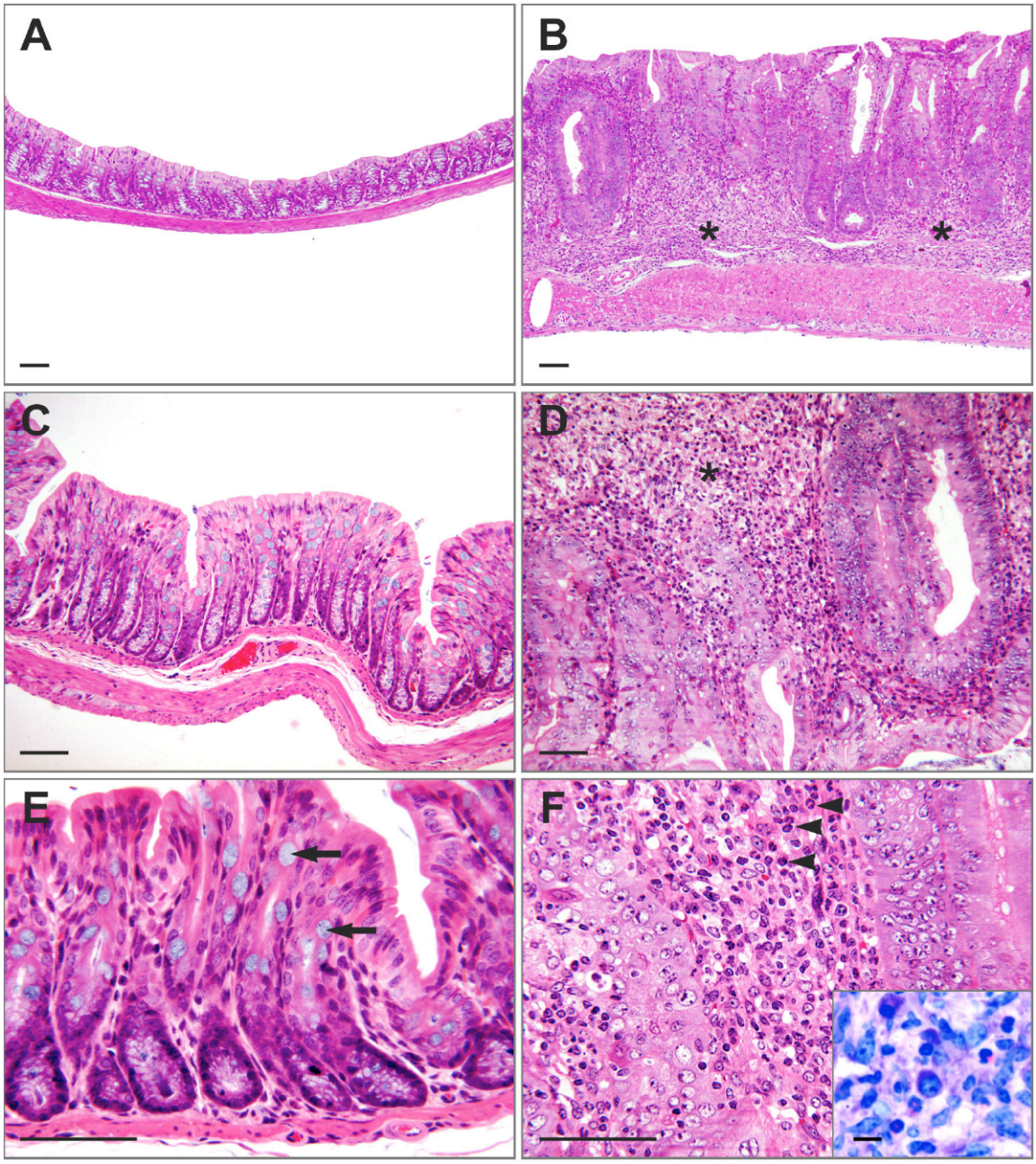

Figure 6-11: Intestinal inflammation.

(A), (C), (E): Unaltered colon histology of non-transgenic control mice. Arrows indicate mucosal goblet cells (E). (B), (D), (F): Histopathology of the colon in DC-LMP1/CD40 mice with thickening of the colon wall (B), hyperplasia of stromal cells and infiltration of granulocytes and lymphocytes within the lamina propria (asterisks in B and D) and reduction of goblet cells. Granulocytic cell infiltration is indicated by arrowheads (F). GMA/MMA sections. HE-staining. Bars = 100 µm. Inset in (F): Giemsa-stained GMA/MMA section of the lamina propria with stromal cells, plasma cells, eosinophile and neutrophile granulocytes and lymphocytes. Bars = 10 µm. Histological pictures are representative of n=3 mice per group.

6.4.5 Spleen histology

Following the finding that we detected strong infiltrations of granulocytes in the colon by histology and an expansion of granulocytes in the spleen and blood by flow

cytometry, we next examined the composition of important immune cells such as T, B cells and DCs in the spleen by performing immunofluorescence microscopy of spleen sections. The analysis revealed that B and T cell areas of DC-LMP1/CD40 mice appeared smaller in size compared to control mice at 6 weeks of age and were further reduced at 12 weeks of age, confirming flow cytometric results showing a reduction of B and T cells in the spleen (Fig. 6-12). At 12 weeks of age the T cell areas were interspersed with CD11c+ cells and the typical splenic structure was no longer observable. We concluded that the immunological disorder induced in DC-LMP1/CD40 lead to the loss of normal splenic structure probably due to the extensive infiltrations of granulocytes.

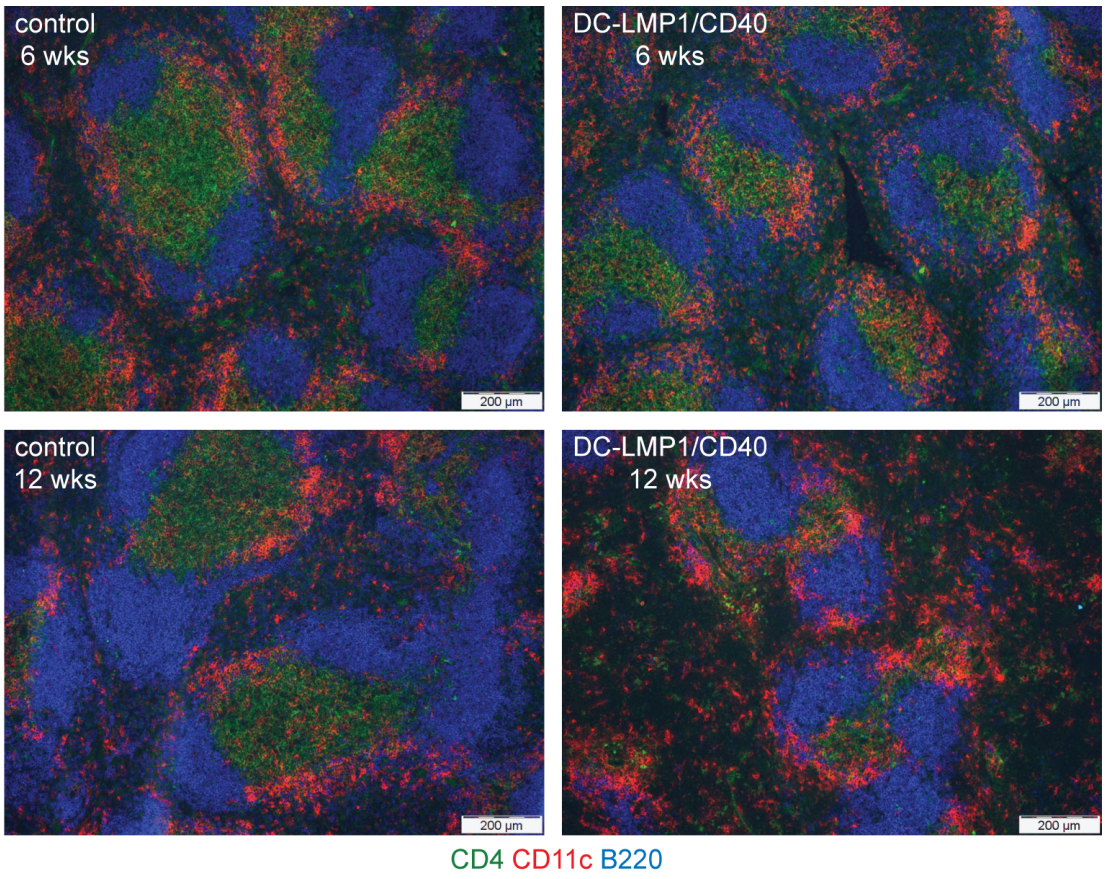

Figure 6-12: Reduction of B and T cell areas after 12 weeks of age.

Immunofluorescence microscopy of spleen sections from DC-LMP1/CD40 mice. Frozen sections from 6 wk and 12 wk old control and DC-LMP1/CD40 mice were immunofluorescently stained for CD4+ T cells (FITC), CD11c+ DCs (PE) and B220+ B cells (AF 647). Bars, 200 µm. Histological pictures are representative of n=3 mice per group.

6.4.6 Langerhans cells

DCs that are found in the epidermis are called Langerhans cells. To determine whether these cells are also affected by CD40 activation, we isolated epithelial layers of the ears of DC-LMP1/CD40 and control mice and stained for MHC-II. We observed a significant reduction of Langerhans cells in DC-LMP1/CD40 mice (Fig. 6-13, A+B). This reduction could be a sign of activation of the Langerhans cells. We had also observed a reduction of migratory DCs in skin-draining lymph nodes (sLN) of DC-LMP1/CD40 mice, possibly meaning that less Langerhans cells had reached the sLN (Fig. 6-3). Following the finding that we also found reduced numbers of Langerhans cells in the epidermis, this could indicate that the epidermis is already seeded with fewer cells maybe due to a reduction of precursors. Another explanation would be that LMP1/CD40-Langerhans cells undergo apoptosis more quickly than control cells possibly because of their higher activation level.

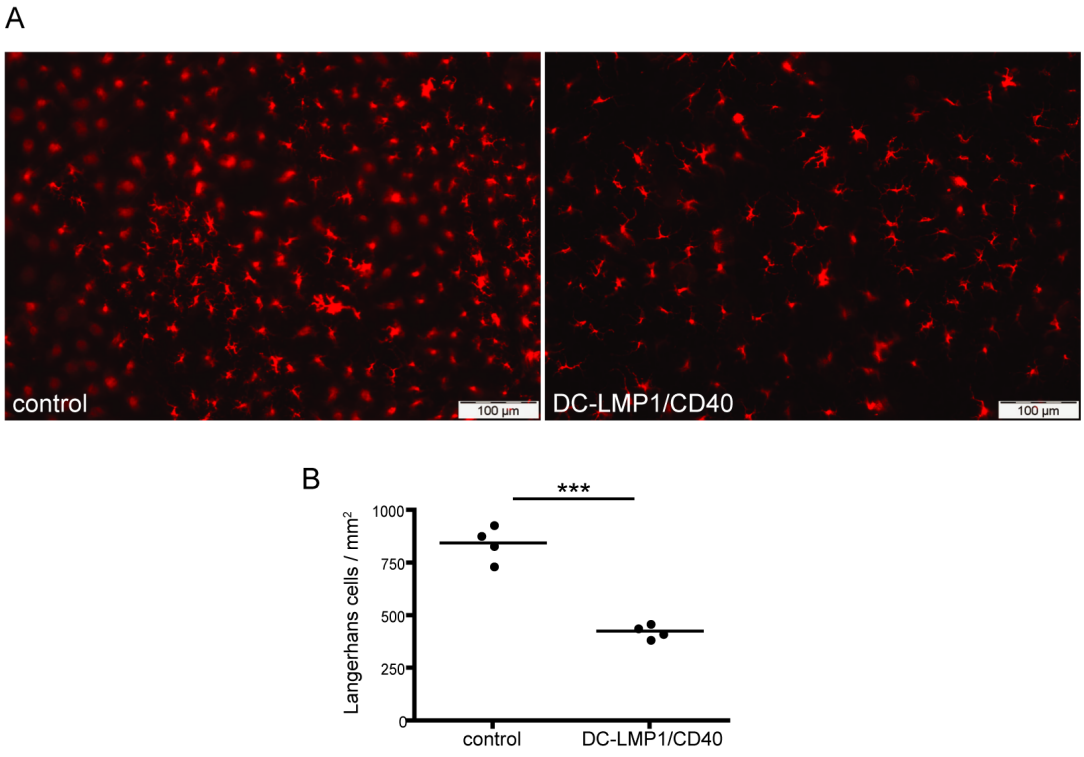

Figure 6-13: Reduced Langerhans cells.

(A) Epithelial layers of the ears of indicated mice were stained for MHC-II (I-A/I-E) to detect Langerhans cells. Bars, 100 µm. Histological pictures are representative of n=3 mice per group. (B) Quantification of Langerhans cells in epidermal sheets. The graph shows the mean of four fields counted from two mice per group.

6.5 Low T cell activation in spleen and lymph nodes

DCs are very potent T cell activators and as we had found intestinal inflammation in the DC-LMP1/CD40 mice, we next analyzed the activation status of T cells in the spleen and in the mesenteric lymph nodes. Flow cytometric analysis revealed that CD4+ and CD8+ T cells did not show a significant increase in activated cells (CD62-CD44+) in the mesenteric lymph nodes. Also in the spleen, we detected a low increase in activated T cells, which was not significant (Fig. 6-14, A), although we detected some individual outliers that showed strong activation.

Intestinal inflammation is often associated with high IL-17 expression; therefore we tested the potential of CD4+ T cells to secrete this cytokine. Intracellular cytokine staining of PMA/ionomycin-restimulated CD4+ T cells showed no significant increase of IL-17+ and IL-17+ IFN-γ+ cells in the mesenteric lymph nodes and the spleen of DC-LMP1/CD40 mice (Fig. 6-14, B), although we detected a non-significant tendency of the T cells to produce more IL-17. However, in contrast to CD4+ T cells, the production of IFN-γ by CD8+ T cells in the mesenteric lymph nodes was significantly increased (Fig. 6-14, B).

We concluded that T cell activation in lymph nodes and spleen was not consistent and could not be detected in every DC-LMP1/CD40 mouse indicating that T cell activation found in these organs was probably not induced by LMP1/CD40-expressing DC present in these organs but by side effects of the advanced disease.

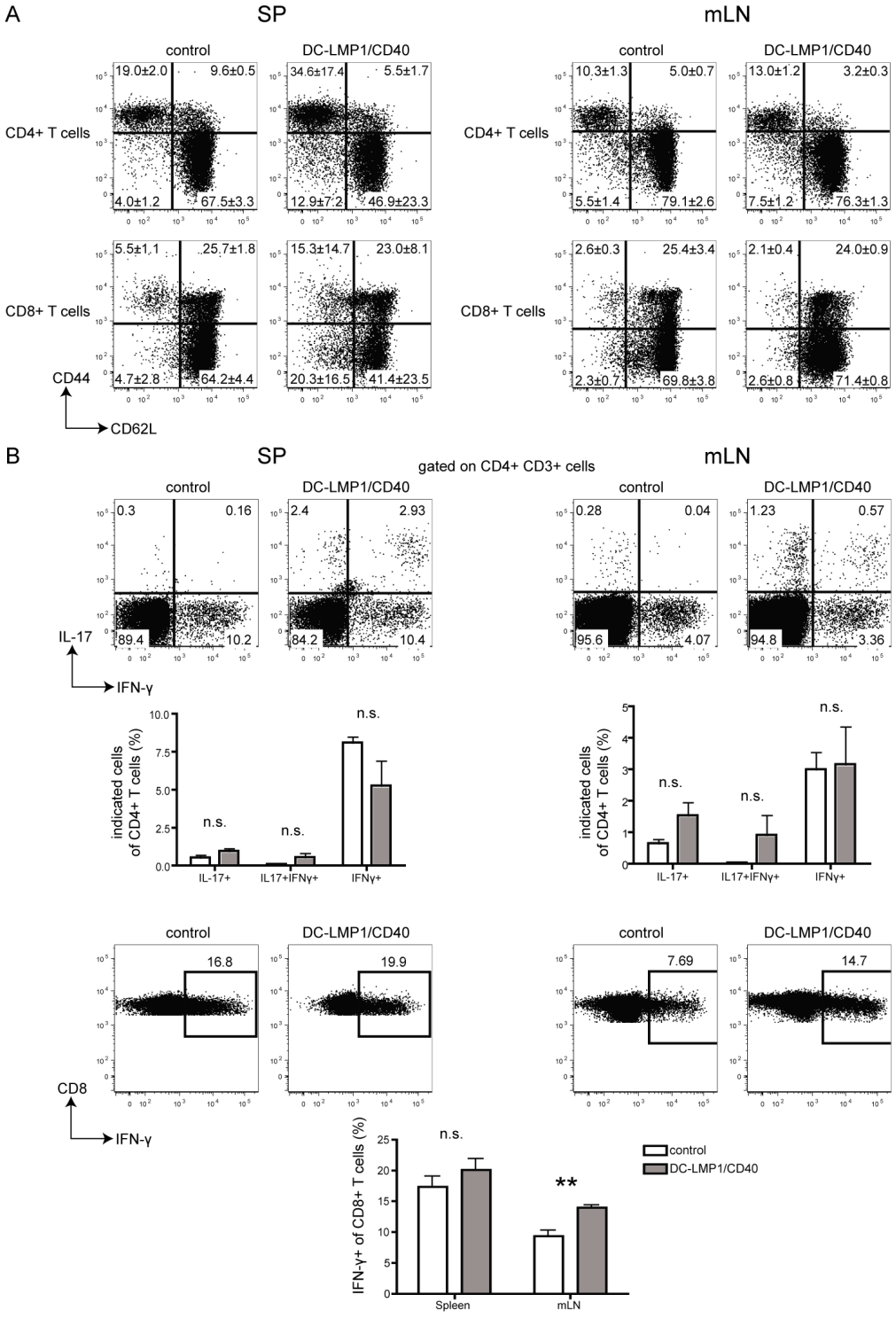

Figure 6-14: Weak T cell activation in spleens and lymph nodes.

(A) Single cell suspensions of spleen (SP) and mesenteric LN (mLN) from control and DC-LMP1/CD40 mice were stained for CD3, CD4, CD8, CD44 and CD62L. The results are representative of two independent experiments, n=3. (B) Intracellular cytokine staining of PMA/ionomycin restimulated CD4+ and CD8+ T cells isolated from mesenteric LNs or spleens of indicated mice. Data pooled from two independent experiments, n=3-5.

6.6 Autoantibodies and elevated IgA and IgM levels in the blood

In order to determine the degree of activation of the humoral immune response in our transgenic mice, we measured the concentration of immunoglobulin isotypes in the serum. Because of the severe pathology we observed in the DC-LMP1/CD40 mice, we additionally determined whether the mice generated autoantibodies.

We found significantly high levels of IgM and IgA in the serum of DC-LMP1/CD40 mice (Fig. 6-15, A). IgA plays an important role in mucosal immune responses and may be elevated because of the colitis that was detected by histology.

Next, we tested if these increased antibody levels also contain anti-nuclear antibodies (ANA). ANAs are antibodies directed against the cell nucleus that destroy specific tissue in the body, and are therefore considered a sign for autoimmunity. By staining HEp-2 cells with sera from control and DC-LMP1/CD40 mice, we found a significant increase of ANAs in DC-LMP1/CD40 mice (Fig. 6-15, B). ANAs are generally capable to contribute to the onset of an autoimmune disease or can be one of the side effects of a disease.

Here we conclude, that elevated levels of ANAs were likely to be a sign of autoimmunity and were of the IgM or IgA isotype as these two isotypes were significantly elevated in the serum.

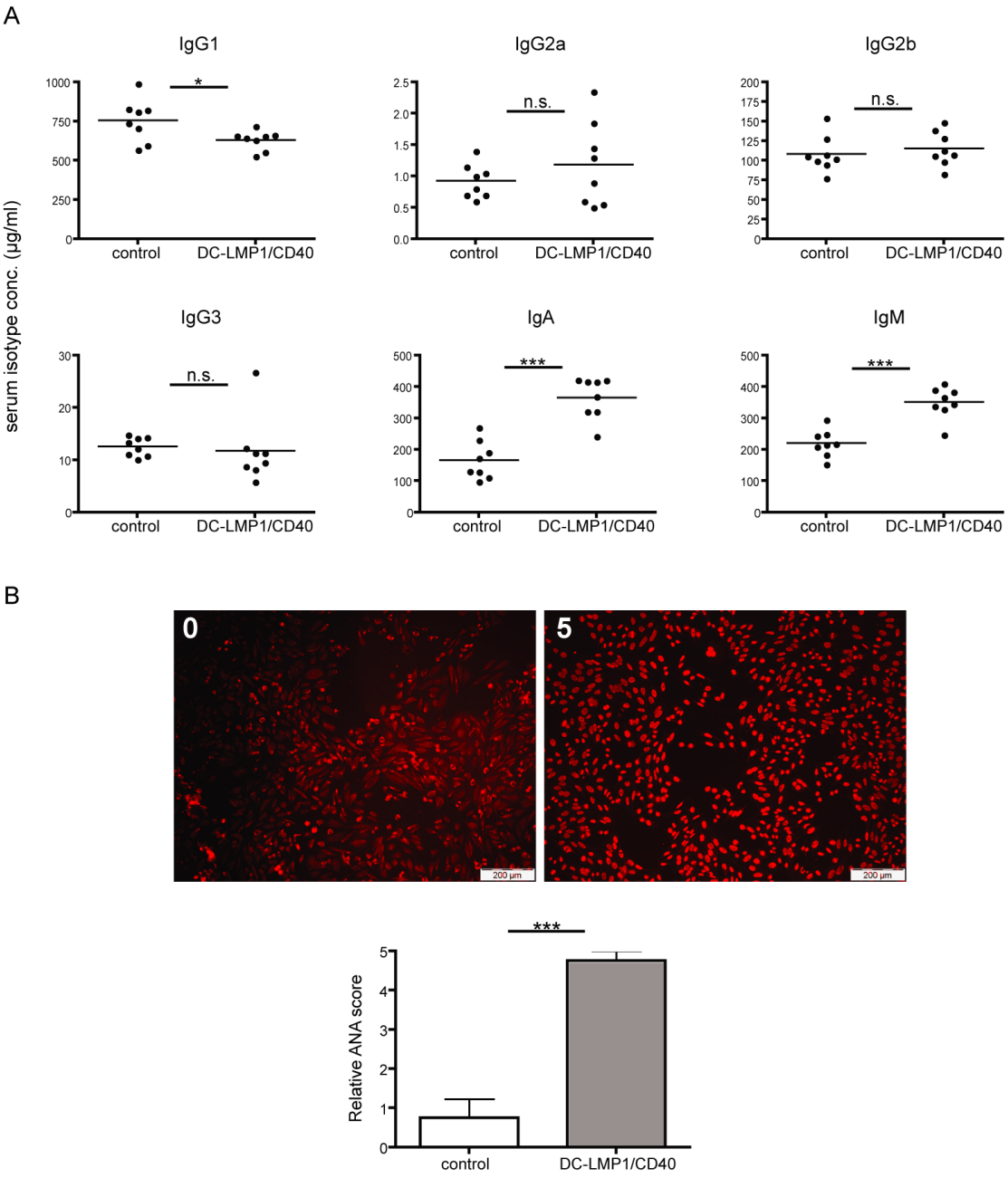

Figure 6-15: Elevated levels of IgA and IgM and autoantibodies.

(A) Immunoglobulin isotype concentrations in the serum of control and DC-LMP1/CD40 mice; n=8. (B) Anti-nuclear antibodies (ANA) were scored between 0 and 5 by determining the staining intensity on HEp-2 cells; bar, 200µm; p=0.0003; n=4.

6.7 Increase of inflammatory cytokines in the serum, spleen and colon

After having detected elevated ANA levels and intestinal infiltrations, we were interested in other inflammatory factors such as cytokines. We therefore examined the

mice's sera, spleens and colons for inflammatory cytokines. For this purpose we used the BD™ Cytometric Bead Array (CBA), which is a flow cytometry application that allows simultaneous quantification of multiple proteins.

Sera were taken from control and DC-LMP1/CD40 mice and analyzed for six different cytokines. We found a significant increase in IFN-γ, TNF-α and IL-6 in comparison with the control (Fig. 6-16, A). All three factors are important pro-inflammatory cytokines and are associated with autoimmune diseases such as inflammatory bowel disease.

In order to determine the cytokine milieu in tissues, we performed CBA on splenic and colonic homogenates. Cytokine concentrations were normalized to the total protein content of each sample. In the colon of DC-LMP1/CD40 we detected an increase in the same three cytokines that were elevated in the serum (Fig. 6-16, B). In the spleen, none of the tested cytokine concentrations were altered compared with control mice.

In summary, these results indicate a local inflammation in the colon, concomitant with an increase of inflammatory cytokines in the serum.

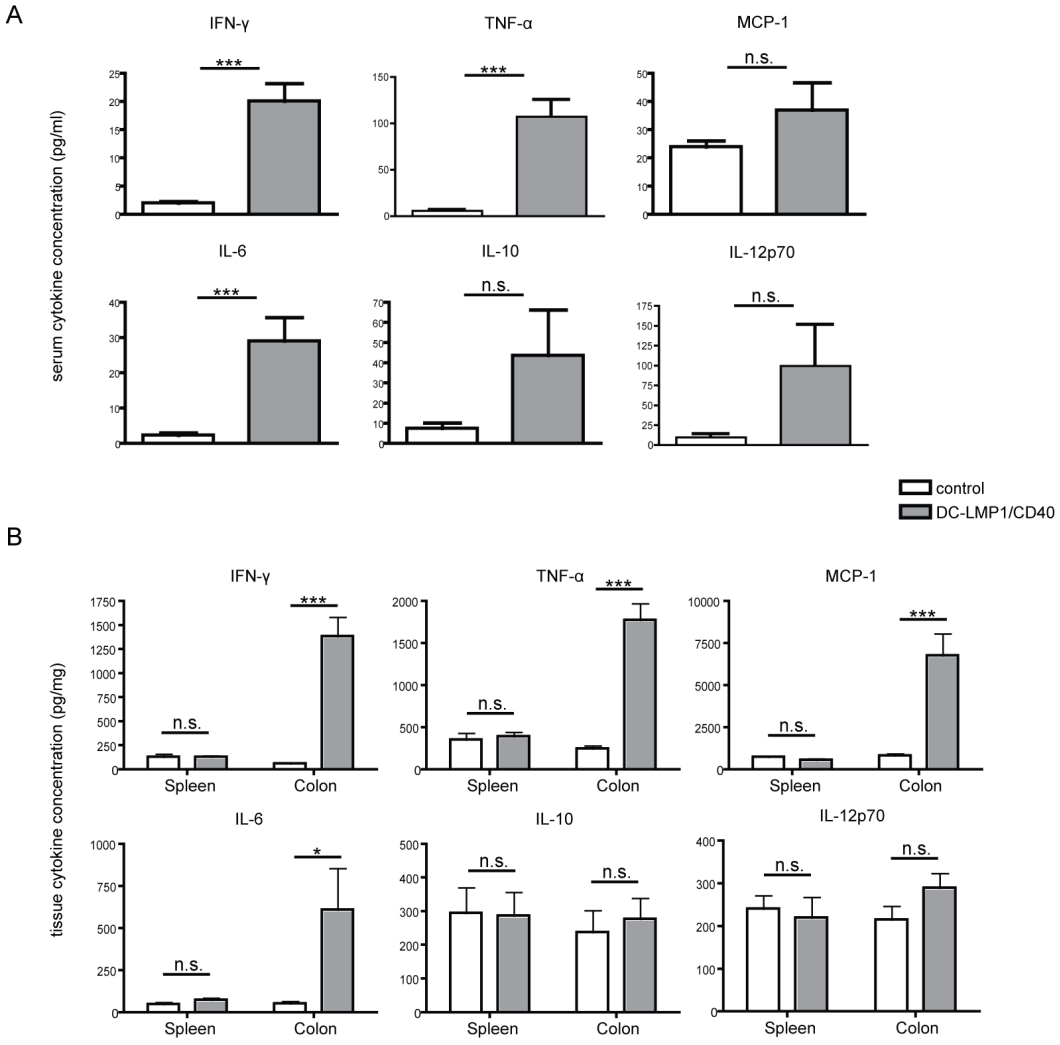

Figure 6-16: Increase of inflammatory cytokines in the serum and colon.

(A) IFN-γ, TNF-α, MCP-1, IL-6, IL-10 and IL-12p70 serum concentration from 6-12 week old control (open bars) and DC-LMP1/CD40 (filled bars) mice, n=22-24. (B) Cytokine concentrations in colon and spleen homogenates from 8-12 week old mice were measured by CBA and normalized to total protein content for each sample, n=5-10.

6.8 Characterization of colonic DC

As we had found an increase of inflammatory cytokines in the gut, we decided to characterize the colonic DCs more in detail.

6.8.1 Reduced CD103+ DC

We stained single cell suspensions of the colons of DC-LMP1/CD40 and control mice for common DC markers. A strong reduction of CD103+ DCs of DC-LMP1/CD40 mice was detected (Fig. 6-17, A). Similar results were obtained in a study in which sorted wildtype DCs were stimulated *in vitro* with anti-CD40, resulting in a genuine

down-regulation of CD103 not caused by cell death (Annacker, 2005). In addition, we stained CD103- DCs for activation markers and detected an increase in CD70, CD80 and CD86 compared to control (Fig. 6-17, B).

These results indicated that DCs in the colon displayed an activated phenotype and did not express CD103 due to loss of the marker or loss of the cells.

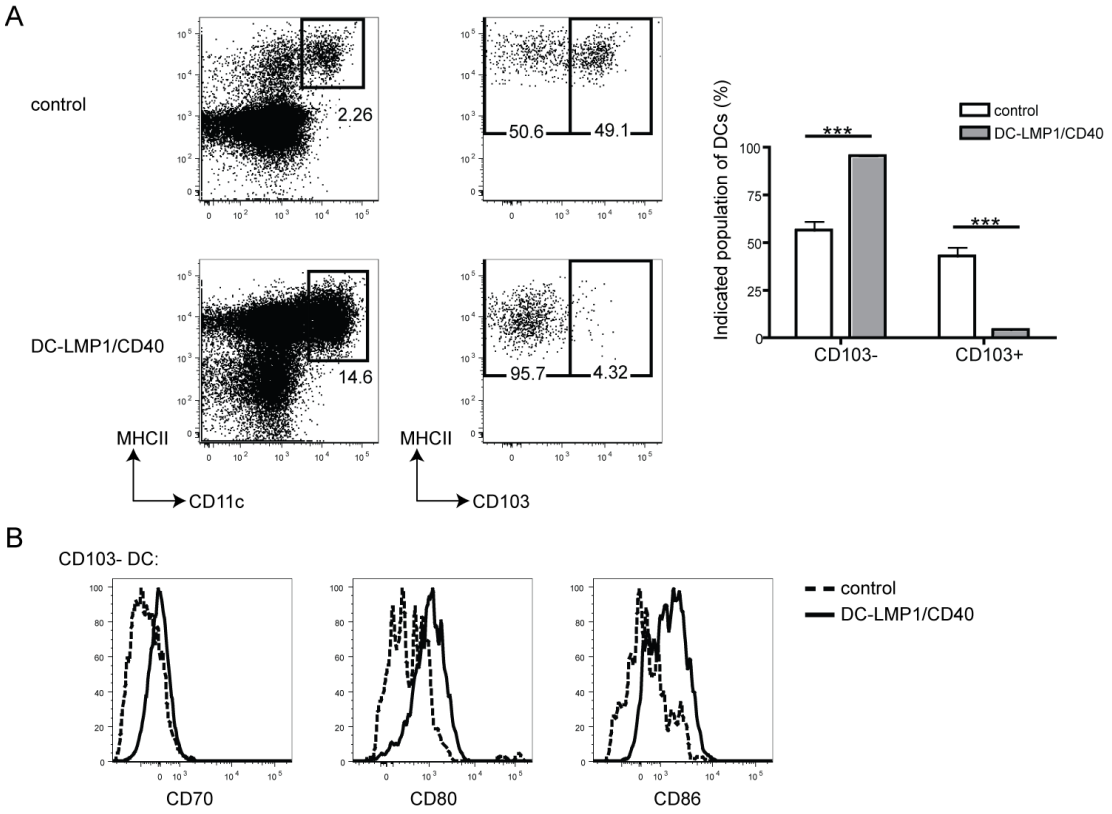

Figure 6-17: Reduction of CD103+ colonic DCs.

Single cell suspensions of the colon from DC-LMP1/CD40 and control mice were stained for (A) CD11c, MHC-II (I-A/I-E), CD103 and (B) for CD70, CD80 and CD86. The results are representative of two independent experiments, n=3.

6.8.2 Increase of IL-23 and other cytokines in colonic DC

Having found up-regulation of activation markers on colonic DCs, we next analyzed cytokine production of splenic or colonic DCs in DC-LMP1/CD40 mice, and compared this with the respective DCs of control mice. CD11c+ cells were sorted by MACS-beads from colons and spleens of DC-LMP1/CD40 and control mice. After sorting, RNA was isolated and transcribed into cDNA in order to perform quantitative PCR (qPCR) for a selection of cytokines. The most prominent difference in cytokine

mRNA expression was found in IL-23, which is composed of the subunits IL-23 p19 and IL-12 p40. IL-23 p19 mRNA was significantly elevated in colonic DCs of DC-LMP1/CD40 mice compared to colonic DCs of control mice (Fig. 6-18, upper row). IL-23 plays a key role in the intestine (Ahern et al., 2008; Sarra et al., 2010) by promoting T_H17 cell responses and gut inflammation. It was also found to be elevated in colons in a study where anti-CD40 antibody was injected into Rag-deficient mice where IBD is induced after antibody injection (Uhlig et al., 2006). In the same study, Uhlig et al. detected a decrease in IL-12 p40 and increase in IL-12 p35; the same pattern that we observed in DCs of DC-LMP1/CD40 mice (Fig. 6-18, upper row). IL-12 p40 and IL-12 p35 are the subunits of the cytokine IL-12.

In addition, mRNA of the pro-inflammatory cytokine IL-1β was significantly increased in colonic DCs of DC-LMP1/CD40 mice compared to control mice. IL-6 mRNA was also elevated, although this did not reach statistical significance.

We also found an increase in IL-10, TGF-β and IDO mRNA in the colonic DCs of DC-LMP1/CD40 mice (Fig. 6-18, middle and lower row). IL-10 is know to play a suppressive role in immune responses, nevertheless IL-10 also has immunostimulatory properties, and can induce recruitment, cytotoxicity and proliferation of CD8+ T cells (Moore et al., 2001). TGF-β is a potent suppressor of inflammation *in vivo*, but may also exhibit some pro-inflammatory effects (Hill and Sarvetnick, 2002). An increase of IDO, which is known to have immunosuppressive activity, upon CD40 stimulation has previously been shown (Hwu et al., 2000). In addition, non-canonical NF-κB signaling in dendritic cells is required for IDO induction and immune regulation (Tas et al., 2007). The induction of the non-canonical NF-κB signaling pathway was also shown in B-cell specific LMP1/CD40 expression (Homig-Holzel et al., 2008).

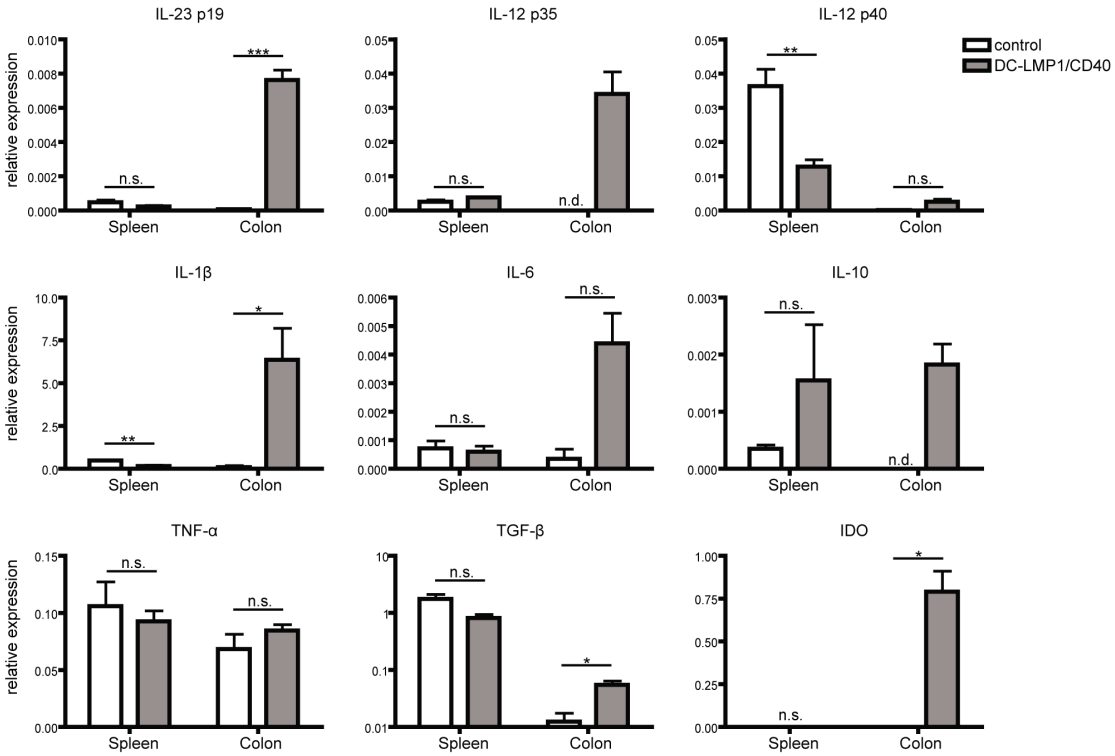

Figure 6-18: Increase of inflammatory cytokines in colonic DCs.

Expression of cytokine mRNA in MACS-sorted (CD11c) DCs of control (open bars) and DC-LMP1/CD40 (filled bars) mice. Data were normalized to Ubiquitin C, n=4-6.

6.8.3 CD103+ DC in the spleen

Following the finding that CD103+ DCs are missing in the colon of DC-LMP1/CD40 mice, we analyzed the expression of CD103 on splenic DCs from these mice. As already noted, we detected a reduction of CD8+ splenic DCs (Fig. 6-9). Approximately 42 % of CD8+ DCs were CD103+ in the control and DC-LMP1/CD40 mice (Fig. 6-19). Proportion wise, no difference was detected in CD103 expression among CD8+ DCs in the spleen.

These results indicated that the loss of CD103 on DCs was selectively observed in the colon. LMP1/CD40 expression was therefore not the only reason that CD103 expression disappeared in the colon as also splenic DCs expressed LMP1/CD40. Side effects of the intestinal inflammation could possibly play a role in the down-regulation of CD103 in the colon.

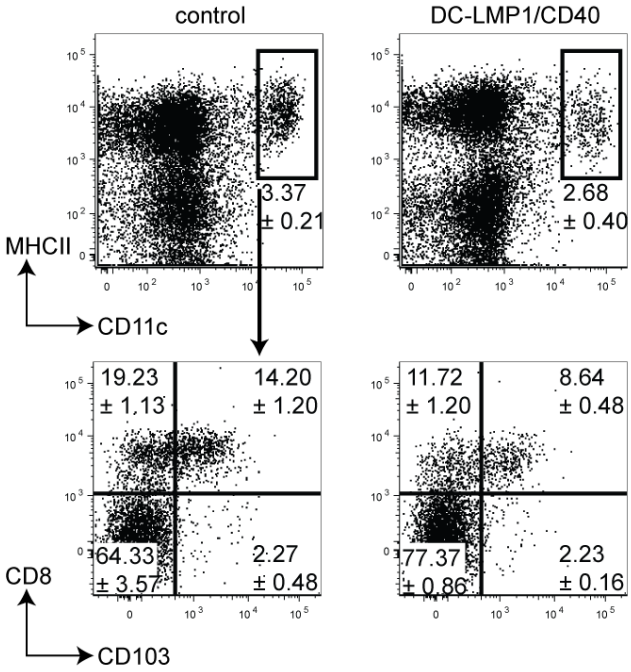

Figure 6-19: CD103 expression of splenic DCs.

Single cell suspensions of spleen from control and DC-LMP1/CD40 mice were stained for CD11c, MHC-II (IA/IE), CD8 and CD103. Percentages of cells in each gate (mean ± SEM, n=3) are indicated.

6.9 Intrinsic DC activation and lack of colonic CD103+ DC development

In order to assess whether the effects we observed in the DC-LMP1/CD40 mice are of intrinsic nature, we generated bone marrow (BM)-chimeras. Irradiated B6 mice, expressing the congenic marker Ly5.1, were reconstituted with 50% of Ly5.1+ B6 bone marrow and 50% of control (Ly5.2+) or DC-LMP1/CD40 (Ly5.2+) bone marrow.

After gating on the congenic markers in control and DC-LMP1/CD40 chimeras, we gated on DCs (CD11c+MHC-II+) and examined the activation markers CD70 and CD80. Only Ly5.2+ DCs, which were derived from BM of DC-LMP1/CD40 mice, showed up-regulation of these activation markers (Fig. 6-20, A), suggesting that this activation was intrinsic but not acting in “trans”. We next gated on T cells and found no difference between CD44 up-regulation of Ly5.1+ and Ly5.2+ T cells within the

same group of chimeras (Fig. 6-20, B), indicating that T cells derived from DC-LMP1/CD40 BM did not show an altered phenotype.

As we observed an increase of granulocytes in DC-LMP1/CD40 mice, we also stained them in the chimeras. In the chimeras composed of DC-LMP1/CD40 and B6 BM, both Ly5.1+ and Ly5.2+ granulocytes accumulated, showing that this effect is not specific for granulocytes derived from DC-LMP1/CD40 BM (Fig. 6-20, C). Granulocytes did not accumulate in the control chimeras (composed of control and B6 BM).

In the colon, only the CD103+ DCs derived from DC-LMP1/CD40 BM were lacking (Fig. 6-20, D), although also Ly5.1+ CD103+ DCs were reduced in comparison to the control chimeras, suggesting that the reduction might be a side effect of the pathology.

Taken together, this experiment showed that DC activation and the reduction of CD103 expression was intrinsically induced by LMP1/CD40 expression. Other effects including reduction of CD103 expression were side effects of the immune pathology because they were also observed in control cells within the DC-LMP1/CD40 chimeras.

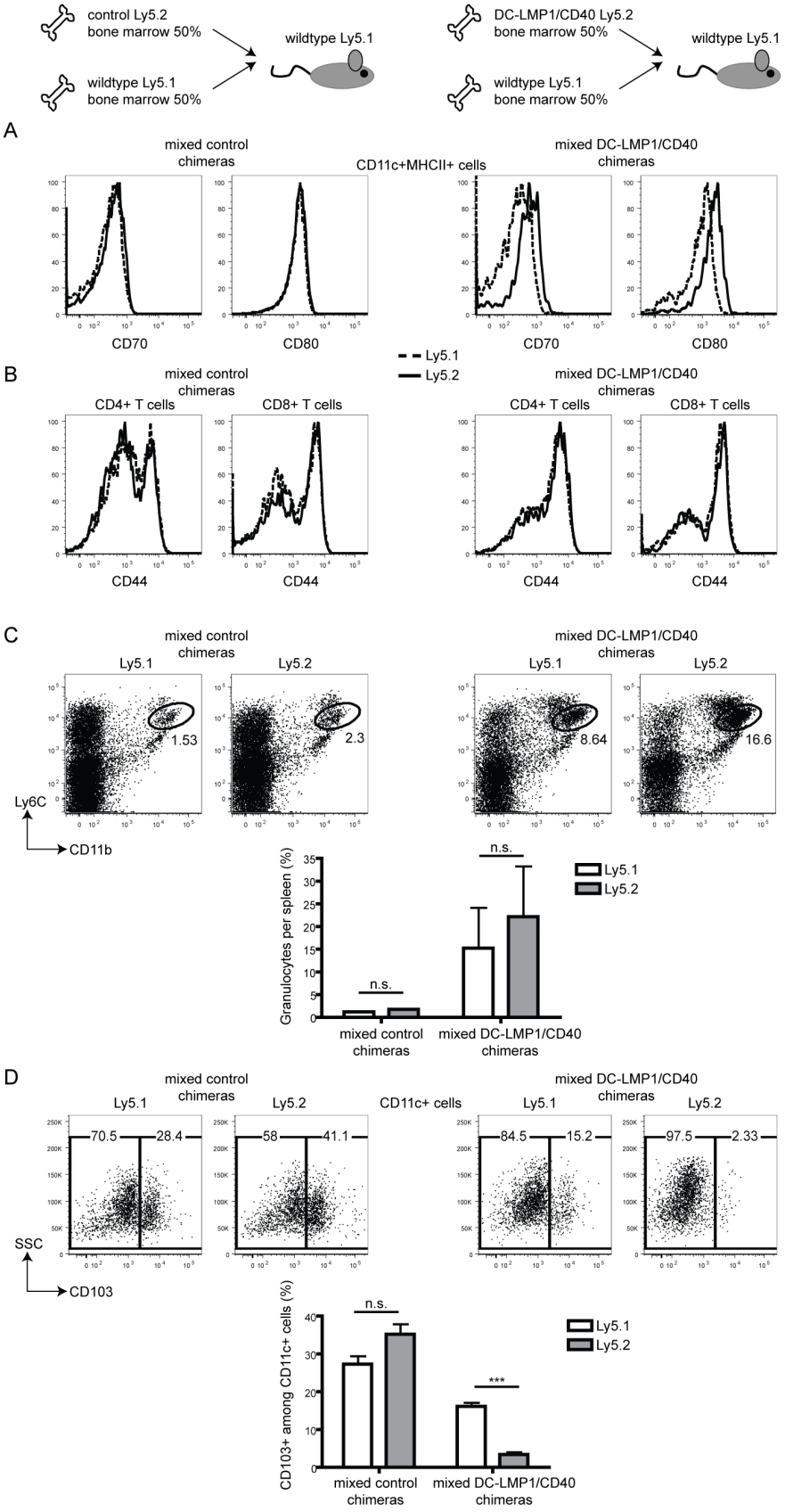

Figure 6-20: Intrinsic effects shown in mixed bone marrow chimeras.

Irradiated Ly5.1+ B6 mice were reconstituted with 50% Ly5.1+ B6 bone marrow and 50% control or DC-LMP1/CD40 (Ly5.2+) bone marrow. Single cell suspensions of spleens were stained for Ly5.1, Ly5.2 and (A) CD11c, MHC-II (IA/IE), CD70, CD80 or (B) CD3, CD4, CD8, CD44 or (C) Ly6C, CD11b. (D) Single cell suspensions of colons were stained for Ly5.1, Ly5.2, CD11c and CD103. The results are representative of two independent experiments, n=5.

6.10 Increased T_H17 cytokines in whole colon tissue

Immune pathologies with involvement of IL-23 are thought to be associated with the induction of IL-17 expression (Sarra et al., 2010). We therefore investigated whether IL-17 production was induced in the colon. Gene expression was determined in colon tissue homogenates of 3 week and 8-11 week old mice by qPCR.

T_H17 cells produce IL-17A, IL-17F, IL-21 and IL-22. All four cytokine mRNAs were more highly expressed in older DC-LMP1/CD40 mice than in controls, although only IL-17A and IL-21 mRNA were statistically significantly elevated (Fig. 6-21). In young mice in which pathology had not yet evolved, IL-17F was the only T_H17 cell cytokine for which a significant increase was detected. From these findings, we concluded that IL-17 expression evolves with pathology, probably due to increase in factors that promote IL-17 expression, such as IL-23, IL-6 and TGF-β.

We also monitored IL-4 and Foxp3 expression. Both are also expressed by CD4+ T cells and are associated with T_H2 and Treg cell induction, respectively. No difference was found in IL-4 expression, neither in young nor in older mice (Fig. 6-21), but Foxp3 was found to be up-regulated in colons of older DC-LMP1/CD40 mice compared to controls (Fig. 6-21). These results indicated that pathology was not associated with T_H2 induction and Foxp3 mRNA was induced during pathology progress. Treg cells have possibly infiltrated the tissue or were induced by factors within the inflamed colon.

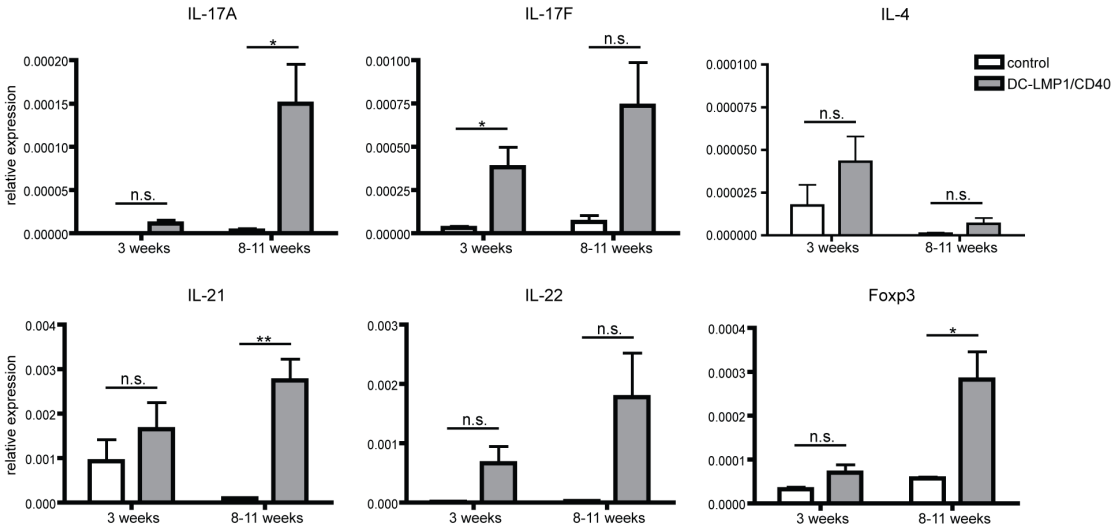

Figure 6-21: Increase of IL-17-A, IL-21 and Foxp3 mRNA in colons of DC-LMP1/CD40 mice.

Expression of cytokine mRNA in colon tissue homogenates. Data were normalized to Ubiquitin C, n=4-6.

6.11 The DC-LMP1/CD40 phenotype is T/B-cell dependent

It has been shown that Rag-deficient mice develop colitis upon anti-CD40 antibody injection (Uhlig et al., 2006). We wondered if DC-LMP1/CD40 mice would also develop pathology independently of T and B cells. We therefore crossed DC-LMP1/CD40 mice with Rag1-deficient mice and measured their weight once a week. The DC-LMP1/CD40-Rag$^{-/-}$ mice gained weight at the same level as control-Rag$^{-/-}$ mice did (Fig. 6-22), but did not gain as much weight as control mice, in a manner that is normal for Rag-deficient mice. Both groups looked healthy, appeared normal in size and did not develop wasting disease. From these results we concluded that the development of pathology we observe in these mice is T- and/or B-cell dependent.

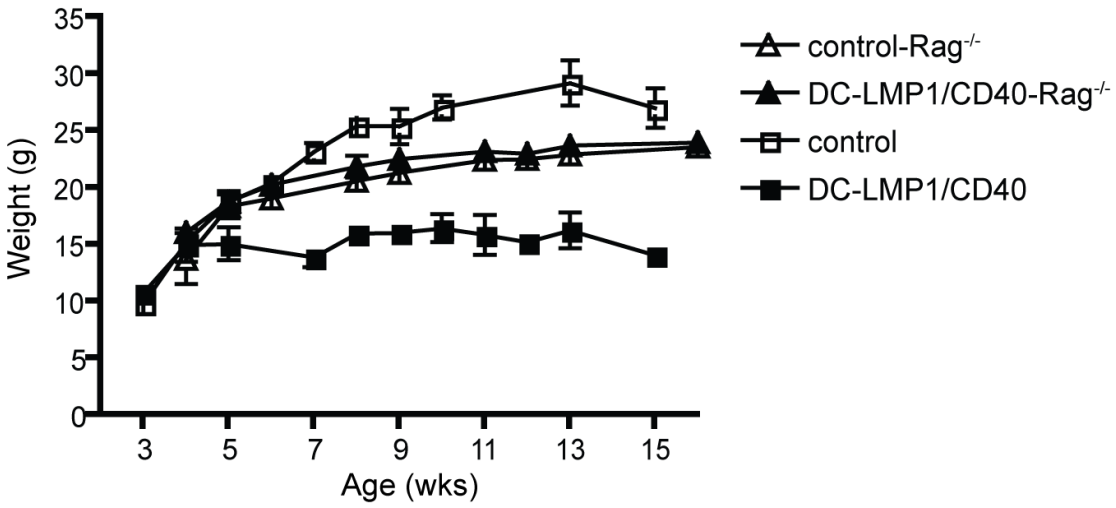

Figure 6-22: Weight loss is T/B-cell-dependent.

Body weight of control-Rag$^{-/-}$ (open triangles) and DC-LMP1/CD40-Rag$^{-/-}$ (filled triangles) in comparison to control (open squares) and DC-LMP1/CD40 (filled squares) mice between 3 and 16 weeks of age. n=2-6 per time point.

6.12 The DC-LMP1/CD40 phenotype is dependent on commensal bacteria

We found strong DC activation in DC-LMP1/CD40 mice in the colon. Therefore, we speculated that commensal bacteria might be an additional trigger for the CD40-activated DCs to induce up-regulation of activation markers and cytokine production. To investigate this, we added four different antibiotics to the mice's drinking water starting at the age of three weeks onwards in order to deplete commensal bacteria. As DC-LMP1/CD40 mice looked healthy and gained weight to the same extent as control mice, we stopped the treatment at week 10 to determine if the effect is reversible. Then they started to loose weight immediately (Fig. 6-23) but still looked healthy for approximately 10 weeks, after which they began to assume a hunched posture and scrubby fur and lost weight more rapidly, necessitating sacrifice at week 28. These experiments indicate that the development of pathology is strictly dependent on commensal bacteria.

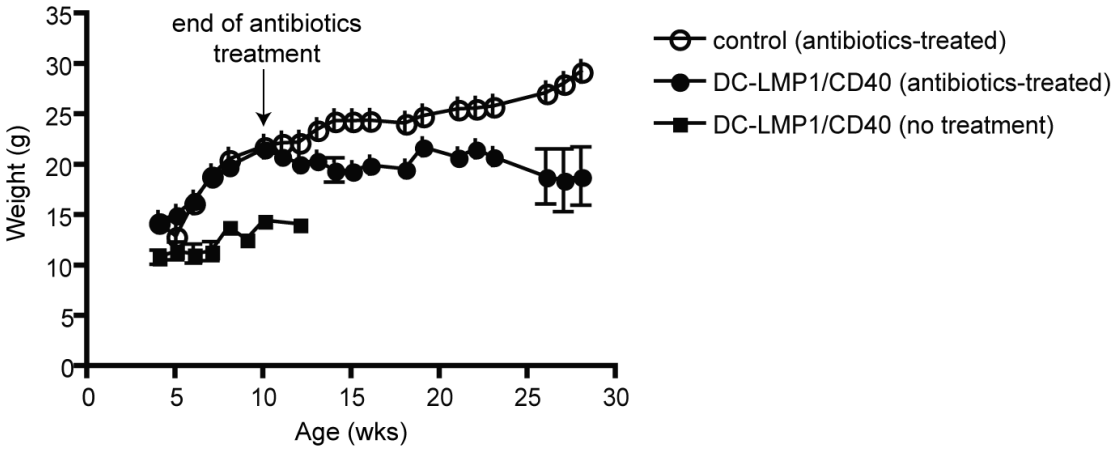

Figure 6-23: DC-LMP1/CD40 phenotype is dependent on commensal bacteria.

Mice were treated with antibiotics for commensal bacteria depletion from week 3 to 10. Body weight of antibiotics-treated control (open circles) and DC-LMP1/CD40 (filled circles) mice in comparison with untreated DC-LMP1/CD40 (filled squares) mice between 4 and 28 weeks of age. n=2-6 per time point.

6.13 BM-DC stimulation

We found an important role of commensal bacteria in the onset of pathology. These additional triggers derived from bacteria might be TLR signals. We therefore tested how sensitive LMP1/CD40-DCs respond to TLR stimulation. For this purpose we used Lipopolysaccharide (LPS), which binds to TLR4. We stimulated BM-DCs from control and DC-LMP1/CD40 mice with 0.4 ng/ml LPS for 3 h and stained for CD11c, MHC-II, CD70 and CD80 expression before and after stimulation. The ratio of stimulated and unstimulated cells was calculated by the division of the percentage of marker^{high} stimulated cells by the percentage of marker^{high} unstimulated cells.

We found that unstimulated BM-DCs of DC-LMP1/CD40 mice contained fewer MHC-II^{high}, fewer CD70^{high} and fewer CD80^{high} DCs than the BM-DCs of control mice (Fig. 6-24, upper part). After 3 h of LPS stimulation, BM-DCs of LMP1/CD40 mice did not express higher amounts of the activation markers than the control DCs (Fig. 6-24, middle part). However, when calculating the ratio of marker^{high} cells of stimulated cells and the marker^{high} cells of unstimulated cells, we detected a significantly greater increase in DC-LMP1/CD40 BM-DCs, suggesting that DCs were more sensitive to LPS stimulation despite their lower activation level (Fig. 6-24, lower part). From these results we conclude that constitutive CD40 signaling in DCs converts them into more sensitive DCs that respond to LPS more strongly.

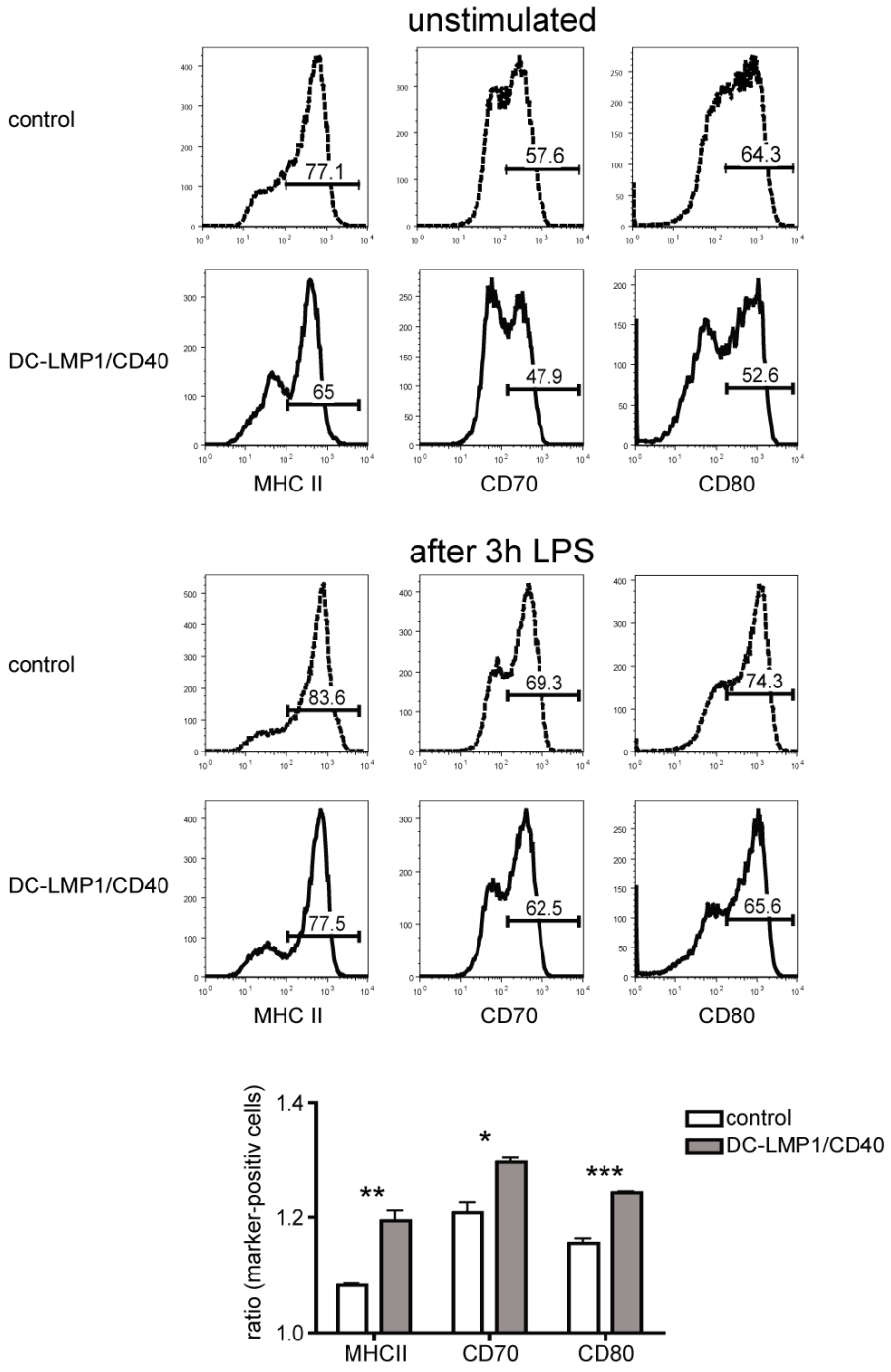

Figure 6-24: Higher sensitivity of DCs for LPS stimulation.

BM-DCs of control and DC-LMP1/CD40 mice were stimulated with 0.4 ng/ml LPS for 3 h and stained for CD11c, MHC-II, CD70 and CD80 expression (A) before and (B) after stimulation. (C) The ratio was calculated by the division of marker^{high} stimulated cells (%) by marker^{high} unstimulated cells (%). The results are representative of one experiment, n=3.

In summary, CD40-activated DCs do not fully mature, confirming previous data (Schulz et al., 2000), and instead achieve an activation level at which they become

very sensitive to additional triggers, like such that are present in the intestine. They are no longer able to induce tolerance locally, which is normally a very important feature of DCs in the gut. However, CD40-activated DCs in the spleen did not produce pro-inflammatory cytokines and did not induce T cell proliferation and effector function.

7 Discussion

The tolerogenic phenotype of DCs remains a topic of debate; in order to influence the maturation state of DCs we expressed a constitutively active CD40 molecule under the CD11c promoter. These DC-LMP1/CD40 mice developed colitis, wasting disease, splenomegaly, increased levels of serum pro-inflammatory cytokines and increased levels of serum IgA and IgM. Fully matured DCs were detected in the intestines but not in the spleens of these mice, suggesting that additional triggers in the gut may lead to complete maturation of the DCs and be the cause of intestinal pathology. However, tolerance was induced in transfer experiments where OT-I T cells were administered systemically. We therefore only found a local activation of DCs accompanied by loss of tolerance.

7.1 Generation of DC-LMP1/CD40 mice

Experiments studying CD40 stimulation of DCs *in vivo* are typically conducted using an agonistic anti-CD40 antibody. However, as already mentioned, the problem with this approach is that also B cells and macrophages are also triggered by this stimulation and that a necroinflammatory liver disease can be induced (Kimura et al., 2006). *In vitro* studies with CD40 ligation on DCs are also problematic because of reasons already discussed in the introduction (see section 3.3.1). In addition, it has been shown that DCs show different activation marker kinetics whether anti-CD40 antibody is given *in vivo* or *in vitro* (Frleta et al., 2003). Our DC-LMP1/CD40 transgenic mouse expresses LMP1/CD40 under the CD11c promoter and thus transmits the chronic CD40 signal primarily to DCs. This DC-restriction is likely to not be complete due to the behaviour of the CD11c promoter; when CD11c-Cre mice were crossed to a EYFP-reporter, EYFP expression was observed in > 95% of splenic DCs, compared with < 10% of lymphocytes and < 1% of myeloid cells such as granulocytes (Caton et al., 2007). Thus it is possible that there is a minor background expression of LMP1/CD40 on other cell types than DCs in our mice, which must be considered. In mixed BM-chimeras we did not detect differences in activation of T cells or in proliferation of granulocytes, suggesting that possible unspecific expression

of LMP1/CD40 expression in cell types other than DCs did not play a role in our system.

By RT-PCR and Western analysis we showed that the STOP-cassette was successfully deleted in BM-DCs of DC-LMP1/CD40 mice and RNA and protein of LMP1/CD40 was detected, respectively. In DC-LMP1/CD40^{flSTOP} mice in which the STOP-cassette is still present, we did not see any transcripts or protein of LMP1/CD40, demonstrating that transcription does only take place when Cre recombinase is expressed.

7.2 Impaired T cell activation, expansion and effector function in the periphery

We did not detect a significant up-regulation of costimulatory molecules on DCs in the spleen and the skin-draining lymph nodes of DC-LMP1/CD40 mice; only CD80 was increased to a small extent. It has been previously observed that *in vivo* CD40 stimulation of DCs via anti-CD40 antibody resulted in a 2-3-fold increase in CD80 and CD86 expression, but this was not sustained over 5 days (Frleta et al., 2003). In fact, we even observed down-regulation of MHC-II in the DC-LMP1/CD40 mice, which corresponds to similar observations upon application of an anti-CD40 antibody *in vivo* (Frleta et al., 2003). Frleta et al. suggested that the down-regulation of MHC-II may be a means to select for high affinity CD4+ T cells by limiting the pool of MHC-II complexes, as this phenomenon has previously been reported for CTLs and MHC-I complexes (Kedl et al., 2002).

When we transferred OT-I T cells into DC-LMP1/CD40 mice, the CD40 stimulus via LMP1/CD40 on DCs and the resulting DC activation was not sufficient to induce substantial proliferation and effector functions of these cells. In the positive control group of wildtype mice injected with an agonistic anti-CD40 antibody, OT-I T cells expanded to a greater extent and developed effector functions. However, the use of the anti-CD40 antibody activates B cells and macrophages as well as DCs, and therefore a combination of secreted factors by different cell types may be the reason for the strong T cell proliferation and killing capacity.

Endogenous T cells in the spleen and mesenteric lymph nodes of DC-LMP1/CD40 mice did not show significant activation in our mice, indicating that DCs in the

periphery were not fully matured. Only CD8+ T cells in the mesenteric lymph node secreted higher amounts of IFN-γ. In the mesenteric lymph nodes, predominantly CD103- DCs, derived from the lamina propria, induce IFN-γ production by CD8+ T cells, which suggests that activated DCs had reached the mesenteric lymph nodes in the DC-LMP1/CD40 mice.

7.3 Inflammatory cytokines in DC-LMP1/CD40 mice

In the serum of DC-LMP1/CD40 mice, we found a significant increase of IFN-γ, TNF-α and IL-6, indicating inflammation. However, when we measured whole colon and whole splenic tissue cytokines, we found elevated levels of serum cytokines and MCP-1 only in the colon. MCP-1 is mainly secreted by monocytes, macrophages and dendritic cells and attracts memory T cells, monocytes and dendritic cells to sites of inflammation. These findings, together with the microscopic observations of the intestinal infiltrations, indicated that a local inflammation in the colon of DC-LMP1/CD40 mice was induced.

In the colon, but not in the spleen, we demonstrated that DCs expressed high amounts of the pro-inflammatory cytokines IL-23, IL-12 and IL-1β. IL-6 was also elevated. We also showed an increase of T_H17 cytokines in whole colon tissue, which could be the result of T_H17-inducing cytokines, such as TGF-β, IL-6 and IL-23, secreted by colonic DCs. We cannot exclude that IL-17 is also produced by cell types other than T_H17 cells, because it has been shown that there can be also T-cell independent IL-17 production in the gut (Uhlig et al., 2006). In support of this, it has been demonstrated that CD40 plays a pivotal role in T_H17 induction, and that it acts to trigger IL-6 release from DC in the context of bacterial infection (Perona-Wright et al., 2009). It has also been shown that T_H17 cells are associated with autoimmune disorders (Hemdan et al., 2010) and promote granulopoiesis (Smith et al., 2007).

In fact, we detected massive infiltrations of granulocytes in the colon of DC-LMP1/CD40 mice and an increase of granulocytes in the blood over time. We also observed an expanded granulocyte population in the spleen.

However, not only inflammatory, but also immunosuppressive cytokines were expressed in DC-LMP1/CD40 mice. IDO exerts an immunosuppressive role. The expression of IDO by DCs was shown to be induced by IFN-γ and CD40 ligation (Hwu et al., 2000). In DC-LMP1/CD40 mice, IFN-γ was present in high amounts in the colon and IDO expression was detected in DCs. Presumably, production of IDO by activated DCs contributes to a mechanism to self-limit immune responses (Hwu et al., 2000). A similar regulatory role might be possible for IL-10. It has been shown that IL-10 is secreted by DCs upon microbial stimuli and CD40 (Edwards et al., 2002). In the context of DC-LMP1/CD40 mice IL-10 was induced in colonic DCs, suggesting that CD40 stimulated colonic DCs tried to self-limit immune responses.

7.4 Role of CD103+ DCs in the gut

In the steady state, colonic DCs constantly migrate to the mesenteric LN (mLN), where they present antigens, such as those sampled from commensal bacteria (Macpherson and Uhr, 2004) and apoptotic epithelial cells (Huang et al., 2000), to T cells for tolerance induction. The CD103+ DCs are mainly migrating to the mLN and play an important role for induction of tolerance. In mice in which constitutive DC trafficking to the mLN is impaired because of CCR7 deficiency, induction of tolerance to oral antigens is defective (Worbs et al., 2006). Intestinal CD103+ DCs can induce expression of the chemokine receptor CCR9 and $\alpha_4\beta_7$ integrin, both known as gut-homing receptors. CD103+ DCs also contribute to control inflammatory responses and intestinal homeostasis by inducing Foxp3+ Treg cells (del Rio et al., 2010; Ng et al., 2010). As CD103+ DCs are strongly reduced in the colon of DC-LMP1/CD40 mice, it is possible that this deficiency may contribute to loss of tolerance. However, it has been shown that, upon CD40 ligation, CD103 is down-regulated on DCs (Annacker, 2005) and we do not know whether DCs with characteristics of CD103+ DCs may still reside within the CD103- population. The down-regulation of CD103 by CD40 ligation might by a mechanism for CD40L+ T cells to overcome suppression by Treg cells. Nevertheless, we detected Foxp3 expression in whole colon homogenates of 3 and 8-11 week old mice. In support of this, Treg cells are found in normal numbers in Batf3$^{-/-}$ mice that lack intestinal

CD103+CD11b- DCs (Edelson et al., 2010). The results suggested that CD103 expression by DCs was dispensable for the presence of Foxp3 expressing cells.

In mixed bone marrow chimeras we showed a reduction of colonic CD103+ DCs derived from DC-LMP1/CD40 BM in comparison to wildtype DCs present in the same mouse, allowing us to assume that the reduction of CD103+ DCs in the transgenic mice is an intrinsic effect that is not mediated *in trans*. To our surprise, the CD103+ wildtype DCs within the DC-LMP1/CD40 chimeras were reduced in comparison to the CD103+ DC in the control chimeras, possibly due to the onset of disease. It is possible that activated CD40L+ T cells in the gut may interact with the CD103+ wildtype DCs and induce CD103 down-regulation, as speculated above. However, further experiments are needed to clarify the mechanism by which CD103 was down-regulated in DC-LMP1/CD40 mice.

7.5 Constitutive CD40 signaling in DCs does not induce immune pathology without microbial stimulation

In view of the fact that we detected local inflammation in the gut, we investigated the outcome of depleting commensal bacteria in DC-LMP1/CD40 mice by antibiotic treatment in the drinking water. Although we did not test the depletion efficacy, the protocol we used for commensal-depletion has been employed previously (Rakoff-Nahoum et al., 2004; Xiao et al., 2007) and was shown to reduce commensal bacteria to a non-detectable level (Rakoff-Nahoum et al., 2004). Unlike their non-treated transgenic littermates, the antibiotic-treated DC-LMP1/CD40-mice were healthy and did not loose weight until the end of treatment. This finding suggests that gut DCs of DC-LMP1/CD40 mice with intact gut flora are no longer able to maintain tolerance due to bacterial recognition. In wildtype mice, intestinal DCs engulf commensal bacteria and present them to T cells in a tolerance-inducing manner (Iwasaki, 2007; Rescigno et al., 2001). The CD40 signal may convert DCs into a semi-mature state in which they cannot tolerate additional triggers and start to produce pro-inflammatory cytokines. These data confirm studies showing that DCs can only fully mature when a microbial trigger is added to CD40 ligation (Schulz et al., 2000).

7.6 Development of disease is T and B cell dependent

In order to investigate if the onset of disease in DC-LMP1/CD40 depends on T and B cells, we crossed the mice to $Rag^{-/-}$ mice, which lack mature T and B cells. Interestingly, as already mentioned, it has been shown that injection of an agonistic CD40 mAb to T and B cell deficient mice was sufficient to induce a pathogenic innate inflammatory response systemically and in the intestine that was functionally dependent on TNF-α, IFN-γ, IL-12p40 and IL-23p40 (Uhlig et al., 2006). Considering these results, we expected the DC-LMP1/CD40-$Rag^{-/-}$ mice to develop colitis. Surprisingly, they developed normally and were disease free. The difference between the two systems is that, upon anti-CD40 antibody injection into $Rag^{-/-}$ mice, the antibody also binds other cells than DCs, such as monocytes, activated macrophages, endothelial and epithelial cells (Diehl et al., 2000). In the DC-LMP1/CD40 mice, only the DCs were activated via CD40. In the case of the antibody study, non-DC cell types may produce inflammatory cytokines that lead to inflammation. In contrast, in DC-LMP1/CD40 mice activated DCs can act only via T cells for disease development, which in turn secrete inflammatory mediators like IFN-γ and IL-17.

7.7 Increased sensitivity for microbial molecular patterns of CD40 stimulated DCs

In order to test how DCs of DC-LMP1/CD40 mice up-regulate costimulatory molecules in response to a TLR ligand, we stimulated BM-DCs with LPS. Intestinal DCs typically encounter TLR stimuli as they sample bacteria through the intestinal epithelium. We found that LMP1/CD40-DCs up-regulated MHC-II, CD70 and CD80 stronger than control DCs in response to LPS, indicating that LMP1/CD40-DCs exhibited a lower maturation threshold. This higher increase of costimulatory molecules might be an indicator of an enhanced sensitivity to TLR triggering that may explain why DCs in the gut of DC-LMP1/CD40 are not able to stay immature or semi-mature but rather become fully matured, immunogenic DCs.

7.8 Conclusion

In this study we wanted to address the question of how important regulation of DC activation is and whether other maturation states, in addition to immature and mature DCs, exist which can have an impact on tolerance induction. For this purpose we generated transgenic mice with a DC-specific constitutive CD40 signal.

From our results, we conclude that CD40-triggering of DCs *in vivo*, in absence of additional activators such as bacterial TLR-ligands derived from the gut, do not secrete critical levels of pro-inflammatory cytokines and are not immunogenic. However, LMP1/CD40-DCs in the gut secrete high amounts of IL-23, IL-6, IL-12 and IL-1β, inducing expression of IFN-γ and IL-17, probably due to T_H17 and T_H1 activation. In this cytokine milieu, granulocytes infiltrate the colon causing tissue damage that results in colitis (Fig. 7-1).

We showed that the constitutive CD40 signal lowers the activation threshold of DCs, which does not result in any harmful impact on most organs. Only additional stimuli at borders of the body, such as the gut and the skin, strongly activate DCs, inducing DC migration and triggering inflammatory autoreactive immune responses. In the skin we did not find any pathological consequences such as psoriasis. The behaviour of these DCs in the lung, which represents another body border, remains to be investigated.

Thus, we show for the first time that CD40 crosslinking is an important mediator for the amplification of other DC activation signals, such as microbial stimuli *in vivo*. As a consequence, otherwise tolerating DCs become immunogenic, causing autoimmunity. These findings demonstrate that DC maturation must be tightly regulated in order to avoid autoimmune disorders.

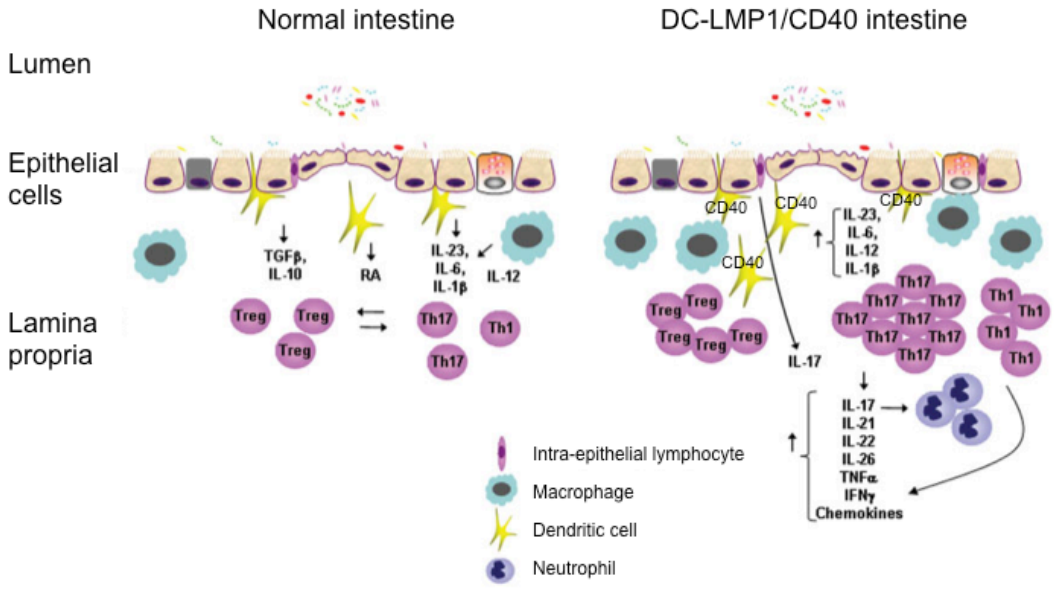

Figure 7-1: Dysregulation of intestinal immune homeostasis in DC-LMP1/CD40 mice.

During homeostasis the intestine holds a balance between pro-inflammatory cytokines from innate (e.g., IL1β, IL-6, IL-12, IL-23) and effector T cell populations (e.g., T_H1 and T_H17 cells), relative to regulatory T cell populations that are influenced by factors such as IL-10, TGF-β and retinoic acid (RA) in the environment. In DC-LMP1/CD40 mice there is dysregulation of this balance, with an increased proportion of granulocytes and IL-17-producting cells. Modified from (Abraham and Cho, 2009).

7.9 Outlook

Dendritic cells play a crucial role in priming adaptive immune responses and in the induction of self-tolerance. The general understanding is that immature DCs induce tolerance and mature DCs induce immunity. Furthermore, it has been suggested that there might be also mature DCs with tolerogenic potential (Reis e Sousa, 2006). Our study aimed to further the understanding of DC maturation and tolerance induction.

The presented work highlights the importance of tight regulation of DC maturation. CD40-activated colonic DCs have a lower activation threshold and are therefore more sensitive to TLR stimulation. Further work is needed to identify the precise mechanism by which CD40-stimulated colonic DC induce pathology and down-regulate CD103. In addition, the induction of Treg cells in the gut is of great interest, as it has been claimed that Treg cell induction is dependent on CD103+ DCs (Siddiqui and Powrie, 2008), and CD103 expression is lacking in the colon of DC-LMP1/CD40 mice, although Foxp3 expression is still detected.

These findings may contribute to a better understanding of DC involvement in tolerance induction and therefore translate into more precise therapeutic control over autoimmune diseases. Furthermore, DC vaccination is a promising immunological approach against tumors, although only limited success has so far been achieved with this methodology. An enhanced understanding of DC maturation mechanisms is crucial for improving such potential therapies.

8 References

Abraham, C., and Cho, J. (2009). Interleukin-23/Th17 pathways and inflammatory bowel disease. Inflammatory Bowel Diseases *15*, 1090-1100.

Ahern, P.P., Izcue, A., Maloy, K.J., and Powrie, F. (2008). The interleukin-23 axis in intestinal inflammation. Immunological Reviews *226*, 147-159.

Ahonen, C.L., Doxsee, C.L., McGurran, S.M., Riter, T.R., Wade, W.F., Barth, R.J., Vasilakos, J.P., Noelle, R.J., and Kedl, R.M. (2004). Combined TLR and CD40 triggering induces potent CD8+ T cell expansion with variable dependence on type I IFN. Journal of Experimental Medicine *199*, 775-784.

Albert, M.L., Jegathesan, M., and Darnell, R.B. (2001). Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. Nature Immunology *2*, 1010-1017.

Aliberti, J., Schulz, O., Pennington, D.J., Tsujimura, H., Reis e Sousa, C., Ozato, K., and Sher, A. (2003). Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells. Blood *101*, 305-310.

Annacker, O. (2005). Essential role for CD103 in the T cell-mediated regulation of experimental colitis. Journal of Experimental Medicine *202*, 1051-1061.

Berberich, I., Shu, G.L., and Clark, E.A. (1994). Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. Journal of Immunology *153*, 4357-4366.

Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., Liu, K., Jakubzick, C., Ingersoll, M.A., Leboeuf, M.*, et al.* (2009). Origin of the lamina propria dendritic cell network. Immunity *31*, 513-525.

Bonasio, R., Scimone, M.L., Schaerli, P., Grabie, N., Lichtman, A.H., and von Andrian, U.H. (2006). Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. Nature Immunology *7*, 1092-1100.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M.C., and Steinman, R.M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. Journal of Experimental Medicine *196*, 1627-1638.

Brocker, T., Riedinger, M., and Karjalainen, K. (1997). Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic

cells can induce negative but not positive selection of thymocytes in vivo. Journal of Experimental Medicine *185*, 541-550.

Busch, L.K., and Bishop, G.A. (1999). The EBV transforming protein, latent membrane protein 1, mimics and cooperates with CD40 signaling in B lymphocytes. Journal of Immunology *162*, 2555-2561.

Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. Journal of Experimental Medicine *204*, 1653-1664.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., and Banchereau, J. (1994). Activation of human dendritic cells through CD40 cross-linking. Journal of Experimental Medicine *180*, 1263-1272.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. Journal of Experimental Medicine *184*, 747-752.

Cisse, B., Caton, M.L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., den Hollander, N.S., Kant, S.G.*, et al.* (2008). Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. Cell *135*, 37-48.

Clark, E.A., and Ledbetter, J.A. (1994). How B and T cells talk to each other. Nature *367*, 425-428.

Coombes, J.L., and Powrie, F. (2008). Dendritic cells in intestinal immune regulation. Nature Reviews Immunology *8*, 435-446.

Coombes, J.L., Siddiqui, K.R.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF- and retinoic acid dependent mechanism. Journal of Experimental Medicine *204*, 1757-1764.

Coope, H.J., Atkinson, P.G., Huhse, B., Belich, M., Janzen, J., Holman, M.J., Klaus, G.G., Johnston, L.H., and Ley, S.C. (2002). CD40 regulates the processing of NF-kappaB2 p100 to p52. EMBO Journal *21*, 5375-5385.

del Rio, M.L., Bernhardt, G., Rodriguez-Barbosa, J.I., and Forster, R. (2010). Development and functional specialization of CD103+ dendritic cells. Immunological Reviews *234*, 268-281.

den Haan, J.M., Lehar, S.M., and Bevan, M.J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. Journal of Experimental Medicine *192*, 1685-1696.

Diehl, L., Den Boer, A.T., van der Voort, E.I.H., Melief, C.J.M., Offringa, R., and Toes, R.E.M. (2000). The role of CD40 in peripheral T cell tolerance and immunity. Journal of Molecular Medicine *78*, 363-371.

Dudziak, D., Kamphorst, A.O., Heidkamp, G.F., Buchholz, V.R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H.W., Park, C.G.*, et al.* (2007). Differential Antigen Processing by Dendritic Cell Subsets in Vivo. Science *315*, 107-111.

Edelson, B.T., Kc, W., Juang, R., Kohyama, M., Benoit, L.A., Klekotka, P.A., Moon, C., Albring, J.C., Ise, W., Michael, D.G.*, et al.* (2010). Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8 + conventional dendritic cells. Journal of Experimental Medicine *207*, 823-836.

Edwards, A.D., Manickasingham, S.P., Sporri, R., Diebold, S.S., Schulz, O., Sher, A., Kaisho, T., Akira, S., and Reis e Sousa, C. (2002). Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. Journal of Immunology *169*, 3652-3660.

Fleige, S., Walf, V., Huch, S., Prgomet, C., Sehm, J., and Pfaffl, M.W. (2006). Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. Biotechnology Letters *28*, 1601-1613.

Freudenthal, P.S., and Steinman, R.M. (1990). The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proceedings of the National Academy of Sciences of the United States of America *87*, 7698-7702.

Frleta, D., Lin, J.T., Quezada, S.A., Wade, T.K., Barth, R.J., Noelle, R.J., and Wade, W.F. (2003). Distinctive maturation of in vitro versus in vivo anti-CD40 mAb-matured dendritic cells in mice. Journal of Immunotherapy *26*, 72-84.

Ginhoux, F., Liu, K., Helft, J., Bogunovic, M., Greter, M., Hashimoto, D., Price, J., Yin, N., Bromberg, J., Lira, S.A.*, et al.* (2009). The origin and development of nonlymphoid tissue CD103+ DCs. Journal of Experimental Medicine *206*, 3115-3130.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X.M., Stanley, E.R., Randolph, G.J., and Merad, M. (2006). Langerhans cells arise from monocytes in vivo. Nature Immunology *7*, 265-273.

Gires, O., Zimber-Strobl, U., Gonnella, R., Ueffing, M., Marschall, G., Zeidler, R., Pich, D., and Hammerschmidt, W. (1997). Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. EMBO Journal *16*, 6131-6140.

Graham, J.P., Arcipowski, K.M., and Bishop, G.A. (2010). Differential B-lymphocyte regulation by CD40 and its viral mimic, latent membrane protein 1. Immunological Reviews *237*, 226-248.

Gupta, S., Termini, J.M., Niu, L., Kanagavelu, S.K., Schmidtmayerova, H., Snarsky, V., Kornbluth, R.S., and Stone, G.W. (2011). EBV LMP1, a viral mimic of CD40, activates dendritic cells and functions as a molecular adjuvant when incorporated into an HIV vaccine. Journal of Leukocyte Biology *90*, 389-398.

Hacker, C., Kirsch, R.D., Ju, X.S., Hieronymus, T., Gust, T.C., Kuhl, C., Jorgas, T., Kurz, S.M., Rose-John, S., Yokota, Y.*, et al.* (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. Nature Immunology *4*, 380-386.

Harnett, M.M. (2004). CD40: a growing cytoplasmic tale. Science's STKE *2004*, pe25.

Hashimoto, D., Miller, J., and Merad, M. (2011). Dendritic cell and macrophage heterogeneity in vivo. Immunity *35*, 323-335.

Hatzivassiliou, E., Miller, W.E., Raab-Traub, N., Kieff, E., and Mosialos, G. (1998). A fusion of the EBV latent membrane protein-1 (LMP1) transmembrane domains to the CD40 cytoplasmic domain is similar to LMP1 in constitutive activation of epidermal growth factor receptor expression, nuclear factor-kappa B, and stress-activated protein kinase. Journal of Immunology *160*, 1116-1121.

Hatzivassiliou, E.G., Kieff, E., and Mosialos, G. (2007). Constitutive CD40 signaling phenocopies the transforming function of the Epstein-Barr virus oncoprotein LMP1 in vitro. Leukemia Research *31*, 315-320.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M., and Nussenzweig, M.C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. Journal of Experimental Medicine *194*, 769-779.

Helft, J., Ginhoux, F., Bogunovic, M., and Merad, M. (2010). Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice. Immunological Reviews *234*, 55-75.

Hemdan, N.Y., Birkenmeier, G., Wichmann, G., Abu El-Saad, A.M., Krieger, T., Conrad, K., and Sack, U. (2010). Interleukin-17-producing T helper cells in autoimmunity. Autoimmunity Reviews *9*, 785-792.

Hermanns, W., Liebig, K., and Schulz, L.C. (1981). Postembedding immunohistochemical demonstration of antigen in experimental polyarthritis using plastic embedded whole joints. Histochemistry *73*, 439-446.

Hildner, K., Edelson, B.T., Purtha, W.E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B.U., Unanue, E.R., Diamond, M.S.*, et al.* (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science *322*, 1097-1100.

Hill, N., and Sarvetnick, N. (2002). Cytokines: promoters and dampeners of autoimmunity. Current Opinion in Immunology *14*, 791-797.

Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17-27.

Hömig, C. (2005). Einfluss der Epstein-Barr-Virus Proteine LMP1 und EBNA2 auf die B-Zellentwicklung in vivo. Dissertation, Fakultät für Biologie (München, Ludwig-Maximilians-Universität).

Homig-Holzel, C., Hojer, C., Rastelli, J., Casola, S., Strobl, L.J., Muller, W., Quintanilla-Martinez, L., Gewies, A., Ruland, J., Rajewsky, K.*, et al.* (2008). Constitutive CD40 signaling in B cells selectively activates the noncanonical NF- B pathway and promotes lymphomagenesis. Journal of Experimental Medicine *205*, 1317-1329.

Huang, F.P., Platt, N., Wykes, M., Major, J.R., Powell, T.J., Jenkins, C.D., and MacPherson, G.G. (2000). A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. Journal of Experimental Medicine *191*, 435-444.

Hwu, P., Du, M.X., Lapointe, R., Do, M., Taylor, M.W., and Young, H.A. (2000). Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. Journal of Immunology *164*, 3596-3599.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. Journal of Experimental Medicine *176*, 1693-1702.

Iwasaki, A. (2007). Mucosal dendritic cells. Annual Review of Immunology *25*, 381-418.

Joffre, O., Nolte, M.A., Sporri, R., and Reis e Sousa, C. (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunological Reviews *227*, 234-247.

Kapsenberg, M.L. (2003). Dendritic-cell control of pathogen-driven T-cell polarization. Nature Reviews Immunology *3*, 984-993.

Kedl, R.M., Schaefer, B.C., Kappler, J.W., and Marrack, P. (2002). T cells down-modulate peptide-MHC complexes on APCs in vivo. Nature Immunology *3*, 27-32.

Kiener, P.A., Moran-Davis, P., Rankin, B.M., Wahl, A.F., Aruffo, A., and Hollenbaugh, D. (1995). Stimulation of CD40 with purified soluble gp39 induces

proinflammatory responses in human monocytes. Journal of Immunology *155*, 4917-4925.

Kilger, E., Kieser, A., Baumann, M., and Hammerschmidt, W. (1998). Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. EMBO Journal *17*, 1700-1709.

Kimura, K., Moriwaki, H., Nagaki, M., Saio, M., Nakamoto, Y., Naito, M., Kuwata, K., and Chisari, F.V. (2006). Pathogenic Role of B Cells in Anti-CD40-Induced Necroinflammatory Liver Disease. American Journal of Pathology *168*, 786-795.

Kleindienst, P., Wiethe, C., Lutz, M.B., and Brocker, T. (2005). Simultaneous induction of CD4 T cell tolerance and CD8 T cell immunity by semimature dendritic cells. Journal of Immunology *174*, 3941-3947.

Lam, N., and Sugden, B. (2003). CD40 and its viral mimic, LMP1: similar means to different ends. Cell Signal *15*, 9-16.

Leenen, P.J., Radosevic, K., Voerman, J.S., Salomon, B., van Rooijen, N., Klatzmann, D., and van Ewijk, W. (1998). Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. Journal of Immunology *160*, 2166-2173.

Liu, K., Iyoda, T., Saternus, M., Kimura, Y., Inaba, K., and Steinman, R.M. (2002). Immune tolerance after delivery of dying cells to dendritic cells in situ. Journal of Experimental Medicine *196*, 1091-1097.

Liu, K., and Nussenzweig, M.C. (2010). Origin and development of dendritic cells. Immunological Reviews *234*, 45-54.

Luckashenak, N., Schroeder, S., Endt, K., Schmidt, D., Mahnke, K., Bachmann, M.F., Marconi, P., Deeg, C.A., and Brocker, T. (2008). Constitutive crosspresentation of tissue antigens by dendritic cells controls CD8+ T cell tolerance in vivo. Immunity *28*, 521-532.

Lutz, M.B., and Schuler, G. (2002). Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends in Immunology *23*, 445-449.

Macpherson, A.J., and Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science *303*, 1662-1665.

Menges, M., Rossner, S., Voigtlander, C., Schindler, H., Kukutsch, N.A., Bogdan, C., Erb, K., Schuler, G., and Lutz, M.B. (2002). Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. Journal of Experimental Medicine *195*, 15-21.

Merad, M., Ginhoux, F., and Collin, M. (2008). Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. Nature Reviews Immunology *8*, 935-947.

Merad, M., and Manz, M.G. (2009). Dendritic cell homeostasis. Blood *113*, 3418-3427.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annual Review of Immunology *19*, 683-765.

Naik, S.H. (2008). Demystifying the development of dendritic cell subtypes, a little. Immunology and Cell Biology *86*, 439-452.

Nathan, C. (2006). Neutrophils and immunity: challenges and opportunities. Nature Reviews Immunology *6*, 173-182.

Ng, S.C., Kamm, M.A., Stagg, A.J., and Knight, S.C. (2010). Intestinal dendritic cells: their role in bacterial recognition, lymphocyte homing, and intestinal inflammation. Inflammatory Bowel Diseases *16*, 1787-1807.

O'Sullivan, B., and Thomas, R. (2003). CD40 and dendritic cell function. Critical Reviews in Immunology *23*, 83-107.

Ohnmacht, C., Pullner, A., King, S.B.S., Drexler, I., Meier, S., Brocker, T., and Voehringer, D. (2009). Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. Journal of Experimental Medicine *206*, 549-559.

Perez, V.L., Van Parijs, L., Biuckians, A., Zheng, X.X., Strom, T.B., and Abbas, A.K. (1997). Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity *6*, 411-417.

Perona-Wright, G., Jenkins, S.J., O'Connor, R.A., Zienkiewicz, D., McSorley, H.J., Maizels, R.M., Anderton, S.M., and MacDonald, A.S. (2009). A Pivotal Role for CD40-Mediated IL-6 Production by Dendritic Cells during IL-17 Induction In Vivo. Journal of Immunology *182*, 2808-2815.

Pinchuk, L.M., Polacino, P.S., Agy, M.B., Klaus, S.J., and Clark, E.A. (1994). The role of CD40 and CD80 accessory cell molecules in dendritic cell-dependent HIV-1 infection. Immunity *1*, 317-325.

Probst, H.C., McCoy, K., Okazaki, T., Honjo, T., and van den Broek, M. (2005). Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nature Immunology *6*, 280-286.

Proietto, A.I., van Dommelen, S., Zhou, P., Rizzitelli, A., D'Amico, A., Steptoe, R.J., Naik, S.H., Lahoud, M.H., Liu, Y., Zheng, P.*, et al.* (2008). Dendritic cells in the thymus contribute to T-regulatory cell induction. Proceedings of the National Academy of Sciences of the United States of America *105*, 19869-19874.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell *118*, 229-241.

Rastelli, J., Homig-Holzel, C., Seagal, J., Muller, W., Hermann, A.C., Rajewsky, K., and Zimber-Strobl, U. (2008). LMP1 signaling can replace CD40 signaling in B cells in vivo and has unique features of inducing class-switch recombination to IgG1. Blood *111*, 1448-1455.

Reis e Sousa, C. (2006). Dendritic cells in a mature age. Nature Reviews Immunology *6*, 476-483.

Reizis, B., Colonna, M., Trinchieri, G., Barrat, F., and Gilliet, M. (2011). Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? Nature Reviews Immunology *11*, 558-565.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., and Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nature Immunology *2*, 361-367.

Romani, N., Lenz, A., Glassel, H., Stossel, H., Stanzl, U., Majdic, O., Fritsch, P., and Schuler, G. (1989). Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. Journal of Investigative Dermatology *93*, 600-609.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. Journal of Experimental Medicine *179*, 1109-1118.

Sanchez, P.J., McWilliams, J.A., Haluszczak, C., Yagita, H., and Kedl, R.M. (2007). Combined TLR/CD40 stimulation mediates potent cellular immunity by regulating dendritic cell expression of CD70 in vivo. Journal of Immunology *178*, 1564-1572.

Sarra, M., Pallone, F., MacDonald, T.T., and Monteleone, G. (2010). IL-23/IL-17 axis in IBD. Inflammatory Bowel Diseases *16*, 1808-1813.

Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H.C., 3rd, Belardelli, F., and Gabriele, L. (2002). ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. Journal of Experimental Medicine *196*, 1415-1425.

Schmid, M.A., Kingston, D., Boddupalli, S., and Manz, M.G. (2010). Instructive cytokine signals in dendritic cell lineage commitment. Immunological Reviews *234*, 32-44.

Schonbeck, U., and Libby, P. (2001). The CD40/CD154 receptor/ligand dyad. Cellular and Molecular Life Sciences *58*, 4-43.

Schulz, O., Edwards, A.D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A., and Reis e Sousa, C. (2000). CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. Immunity *13*, 453-462.

Schulz, O., Jaensson, E., Persson, E.K., Liu, X., Worbs, T., Agace, W.W., and Pabst, O. (2009). Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. Journal of Experimental Medicine *206*, 3101-3114.

Siddiqui, K.R., and Powrie, F. (2008). CD103+ GALT DCs promote Foxp3+ regulatory T cells. Mucosal Immunology *1 Suppl 1*, S34-38.

Smith, C.M., Wilson, N.S., Waithman, J., Villadangos, J.A., Carbone, F.R., Heath, W.R., and Belz, G.T. (2004). Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. Nature Immunology *5*, 1143-1148.

Smith, E., Zarbock, A., Stark, M.A., Burcin, T.L., Bruce, A.C., Foley, P., and Ley, K. (2007). IL-23 is required for neutrophil homeostasis in normal and neutrophilic mice. Journal of Immunology *179*, 8274-8279.

Steinman, R.M., and Cohn, Z.A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. Journal of Experimental Medicine *137*, 1142-1162.

Steinman, R.M., and Idoyaga, J. (2010). Features of the dendritic cell lineage. Immunological Reviews *234*, 5-17.

Steinman, R.M., Turley, S., Mellman, I., and Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. Journal of Experimental Medicine *191*, 411-416.

Steinman, R.M., and Witmer, M.D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proceedings of the National Academy of Sciences of the United States of America *75*, 5132-5136.

Sun, C.M., Hall, J.A., Blank, R.B., Bouladoux, N., Oukka, M., Mora, J.R., and Belkaid, Y. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. Journal of Experimental Medicine *204*, 1775-1785.

Sung, S.S., Fu, S.M., Rose, C.E., Jr., Gaskin, F., Ju, S.T., and Beaty, S.R. (2006). A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. Journal of Immunology *176*, 2161-2172.

Swiecki, M., Gilfillan, S., Vermi, W., Wang, Y., and Colonna, M. (2010). Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. Immunity *33*, 955-966.

Tailor, P., Tamura, T., Morse, H.C., 3rd, and Ozato, K. (2008). The BXH2 mutation in IRF8 differentially impairs dendritic cell subset development in the mouse. Blood *111*, 1942-1945.

Tas, S.W., Vervoordeldonk, M.J., Hajji, N., Schuitemaker, J.H., van der Sluijs, K.F., May, M.J., Ghosh, S., Kapsenberg, M.L., Tak, P.P., and de Jong, E.C. (2007). Noncanonical NF-kappaB signaling in dendritic cells is required for indoleamine 2,3-dioxygenase (IDO) induction and immune regulation. Blood *110*, 1540-1549.

Uchida, J. (1999). Mimicry of CD40 Signals by Epstein-Barr Virus LMP1 in B Lymphocyte Responses. Science *286*, 300-303.

Uhlig, H.H., McKenzie, B.S., Hue, S., Thompson, C., Joyce-Shaikh, B., Stepankova, R., Robinson, N., Buonocore, S., Tlaskalova-Hogenova, H., Cua, D.J.*, et al.* (2006). Differential Activity of IL-12 and IL-23 in Mucosal and Systemic Innate Immune Pathology. Immunity *25*, 309-318.

van Kooten, C., and Banchereau, J. (2000). CD40-CD40 ligand. Journal of Leukocyte Biology *67*, 2-17.

Varol, C., Vallon-Eberhard, A., Elinav, E., Aychek, T., Shapira, Y., Luche, H., Fehling, H.J., Hardt, W.D., Shakhar, G., and Jung, S. (2009). Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity *31*, 502-512.

Worbs, T., Bode, U., Yan, S., Hoffmann, M.W., Hintzen, G., Bernhardt, G., Forster, R., and Pabst, O. (2006). Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. Journal of Experimental Medicine *203*, 519-527.

Wu, L., and Shortman, K. (2005). Heterogeneity of thymic dendritic cells. Seminars in Immunology *17*, 304-312.

Xiao, H., Gulen, M.F., Qin, J., Yao, J., Bulek, K., Kish, D., Altuntas, C.Z., Wald, D., Ma, C., Zhou, H.*, et al.* (2007). The Toll–Interleukin-1 Receptor Member SIGIRR Regulates Colonic Epithelial Homeostasis, Inflammation, and Tumorigenesis. Immunity *26*, 461-475.

Yasui, T., Muraoka, M., Takaoka-Shichijo, Y., Ishida, I., Takegahara, N., Uchida, J., Kumanogoh, A., Suematsu, S., Suzuki, M., and Kikutani, H. (2002). Dissection of B cell differentiation during primary immune responses in mice with altered CD40 signals. International Immunology *14*, 319-329.

Zimber-Strobl, U., Kempkes, B., Marschall, G., Zeidler, R., Van Kooten, C., Banchereau, J., Bornkamm, G.W., and Hammerschmidt, W. (1996). Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival. EMBO Journal *15*, 7070-7078.

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