Subsets of intestinal dendritic cells and their role in orally-induced immune responses

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Cover illustration: Schematic view of intestinal dendritic cell migration

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Abstract

Vaccination is the most effective means of preventing infectious diseases and improving global health. However, few vaccines have successfully been developed for protection at mucosal surfaces where most infectious pathogens enter our body. One major reason for this is the lack of adjuvants, immune enhancing agents, that can be administered together with the vaccine. The enterotoxin cholera toxin (CT) is a potent mucosal adjuvant but the toxicity precludes its use in humans. Derivatives of enterotoxins with reduced toxicity are today the most promising candidates for safe and efficient oral adjuvants. However, the underlying mechanisms for the adjuvant activity of enterotoxins are still not fully known.

Dendritic cells (DCs) are immune cells that sense the microenvironment and confer T cells with ability to help B cells differentiate into antibody-producing plasma cells, necessary for vaccine-induced protection. Intestinal DCs are important both for immunity and tolerance. However, intestinal DCs constitute a heterogeneous population of cells. The function of intestinal DC subsets therefore needs to be defined further to understand how these contribute to tolerance under steady state and to induce immunity during infection or following oral immunization.

In this thesis the role of intestinal DC subsets, in the induction of immune responses following oral administration of antigen, with or without CT as adjuvant, was elucidated. This was done after developing a microsurgical technique in mice that by cannulation of lymphatic vessels allows the direct collection of DCs that exit the intestine under steady state and following vaccination. This technique was combined with the use of genetically modified mice 1) in which DCs can be ablated; 2) that lack specific DC subsets; 3) that are deficient in intracellular signaling pathways in DCs or in other immune cells or 4) that lack CD47, a surface receptor known to influence cell migration.

In the thesis we demonstrate the requirement of cDCs for the activation of antigen-specific T cells and the generation of antigen-specific antibodies following oral immunization when using limiting doses of antigen and CT as an adjuvant. In addition, we show *in vivo* that intact signaling through Gsα specifically in cDCs is essential for the oral adjuvant activity of CT. Using the cannulation technique we show that four subsets of DCs migrate from the intestine under steady state and following oral immunization. Selectively the $CD11b^{-}CD8^{+}$ subset does not show signs of activation after oral CT and this subset was also found to be dispensable for the generation of antigen-specific intestinal antibodies using this adjuvant. The necessity for $CD11b^{+}CD8^{-}$ cDCs could not be establish in CD47 deficient mice, although these mice display significant reduction of this subset in intestinal tissues. Rather, expression of CD47 by non-hematopoietic cells is pivotal for intestinal antibody generation after oral immunization. Finally, signaling pathways involved in CT's adjuvanticity were addressed and shown to be independent of classical TLR-signaling. Moreover, caspase 1/11 activity was not necessary for the generation of antigen-specific serum IgG but for intestinal IgA following oral immunization with CT.

In conclusion, we have shown a requirement for cDCs and an intact signaling specifically in these cells for the oral adjuvant activity of CT. Furthermore we have identified that the generation of intestinal and systemic antibodies following oral immunization with CT are differentially regulated. These results may therefore have important implications for the development of improved oral vaccines.

Keywords: dendritic cells, oral vaccination, intestine, antibody responses, cholera toxin, Gut-associated lymphoid tissue
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Original papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Fahlén-Yrlid L, Gustafsson T, Westlund J, Holmberg A, Strömbeck A, Blomquist M, MacPherson G G., Holmgren J, Yrlid U**
CD11c(high) dendritic cells are essential for activation of CD4+ T cells and generation of specific antibodies following mucosal immunization
Journal of Immunology, 2009, vol. 8, Issue 183, pages: 5032-41

II. **Westlund J, Livingston M, Fahlén-Yrlid L, Oldenborg P-A, Yrlid U**
CD47-deficient mice have decreased production of intestinal IgA following oral immunization but a maintained capacity to induce oral tolerance
Immunology, 2012, vol. 3, Issue 3, pages: 236-244

III. **Westlund J, Capar S, Fahlén-Yrlid L, Livingston M, Ekman L, Lycke N Y., Yrlid U**
Oral adjuvant activity of cholera toxin is independent of classical toll-like receptor and inflammasome pathways but requires G_sa expression in $CD11c^+$ dendritic cells
Manuscript

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Abbreviations

APC	Antigen presenting cell
BATF3	Basic leucine zipper and transcription factor ATF-like 3
BM	Bone marrow
BMCh	Bone marrow chimeras
BMDC	Bone marrow DC
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDC	Classical dendritic cell
cDC	Conventional dendritic cell
CFSE	5,6-Carboxyfluorescein diacetate succinimidyl ester
CLN	Cervical lymph node
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CT	Cholera toxin
CTA	Cholera toxin subunit A
CTB	Cholera toxin subunit B
CTL	Cytotoxic T lymphocyte
CP	Cryptopatches
DC	Dendritic cell
DTR	Diphtheria toxin receptor
DTx	Diphtheria toxin
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicular associated epithelium
FLT3L	FMS-like tyrosine kinase 3 ligand
GALT	Gut-associated lymphoid tissue
GC	Germinal centre
GM-CSF	Granulocyte monocyte-colony stimulating factor
HEV	High endothelial venules
i.n.	Intra nasal
i.v.	Intravenous
IFA	Incomplete Freud´s adjuvant
IFN-γ	Interferon-γ
IFR	Interfollicular (T cell) regions
IL-DCs	Intestinal lymph DCs
ILF	Isolated lymphoid follicle
Irf	Interferon regulatory factor
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
KO	Knock-out
LP	Lamina propria
LPS	Lipopolysaccharide
M cell	Microfold cell
MDP	Macrophage DC progenitor
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MLNX	Mesenteric lymphadenectomy
NALT	Nasal associated lymphoid tissue
NOD	Nucleotide-binding oligomerization domain (as in NOD-like receptor)
OT-I /OT-II	OVA-transgenic $CD8^+$/ $CD4^+$ T cells
OVA	Ovalbumin
p.o.	Per os (oral)
PAMPs	Pathogen-associated molecular patterns
pDC	Plasmacytoid DC

PLO	Primary lymphoid organ
PP	Peyer's patches
PRRs	Pathogen recognition receptors
s.c.	Subcutaneous
SED	Subepithelial dome
SHP	Src homology domain 2 containing phosphatases
SILT	Solitary intestinal lymphoid tissue
SIRP-α	Signal-regulatory protein α
SLOs	Secondary lymphoid organs
STAT	Signal transducer and activator of transcription
TD	Thoracic duct
TDC	Thoracic duct cannulation
TF	Transcription factor
Tfh	T follicular helper cells
Th1/2/17	T helper cells type 1/2/17
TLRs	Toll-like receptors
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumour necrosis factor- alpha
WT	Wild type

Introduction

General introduction

The importance of our immune system is well known to us for defending us against dangerous microorganisms. The majority of such pathogens enter our body through mucosal surfaces, such as the lining of nose, mouth and intestine. The oral route in particular is one of the major points of entry for pathogen invasion[1]. The intestinal mucosa and its integral immune compartment has an intricate and dual role in protecting us from pathogens in an environment in which the majority of the antigens derive from beneficial commensal bacteria, harmless food derivatives and the body's own proteins which need to be tolerated by the host. To generate tolerance towards self and non-harmful substances is as important as inducing immunity to pathogens. If either fails, pathology results. Throughout the intestine and other mucosal surfaces, dendritic cells (DCs) are positioned. They internalize proteins at the mucosal site, process the protein and migrate to draining lymph nodes (LNs) where they present the processed protein to T lymphocytes. The outcome of this T-DC interaction results either in induction of tolerance or an active immune response with effector cells specifically to function to eradicate the pathogen and to help B cells become antibody-producing plasma cells. Generation of high-affinity antibodies are crucial for a memory response in case of reinfection, and the goal with vaccinations, i.e. to give prophylactic protection. At the onset of this thesis, the role of intestinal DCs in either setting was incompletely studied. In particular, the specific role of the emerging subsets of intestinal DCs was not fully known.

During my PhD, I have therefore studied subsets of intestine-derived DCs and their role in immune responses induced by the oral route. In the following sections I will give a general overview of the field of research of DC biology and present aspects of immunological mechanisms key to this thesis work.

Two sides of the immune system – tolerance and immunity

The gut is home to a tremendous amount of commensal bacteria that together with food and the body's own proteins, constitute antigens that must be tolerated by the immune system. Therefore, the steady state of the T-DC interaction in intestinal draining LNs (mesenteric LNs; MLNs) is tolerance. However, during an infection this tolerogenic state has to be overridden to elicit immunity against the intruding pathogen, in which antibody production and microbial killing is initiated.

Tolerance – central, peripheral and oral

All nucleated cells express major histocompatibility complex class I (MHC-I) to enable presentation of endogenously expressed proteins. In case of infection, viral proteins transcribed by the cell will be processed and presented on MHC-I molecules. However, during the steady state, the majority of antigens presented on MHC-I are self-antigens and various mechanisms operate so that lymphocytes do not start an immune reaction towards these antigens. Mechanisms to ensure self-tolerance include clonal deletion, clonal diversion, receptor editing, and anergy[2]. Central tolerance is the term for tolerance induced in the primary lymphoid organ (PLO) during the maturation of B and T lymphocytes (henceforth termed B and T cells). Tolerance induced outside PLO is termed peripheral tolerance. Cells with high affinity to self-antigens are eliminated either by clonal deletion (apoptosis), or induced to a state of unresponsiveness; anergy, within the B and T cells. The former mainly applied for central tolerance, while anergy occur more frequently also in peripheral tolerance.

Self-reactive cells, mainly B cells, can escape elimination by a process called receptor editing, in which the affinity of its B cell receptor (BCR) is changed. Self-reactive T cells within the thymus may be selected by clonal diversion with imprints for suppressor or regulatory function, i.e. to become regulatory T cells (Treg)[2].
In the periphery/systemically, another type of tolerance can be generated, in fact toward antigens in combination with immunostimulatory agents. In order for this to occur, the antigen must have entered via the oral route in the initial encounter with the host. Hence, the term for this type of tolerance is oral tolerance. The reason is that oral delivered substances are prone to elicit tolerance as the majority of substances within the gut are beneficial commensals or food derivatives, important to tolerate. In contrast, an immunostimulatory antigen in the periphery elicit antibody responses upon the second encounter if the first encounter also was peripheral/systemical.

Immunity – innate and adaptive

The immune system can be divided into two parts; the innate and the adaptive, or native and acquired, immune system. The innate immune system gives a very rapid response with specificity receptors covering a broad yet limited spectrum. The receptors involved in adaptive immune system display nearby unlimited amount of specificities. These are screened to identify a certain motif and, hence the name, adapt the response to an re-infection, with high affinity receptors with adapted functional specificity.

The innate immune system acts as a first line of defence, together with the epithelial cells lining body surfaces, as the first barrier against invading pathogens. The innate immune system mounts a very rapid response that is initiated within hours or even minutes of infection or trauma. Cells of the innate immune system recognize structures common for different pathogens, usually involved in specific functions, such as motility, and thus distinct from the host. These structures are conserved between different pathogens and are integrated in the term pathogen associated molecular patterns (PAMPs). They are recognised by pathogen recognition receptors (PRRs) on innate immune cells, for example antigen presenting cells (APCs) such as DCs, which express a large variety of these receptors. The innate immune response is similar to all pathogens at all time. In contrast, the adaptive immune system involves cells with highly specific receptors recognising specific sequences and, most importantly, results in a memory. This memory lies in the ability to mount a more rapid response upon re-encounter, to produce antibodies with a higher affinity and functional specificity, from memory cells generated at the first encounter. Due to the increased multitude of specificities among lymphocyte receptors within the adaptive immune system, the machinery is slow in the initial encounter but ensures a more rapid and effective response upon re-infection.

Although innate and adaptive immune responses are usually treated as separate divisions of the immune system, they are intimately connected. DCs function as an important link between the two. In peripheral tissue, such as the intestine, DCs scan the tissue for antigens and migrate to draining LNs. In the LNs processed antigen is presented to T cells, belonging to the adaptive immune response, which respond cognately to the presented antigen - either with tolerance or immunogenic reaction.

Anatomy of the immune system

Structurally, three different anatomical regions are of importance for an appropriate immune response. Firstly, the primary lymphoid organ (PLO) in which immune cells are generated and

matured. Secondly, the secondary lymphoid organs (SLOs), (inductive sites), in which migratory DCs present the captured antigen to T cells, in a process called priming - to initiate a response. Finally, the effector site, i.e. the site of infection in which DCs captured the antigen and to which lymphocytes home after priming, becoming fully activated upon re-encounter of the antigen in order to eliminate the infection. Thus DCs migrate from bone marrow to peripheral tissue, e.g., intestine, and finally to SLO.

Primary lymphoid organs

All immune cells are of hematopoietic origin, thus originating from a precursor in the bone marrow (BM) (Figure I1). The BM is thus a primary lymphoid organ, further it is responsible for all the steps generating mature B cells. T cells arise from BM-derived progenitors that home to the thymus. Thymus is thus the PLO for T cells. After T lineage commitment and expansion, T-cell receptor (TCR) gene rearrangement follows to generate CD4 and CD8 double-negative cells. Double-negative cells give rise to a large number of CD4 and CD8 double-positive thymocytes. CD4 and CD8 are glycoproteins that functions as co-receptors for the recognition of either MHC-II or MHC-I, respectively[3]. Somatic recombination of TCR genes results in a tremendous repertoire of distinct TCRs with random specificity. Before leaving the thymus, the T cells have matured into single-positive cells with non-self restricted antigenic specificity by selection processes mediated by APCs. Positive selection is mediated by thymic epithelial cells and ensures that T cells can respond to self-MHC[4]. T cells that recognize antigen-MHC-II-complex on the epithelial cell, and at the same time bind to the complex with the CD4 co-receptor, receive a survival signal trough the TCR-complex that allows further maturation into single-positive, $CD4^+$ T cells. A reciprocal selection process leads to MHC-I restricted CD8 expressing T cells. Double-positive T cells expressing TCRs that do not bind antigen-MHC complexes die by neglect[2]. However, the antigen presented in the thymus is self-derived and thus T cells with strong avidity to such antigen must be regulated to ensure self-tolerance. Self-tolerance induced in the thymus is referred to as central tolerance as depicted earlier. Thymic APCs, preferentially DCs, are the responsible cells for the process in which T cells with a too strong binding to antigen-MHC-complex are eliminated by induction of apoptosis (clonal deletion) or selected for imprints to become regulatory T cells (clonal diversion)[4]. Clonal deletion is also denoted as negative selection within the T cell repertoire and can occur either in the stage of double positive or single positive thymocytes.

Small intestinal lamina propria

The small intestinal lamina proria is the effector site in orally induced immune responses. The wall of the small intestine consist of four layers, closest to the gut lumen is the mucosa, after which follows; submucosa, muscularis externa and serosa. A single epithelial layer, organized into crypts and villi lines the mucosa in the small intestine and is protected with a mucus layer. Underlying the epithelial cells, the basal lamina, a layer of extracellular matrix separates the epithelial cells from the lamina propria, the effector site of gut immunity[5]. A thin smooth muscle layer, the muscularis mucosa, separates the mucosa from the submucosa. Within the submucosa, a network of lymphatics (together with blood vessels and nerves) reside[6]. DCs arrive into the submucosa via the blood, and migrate toward epithelial cells close to the gut lumen to sample the environment.

Peyer's patches

Throughout the intestine a number clusters of lymphoid follicles, Peyer's patches, are dispersed. Peyer's patches (PP) are organized lymphoid follicles within the intestine which act as inductive sites for gut immune responses[7]. They usually consists of several follicles per patch, and have a specialized epithelial layer, called the follicle-associated epithelium (FAE),

an underlying subepthelial dome (SED) under which the B cell follicles and interfollicular T cell regions (IFR) are located. Anatomically, PP can stretch down through muscularis mucosa into the submucosa[8]. FAE is clearly distinct from regular epithelium in the LP, containing specialized microfold (M) cells with shorter and fewer microvilli[8]. The M cells are specialized to take up luminal antigens for transport to DCs in the SED[7,9].

Cryptopatches and isolated lymphoid follicles

Cryptopatches (CP) were first identified by Kanamori and collegues in murine intestinal wall as clusters of innate lymphoid tissue cells surrounded by DCs[10]. The clusters contain cells expressing stem cell growth factor receptor (C-kit) and are negative for lineage markers, and are located at the base of the intestinal crypts. CP have been described in mice but originally reported to be lacking in humans[11], which recently, has been challenged by Lügering and collegues[12]. One function of CP was suggested to be the generation of intraepithelial lymphocytes (IELs) in the overlying epithelium. This was supported by the increased number of IELs upon transfer and tissue graft of $Lin^{-}c\text{-}kit^{+}$ CP cells[13]. However, CPs was later shown to be dispensable for IEL generation[11]. Isolated lymphoid follicles (ILF) are another form of organised gut-associated lymphoid tissues (GALT), comprising single B cell follicles, sometimes including a germinal center (GC) with IgD^{+} and IgA^{+} B cells, underlying an epithelium containing M cells[14]. Microbes such as *Salmonella enterica* and *Yersinia* have both reported to infect through ILFs[15,16]. ILFs are microbiota-induced structures whereas CPs develop in germ-free mice. An emerging concept suggests that CP are precursors for ILF[17]. In line with this, the colonization of germ-free mice induces profound changes within the solitary intestinal lymphoid tissues (SILT)[18], increasing the number of ILFs with reduced numbers of CP[18]. Suggestively, this modulation involves the recognition of peptidoglycan, by the innate receptor NOD1 in epithelial cells with upregulation of *ccl20* and subsequent activation of CCR6, critical for ILF formation[19].

The lymphatic system

The lymphatic circulation is a system consisting of lymphatic vessels that drain peripheral tissues of excessive interstitial fluid, which is later returned to the blood. Thus, lymphatics function as a reservoir to adjust the blood volume. The lymph draining a tissue will contain soluble antigens. DCs with captured antigens also use the lymphatic drainage to migrate to the nearest LN. Several afferent lymphatic vessels join in one LN, which is drained by a single efferent lymph vessel. This progressive assembly leads to larger and larger vessels, ultimately leading to a point where the fluid returns to the blood circulation. The thoracic duct (TD), or left lymphatic duct, is the largest lymphatic vessel collecting most of the body's drained lymph, including intestine-derived lymph. TD empties back to the blood in the left internal jugular vein[20]. Several mesenteric lymphatic vessels drain the small intestine as it descends and transitions into colon. The mesenteric lymph vessels drain to the MLN, which consist of several LNs adjacent to one another (like pearls in a necklace)[6]. DCs role as the primary inducer of naïve T cells endows them to migrate from intestinal LP and PP in lymph to draining MLN[21,22], to exert their function as APC after which they die by apoptosis. The LNs are continuously flushed with lymph from the periphery that continues its flow through the LNs, however virtually no DCs continue in the efferent lymphatics.

Mesenteric lymph nodes

MLNs presumably together with PP are the SLOs most important for gut immunity. The MLN drain different parts of the intestine and are connected to each other in a chain[6]. Afferent lymphatics drain the intestinal tissues into the MLN subcapsular sinus. DCs entering the LN from the intestine enter via the subcapsular sinus and translocate into the cortex. Internal to the cortex is the medulla. Several afferent lymphatics are scattered throughout the fibrous capsule

covering the LN, but only one efferent lymphatic vessel leaves each LN, from the hilum. Lymph nodes are provided with blood from an artery entering through the hilum. The artery branches in the outer cortex, converges to one vein and leaves the LN through the hilum. T cells (and B cells) arrive into the LN from the blood, via specialized vessels called high endothelial venules (HEV) and migrate to the cortex. Resident DCs in MLN originate from the blood and hence also arrives to the LNs via HEV. Migration to the cortex is mediated by secretion of the chemokines, CCL19 and CCL21, expressed by cortical stromal cells [23]. In peripheral tissues, DC express CCR6, the receptor for CCL20, but on migration to the draining lymph node, they downregulate CCR6, and upregulate CCR7 [24,25], allowing them to respond to CCL19 and CCL21. Following chemotactic gradients, both DCs and T cells migrate to the interfollicular areas of the paracortex of the MLN to interact with each other. The interaction results in activated T cells with diverse functions in SLOs and peripheral tissues.

Dendritic cells

Dendritic cells (DCs) were first described in 1973 by Ralph M. Steinman and Zanvil A. Cohn [26]. Notably, in 2011 Steinman received (post mortem) the Nobel Prize in medicine “for his discovery of the DC and its role in adaptive immunity”. Indeed, DCs are now recognized as the most important APCs with an exceptional ability to migrate in afferent lymphatics and to prime naïve T cells in SLOs [27,28]. Heterogeneity within the DC population is classified by surface expression of distinct proteins, but all DC share a common ancestor. Transcription factors and other proteins can modulate the development and function of specific DC subsets. Mice harbouring defect in these proteins may function as useful model systems to study the role of specific DC subsets[29].

Classification of DCs

DCs are heterogeneous and the classification of DCs is often ambiguous. However, in mice, all DCs express the integrin CD11c and MHC class II (MHC-II), although to a variable extent[30]. The most obvious distinction can be made between the majority of DCs being conventional, also called classical DCs (cDCs), and plasmacytoid DCs (pDCs)[31]. pDCs are also called natural interferon producing cells for their great ability to secrete type I interferons, which makes them specialized for immune response towards viral antigens[32]. pDCs express lower levels of CD11c and MHC-II than cDCs and additionally they express intermediate levels of B220 (CD45R), a commonly used B cell marker. To further classify the heterogeneic population of cDCs, division into migratory or tissue-resident cDCs is used[33-35]. However, the ability of associating this feature with phenotype by surface expression is limited. Sub-classification of cDCs is instead usually made on the basis of surface expression of different proteins, most commonly CD8α and CD11b, but also additional markers such as SIRP-α, CD103 (integrin α-E) and CD207 (Langerin), differentially expressed depending of the tissue site of the DCs[30,33,35]. DC subsets will be further discussed in later sections in relation to their localization.

DC ontogeny

As previously mentioned, all DCs originate from the bone marrow (BM) and arise during hematopoiesis. During this process a progenitor cell differentiates with gradual maturation and specification towards a certain cell type and concurrent reciprocal loss of ability to become other cell types (In Figure I1 the steps of DC ontogeny is outlined). The ultimate progenitor of immune cells is a hematopoietic stem cell[36-38], which gives rise to the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP)[30,39]. These two separate progenitors account for all subsequent immune cells. B and T cells, among others, evolve from the CLP, whereas the CMP gives rise to a macrophage/DC progenitor (MDP). The next stage of maturation in the MDP-linage results in a loss of ability to generate cells of monocyte phenotype and thus exclusively generate DCs, therefore called the common DC progenitor. Both cDCs and pDCs arise from this progenitor, and seed the blood (Figure I1 [40]).
The generation of DC from the BM is stimulated by hematopoietic growth factors. BM-cultures supplemented with granulocyte-monocyte-colony stimulating factor (GM-CSF) generate DCs[41]. GM-CSF is also involved in the generation of inflammatory monocyte derived DCs and tissue DCs in the intestine and skin[35,42,43]. Upon inflammation, enhanced production of GM-CSF, which is secreted by various cell types, including stromal cells, endothelial cells, activated T cells and macrophages, may contribute to DC generation[44]. However, the role of GM-CSF in DC generation during homeostasis seems minor as mice deficient for GM-CSF or its receptor have normal or only marginally decreased levels of DCs during steady homeostasis[45]. In contrast, both pDC and cDC generation during steady state have shown to be dependent on the hematopoietic growth factor, Fms-like tyrosine kinase 3 ligand (FLT3L)[37,38], with significantly reduced numbers of pDC and cDCs in FLT3$^{-/-}$ mice[46]. A combined lack of GM-CSF and Flt3L reduces the number of DC progenitors and skin DC further[47]. In contrast, mice with a combined lack of GM-CSFR and FLT3 do not have additional reduction in comparison with FLT3$^{-/-}$ mice[48].

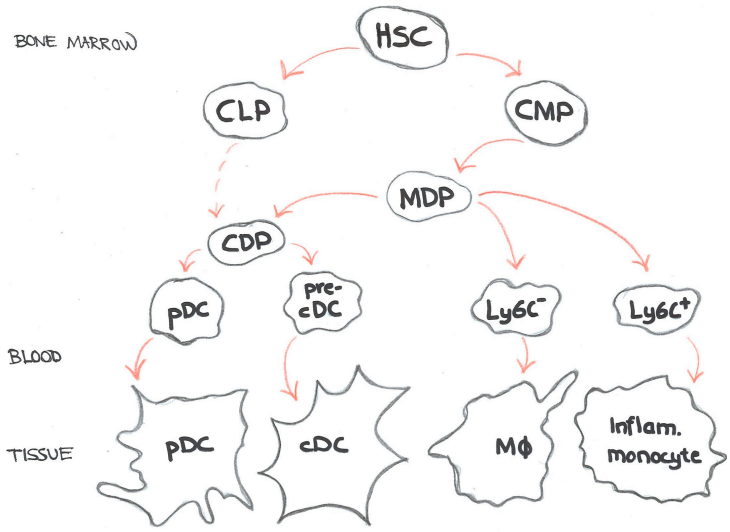

Figure I1. The development of DCs originates from a hematopoetic stem cell (HSC). CLP:common lymphoid progenitor; CMP:common myeloid progenitor; MDP:Macrophage/DC progenitor; CDP: common DC progenitor; Mϕ: Macrophage

Additionally, the ability of progenitors to develop DCs, regardless of lineage, was linked to expression of FLT3[49]. Moreover, addition of FLT3L, both *in vitro*[50,51] and *in vivo*[52]

results in vastly increased numbers of both pDCs and cDCs. In contrast, BM cultures supplemented with GM-CSF promote generation of $CD11b^+$ DCs, but inhibit the generation of pDC[53]. The signaling pathway for FLT3L involves the TF (transcription factor) signal transducer and activator of transcription 3 (STAT3), which is essential for the efficient generation of pDCs (and cDC) from FLT3L BM cultures[54].

In order to study the role of DCs and other immune cells of hematopoetic origin, in relation to non-hematopoietic cells, such as stromal or parenchymal cells, e.g. epithelial cells, bone marrow chimeras (BMCh) are used as a tool. BMCh are generated by lethal irradiation of the host's own bone marrow and replacement with new BM substituting the whole hematopoietic compartment, for more than 6 months[55]. However stromal cells and other low-dividing cells survive irradiation.

DC subsets

In the following section, the subsets of DCs most relevant for the thesis will be described. Although pDC clearly represent a DC subset, the focus on this subset has been minor during this thesis and pDCs are therefore very briefly discussed. Although, many DC subsets share expression of membrane markers and may share some functions, for example, being migratory or tissue-resident, most probably they have distinct functions[34] and therefore should be differentiated when possible. However, based solely on surface expression the most commonly used distinction between cDC subsets in lymphoid tissues is made on the expression of CD8α and CD11b, originally also including CD4. Based on this, three cDC subsets are present in the spleen; $CD8\alpha^+CD11b^-$, $CD8\alpha^-CD11b^+CD4^-$ and $CD8\alpha^-CD11b^+CD4^+$, in addition to pDCs[27,56,57](Figure I2). In MLN and PP, an additional, triple negative population exists[58,59] along with the $CD8\alpha^+CD11b^-$ and $CD8\alpha^-CD11b^+CD4^+$ cDC subset (Figure I2). In non-lymphoid tissues, the heterogeneous DC population consists of

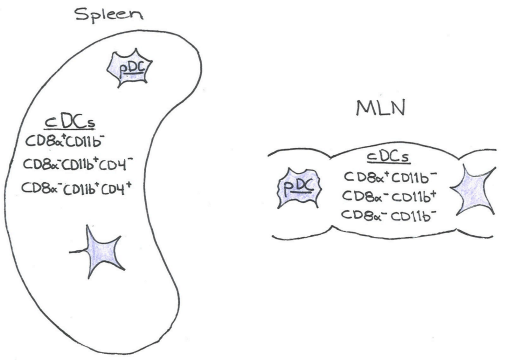

Figure I2. DC subsets in lymphoid tissues

several subsets divided on the basis of tissue specific markers in addition to CD8α and CD11b e.g. Langerin[60] and CD103, preferentially in the skin and in the intestine[61], respectively.
Langerin, a transmembrane lectin, was originally identified as a marker for a DC subset, in mouse and human epidermis, called Langerhans cells(LCs)[60]. Newer mouse model systems have, however, demonstrated $Langerin^+$ cells distinct from LCs, in dermis and skin-draining LNs[62-64]. LCs are, in contrast to other DC subsets, radioresistant, due to self renewal from a local precursor, independent of blood and BM [65]. Although the mechanisms behind the generation of LC during steady state are incompletely known, $Gr1^+$ monocytes have been shown to migrate to inflamed skin and proliferate locally and differentiate into LCs[66]. LCs do migrate to skin-draining LNs but at arrive much later than dermal $Langerin^+$ DCs, thus their role for induction of immunity in skin-draining LNs is doubtful[67]. In contrast to most DCs, LC evolve independently of FLT3 and FLT3L[68].

Transcription factors important for selective DC subsets

The generation of DCs has been shown to be dependent on different TF, some with differential importance for different DC subsets[69]. TFs of the interferon regulatory factor (IRF) family have shown to be predominantly expressed in immune cells and many of the known IRFs have a profound effect on immune regulation. IRF2 deficiency leads to decreased numbers of $CD11b^+$ cDCs and, to a certain degree, Langerhans cells[70]. Mice deficient in IRF4 have reduced splenic $CD11b^+$ cDCs and pDC[71]. In contrast, $CD8\alpha^+$ cDCs, and pDCs as well as Langerhans cells are reduced in mice lacking IRF8 [72]. Other TF affecting $CD11b^-$DC development include RelB [73] and TRAF6 (TNF receptor-associated factor 6) [74] involved in nuclear factor kappa beta signaling pathway. The TF Id2 is of special importance for $CD8\alpha^+$ DCs. A more general developmental defect among all cDCs and pDCs is seen in mice deficient for PU.1 [75] and Ikaros [76].

To elucidate the function of different DC subsets, models in which selective subset defects have been shown, would be of great interest. Mice deficient in selective IRFs could be used. However, in $IRF8^{-/-}$ mice lacking $CD8\alpha^+$ DCs but with normal numbers of $CD4^+$ DCs, these "remaining" DCs do not upregulate MHC-II or co-stimulatory molecules [72]. In addition, migration of DCs to T cell areas of SLOs does not occur, showing a broader functionality of IRFs and making conclusions regarding DC function in $IRF8^{-/-}$ mice difficult.

BATF3

Another TF separate from IRFs, with a more restricted expression profile and with profound effects on the generation of DC subset is the basic leucine zipper transcription factor, AFT-like 3 (BATF3). BATF3 is an activator protein 1 TF[77] that is expressed at high levels selectively in DCs, with low or no expression in other immune and non-immune cells[78]. In particular, $CD8\alpha^+$ DCs have a high expression of *Batf3* and show a specific dependency on this TF for their generation ($Batf3^{-/-}$ mice)[78]. $CD8\alpha^+$ DCs are the main cross-presenting APC, i.e have the ability to present antigens from another cell on its own MHC-I. Thus, as $Batf3^{-/-}$ mice specifically lack this subset of DCs, they show an impaired ability to activate $CD8^+$ T cells and to induce a cytotoxic T lymphocyte (CTL) response and thereby combat viral infections, such as West Nile virus, as demonstrated by Hildner et al[78]. In addition to $CD8\alpha^+$ DCs, $CD103^+$ DCs in peripheral lymphoid and non-lymphoid tissues, eg, MLN, LP, lung and dermis, are dependent on BATF3 for their generation[79]. However, B and T cells are apparently unaffected, with T cells fully capable of expressing the gut-homing molecules $\alpha4\beta7$ and CCR9[79]. The requirement for *batf3* during $CD8\alpha^+$ DCs development varies in mice bred on different backgrounds, demonstrating a non-universal effect[80,81]. Notably, the depletion $CD8\alpha^+$ DCs is not irreversible in $Batf3^{-/-}$ mice and can be regenerated upon microbial stimulus or IL-12 administration[81]. $CD8\alpha^+$ DCs are important for the generation of IL-12 and for control of infectious agents such as *Toxoplasma gondii*[82] and *Mycobacterium tuberculosis*[81]. Consequently, the level of IL-12 is low during the first three weeks after infection with *Mycobacterium tuberculosis,* but increases to approximately half that seen in WT mice, with a concomitant regeneration of $CD8\alpha^+$ DCs by the end of the experiment. Injections of IL-12 also increase the frequency of $CD8\alpha^+$ DCs and these DCs generated in $Batf3^{-/-}$ mice are as efficient as WT $CD8\alpha^+$ DCs in initiating responses in antigen-specific $CD8^+$ T cells on a cell to cell basis. Thus, a positive feedback loop seems to exist between IL-12 and $CD8\alpha^+$ DCs. This "de novo-generation" in $Batf3^{-/-}$ mice was elegantly shown to be dependent on the TF IRF8 via a leucin Zipper DNA domain shared among *Batf3* and *Batf* [82].

CD47 and CD172a

Proteins other than TFs have been shown to have an effect on development or function of cDC subsets. The glycoprotein CD47 is such an example. CD47 (also called integrin-associated protein[83]) is a ubiquitously expressed glycoprotein belonging to the Ig superfamily[84]. It associates with several integrins, collagen receptor, fibrinogen receptor and thrombospondins[85]. Additionally, CD47 is the major receptor for signal-regulatory protein alpha (SIRP-α/CD172a)[86]. Whereas the interactions between CD47 and integrins appear to be predominantly in *cis*, resulting in a plasma membrane complex, the interaction with CD172a can function both with integrins and independently of integrins, in *trans*[87,88]. Ligation of CD172a and subsequent signaling induces phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on its cytoplasmatic tail leading to the recruitment and activation of Src homology domain 2 containing phosphatases -1 and -2, (SHP-1, and SHP-2, respectively). The outcome of CD172a ligation results in both positive and negative regulation of diverse functions such as phagocytosis, cell migration, and cellular response to growth factors[85,86,88]. A certain interaction between CD172a and CD47 is of major immunological importance as CD47 on any cell binds to CD172a on macrophages delivering a negative signal for phagocytosis, thus functioning as a marker of self[89]. CD47 has also been implicated to be involved in cytokine induction. However, both immune stimulatory and dampening modulations have been reported[90,91].

CD47 deficiency results in reduced numbers of cDCs in the marginal zone of the spleen, corresponding to CD172a-expressing $CD8\alpha^{-}CD11b^{+}$ DCs[92]. CD47 has also been shown to be important for migration to skin draining LNs, despite normal numbers of tissue DCs in $CD47^{-/-}$ mice and normal CCR7 expression[92,93]. The i*n vivo* competitive migration assay, with $CD47^{-/-}$ and $CD47^{+/+}$ BM-DCs revealed impaired migration of $CD47^{-/-}$ BM-DCs to draining LN, suggesting an intrinsic defect in the DC. Additionally, BMCh constructed from irradiated $CD47^{-/-}$ hosts grafted with $CD47^{+/+}$ DC behaved as normal mice in regards to generation of T cell proliferation[93]. Due to the role of CD47 as a marker of self, the opposite BMCh cannot be generated as $CD47^{-/-}$ cells are readily phagocytosed by the host.

Whether CD47 also affects DC subsets in the gut and whether this affects intestinal immune responses had not been carefully addressed when the second study of this thesis was initiated. The only study available was with regard to trinitrobenzene sulfonic acid (TNBS)-induced colitis. In this study, Fortin et al showed that TNBS induced colitis is associated with increased migration of $CD103^{-}SIRPa^{+}(CD11b^{+})$ DC from the intestine to the MLN. Furthermore, CD47 deficient mice, harbouring a lower frequency of this DC subset and concomitant reduced migration to the MLN, are protected from TNBS-induced colitis[94]. In addition, CD172a, one of the receptors for CD47, has also been reported to modulate DC function and specifically their migration from the skin[95]. CD172a is differentially expressed, with highest expression seen in spleen, but other lymphoid and non-lymphoid organs such as LNs, brain and ovaries also express CD172a[96]. Cellular expression is highest in cDCs and pDC in spleen, but also NK cells and mature macrophages preferentially express CD172a[96]. Further, within the cDC population, highest expression is seen in $CD4^{+}$ DCs and to a lesser extent double negative (DN) DCs[96]. In mice lacking the cytoplasmic region of CD172a, a decreased proportion of total cDCs, and more specifically $CD4^{+}$ DC (and not $CD8\alpha^{+}$) is observed[97]. This is most likely not due to a generational defect from BM, as *in vitro* culture of CD172a mutant mice with FLT3L (and GM-CSF) results in normal numbers of cDCs. Rather, an intrinsic defect resulting in shortened half-life specifically in CD172a mutant $CD4^{+}$ DCs has been detected and suggested as an explanation for the DC subset-specific deficiency [97].

Intestinal DC subsets

Small intestinal lamina propria

The generally accepted view of DC function in the intestine is that DCs capture antigens and migrate to draining LN to exert their role as APC. In addition to the major $CD11b^+$ $CD8\alpha^-$ and $CD11b^-CD8\alpha^+$ DC subsets present in the lamina propria, expression of the integrin CD103 can be used to further divide the cells (Figure I3). Furthermore, cells situated in the gut epithelium with protrusions into the gut lumen have been identified as $CD11c^+CX3CR1^+$[98-100]. With the use of the $CX3CR1^{gfp/+}$ reporter mouse, the $CX3CR1^{hi}$ cells have been clearly distinguished from the $CD103^+$ population[101,102]. $CX3CR1^{hi}$ have an inability to migrate in lymph, both during steady state[102] and upon administration of the TLR7/8-ligand, R848 (Resiquimod)[101]. This is consistent with a inability to up-regulate CCR7[98,101]. This is in contrast to a study by Diehl et al[103] that suggests that $CX3CR1^+$ cells migrate in afferent lymph, particularly upon infection of antibiotic treated (microbiota-deprived) mice, with non-invasive *Salmonella*. Unfortunately, the authors did not clearly distinguish $CX3CR1^{int}$ cells from $CX3CR1^{hi}$ cells, two highly diverse populations, with the former previously detected, although rarely, in lymph during steady-state[101]. Notably, the suggested migratory ability of $CX3CR1^+$ (Diehl et al., 2013) is in contrast not only to data obtained using the same method[101], but also pseudo-afferent lymph[102]. Furthermore, the differential origins of $CX3CR1^{hi}$ and $CD103^+$ cells have been clearly demonstrated in several reports[42,98,101,104], implying a common origin of $CX3CR1^{hi}$ to macrophages. Mice lacking the receptor for monocyte colony stimulating factor, have a reduced population of CX3CR1, but not CD103[42]. Although not depicted in the original paper[98], in subsequent papers the expression of F4/80, a common marker for macrophages, is elevated in cells expressing high levels of CX3CR1[101,102]. Fluorescence-labelled OVA was readily taken up by $CX3CR1^+$ cells, in fact more efficiently than by $CD103^+$. However, only $CD103^+$ induced the proliferation of $CD4^+$ T cells[101].

$CX3CR1^+CD11c^+$ cells identified in spleen and lymph nodes express CD8 but do not produce IL-12, are poor at cross-presenting and hence therefore distinct from classical $CD8\alpha^+$ DCs. Additionally, $CX3CR1^+$ DCs do not secrete type 1 interferons in response to virus and thus are distinct from pDCs [98]. DCs, originating from the gut also transmit information to responding T cells the tissue location of antigen. This gut homing feature can be measured by the ability to up-regulate CCR9 in responding T cells and correspondingly aldehyde dehydrogenase (ALDH) activity, is lower in $CX3CR1^+$ cells than $CD103^+$ DCs[101]. Reports of the proportion of CD11c-expressing cells in the small intestine of either cell type are contradictory making definition of the major cell population difficult [98,101,105].

Small intestinal lymph

In my PhD work I set up a system to specifically study DCs migrating from the murine intestine towards the MLN (by thoracic duct cannulation (TDC); described more in detail in key methodologies). The TD is, as mentioned, an efferent vessel and as such contains virtually no DCs. Removal of the MLN and subsequent cannulation of the thoracic duct thereby provides pseudo-afferent intestinal lymph, containing intestinal lymph DC (IL-DCs).

In rodents, cDCs but not pDCs migrate in intestinal lymph of mesenteric lymphadenectomized rats (MLNX)[106]. In larger animals, such as sheep and mini-pigs, pDCs or pDC-like cells have been retrieved from afferent skin lymph[107]. Although pDCs do not migrate in murine intestinal lymph[106], they have been shown to be of particular importance in activation and release of cDCs from the intestine via their production of cytokines in response to TLR7/8 stimuli[108]. Recently, further characterization of DC subsets have been made in murine intestinal lymph [102] revealing four subsets of cDCs with regard to CD103 and CD11b

expression, I) $CD103^+CD11b^+$, II) $CD103^+CD11b^-(CD8\alpha^+)$, III) $CD103^-CD11b^+$ and IV) $CD103^-CD11b^-$ (Figure I3). While populations I and II were expected to be found, as CD103 was used as a marker for intestinal migratory DCs, this was the first report showing migration of DCs not expressing CD103 from the gut. The $CD103^-$ DCs constitute approximately 15% of all intestinal lymph DCs (IL-DCs). As with $CD103^+$ IL-DC, $CD103^-$ express MHC-II and co-stimulatory molecules CD80, CD86 and CD40, but not the macrophage marker F4/80. $CD103^+CD8\alpha^+$ DCs are the most prominent cell type to present antigen to $CD8^+$ T cells. Nonetheless, at higher DC:T cells ratios $CD103^-$ DCs have a similar capacity. With regard to priming of CD4 cells, OVA transgenic $CD4^+$ T cells (OT-II) cultured with OVA-pulsed $CD103^-$ DCs proliferated to a higher extent than OT-II cells cultured with either $CD11b^+$ or $CD8\alpha^+$ $CD103^+$cDCs. Previous *in vitro* studies have, despite a similar induction of proliferation within responding T cells of different DC subsets, shown that the ability to induce the gut homing molecule CCR9 is restricted to $CD103^+$ DCs in MLN[105,109]. However, $CD103^-$ IL-DCs showed a similar ability to induce gut homing as both $CD103^+$ subsets, concomitant with the fact that they also have ALDH activity[102]. Taken together, this demonstrates that the $CD103^-$ cells present in murine intestinal lymph are, in fact, *bona fide* DCs. Importantly, only the $CD103^-$ IL-DCs induced IFN-γ and IL-17 secretion by OT-II cells, after co-culture with the four IL-DC subsets collected under steady state[102]. Similar findings were reported when $CD103^-$ and $CD103^+$ MLN DCs were separated[110]. However, in these experiments the $CD103^-$ DCs were not only gut derived, but would also include blood-derived DCs in this population of DCs from the MLN.

Mesenteric lymph node

MLN is supplemented with both blood and intestinal lymph, resulting in blood-derived resident and gut-derived migratory DCs. Despite many efforts, there is as yet no single marker to distinguish between blood-derived and intestinal derived DCs within MLN. CCR7 is important for migration of intestinal DCs to MLN[111,112] and IL-DCs express high levels of CCR7 mRNA[102]. $CCR7^{-/-}$ have reduced numbers of DCs in MLN [105],[113] and p.o. immunization with OVA in CCR7 deficient mice does not induce proliferation of responding T cells, neither $CD8^+$ nor $CD4^+$ [105]. Staining with CCR7 does unfortunately not result in a reliable differentiation of DC subsets by flow cytometry. However, with the knowledge obtained from the cannulation experiments described above, the same division into four DC subsets as for IL-DCs can be applied to the MLN when DCs expressing high levels of MHC-II are analyzed (Figure I3 and D1). Additionally, DCs that have migrated from PP may contribute to the DC populations in the MLN[6,21,22].

The ability of inducing the gut homing molecules CCR9 and $\alpha4\beta7$ on $CD8^+$ T cells has previously been shown to be exclusive for the $CD103^+$ DCs within LP and MLN during steady state[105]. Of note, LP DCs but not MLN DCs, are the most prominent gut-homing inducers regarding CCR9 and $\alpha4\beta7$ induction. PP also have this ability, but splenic DCs do not[105]. It has been shown that the property of induction of gut homing is not associated only with expression of CD103, as splenic $CD103^+$ DCs do not induce CCR9 expression. Later *in vitro* experiments confirm that $CD103^{-/-}$ DCs are able to induce CCR9 expression in responding T cells[109], endorsing the finding by Cerovic et al regarding lymph $CD103^-$ DCs [102]. Interestingly, elegant studies with LN transplantations demonstrated the importance of stromal cells within MLN for the induction of gut homing properties of T cells[114]. Peripheral, i.e. axillary, inguinal and brachial LNs transplanted into MLNX mice could not confer gut-homing properties to T cells[114].

The gut homing ability is linked to ALDH activity. Additionally, CCR9 expression is induced only after p.o. immunization[105]. CCR9 is not, however, required for the actual activation of effector T cells entering the intestine, as CCR9-deficient mice induce proliferation of $CD8^+$ OVA transgenic cells, equivalent to the level of which WT mice induce proliferation. However, the ability to home back to the intestine fails in these mice[105].

Peyer's patches

Three different subtypes of cDCs have been reported within the PP: $CD11b^+CD8\alpha^-$, $CD8\alpha^+CD11b^-$ and $CD11b^-CD8\alpha^-$[27,30,58](Figure I3). $CD11b^+$ cDCs preferentially localize within the SED; $CD8\alpha^+$ cDCs in IFR; the double negative are found throughout both the SED and IFR[58]. Upon microbial stimulation, the SED becomes devoid of $CD11b^+$ cDCs, most likely due to an efficient migration into IFR[58]. These different cDC subsets do not just show preferential localization, but also differential cytokine production. $CD11b^+$ cDC is the only IL-10-producing cDC in PP. Additionally, the cytokine production from the responding T cells is different accordingly to the different priming cDC subset. T cells primed by CD11b expressing DCs preferentially produce IL-4 and IL-10, while IFN-γ was produced by T cells co-cultured with $CD8\alpha^+$ DCs and $CD11b^-CD8\alpha^-$ cDCs[59]. Also, $CD11b^+$ DCs within the SED express CCR6 and thus show a more immature phenotype[58]. A later study using oral administration of labelled OVA, showed that DCs within the SED migrated with internalized OVA to T cell areas to initiate DC-T cell interactions[115]. Suggestively, immature DCs within the SED capture antigen with or without microbial stimuli[58,115]. Without microbial stimuli, the responding T cells show an immature phenotype; i.e. they do not express CCR7, but express CCR4, CxCr3, CCR9. Interestingly, relatively high mRNA levels of FoxP3 were also expressed by these T cells suggesting a potential induction of regulatory T cells[115].

The specific role of PP in gut immunity is still controversial. Mice lacking PP due to *in utero* treatment with lymphotoxin receptor antibodies, are still able to generate IgA response[116]. However, several *in vitro* co-culture studies have shown the vital role of PP-DCs to generate IgA[117,118]. In addition, Germ-free mice with underdeveloped PP show an increase in IgA and IgG-titers following maturation of PP upon antigenic stimuli[119]. Previous studies have also indicated that PP is the main site for the generation of IgA-producing precursor cells[58].

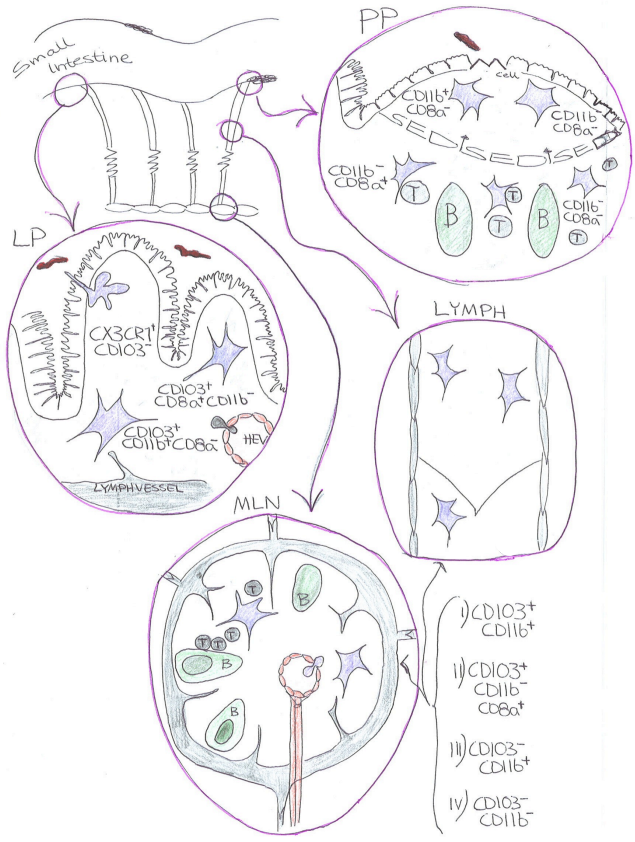

Figure I3. DC subsets within amll intestinal lamona propria (LP), Peyer's patch (PP), lymph and mesenteric lymph node (MLN).

Antigen processing and presentation by DCs

DCs express a variety of receptors to capture antigens, and differential endosomal processing within the DC ensures accurate presentation to T cells. Antigens taken up by the DCs are processed within exosomal or endosomal compartments followed by presentation on differential MHC-complexes. Exogenous particles taken up into the cell are degraded and loaded onto MHC-II in cytosolic compartments called lysosomes. MHC-II molecules arrive to the lysosome from the endoplasmatic reticulum (ER) with invariant chain bound the antigen cleft, also holding the MHC complex together and guides the MHC-II towards the lysosome[120,121]. In late endosomal compartments invariant chain is degraded to CLIP, which in the lysosomes is exchanged for peptides with higher affinity. Peptide-loaded MHC-II translocate to the surface and are recognized by $CD4^+$ T cells[122]. $CD4^+$ T cell help B cells to become antibody-producing plasma cells and are therefore most important for immunity against extracellular pathogens.

All cells should signal to declare their health status, as peptides from endosomal antigens are associated with MHC-I expressed on all nucleated cells. Cytosolic proteasomes degrade ubiquitin marked misfolded proteins, alternative transcripts, as well as functional proteins; cytosolic or derived from endosomal compartments[123-125]. Further digestion by cytosolic peptidases yield peptides for transport to the ER performed by TAP[120,124-126]. The degraded antigen is loaded onto newly synthesized MHC-I molecules in the ER and transported to the surface[124-126]. A TAP independent pathway is suggested where endocytosed antigens are loaded onto recycled MHC-I molecules[120]. At the surface, MHC-I/peptide complexes are selectively bound by $CD8^+$ T cells. These $CD8^+$ T cells are needed for effective eradication of intracellular pathogens by cytotoxic mechanisms. In order for the $CD8^+$ T cell to become "mature" and respond to antigen bund to MHC-I in the periphery, it needs to be primed by APCs. APCs thus need to be able to "cross-present" antigens produced by other cells on its own MHC-I. DCs and in particular $CD8\alpha^+$ DCs have *in vitro* and *in vivo* proved to be the major cross-presenting cell[127,128]. In contrast, when targeted with antigen *in vivo* $CD11b^+$ DCs preferentially stimulate proliferation of $CD4^+$ T cells, whereas antigen-targeted $CD8\alpha^+$ DCs have the ability to present not just on MHC-II but also MHC-I and thus activate $CD8^+$ T cell [127].

Uptake of Ag by intestinal APCs

DCs sample their environment by two broad mechanisms: phagocytosis and pinocytosis. Pinocytosis, an important feature in DC biology, can be further divided into macro-pinocytosis and receptor-mediated endocytosis[129]. Mature DCs leave the intestine with captured antigen and, if receiving microbial stimuli, become activated with a subsequent increase in the levels of MHC and co-stimulatory molecules during the course of migration. Intestinal antigens are readily taken up by M cells of PPs for transport to DCs in the SED. Alternatively luminal antigens are taken up by absorptive epithelial cells and then endocytosed by the DCs present within the LP. Pathogens, like *Salmonella* and *Shigella* reaching the intestinal lumen predominately gain access to the host via M cells in FAE of the PP[130]. Additionally, lamina propria $CD11c^+$ cells have been shown to extend intraepithelial dendrites into the gut lumen[99] and it is suggested that they thereby sample luminal antigens[131]. These interepithelial dendrites have been shown by intra vital microscopy to be generated by $CX3CR1^+$ CD11c expressing cells[100]. The contribution of this route to total uptake of luminal antigen into DC is unclear, as non-invasive pathogens still gain access to gut mucosa in CX3CR1 deficient mice that lack luminal dendrites[132]. In addition, the designation of $CX3CR1^+$ cells as DCs, has more recently been questioned, as they in fact more resemble macrophages than DCs, as mentioned previously. Moreover, *in vivo* imaging has recently

visualised the transfer of intestinal antigen absorbed by goblet cells to underlying $CD103^+$ LP-DCs [133].

Pathogen recognition receptors

As mentioned previously, the innate immune system is characterized by response against commonly shared features among classes of pathogens. Different pathogen associated molecular patterns (PAMPs), are recognized by different classes of PRRs. These shared features comprise bacterial or viral nuclei acids, e.g. double stranded RNA, components of the bacterial cell wall e.g. peptidoglycan or lipopolysaccharide (LPS) and are recognized by different PRRs. Various types of PRRs are expressed by a phagocytic cell, such as the DC, including Scavenger receptors, mannose receptors, and most relevant for the work in my thesis; Toll-like receptors (TLRs) and NOD-like receptors (NLRs).

Toll-like receptors

TLRs are the best-characterized PRRs. TLRs are a family of membrane receptors with differential expression between the surface membrane and endocytic membranes. Differential expression of TLRs is also seen between pDC and cDCs as well as between different cDC subsets. Intracellular TLR3, TLR7 and TLR9, identify viral double stranded RNA, viral single stranded DNA and viral and bacterial unmethylated CpG in DNA, respectively. TLR7 and TLR9 are mainly expressed by pDCs, explaining their central role in anti-viral responses[134]. In cDCs, the expression of TLRs is concentrated on the surface membrane: TLRs such as TLR2, specific for, e.g., bacterial peptidoglycan; TLR4, specific for LPS; and TLR 5, sensing flagellin; but also intracellular TLRs like TLR3, TLR9 and TLR13 are expressed[134]. Also within the cDC population, expression of TLR varies, $CD8^+$ DCs preferentially express TLR3, but in contrast to $CD11b^+$ lack expression of TLR7 [135]. Signaling via TLRs starts by dimerization of two hetero or homo dimers of TLRs. All TLRs employ the adaptor protein MyD88, with the exception of TLR3 and TLR4. TLR4 has both a MyD88-dependent and a MyD88$^-$independent pathway, the TRIF pathway which is also employed in TLR3 signaling. The major signaling steps exemplified by TLR4, are outlined in Figure I4a. TLR4 ligation results in the activation of the TFs NFκβ, AP-1 and Irf3, orchestrating a pro-inflammatory response by transcription of a diverse range of genes important for proliferation and differentiation and lead to downstream activation of pro-inflammatory cytokines. IFN-β is produced upon TLR4 ligation, and by a feedback loop, results in TRIF or Myd88 dependent induction of IFN-α, further activating the cell[136].

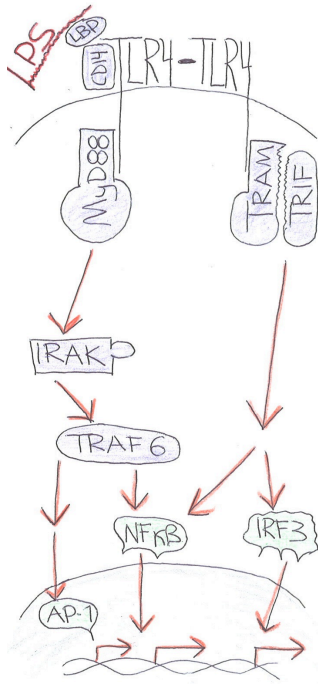

Figure I4. LPS ligation to TLR4 induces MyD88- and TRIF signaling pathways.

NOD-like receptors and their involvement in inflammasome signaling

NLRs compromise various intracellular PRRs involved in microbial/viral recognition. NLR-related protein 3 (NLRP3/ also denoted NALP3) is of particular interest as it has been shown to be involved in the response towards diverse viruses and bacteria, as well as fungi [137]. However, non-pathogenic stimuli such as stress mediated by reactive oxygen species can also lead to activation of this receptor[138]. Upon activation, a signaling protein complex called the inflammasome is assembled. The cardinal feature of the inflammasome is the activation of caspase-1 which catalyses the cleavage of proIL-1β and proIL-18 to yield the active IL-1β and

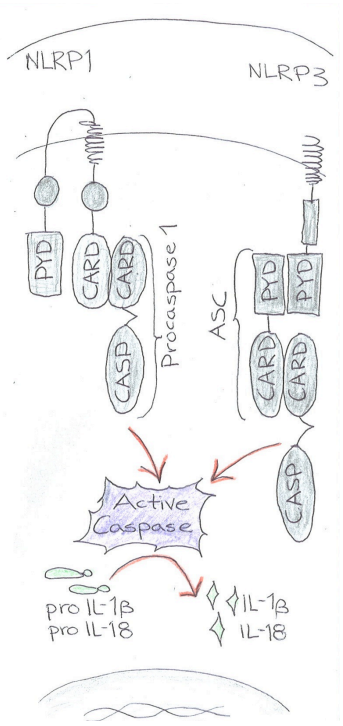

Figure I5. Signaling pathways for NLRP1/NALP1 and NLRP3/NALP3 inflammasome

IL-18 proinflammatory cytokines. Both macrophages and DCs, but also intestinal epithelial cells have expression of all the inflammasome compartments involved in NLRP3 inflammasome signaling. [139]. The inflammasome is not a static complex. Several receptors with different specificities have been reported to induce IL-1β and IL-18 secretion in a caspase-1 dependent manner, although NLRP3 is the best described. RIG-1 and AIM2 are two receptors involved in viral recognition, while NLRP1 and IPAF both have bacterial specificities[137]. The signaling leading to recruitment, and thereby autocleavage, of caspase 1, involves caspase activator and recruitment domain (CARD) directly on the receptor, or via the adaptor protein apoptosis-associated speck like protein and containing CARD (ASC), which also contains a pyrin-containing domain (PYD) for binding to PYD on the receptor (Figure I4b)[140]. Initial studies of caspase $1^{-/-}$ ascribed caspase 1 certain functions. However, recently caspase 1 and caspase 11 have shown to be to closely mapped in the genome to be segregated by Kayagaki et al, suggested that meanwhile canonical inflammasome activation of caspase 1 induces programmed cell death, in addition to IL-1β and IL-18 activation, a non-canonical signaling pathway exist with caspase 1 as activator of IL-1β and IL-18 but caspase 11 as responsible for the programmed cell death. Interestingly, Kayagaki et al found CTB as activator of the non-canonical signaling pathway in LPS-primed macrophages[141].

A study in mice generated by gene targeting to lack the TLR-adaptor protein Myd88, revealed that Myd88 is an important adaptor in the signaling of IL-1R and IL-18R pathways[142]. Effector functions of IL-1β, including induction of acute phase proteins and IL-1β-specific cytokine were impaired in $Myd88^{-/-}$ mice. These mice were also unresponsive to IL-18-stimulation leading to impaired IFN-γ production by natural killer cells[143].

T cell responses – CTLs and T helper cells

The differentiation of T cells after priming results in effector $CD8^+$ T cells ready to respond to cognate antigen when in the periphery and effector $CD4^+$ T cells, of several possible T helper cell subclasses, with diverse function and cytokine production profiles. Generation of mature effector $CD4^+$ T cells is dependent on three signals for full activation. The first signal is through the T cell receptor/CD4 and MHC-II/antigen complex. The second signal is through CD28 ligated by a co-stimulatory molecule, CD86 (or CD80), on the DC. The third is mediated by secreted cytokines. Without the co-stimulatory signal via $CD28^-$ CD80/CD86[144], DC-T cell interaction lead to T cell anergy, a state of unresponsiveness and inability to proliferate[145]. The differentiation of $CD4^+$ T cells is essential for the generation of high affinity antibodies with increased specificity, but also for proliferation of $CD8^+$ CTLs upon re-encounter with an antigen[146].

In addition to DCs maturation in response to microbial/viral stimuli via PRRs, as mentioned above, secretion of proinflammatory cytokines, such as TNF-α or interleukin IL-1β[147,148] and ligation of CD40[149], also results in activation of the DC[150]. Also non-pathogenic/non-inflammatory actions - for example, during mechanical and enzymatic

purification - can lead to activation of the DC[150]. However, the state of activation is different. DCs activated by non-pathogenic stimuli do not result in efficient upregulation of co-stimulatory molecules[151] and T cells responding to these DCs do not differentiate to effector T cells[152]. DCs activated in the presence of "danger signals" can either become directly activated, that is through ligation to PRRs or indirectly activated by inflammatory signals such as interferons. DCs activated in either situation generate proliferating T cells, but not all become functionally active. Direct activated DCs generates IFNγ-producing T cells, whereas indirect activated DCs do not[153].

Subsequent interaction of activated DCs with $CD4^+$ T cells results in a reciprocal up-regulation of diverse surface proteins leading to cytokine production and effector function of the T cell. Naïve $CD4^+$ T cells (T helper 0) differentiate into different subsets of T helper cells, with distinct specificities characterized by differential cytokine profile[154]. Th1 and Th2 subsets were the first to be identified, but several lineages have recently emerged, including Th17, T follicular helper cell (Tfh) and T regulatory cells [155]. Th1 cells differentiate in response to expression of the TF T-bet and secrete IFN-γ as their signature cytokine. IFN-γ activates STAT1 which induce expression of T-bet, resulting in a positive feedback-loop[156]. Th1 cells are governed by IL-12 secreting macrophages and DCs, and are involved in the immune responses towards virus and intracellular pathogens. Th2 cells are regulated by the TF GATA-3, and IL-4, IL-5 and IL-13 are secreted as the signature cytokines for Th2[157]. Th2 response, classically induced by helminth infection results in IgE antibodies and activation of mast cells and basophils [158]. ROR-γT is the TF important for differentiation into Th17 cells[159]. Th17 cells are identified by their production of IL-17, but also secrete IL-22, both important cytokines in protection against bacteria and fungi[160]. Development of Th17 cells is driven by STAT-3, IL-6 and IL-23. TGF-β in combination with IL-6 induces ROR-γT expression[161]. In contrast, TGF-β in the absence of IL-6 induces Foxp3, the TF crucial for Treg development [162]. The cytokine milieu will also affect the T cell dependent antibody response. IL-4 from Th2 cells results in the generation of IgE and IgG1 while IgG2a is primarily induced by Th1 cell produced IFN-γ. In addition, specialized Tfh cells are located in the B cell follicle and the germinal center to assist in B cell maturation into plasma cells secreting high affinity antibodies. DC-primed $CD4^+$ T cells destined to become Tfh up-regulate CXCR5 and down-regulate CCR7 to enable migration towards B cells at the borders of the B cell follicles[163]. The TF important for Tfh induction is Bcl-6, which serves as a master regulator that also antagonize TF of the other Th cell lineages[164]. Bcl-6 promotes Il-21 secretion, which is crucial for GC formation as mice deficient for IL-21 have deformed GCs.

Primed $CD8^+$ T cells later mature into cytotoxic T lymphocytes (CTLs) with the ability to kill target cells by the delivery of lytic granules[165]. Co-stimulatory signals through CD40/CD40L are necessary for the accumulation of $CD8^+$ T cells in the intestine, as well as for their differentiation into CTLs. In contrast, CD40/CD40L is not required for the actual CTL response[166].

B cell responses - generation of antibodies

Antibodies, are secreted forms of the immunoglobulin of the BCR. Antibodies can be generated independently of T cell help by crosslinking several BCRs, which results in activation of the B cell[167]. Alternatively the generation of antibodies is dependent on $CD4^+$ T cell help. It is generally believed that generation of high affinity antibodies and memory B cells are T cell dependent[168-170]. Most naïve B cells reside in the follicles within the SLO awaiting antigenic encounter. Primed B cells migrate toward the B/T cell zone in the follicular

border by up-regulation of CCR7, the receptor for CCL19, and CCL21 expressed in the T cell zone[171,172]. The continued expression of CXCR5 ensures that the B cell is kept within the follicle through the retained responsiveness toward the follicle-expressed CXCL13[173]. The B cell interacts with the cognate T cell through binding of MHC-II/antigen complex to TCR, ensuring antigen-specificity. The MHC-II/antigen complex- TCR interaction leads to reciprocal activation of the cells by increased expression of CD40 and CD40L, on the B and T cell respectively. CD40L/CD40 ligation is of particular importance as mice deficient in either molecule have defective GC-formation[174,175]. GCs are specialized regions within the B cell follicle in which rapid cell division (clonal expansion) takes place in response to infection or vaccination. The primary purpose of the GC reaction is to generate antibody-producing plasma cells and memory B cells with high affinity[176].

Somatic hypermutation (SHM) and class switch recombination (CSR), both involving the enzyme, activation induced cytidine deaminase (AID)[177] are central for the maturation of antibody responses. Point mutations in the light chain initiated by AID yield, in concert with error prone DNA repair, B cells producing antibodies with higher affinity[178]. CSR involves modification within the heavy chain generating different subclasses or isotypes. There are five classes of antibodies, IgM, IgD, IgG, IgA and IgE, each with different functions and tissue localisation. IgM is the native antibody isotype, expressed by naïve B cells; IgG is important for bacterial and viral defence in systemic infections; IgE is the primary antibody against parasites; and IgA is predominantly expressed in mucosal tissues[179].

70% of all antibodies produced in the body is estimated to be IgA [180]. IgA is important both for immunity against pathogens and to retain commensal bacteria in the gut lumen. Binding with high affinity suggests to mediate neutralization of toxins and pathogens, whereas low affinity binding restricts commensal bacteria to the gut lumen[180]. Two mechanisms have been proposed for generating IgA antibodies. The first is a T cell independent mechanism, driven by commensal bacteria and the second mechanism is driven by T-dependent antigens and initiated in organized lymphoid tissue of the GALT. Gut DC have a unique ability to induce IgA switching *in vitro*, mainly through TGF-β and RA, with contributions by effector cytokines e.g. IL-6, IL-4 and IL-5[180,181]. Specifically, $CD11b^+$ DC in PP induce IgA production by B cells *in vitro*, with contribution of IL-6[118]. The contribution of different cytokines have been addressed *in vivo* by studies in mutant mice specific for selective cytokine, where mice lacking IL-6[182] or IL-5[183] have lead to their suggested contribution to IgA-responses[180]. Two additional factors that initiate IgA CSR are; a proliferation inducing ligand (APRIL) and; B cell activation factor of the tumour necrosis factor family (BAFF)[184,185]. These factors, derived from DCs and epithelial cells may act directly on B cells, independently of T cells. Thus, these factors may promote IgA responses in the absence of organized lymphoid tissues, e.g. in intestinal lamina propria. This serves as the proposed mechanism by which commensal bacteria induce IgA responses. In contrast, CT is highly dependent on $CD4^+$ T cells to generate IgA responses[186]. By using ligated intestinal loops challenged with CT, Husband and Gowans[187], demonstrated PP as inductive sites for IgA responses against CT. Intestinal loops that contain PP induced IgA responses while loops devoid of PP did not[187]. PP were described already 40 years ago as the main inductive site for IgA responses and the predominant site for IgA precursor cells[188] . The precursor cells migrate via TD and home to lamina propria[189].

Vaccines and adjuvants

Vaccination is based on nature's own way to elicit an immune response to deal with infectious agents. Ultimately, the aim of an immune response is not the mere extermination of the invading pathogen, but also, in case of a secondary infection, to respond with an even faster and more precise immune reaction. The cardinal function of such memory responses is to generate antibodies with high affinity (by SHM) and effector specificity (by CSR) i.e. induction of IgA upon mucosal antigenic stimuli, from memory B cells - with the help of memory T helper cells.

The first vaccination is commonly attributed to Dr. Edward Jenner in the classical experiment in 1796. An 8-year old boy was inoculated with lesion material from the severe smallpox, remarkably without catching disease, and due to prior inoculation with matter from a cowpox lesion. Hence, the term vaccine derives from the latin word *vacca* meaning cow, and although this was perhaps not the very first case of vaccination, it became a milestone in immunology[190]. Jenner and colleagues set the basis for what led to eradication of small pox in our time, and to effective control of a number of diseases including polio, diphtheria, tetanus, *Haemophilus influenza* type B, measles, yellow fever, mumps, rubella, typhoid fever and rabies[191]. However, challenging and emerging infections like dengue fever, malaria and HIV raise a demand for better and more effective vaccines.

Overall, vaccines can be divided into 2 different formulations: live attenuated; or killed whole/subunit vaccines. Live attenuated vaccines induce a strong long-term memory response like a natural infection, as all antigens are retained. The down-side is the requirement for a cold-chain and more importantly for the vaccinee, the risk of mutation, reversing the attenuation with regained virulence[192]. A more safe vaccine is based on killed bacteria, virus or their toxins. Killed whole cell/subunit vaccines are weaker and poor inducers of long-term memory and thus need co-administration of adjuvants. Polio and cholera exemplify killed whole vaccines, whereas diphtheria toxin and tetanus toxin are examples of subunit vaccines.

Adjuvants do not elicit immunoreactions by themselves but enhance an immune response towards a co-administered antigen. Importantly in vaccine formulations, adjuvants need to be able to stimulate a long-lived memory response[193]. Benefits from adding adjuvant to a vaccine formulation to enhance immune reactions is that less antigen can be used and that the number and frequency of antigen doses can be reduced. The actions by which adjuvants operate include enhanced antigen processing/presentation, immune activation and by creating a depot for elongated presence of antigen[194,195]. Many of the adjuvants possess known features of PAMPs to activate DCs, or other APCs, via PRRs[196].

TLR agonists

The outer membrane component, LPS of gram-negative bacteria is a TLR4 ligand. LPS activates DC resulting in up-regulation of co-stimulatory molecules, migration to T cell areas within the spleen and increased T cell proliferation[197]. However, LPS is too toxic to be used in humans. Monophosphoryl lipid A (MPLA), is a attenuated version of LPS that induces migration and co-stimulatory molecules similar to LPS, but has a much reduced toxicity[198]. The reduced toxicity is attributed to the mode of action in signaling pathways, biased to recruit the adaptor protein TRIF rather than MyD88[199]. Due to their dependence of MyD88, pro-inflammatory cytokines such as IL-1β, IFN-γ and IL-6 only increases after LPS but not MPLA administration. Furthermore, immunization with MPLA and LPS as adjuvants elicited similar immune responses in MyD88 deficient mice[199]. The discrepancy of decreased toxicity in MPLA compared to LPS and ability to produce IL-1β can further be attributed the impaired

ability of MPLA to activate the NLRP3 inflammasome, activated upon MyD88 signaling[200].
MPLA is used in two vaccines, towards human papilloma virus and hepatitis B, respectively, together with alum[201].

Alum

Alum, widely referred to in the field of medicine, as aluminium salts, is the most commonly used adjuvant in human vaccines. It is included as adjuvant in a range of different vaccines, for example, against diphtheria (*Corynebacterium diphtheriae*), tetanus (*Clostridium tetani*), *Haemophilus influenzae* type B, pneumococcus (*Streptococcus pneumoniae*)[202]. Alum elicits a strong antibody response and is a potent TH2 inducer and thus is highly effective against extracellular bacteria and their toxins[203]. In contrast, due to its attenuated TH1 response, it is a poor adjuvant for intracellular pathogens such as viruses. Alum has been used for over 70 years with an outstanding safety record, but the mode of a function is still puzzling. Alum induces a massive influx of innate immune cells, such as neutrophils, eosinophils, pDC, cDC and inflammatory monocytes[203,204]. The inflammatory monocytes adopt DC phenotype upon stimuli with antigen and alum, and increase the level of CD11c, MHC-II and CD86[203]. Several studies have outlined the role of the NALP3 inflammasome in alum adjuvanticity. Most studies are united in recognising the contribution of NLRP3 for the secretion of IL-1β. However, with regard to antibody responses, the NLRP3 inflammasome is not essential[205], except in IgE and IgG1 responses[204,206].

Mucosal vaccination

The majority of the human vaccines available today are administered via parenteral routes, intramuscularly or subcutaneously.[207]. These are good inducers of systemic immunity, but usually less efficient for mucosal immunity[208]. Mucosal tissues are often a barrier to systemically produced serum antibodies, especially intestinal mucosa, permeable only during inflammation[207]. However, mucosal sites are ports of entry for a majority of infectious agents, so a local immune response is desirable. Mucosal vaccination yields a more effective vaccination because systemic immunity is also induced in concert with local immune responses[209]. Furthermore, mucosal immunization has the advantages over parenteral immunization of ease of administration and being needle free – with a lesser risk of transmitting infection in addition to improved compliance in, e.g., children.

On the market today, there is only a hand full mucosal vaccines: orally administered vaccines against cholera, polio and against typhoid fever, and one vaccine against rotavirus, administered intranasally[207]. This reflects the caveat with oral in contrast to systemic delivery. Orally administered vaccines have a risk of degradation in the digestive system, in contrast to systemic delivered vaccines. In addition, in order to generate immunity, oral vaccines demands a strong adjuvant included in the vaccine-formulation, in order to override the mechanism of oral tolerance.

Cholera toxin

Cholera toxin (CT) is the powerful toxin released by the Gram-negative bacterium *Vibrio cholerae*, the causative agent of cholera, resulting in massive loss of water by diarrhea. Cholera and the related heat-labile toxin (LT) from *Escherichia coli* are among the most powerful toxins known affecting millions of people annually, thousands with lethal outcome[210]. CT together with e.g. heat-labile toxin from *Escherichia coli* belongs to the family of AB_5 toxins, constructed of one enzymatic active A component (CTA) and a pentameric ring of B components (CTB) with receptor binding properties[211,212]. The A

component consists of a enzymatically active A_1 linked by a disulphide bond to the A_2 subunit, the latter linking the A units to the B subunits[212].

The only known receptor for CT is the ganglioside GM1[213] present on all nucleated cells[214,215]. Upon binding of the CTB to GM1, the holotoxin is endocytosed and transported by retrograde transport from Golgi to the ER[210,213,216,217](Figure I6). In the ER the disulphide bond between the two A subunits is reduced, separating the A1 from the A2 and B subunit. A1 translocates to the cytosol via ER-associated degradation pathway, usually mediating the proteosomal degradation of misfolded proteins[218]. The A1 unit evades degradation by unknown mechanisms but, proposed by natural low lysine content, and instead refolds in the cytoplasm where it acts in ADP-ribosylation of the α subunit of the G protein Gs at the cell membrane. Following ADP-ribosylation, $G_s\alpha$ continuously activate adenylate cyclase, increasing the levels of cyclic-AMP[210,219]. One of the actions of cyclic-AMP is facilitation of the efflux of Cl^- with accompanying water into the gut lumen, causing the watery diarrhea that can reach up to 500-1000mL/h[220](Figure I6).

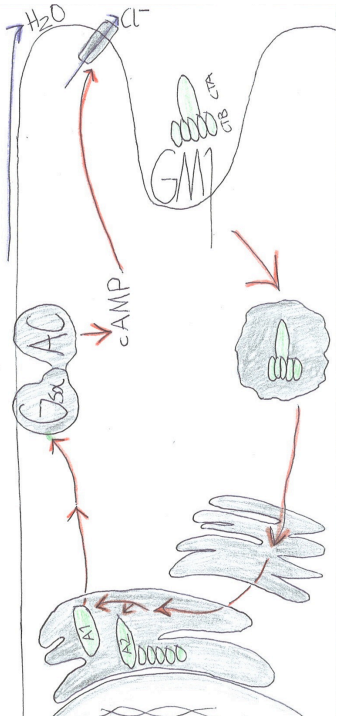

Figure I6. Transmigration of cholera toxin from gut lumen into intracellular compartements

In addition to its toxicity, CT also provides strong adjuvanticity when administered per os[221,222] or other mucosal and systemic routes[223-225]. Cholera toxin is the most potent oral adjuvant in mice. However, due to its toxic effect it is precluded from human use. In trials to separate the toxicity from the adjuvanticity, recombinant versions of CT and LT have been produced. One such, is the CTA1-DD fusion protein which links the enzymatically active CTA1 subunit with a dimer of the D-fragment from *Staphylococcus aureus* protein A[226]. This fusion protein retains the adjuvant effect but is devoid of the toxic effects generated by CT holotoxin[226]. CTA1-DD activates complement, through its DD-domain, enabling interaction with complement receptors on follicular DCs [227]. Immunization with CTA1-DD as adjuvant co-administered with T dependent antigens augments the GC formation and serum antibody production and generation of long-term memory response[227]. Successfully modified versions of LT, have also been made. In particular, the double mutant LT (dmLT) with 2 point mutations leading to inability to release the A1 subunit, results in effective protection without toxic effects and is included in human clinical trails[228,229].

Activation of intestinal DC by CT

CT induces various responses in DCs including migration and activation and its adjuvanticity is believed to work through interactions with DC[203[230,231]. DCs accumulate in MLN 24h after oral administration of CT[232,233]. Preceding this, the number of DCs are increased in the small intestinal lamina propria already with the first to second hour after the administration of CT [232]. The influx of DCs to MLN results in elevated proliferation of T cells[232,233]. The activated T cells secrete IL-4, IL-13 and to a lower extent IFN-γ[232,233]. CT have generally been considered as a Th2 adjuvant but more recent studies have shown a mixed Th1/Th2/Th17 type of response[234].

In contrast to MLN, the number of DC in PP remains stable during 48h after p.o. CT. Although DC numbers in PP may stay the same, intra gastric administered CT induces a rapid migration of DCs into the FAE, in particular[235]. This is transient and within 12-24h the efflux of DC brings the DC levels back to normal. A more sustained response of oral immunisation with CT, is the migration of DC from the SED into the IFR[230].This migration is seen in both the $CD11b^{+}$ and DN cDC populations within the SED[58,230,236]. Of note, $CCR6^{-/-}$ mice lack the SED populated $CD11b^{+}$ cDCs specifically and generate a lower antibody response exclusively after oral, but not systemic, immunization with CT[236].

Traditionally vaccines have been used based on empirical results, reflected by the abundant use of alum as an adjuvant with action mechanism that are still incompletely understood. However, more recently, increased knowledge of the mechanisms of adjuvanticity have led to the rational design of vaccines[237]. In order to induce successful vaccination, memory cells in both humoral and cellular immunity is needed. DC as the main APC to prime naïve lymphocytes, thus function as the main inducer of these adaptive immune responses necessary to elicit the long-term memory. Hence, efforts in gaining understanding of DCs may contribute to knowledge important in vaccinology

Aim

The overall aim of this thesis work was to elucidate the role of intestinal DC subsets in the induction of immune responses following oral administration of antigen, with or without CT as adjuvant.

The specific aims were:

- To determine the requirement of cDCs for $CD4^+$ T cell activation and subsequent antibody production following oral immunization
- To develop a microsurgical technique to characterize cDC migrating in murine intestinal lymph under steady state and following oral immunization
- To assess the role of cDCs subsets in the generation of antigen-specific immune responses following oral immunization with CT
- To elucidate the signaling pathway involved in intestinal DCs activation and induction of antigen-specific antibody responses following oral immunization with CT.

Key methodologies

Mice

Wild-type (WT) BALB/c (paper II) were purchased from Taconic, Denmark and WT C57Bl/6 (paper I and III) were purchased from Taconic, Denmark or Harlan, Netherlands. All transgenic and knockout (KO) mice were bred on the C57Bl/6 background, with the exceptions of $CD47^{-/-}$ and DO11.10 mice, which were on BALB/c background. All mice were housed under specific pathogen-free conditions at the Experimental Biomedicine Animal Facility, University of Gothenburg, Gothenburg, Sweden. Unlike humans, mice do not naturally express the receptor for diphtheria toxin (DTR), so transgenic CD11c-DTR (B6.FVB-Tg Itgax-DTR/GFP 57Lan/J) mice were constructed, expressing DTR under control of the CD11c promoter, enabling conditional CD11c-expressing DC ablation upon diphtheria toxin (DTx) administration. An additional technique specifically targeted to cDCs was the use of the cre-lox system to evaluate the importance of $G_s\alpha$ specifically in cDCs (Fahlén-Yrlid et al submitted). Mice in which $G_s\alpha$ was floxed[238] were crossed with mice with the enzyme Cre targeted to the CD11c locus [239]. In the offspring, when Cre is actively expressed, i.e in cDCs, a loop is formed between the two loxP loci and the intervening $G_s\alpha$ is enzymatically deleted.

Immunizations (paper I-III)

For measurements of antibody responses, the model antigen, ovalbumin (OVA), was administered per os (p.o.) at 300 μg/mouse, with or without 10 μg/mouse of cholera toxin (CT) on four occasions ten days apart. For DC activation measurements, 10 μg/mouse of CT was administered p.o. 24h before sacrificing the mice.

Oral tolerance (paper I)

For induction of oral tolerance, 50 mg (or 5 mg) of OVA, or PBS as control, was administered p.o. to $CD47^{-/-}$ and BALB/c mice. Ten days later, all mice were challenged with 100 μg OVA in incomplete Freud´s adjuvant (IFA) subcutaneously. Draining inguinal LN were excised after one week and harvested cells were restimulated with OVA, 10-fold titrations up to 1mg, for three days. Proliferation of cells was assessed by [^{3}H]thymidine incorporation the last 6 h.

Bone marrow chimeras (paper I-III)

CD11c-DTR transgenic mice given DTx injection will respond by efficient ablation of DCs *in vivo* [240]. Unfortunately, the injection results in death of the mice within 6-7 days, due to the loss of CD11c-expressing, radiation-resistant, non-hematopoietic cell type/s [241]. Therefore, generating BM-chimeras from irradiated WT mice, reconstituted with CD11c-DTR BM, enables study of the mice for longer periods after injection of DTx. Bone marrow (BM) chimeras were generated by lethal irradiation (1000rad) of mice and reconstitution with BM from a donor with genome diverse from the host. The approach of BM-chimeras was employed to elucidate the importance of CD47 in different cell types. $CD47^{-/-}$ mice were reconstituted with WT bone marrow after irradiation, but the reverse chimerism was not possible as $CD47^{-/-}$ cells would be eradicated by WT ($CD47^{+/+}$) macrophages. This is due to that CD47 expressing cells deliver inhibitory phagocytic signals to macrophages via the ligand CD172a. Thus, CD47-deficient cells in a CD47 competent host cannot deliver these inhibitory signals and will hence be phagocytosed.

Bone marrow DC culture (paper I and III)

Bone marrow was flushed from femurs and tibias from BM donors and resulting BM cells were filtered. After red blood cell lysis, remaining cells were established in culture in ISCOVEs complete medium supplemented with murine FMS-like tyrosine kinase 3 ligand (m-FLT3L). FLT3L was generated from supernatants of CHO cells transfected with a plasmid containing m-FLT3L and a FLAG peptide followed by purification in anti-FLAG columns [242].

Adoptive transfer

Spleens and LNs from mice harbouring OVA-specific $CD4^+$ T cells, OT-II or DO11.10, were harvested, red blood cells were lysed and remaining cells washed. The $CD4^+$ T cell fraction was enriched by magnetic separation and stained with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) before intravenous (i.v.) injection into recipients, $2.5\text{-}5x10^6$ cells/mouse. CFSE is preserved in the cell cytoplasm during cell divisions. Thus, the proliferation of CFSE labeled T cells, can be followed as the CFSE content is halved for every division. This technique is commonly used to study antigen specific T cell activation. To induce T cell proliferation in adoptively transferred mice, the day following adoptive transfer of the T cells mice were immunized with OVA p.o., intra nasal (i.n.), or i.v. - with or without CT 10μg p.o. Lymph nodes and lymphoid organs were harvested for analysis three days later.

Antibody assay (paper I-III)

Intestines and serum were collected one week after the last immunization, for measurements of antibody responses. The mice were perfused with heparin-containing PBS before extraction (PERFEXT-samples) of the small intestine. Antigen-specific antibody titers in the samples were measured using enzyme-linked immunosorbent assay (ELISA). The plates were coated with OVA and blocked for non-specific binding with bovine serum albumin (BSA). Samples and a positive control were added and 3-fold dilution series were made. Bound antigen-specific IgA and IgG in the samples was detected by adding a secondary antibody, conjugated with horseradish peroxidase (HRP). O-phenylene-diamine (OPD) dihydrochloride substrate was added and antigen titers were assessed by measuring the highest concentration giving an optical density with a value of 0.4 above background.

Flow cytometry and cell sorting (paper I-III)

The characterization and analysis of different properties of cells have been a major methodology throughout the work described in the papers for this thesis. For this, the technique of flow cytometry has been applied. Flow cytometry is a method in which cells in single cell suspension travel through a nozzle, and diffract laser light, which is collected by detectors. Scattered light gives information on granularity and size. Additional detectors sense the emitted light from laser-excited fluorochrome-conjugated antibodies - directed to epitopes of interest. Expression of both cell-surface and intracellular proteins can be detected. Additionally, live cells with different properties can be sorted from complex populations and can be included in further experiments, by fluorescence activated cell sorting, FACS, whereby an electrical charge is placed on the cells according to chosen properties, and cell populations are sorted into separate tubes, directed by magnetic fields. For FACS, a FACSAria II was used to sort DC subsets and OT-II cells for co-culture, as well as for analysis. Cell phenotype analysis and cytokine production by OT-II cells were performed using a LSR-II flow cytometer in conjunction with cytometric bead assays.

Thoracic duct cannulation (paper III)

Extensive work has been carried out on the technique to collect murine intestinal lymph, by cannulation of the thoracic duct (TD). As DCs within MLN, are not conclusively distinguishable if they have derived from blood or mesenteric lymph, this technique was set up to enable study of known intestine-derived DCs. In addition this technique has the advantages of minimal physical or enzymatic manipulation. At four weeks of age MLNs were surgically removed by mesenteric lymphadenetomy, MLNX. After six weeks afferent and efferent lymphatic vessels (leading to and from MLN, respectively) had anastomosed. Mice then underwent thoracic duct cannulation (TDC), in which lymph, now containing DCs from the intestine, was collected for up to 24 h, before performing further analysis. Cannulation of mice with intact MLNs give virtually no DCs as all DCs are trapped in the draining LNs (Figure D1A; in the discussion). In contrast, in MLNX mice a clear DC population derived from the intestine, that no longer is trapped in MLN, is present (Figure D1B). In MLN, the same $CD11c^{+}MHC\text{-}II^{bright}$ population of intestinal migratory DCs is present along with an additional blood-derived tissue-resident $CD11c^{+}MHC\text{-}II^{+}$ cell population (Figure D1C). The two techniques used for MLNX and TDC within the thesis are described below. In both techniques, isoflurane anaesthesia was used, with Temgesic (buprenofen) and Marcain (bupivacaine) given for systemic and local analgesia, respectively.

For MLNX, the abdomen was shaved and after incision along the *linea alba* the intestine was exteriorised to expose the MLNs. Using fine forceps, MLNs were removed by gentle pinching. The intestines were replaced and the muscular layer and skin were sutured. Surgery and recovery were carried out on a heat pad. Mice were maintained for at least six weeks before TDC.

For TDC, the incision was made just below the ribs on the left abdomen. The TD is situated proximal of the adrenal gland and dorsal (but adjacent to) the abdominal aorta. To better visualize the TD and the point of incision, the procedure was performed under a 10x magnification microscope. A small incision, using either microscissors or needle, was made to allow cannulation with a polyurethane cannula (outer diameter of 0.6mm). For optimal flow, the cannula was filled with heparin-supplemented PBS. When inserted, the cannula was glued into place. The cannula exited through the muscle and along the back of the mouse and out through the skin in the neck. The cannula was then inserted into a metal spiral in a harness to allow the collection of intestinal lymph during normal activity. The normal activity of the mouse ensured good lymphatic flow and, thus, higher DC yield than a mouse under anaesthesia, which was the standard protocol used initially during establishment of the TDC experiments.

Results

Paper I: CD11chigh Dendritic Cells Are Essential for the Activation of CD4+ T Cells and the Generation of Specific Antibodies following Mucosal Immunization

The development of CD11cDTR mice[240] introduced a new ability to specifically study the role of DCs in different settings. Initially, the majority of studies had focussed on the role of DCs in parenteral immunization, most often related to $CD8^+$ T cell activation. Parenteral immunization is effective for the induction of systemic protection, but the majority of pathogens use mucosal surfaces as the point of entry into the body and many have adapted for replication within mucosal tissues. Hence, mucosal immunizations are more effective than parenteral at a local level and, in addition, are also good inducers of systemic protection. The role of DCs in mucosal immunization, particularly with regard to their interaction with $CD4^+$ T cells, had not been studied at the onset of the PhD studies and is the focus of paper I.

Conventional DCs are essential for the activation of $CD4^+$ T cells

In paper I, BM-chimeras were used, in which the WT hematopoietic cell compartment was exchanged for CD11cDTR BM (CD11cDTR/WT). Firstly, it was established that $CD11c^{high}$-expressing DCs (conventional DCs; cDCs) were efficiently ablated after injection of DTx into both CD11cDTR/WT and CD11cDTR mice. Although more efficient in some tissues than in others, the depletion of cDCs was evident in all organs analysed: spleen, MLN, LP, PP, peritoneal exudate cells, nasal associated lymphoid tissue (NALT) and cervical LN (CLN). To test the effect on proliferation of $CD4^+$ T cells, adoptive transfer experiments were carried out. Injection of DTx was given 70hrs and 2hrs before i.v. transfer of OVA-specific $CD4^+$ T cells. The mice were immunized 18hrs after depletion and after a further 3 or 5 days the organs were harvested. The administration of DTx and subsequent loss of $CD11c^{high}$ DCs resulted in markedly reduced proliferation of responding T cells after both oral and nasal immunization with OVA. This was not due to altered recruitment or baseline activation of adoptively transferred T cells, as frequency of total and $CD69^+$ OVA transgenic $CD4^+$ T cells retrieved in LNs was unaltered after transfer. Addition of CT as an adjuvant demonstrated a dependence of cDC for proliferation of T cells similar as for OVA without adjuvant. Additionally, the activation of responding T cells, measured by expression of CD69, was decreased in mice where cDC were depleted by DTx injection (Figure R1). Hence, cDCs are vitally important for the activation of $CD4^+$ T cells after oral and nasal administration of OVA, with or without CT.

Conventional DCs are critical for antibody responses

To investigate if the defect in activation of $CD4^+$ T cells resulting from from cDC depletion gave rise to any downstream effect, the generation of antigen-specific antibodies was next assessed. Thus, CD11cDTR/WT were immunized once with OVA and CT, after depleting cDC or not. Following oral and nasal immunization, both anti-OVA and anti-CTB IgG in serum were abrogated in mice devoid of cDCs. Unfortunately, anti-OVA IgA responses in intestines were at undetectable levels, regardless of depletion of cDCs or not. Evidently, a single immunization was not sufficient to induce detectable levels of anti-OVA IgA in the intestine. However, several immunizations with preceding DC depletions would have resulted in depletion of plasmablasts, in addition to DC, as B cells differentiating to plasma cells up-regulate CD11c-expression[243]. Nevertheless, anti-CTB IgA was detected and was at a significantly lower level than for mice with an intact DC pool (Figure R2). Taken together, these data indicate that cDCs are critical for the generation of OVA-specific serum IgG and intestinal IgA following oral immunization.

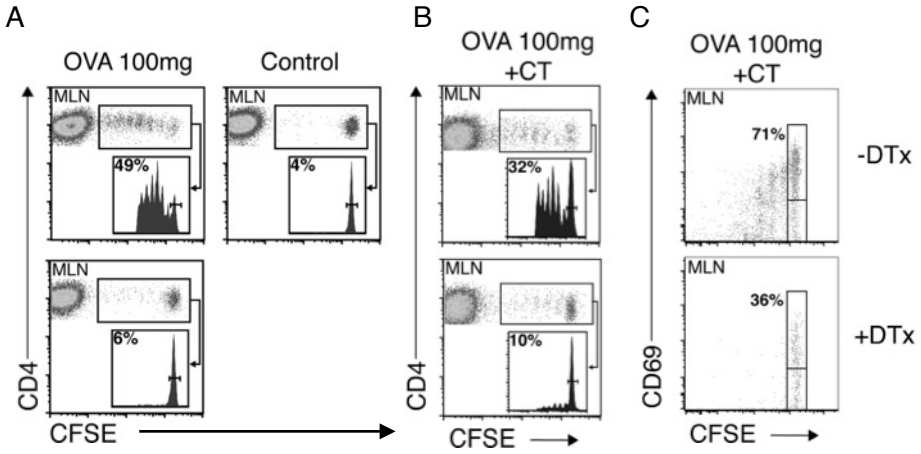

Figure R1. Activation of T cells is dependent on cDCs. Proliferation of OT-II cells in MLN after **A,** oral OVA (left), or PBS administration (right) and **B,** OVA+CT immunization. Numbers indicate the frequency of cells that entered division. **Upper panels,** without DTx injection, **Lower panels,** after DTx injection. **C,** Percentage of $CD69^+$ non-divided OT-II cells.

Conventional DC dependence is overridden by increased antigen dose

Tolerance at mucosal surfaces can be broken by high doses of antigen. It has been suggested that high concentrations of antigen might lead to an altered immune response because antigen may then become accessible to APCs other than cDCs[244]. For this reason, a high dose of antigen, 30 times more than required for detection of T cell proliferation in a control animal, was administered. At this dose of antigen, proliferation of $CD4^+$ T cells was also seen in mice depleted of cDCs, i.e. overriding the dependence for cDCs seen under conditions of limited antigen dose. This prompted a search for the cells responsible for inducing this proliferation. In CD11cDTR/WT mice $CD11b^{high}CD11c^{-/low}$ myeloid cells (mostly Ly6G expressing neutrophils) were recruited after DTx injection. However, as these cells were poor inducers of T cell proliferation *in vitro*, they were most likley not APC candidates in this setting. To study the importance of B cells *in vivo* in DC-depleted mice, mixed BMCh mice were constructed. Thus, B cell-deficient mice (μMT) were bred with CD11cDTR to enable combined B- and DC-deficiency. However, mice lacking all B cells also suffer from structural immune defects presumingly affecting more than B cell immune response. To circumvent this drawback, BM from CD11cDTR/μMT offspring, was mixed with BM from MHC-II deficient ($MHC\text{-}II^{-/-}$)

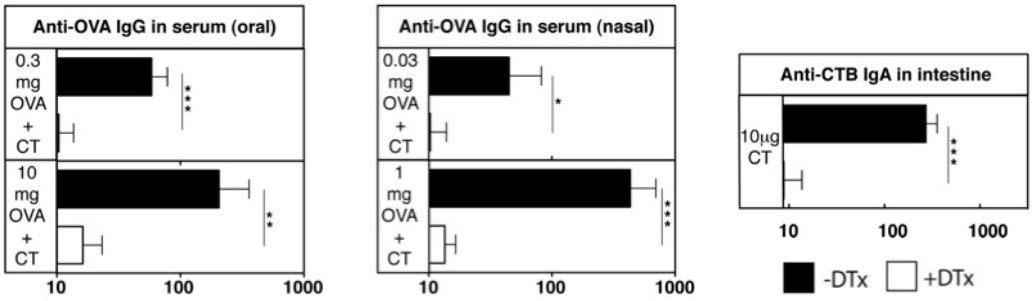

Figure R2. OVA- and CTB-specific antibody responses are dependent on cDCs. Antibody responses towards OVA in serum after **A,** oral and **B**, nasal immunization. **C,** IgA antibodies against CTB, measured in the intestine.

mice, which have B cells but which are unable to present Ag. These mixed BMCh mice showed proliferation of T cells after depletion of the DTx sensitive cDCs, when the high dose of antigen was given, like in CD11cDTR mice. To analyze the possibility that pDCs influence T cell proliferation, CD11cDTR/WT mice was injected with the depleting monoclonal antibody 120.G8, directed against pDCs, for a combined depletion of pDCs and cDCs upon DTx treatment. Although demonstrating cDC dependence at low antigen dose, a 30-fold increase in dose induced T cell proliferation even after addition of the 120.G8 antibody, confirming that pDCs are not required for the proliferation seen at high antigen dose.

In this paper, the dependence of cDCs for $CD4^+$ T cell activation after mucosal immunization, with or without CT, was demonstrated, and it was shown that cDCs were essential for the generation of antigen-specific antibodies. However, a very high dose of Ag still elicits T cell proliferation, but neither pDCs, B cells and most likely not newly recruited $CD11b^{high}CD11c^{-/low}$ myeloid cells are responsible for this proliferation.

Paper II: CD47-deficient mice have decreased production of intestinal IgA following oral immunization but maintain the capacity to induce oral tolerance

Following the confirmation of the importance of cDC in the induction of immune responses to fed antigen in mice, we next wanted to address the role of subsets within the diverse DC population. The maintenance of homeostasis of $CD11b^+$ DCs in lymphoid organs had been reported to be dependent on the expression both of the ubiquitously expressed glycoprotein CD47 and of one of its ligands, namely CD172a (SIRP-a)[97]. Also, using mice deficient for CD47 or in CD172a-signaling, the dependence of these two proteins for the migration of the $CD11b^+$ DCs, to draining LNs from skin, had been shown [92,93,95]. However, the importance of CD47 in both oral tolerance and adaptive immune responses was unknown, so in paper II, CD47-deficient mice were used to determine its importance in orally induced immune responses. At the same time, the role of CD11b-expressing DC subset could be elucidated in this setting, if CD47-dependent migration of $CD11b^+$ DCs would be applicable also to non-skin draining LN, i.e. gut-draining MLN (as this subset would be lacking in herein).

CD47-expression is important for maintaining cell numbers in gut

The basal DC phenotype in CD47$^{-/-}$ mice was firstly established, in comparison with WT mice. Mice deficient in CD47 have as much as a 50% reduction of the number of total leukocytes, selectively within MLN, small intestinal LP and PP, while maintaining normal cell numbers in spleen and skin-draining axillary and inguinal LNs (Table 1).

Table 1. Total number of cells in different organs

	Organ					
Strain	ALN	ILN	Spleen	MLN	LP	PP
Wild-type	6·98	3·51	82·14	25·00	7·60	8·53
	± 2·58	± 1·43	± 16·12	± 7·60***	± 3·75**	± 3·58**
Knockout	6·85	3·18	63·23	14·25	3·25	5·04
	± 2·49	± 0·92	± 19·54	± 3·77	± 2·54	± 3·01

ALN, axillary lymph node; ILN, inguinal lymph node; MLN, mesenteric lymph node; LP, lamina propria; PP, Peyer's patches.
The organs were harvested and digested in liberase or collagenase (LP) after EDTA-treatment (LP + PP) and the total number of live cells in the cell suspensions was determined as non-Trypan blue stained cells. Data shown are median × 10^6 ± SD, and represent data from at least three experiments with four mice in each.
$^{**}P < 0.005$ and $^{***}P < 0.001$ using Student's *t*-test.

For further comparison of DC and DC subset between CD47$^{-/-}$ and WT mice, frequencies and not absolute numbers were used, due to the skewed decrease in total cell numbers within the GALT of CD47$^{-/-}$ mice. cDCs were present at a reduced frequency in MLN but not in PP or LP (Figure R3a). Within MLN, two populations of cDCs are found, CD11c$^+$MHC-II^{bright} and CD11c$^+$MHC-II$^+$ (Westlund et al, manuscript; paper III). The CD11c$^+$MHC-II$^+$ population contains mostly CD103$^-$, DCs while the CD11c$^+$MHC-II^{bright} population contains mostly CD103$^+$. CD103 has been shown to be highly expressed by most but not all DCs migrating in intestinal lymph (IL-DCs) ([102]Westlund et al. manuscript, paper III). Thus, the level of expression of MHC-II rather than CD103 was applied to discriminate IL-DCs from lymph node resident DCs in MLN. Within both CD11c$^+$MHC-II^{bright} and CD11c$^+$MHC-II$^+$ DCs in MLN, the cDC subset expressing CD11b exist at a significantly lower frequency (Figure R3b and c). Also CD11b$^+$ among CD103$^+$ DCs in LP are significantly reduced in frequency (Figure R3d). However, frequency of CD11b-expressing DCs in PP is normal (Figure R3e). cDCs expressing CD11b mutually express CD172a[92,93,97,245].

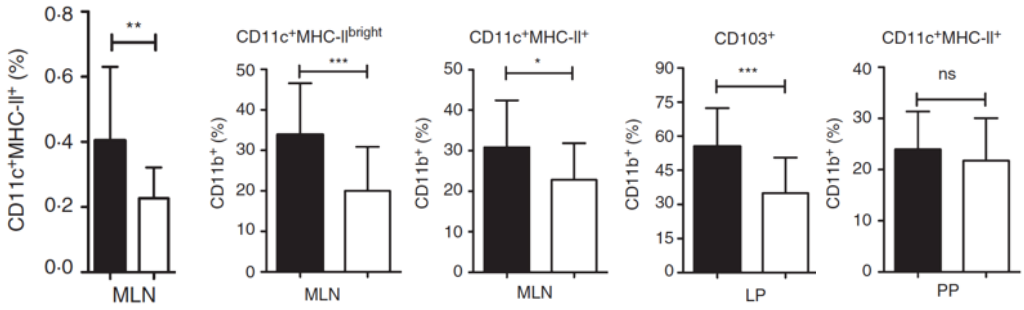

Figure R3. Frequency of cDCs and cDC subsets. **A,** Frequency of CD11c$^+$MHC-II$^+$ cells in MLN among live leukocytes. **B,** Frequency of CD11c$^+$MHC-II^{bright} cells and **C,** CD11c$^+$MHC-II$^+$ cells among total cDCs in MLN **D,** Frequency of CD103$^+$ DCs among total cDCs in LP **E,** Frequency of CD11b$^+$ DCs among total cDCs in PP.

Impaired mucosal but not systemic immune response following oral immunization of CD47$^{-/-}$ mice

The immunological relevance of this cellular defect in CD47$^{-/-}$ mice and, more specifically, the importance of the CD11b-expressing DC-subset, was evaluated next. Mice with MLNs surgically removed (by mesenteric lymphadenectomy) are incapable of establishing oral tolerance (own data and [246]), but still fully capable of generating antigen-specific antibody responses. This underlines the importance of MLN as a site for maintaining the steady state in the gut. Therefore, the proliferation of CD4$^+$ T cells in MLN was assessed after feeding OVA only.

First, the proliferation of adoptively transferred OVA-specific CD4$^+$ T cells was assessed. After feeding OVA, the transferred T cells in CD47$^{-/-}$ mice exhibited a reduced proliferation profile compared to in WT mice. Interestingly this reduced proliferation was a mucosal-restricted phenomenon, as parenteral (i.v.) immunization resulted in equally good proliferation in the two strains of mice (Figure R4).

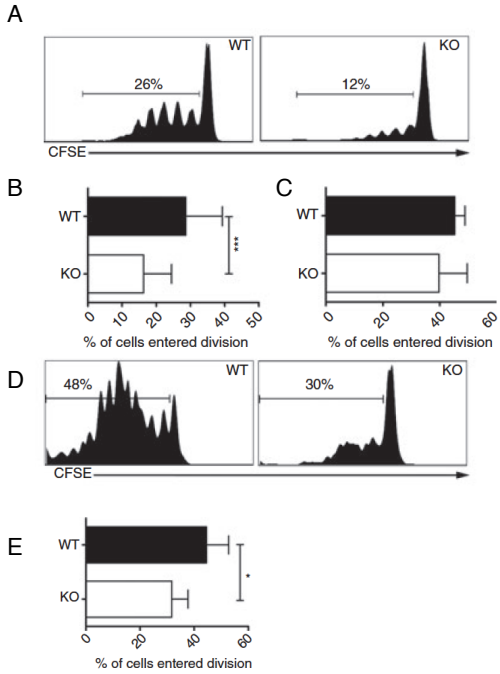

Figure R4. T cell proliferation after oral and i.v. immunization with OVA+/-CT. Frequency of CD4$^+$ OVA transgenic T cells in MLN that entered cell division after p.o. immunization with **A and B,** OVA **D and E** OVA+CT. **C**, Frequency of cells in spleen that entered division after i.v. immunizations with OVA. Pooled data from at least 3 experiments with 2-4 mice in each.

As CD47$^{-/-}$ mice exhibited reduced cell numbers particularly in MLN, within the CD11b-expressing DC population, combined with the fact that these mice showed a reduced proliferation only after mucosal immunization, we hypothesized that the ability to induce oral tolerance would be reduced in these mice. However, induction of oral tolerance was at least equally efficient in CD47$^{-/-}$ mice and WT mice (Figure R5). Next the importance of CD47 expression after including CT as an adjuvant was addressed. The proliferation pattern of adoptively transferred CD4$^+$ OVA-specific T cells was no different from feeding OVA without adjuvant, i.e. exhibited reduced proliferation in CD47$^{-/-}$ mice specifically after mucosal immunization.

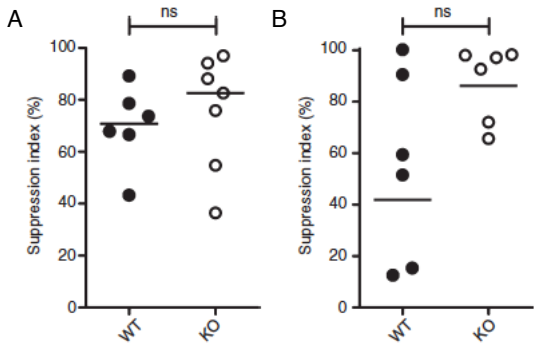

Figure R5. Maintained ability to induce oral tolerance in CD47$^{-/-}$ mice. CD47$^{-/-}$ and wild-type mice were fed PBS or **A,** 50 mg OVA, **B,** 5 mg OVA, challenged with OVA+IFA, s.c. and OVA-specific proliferation was measured in the draining LNs after re-stimulation *ex vivo*. Suppression index is calculated as the proliferation (counts/min) of T cells from OVA-fed mice in relation to PBS-fed animals that were normalized to 0%. Results are **A,** pooled from two experiments with two to four mice in each or **B,** from one experiment with six mice per group. Bars show grand median.

Next, effects on antibody production were examined. After three immunizations with OVA and CT, the OVA-specific antibody responses in CD47$^{-/-}$ mice revealed an impaired intestinal OVA-specific IgA (Figure R6a). In contrast, the generation of serum anti-OVA IgA and IgG titers were comparable in wt and CD47$^{-/-}$ mice (Figure R6c and d). This defect was not due to a general disability to produce IgA in the intestine, as total anti-IgA response herein was normal (Figure R6b). Thus, the defects in these CD47$^{-/-}$ mice were skewed to mucosal tissues, i.e. intestinal antigen-specific antibody generation, and yet induction of oral tolerance was unaffected.

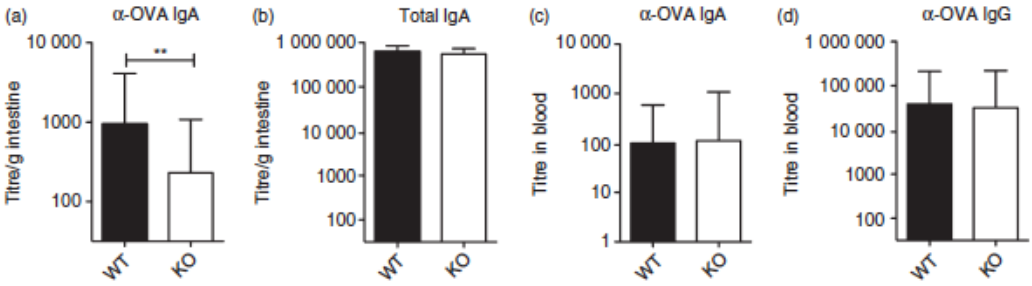

Figure R6. Intestinal OVA-specific IgA titer is reduced in CD47$^{-/-}$ mice. WT and CD47$^{-/-}$ mice were fed OVA+CT three times. One week later **A,** anti-OVA-specific IgA and **B,** total IgA in perfused intestines, or **C,** anti-OVA-specific IgA and **D,** IgG titers in serum were determined. The graphs show titers from four experiments with (a, b, d) at least three mice or (c) two or three mice in each. Error bars show SD.

Non-hematopoietic cells and not the reduced frequencies of CD11b^{+} cDCs are responsible for the impaired mucosal immune response in CD47$^{-/-}$ mice

In order to reveal the ontogeny of the cell responsible for the gut-associated defects in CD47$^{-/-}$ mice, BMCH was used. CD47 is an innate marker of self and, through its ligation to macrophage SIRPα (CD172a), macrophage phagocytosis is prevented by inhibitory signaling through SIRPα [89]. Therefore, only WT BM can be transferred to CD47$^{-/-}$ and not vice versa. When lethally irradiated CD47$^{-/-}$ mice were reconstituted with WT BM, this did not restore either gut cellularity, i.e. number of cells or intestinal antibody production but, however, the frequency of CD11b^{+} DCs was restored. As the frequency of CD11b^{+} DCs is reduced in MLN, and not PP of CD47$^{-/-}$ mice, and the defect lies not in oral tolerance induction, but rather in intestinal antibody generation, CD11b^{+} DCs are seemingly not responsible for the defects in CD47$^{-/-}$ mice. Therefore, we conclude that CD47 is dispensable for oral tolerance induction

and that OVA-specific IgA production is dependent on non-hematopoietic cells expressing CD47 rather than on the decreased frequency of $CD11b^+$ DCs.

Paper III: Oral adjuvant activity of cholera toxin is independent of classical toll-like receptor signaling, but requires $G_s\alpha$ expression in $CD11c^+$ dendritic cells

In paper I we show the dependence of cDCs for $CD4^+$ T cell activation and antibody production after oral immunization adjuvanted with CT. In paper II, the role of $CD11b^+$ cDCs was addressed in CD47 deficient mice. In paper III we extended our analysis of the effect of oral administration of CT to other gut derived subsets of cDC. Further we wanted determine which signaling pathways, primarily in DCs, that were involved in orally induced immune responses after CT vaccination.

The role of different intestinal lymph DC subsets after oral CT administration

To continue the study on gut-derived DC subsets and their role in orally induced immune responses we used the cannulation technique that I have established during my PhD studies. This technique enables the direct study of DCs migrating from the gut; IL-DCs.
At the time for cannulation, mice were fed CT or left unfed as controls. Flow cytometric analysis of IL-DCs in these mice revealed that CT induced a significant increase in the frequency of cDCs migrating in intestinal lymph. (figure R7A). IL-DCs in lymph can be divided into four subsets with regard to the integrins CD103 and CD11b, I) $CD103^+CD11b^+$, II) $CD103^+CD11b^-$, III) $CD11b^-C103^-$ and IV) $CD103^-CD11b^+$ (figure R7A, right panels). Analysis of the subset frequencies, determined that there was no preferential increased exit of either (figure R7A, right panels). Interestingly, all IL-DCs subsets, except for the $CD103^+CD11b^-(CD8\alpha^+)$, showed a significant increase in CD86 expression following CT compared to unimmunized control mice (Figure R7B).

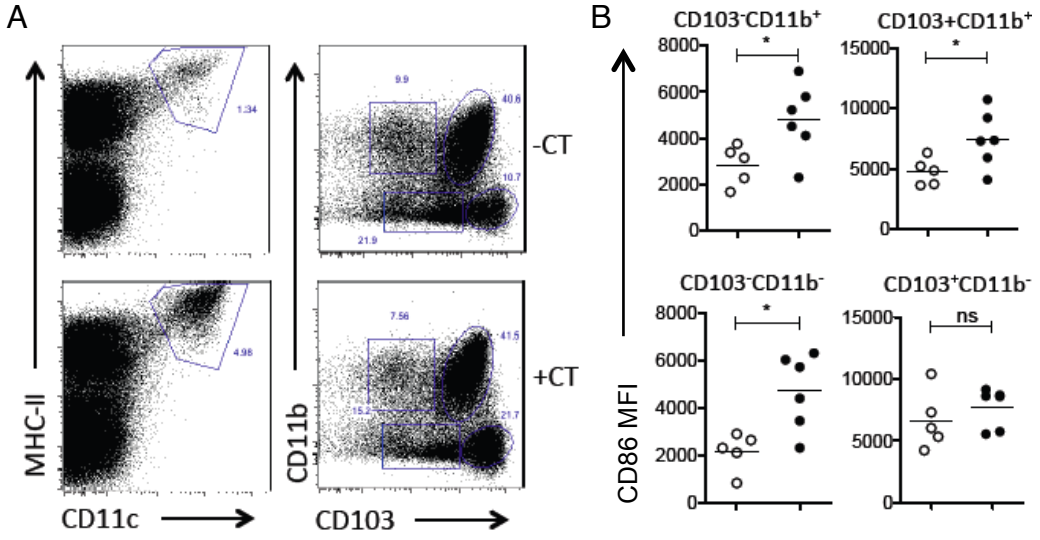

Figure R7. IL-DCs from CT-treated or untreated cannulated MLNX mice. **A,** Frequency of DCs among all live leukocytes (left)**,** the four different IL-DC subsets, in regards to CD11b and CD103 expression (right). Upper panel; non-treated, lower panel; CT-treated. **B,** Expression of CD86 within the different subsets, empty circles; untreated, filled circles; CT-treated.

To examine any differential influence on responding $CD4^+$ T cells in regards to proliferation and induction of cytokine production, the four different IL-DC subsets were sorted, pulsed

with OVA and cultured with CFSE labelled OVA-specific CD4+ T cells (OT-II cells). No obvious difference in proliferation of responding T cells was detected (not depicted), but their cytokine profiles were distinctive. The $CD103^{-}CD11b^{+}$ were major inducers of IFNγ-production in responding T cells during steady state. However, this production was reduced after CT administration, but with a parallel IL-10 production increase.

The role of Batf3-dependent DCs in oral immunization

The lack of up-regulation of CD86 by $CD103^{+}CD11b^{-}$ IL-DCs suggests that this subset might not be responding to CT in vivo. $Batf3^{-/-}$ mice have been reported to have dramatically reduced numbers of $CD103^{+}CD11b^{-}CD8^{+}$ DCs in MLN[79,80]. Interestingly, it was recently shown that these DCs, dependent on the transcription factor BATF3, were required when CT was used as an adjuvant in epicutaneous immunization[224]. To elucidate the importance of the $CD103^{+}CD11b^{-}$ DC subset in the oral adjuvant activity of CT, we took advantage of $Batf3^{-/-}$ mice.

Although the initial reports stated complete loss of the $CD11b^{-}$ DCs in the $Batf3^{-/-}$ mice, subsequent reports have pointed instead towards a marked decrease in $CD103^{+}CD11b^{-}CD8^{+}$ DCs, depending on different tissues and even different strains of mice. Cannulation of the thoracic duct in MLNx $Batf3^{-/-}$ and WT mice yield a similar frequency of total IL-DCs. In PP, the total DC frequency in $Batf3^{-/-}$ was decreased compared to WT controls. The subsequent quantification of subsets within IL-DCs and PP, revealed a dramatic, near, complete loss of the $CD103^{+}CD11b^{-}CD8^{+}$ DC-population (Figure R8A and B). However, $Batf3^{-/-}$ and WT mice showed comparable levels of anti-OVA IgA titers in the intestine after oral immunization with OVA and CT (Figure R8C). This showss that Batf3-dependent $CD103^{+}CD11b^{-}$ DCs cDCs are not essential for the antigen-specific antibody production in the intestine after oral immunization with CT.

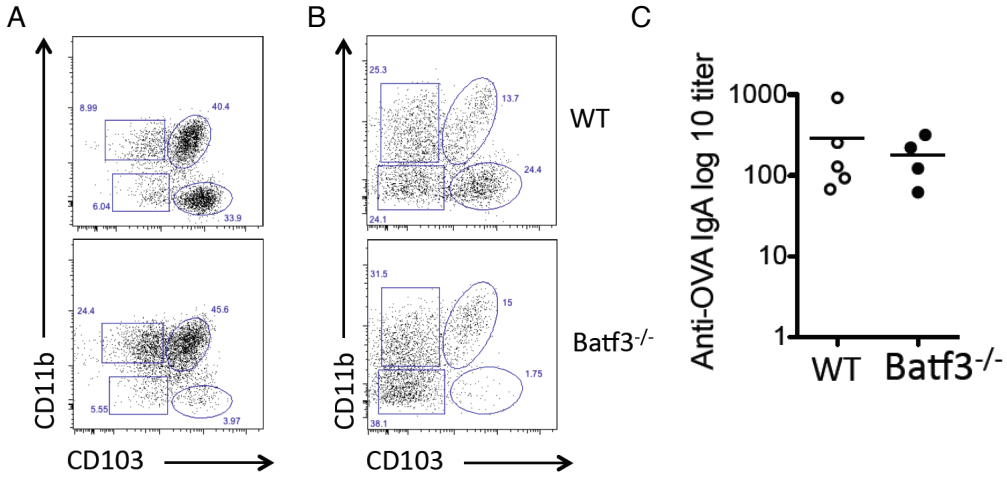

Figure R8. $Batf3^{-/-}$ dependent DCs are not essential for intestinal antibody responses. Frequency of the four subsets in **A,** intestinal lymph and **B,** Peyer's patches. Upper panel; WT, lower panel; $Batf3^{-/-}$. **C,** Intestinal OVA-specific IgA titer following OVA + CT p.o.

$G_s\alpha$-expression in cDCs is necessary for DC-activation and for the oral adjuvant activity of cholera toxin

In contrast to the well-known mechanism by which CT induces diarrhea, the mechanism of action for CT adjuvanticity is incompletely known. The diarrheal response results from the enzymatic activity of the CTA1 subunit and its ribosylation of $G_s\alpha$ G protein, resulting in constitutive production of cAMP and leading to ion efflux with accompanying water loss [247],(Iyengar 1993,[248]. The adjuvanticity of CT is also most likely coupled to the enzymatic activity of CTA1, as most enzymatic inactive mutants of CT do not elicit antibody production. Recent results from our lab show that the ganglioside GM1, the only known receptor for CT, needs to be expressed on DC, in order for orally administered CT to elicit adjuvant activity [249]. We therefore next wanted to explore which signaling pathways in cDCs that are involved in the oral adjuvant activity of CT. Applying cre-lox-technology, mice in which $G_s\alpha$ was deleted specifically in CD11c-expressing cDC, CD11c-cre X flox GsA (CD11cGsa), were generated (Fahlén-Yrlid et al submitted). While oral administration of CT resulted in upregulation of CD86 by gut-derived cDCs in the MLN of littermate controls, CD11cGsa mice failed to do so (Figure R9).

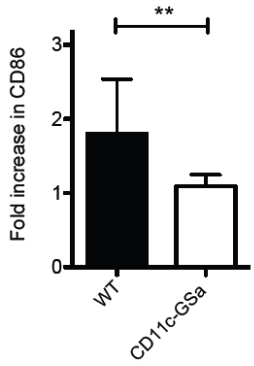

Figure R9. CT-mediated activation of intestinal cDCs *in vivo*. CD11cGsa mice and littermates were orally immunized with PBS or CT and 24h later single cell suspensions from MLNs were analyzed by flow cytometry. The analysis shows gated $7AAD^-CD11c^+CD45R^-$ cells being $CD11c^+MHC\text{-}II^+CD103^+$ and finally analysed for CD86 expression. The error bars show SD.

In addition, BMDC from CD11cGsa mice stimulated either with CT or CTA did not elevate CD86 levels. However, CD11cGsa BMDCs are able to upregulate CD86 in response to lipopolysaccharide (LPS) *in vitro*. Finally, CD11cGsa mice were immunized four times with OVA and CT. Both OVA-specific antibody titers in intestine and serum of CD11cGsa mice were severely impaired compared to littermates (Figure R10). Thereby we conclude that $G_s\alpha$ mediated signaling specifically in cDCs is essential for the oral adjuvant activity of CT.

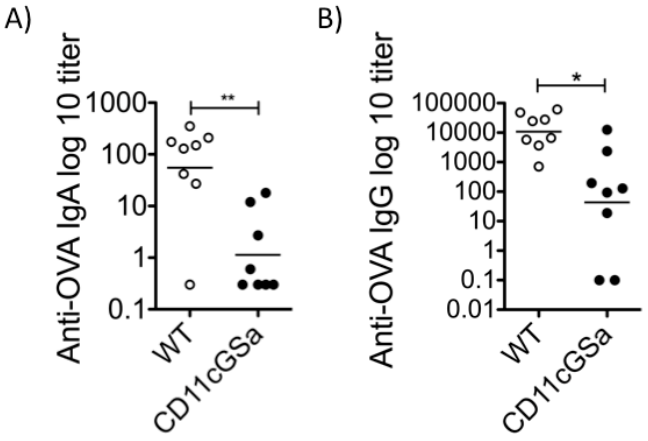

Figure R10. Impaired antibody responses in CD11cGsa mice following oral immunization with CT. CD11cGsa mice and littermate controls were fed OVA +/- CT four times at ten-day intervals. One week after the last immunization intestines and serum were collected and **A,** intestinal anti-OVA Ig A and **B**, serum anti-OVA IgG were determined.

The oral adjuvant activity of CT does not require TLR signaling

To evaluate the involvement of TLR-signaling in the oral adjuvant activity of CT, mice deficient for MyD88 were used. MLN-DCs of MyD88$^{-/-}$ mice, upregulated the co-stimulatory molecule CD86, in response to oral CT, in comparable levels to WT (Figure R11A). Additionally, repeated oral immunization with OVA and CT in MyD88$^{-/-}$ mice generated normal anti-OVA IgA and IgG responses (Figure R11B and C). TRIF signals via IFNαR, and thus mice with a combined deficiency for MyD88 and IFNαR, lacks the two adaptor proteins required for any TLR signaling. Still, MyD88/IFNαR$^{-/-}$ mice displayed similar levels of IgA and IgG to WT mice following oral immunization with OVA + CT (figure R11B and C). Hence, this shows that TLR-signaling is dispensible for the oral adjuvant effect of CT.

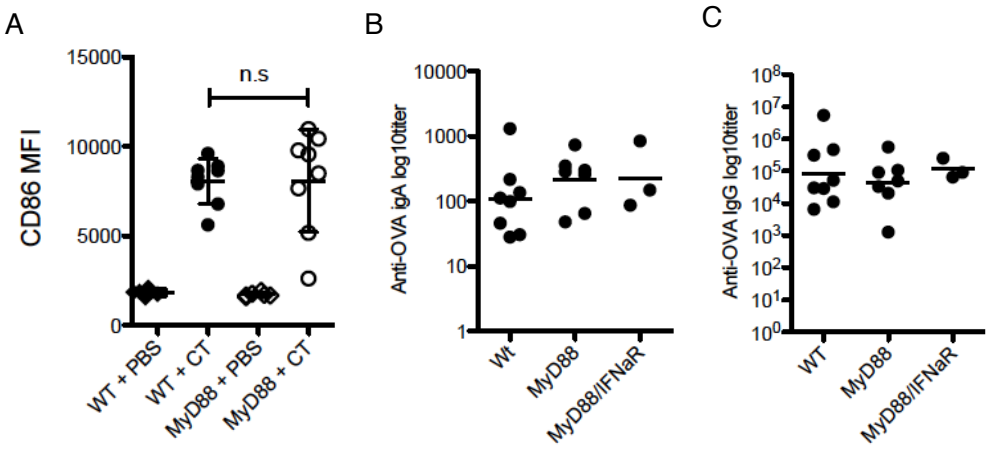

Figure R11. Activation of intestinal DCs and induction of antibodies in mice lacking classical TLR signaling after oral immunization with CT. **A,** WT and MyD88 deficient mice were orally administered PBS or CT. 24h later CD86 expression by intestine-derived cDCs in the MLN was measured by flowcytometry. **B-C,** Anti-OVA **B,** IgA titres in intestinal tissue or **C,** IgG titres in serum one week after the last of three oral immunizations with OVA and CT of WT, MyD88 and MyD88/IFNαR deficient mice. Each symbol represents an individual mouse. Error bars show SD.

Involvement of the inflammasome in the generation of intestinal IgA but not systemic IgG following oral immunization with CT

The cardinal feature in inflammasome signaling is the activation of caspase 1. Hence, mice deficient in caspase 1/11 (Casp1/11$^{-/-}$) were used to determine the role of the inflammasome in CT- induced oral immune responses. Gut-derived MLN DCs from Casp1/11$^{-/-}$ fed CT did not upregulate CD86 (Figure R12A) suggesting an incapacity of CT to activate DCs in vivo. In addition, the ability to generate anti-OVA IgA response in the intestine was after four p.o. immunizations with OVA impaired in Casp1/11$^{-/-}$ mice (Figure R12B and C). Comparing titers with GM1$^{-/-}$ mice, which show a total inability to generate CT-adjuvanted antigen-specific antibody-responses, Casp1/11$^{-/-}$ mice showed an intermediate response in intestinal antibody production (Figure R12B and C).In contrast, the serum anti-OVA IgG response in Casp1/11$^{-/-}$ mice was not impaired compared to WT mice. These results suggests that caspase1/11 dependent signaling is important for the activation of gut-derived DCs following oral immunization with CT. Furthermore they indicate that the inflammasome is involved in

generation of antigen-specific intestinal IgA but not systemic IgG following oral immunization adjuvanted with CT.

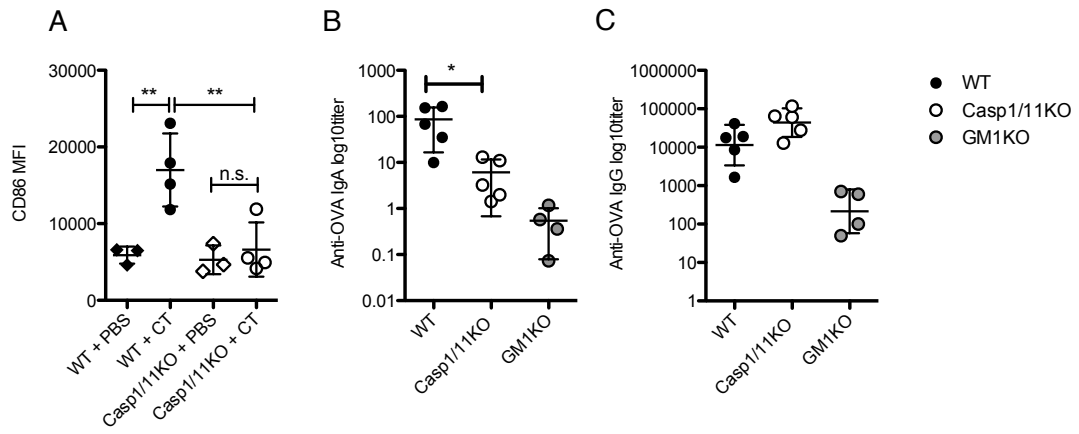

Figure R12. Activation of intestinal DCs and induction of antibodies after oral immunization with CT in Casp1/11$^{-/-}$. Casp1/11$^{-/-}$ and matched WT controls were orally immunized with PBS or CT. 24h later CD86 expression by intestine-derived cDCs in the MLN were gated and analysed. **B-C,** Anti-OVA **B,** IgA titres in intestinal tissue or **C,** IgG titres in serum one week after three oral immunizations with OVA and CT. Error bars show SD. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test where *$p<0.05$.

In this paper we investigated the adjuvant activity of CT with regard to DC signaling and subsets. We conclude that Gsα-expression in cDCs is necessary for activation of DC as well as for antibody response when CT is used as an oral adjuvant. This does not require TLR signaling but need caspase1/11 and hence inflammasome activity for the generation of antigen-specific intestinal IgA. Moreover, we conclude that CT is not dependent on CD103^{+}CD11b^{-} cDCs for its oral adjuvant effect.

Discussion

DCs are phenotypically and functionally heterogeneous. Improved knowledge of the immunobiology of intestinal DCs and their role during homeostasis and following immunization would be useful for vaccinology to mucosal pathogens, but also in the field of food allergies. In this thesis the role of different DC subsets in immune responses towards orally administered antigens has been investigated.

The role of $CD11c^+$ DCs in oral vaccination with CT

In paper I, we sought to elucidate the role of CD11c-expressing DCs in orally-induced immune responses. CD11c-DTR mice, in which cells expressing CD11c are sensitive to DTx and undergo apoptosis after DTx injection, were used for this purpose. Due to the essential expression of CD11c in non-hematopoietic cells, these mice succumb due to DTx-injection within a week. To circumvent this, BMCh mice were generated by irradiation of WT C57Bl/6 mice and re-constitution with BM from CD11c-DTR mice. Activation of $CD4^+$ T cells in response to oral OVA, measured as their proliferation, was abolished after depletion of cDCs. The addition of CT did not alter the cDC dependence for $CD4^+$ T cell activation. Furthermore, cDCs were essential for the generation of antigen-specific mucosal and systemic antibodies to orally (and nasally) administered OVA, adjuvanted by CT. However, when antigen concentration was not limiting, the dependence of cDC could be overridden.

Immediately after DC depletion, cells were recruited to lymphoid tissues. Interestingly, this had not been reported in previous studies with CD11c-DTR mice. Most recruited cells expressed high levels of CD11b, Ly6C and Ly6G, but did not express CD11c, or did so at very low levels. Splenocytes treated with DTx were poor inducers of proliferation of responding $CD4^+$ T cells *in vitro*. Even at 72h after cDC depletion *in vivo*, little T cell proliferation was observed *in vitro,* strongly indicating that the recruited cells do not differentiate into efficient APCs. Hence, the majority of the recruited population most likely represents non-conventional APCs such as neutrophils, not directly responsible for the activation of $CD4^+$ T cells *in vivo*. In unpublished studies, we have inhibited the influx of granulocytes induced by DTx by using depleting antibodies directed against Ly6C. However, this did not reduce the expansion of T cells in DTx-treated mice given a high dose of antigen.

It has been reported that OVA conjugated to pDC-directed antibodies can induce proliferation of OVA-specific $CD4^+$ T cells in cDC-depleted mice after s.c immunization[250], suggesting a role for pDCs as APCs for $CD4^+$ cells. In this study, Sapoznikov et al found no significant depletion of pDCs after DTx injection, while we have consistently observed a partial depletion, also reported by others[244,251]. The pDCs remaining after cDC depletion are fully functional, shown by the requirement for combined DTx treatment and pDC-depleting antibody treatment, for inhibition of OVA-specific antibody generation after a CpG-induced immune response. Nevertheless, proliferation of $CD4^+$ T cells induced by immunization with a high dose of antigen was retained after combined depletion of cDCs and pDCs. Thus, pDCs are not responsible for the activation of T cells observed after mucosal immunization of cDC-depleted mice with a high dose of OVA. Although Sapoznikov et al[250] showed that specific targeting of antigen to pDCs resulted in activation of $CD4^+$ T cells, they did not address whether removal of pDCs abrogated the activation. In addition, pDCs originating from skin (Sapoznikov), versus gut (our studies), could have different capacities to function as APCs. For example, skin pDCs have been shown to migrate in afferent lymph[107] while afferent lymph from mucosal tissues does not contain pDCs[106].

B cells were also excluded as the APC-population responsible for the activation of T cells after cDC depletion under non-limiting antigen conditions. This is consistent with a previous study where B cell-deficient mice retained $CD4^+$ T cell-activating capacity in skin-draining LNs, in the absence of cDCs[250]. Lymphocytes are important for correct DC localisation and function within lymphoid structures[252,253]. Therefore, to exclude influences of changed structure or overall lymphocyte cellularity within LN, our study was conducted in the presence of B cells, though deficient for MHC-II. In contrast, BMCh mice generated by reconstitution of μMT mice with mixed BM from CD11c-DTR mice and B cells deficient in MHC-II showed, after DTx injection, a reduced proliferative response of T cells, in comparison to BMCh mice generated using WT mice as recipients[254]. This may be explained by different routes of immunization and/or antigen dose used in this study compared to our study. Nonetheless, B cells may contribute to T cell proliferation but, if so, then most likely in collaboration with cDCs, or after $CD4^+$ T cells have been primed by cDCs. For example, previous reports have shown that cDCs are sufficient to initiate Tfh maturation, but cognate B cell interactions are necessary for their full differentiation into CXCR5, PD1 and Bcl-6 expressing Tfh[255].

DTx injection into CD11c-DTR mice also depletes marginal zone macrophages and their sinusoidal counterparts in LNs[256]. Therefore, it is unlikely that these cells are responsible for the cDC-independent proliferation of $CD4^+$ T cells after feeding a high dose of OVA. Additionally, it has been reported that mice ablated of macrophages by injection of chlodronate-containing liposomes, have increased rather than reduced number of antibody-secreting cells after T-dependent antigen immunization[257].

CD11cDTR mice are efficiently, although not absolutely, depleted of cDCs by DTx treatment. Thus, it is still possible that the remaining cDCs, although few, present antigen after high antigen dose. However, recent unpublished results from our group show that although T cells are activated after high antigen dose, the DTx-treated mice show a dramatically reduced ability to induce $CD4^+$ T cells secreting IFN-γ. Impaired isotype switching to IgG2c antibodies following immunization also occurs. This shows that although there may have been some residual cDCs, they were not able to perform functionally as when present in normal numbers. In addition, other studies with similar cDC-depletion efficiency as that observed in our study, show an almost complete inhibition of $CD8^+$ T cell responses following DTx treatment[240,251,258,259]. Hence, although we cannot directly address this experimentally, our data strongly suggest that the cDCs remaining after DTx injection are not the main inducers of $CD4^+$ T cell proliferation, even when high doses of antigen are given.

A growing number of reports suggest a capability of inflammatory monocytes to differentiate into DCs under inflammatory conditions. Rivollier et al showed an influx of $Ly6C^{hi}$ monocytes in colitis, differentiating in the inflamed colon into $CX3CR1^{int}$ inflammatory DCs[260]. A more recent report further suggests that this population of inflammatory monocyte-derived DCs can adopt full APC-function, with migratory ability, and capacity to activate T cells[261]. NK-derived IFN-γ has been suggested to be involved in the recruitment of monocytes differentiating into DCs after intraperitoneal infection with *Toxoplasma gondii*[262]. Further, LPS may function as a mediator of recruitment of blood monocytes into LN and to induce subsequent differentiation into DCs[263]. Even though there are no reports confirming the differentiation of inflammatory monocytes into antigen presenting DCs in the small intestine, this could be an explanation for the cDC- independent T cell proliferation after immunization with a high dose of antigen.

Specific involvement of cDC subsets in orally-induced immune responses

After establishing the importance of cDCs for the activation of T cells and reciprocal antibody-production by plasma cells, we initiated further studies to elucidate the role of different subpopulations of DC in this context. We therefore established a cannulation model in mice that had previously been performed in rats and cattle. Using this model, we conducted a study of pseudo-afferent intestinal lymph DCs, following oral immunization with CT. DCs are easily activated and, during isolation from tissues by mechanical and enzymatic disruption, they could potentially become activated. In addition, only a fraction of cDCs are released by

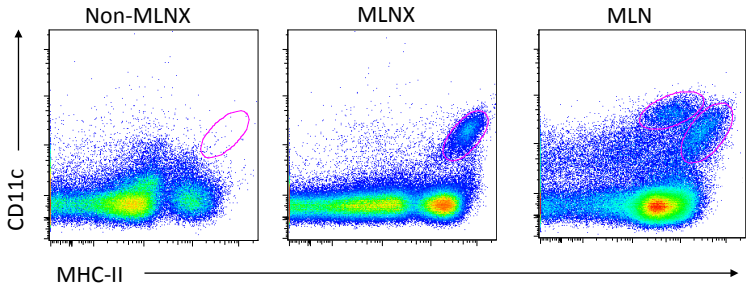

Figure D1. cDC populations in **A,** intestinal lymph of mice with intact MLNs**; B,** intestinal lymph after surgical removal of MLNs, **C,** MLN

such techniques, which may preferentially purify particular subtypes of cells[264]. Collection of IL-DCs by cannulation does not only account for an isolation-method with minimal manipulation of DCs, but also gives a high yield of DCs. Further, cannulation of MLNx mice highlighted the $CD11c^{+}MHC\text{-}II^{bright}$ cDC phenotype as the best way of distinguishing intestinal migratory DCs from resident DCs, rather thanthe $CD11c^{+}MHC\text{-}II^{+}$ phenotype (Figure D1).

As stated previously, four subsets of IL-DCs can be distinguished based on the expression of CD103 and CD11b. The four populations are: I) $CD103^{+}CD11b^{+}$; II) $CD103^{+}CD11b^{-}$; III) $CD103^{-}CD11b^{+}$ and IV) $CD103^{-}CD11b^{-}$. Cannulation of CT-fed mice revealed an interesting finding in that $CD103^{+}CD11b^{-}(CD8a^{+})$, as the only IL-DC subset, did not up-regulate the co-stimulatory molecule CD86. However, the migration of this subset in comparison with the others was not impaired, as all subsets migrated in intestinal lymph in similar frequencies with or without CT feeding. In addition, no obvious difference in proliferation of $CD4^{+}$ T cells in co-cultures with the different subsets was detected, with or without CT. We have recently shown that direct interactions between GM1 and hematopoietic cells and CT is required for activation of gut-derived cDCs in the MLN[265] As all nucleated cells express GM1, a lack of GM1 expression cannot be the reason for the reduced activation. Our results, therefore, suggest that in addition to binding of CT to GM1, some factors, most likely soluble, are released in response to CT and that these must bind receptors expressed by DCs in order for them to upregulate CD86. If $CD103^{+}CD11b^{-}(CD8a^{+})$ IL-DCs do not express such additional receptors then this could explain their incapacity to become fully activated by CT. We have not been able to address what these factors could be, but we have sorted the different populations of IL-DCs and will be conducting comparisons of genes expressed by the subsets. Importantly, in rats all IL-DCs subsets upregulate CD86 in response to R848 administration[108] and in preliminary experiments we have observed the same in mice. Hence $CD103^{+}CD11b^{-}(CD8a^{+})$ are not refractory to direct or indirect effects of microbial stimuli. A third possibility is that the anatomical origin of this subset differs and that CT does not access this subset. Indeed, it was originally suggested that $CD103^{+}CD11b^{-}(CD8a^{+})$ cells originate primarily from PP[266] but recent cannulation studies in mice lacking PP show that

this subset is still present. Hence, although this could be an explanation there is thus far no experimental evidence for differences in the distribution of CD103+CD11b+/- cDCs in the intestine.

The cytokine profiles of the responding $CD4^+$ T cells co-cultured with different subsets from controls or mice fed CT, confirmed that $CD103^-CD11b^+$ cDCs were the major inducers of IFN-γ production by the T cells. $CD103^-CD11b^+$ IL-DCs from mice fed CT, however, displayed a reduced capacity to induce IFN-γ production. This is not due to CT-induced apoptosis in the cells as these IL-DCs induce increased levels of IL-10 in these co-cultures.
To test the role of $CD11b^-(CD8a^+)$ cDCs for CT's adjuvanicity, we used $Batf3^{-/-}$ mice with a reported lack of this specific subset. While $Batf3^{-/-}$ mice displayed a pronounced reduction of $CD103^+CD11b^-$ both in PP and among IL-DCs, they still elicited normal antigen-specific intestinal antibodies following immunization with CT. In contrast, following epicutanous immunization, *batf3*-dependent $CD11b^-$ cDCs were important for CT adjuvanticity[224]. Further, the importance of $CD11b^-$ cDCs against viral infections, such as West Nile virus and Sendai virus, has been established in several reports[78,79], reflecting their role as the major cross-presenting cDC. In contrast, $CD11b^-$ cDCs, as shown in our studies, are dispensible for $CD4^+$ T cell activation and antibody responses. In line with this, $CD4^+$ T cell activation in skin draining lymph nodes are dependent on $CD11b^+$ cDCs[92,93]. Thus, we cannot find a requirement for $CD11b^-(CD8a^+)$ in the induction of $CD4^+$ T cell-dependent antibody responses. This subset is clearly important for activation of $CD8^+$ T cells[78,82,127,128,224]. Whether $CD11b^-(CD8a^+)$ are important for the induction oral tolerance would be interesting to address, but was outside the scope of this thesis.

When we initiated the study that resulted in paper II, few KO-models were available to study the requirement of $CD11b^+$ DC subsets for the induction of oral immune responses. Mice deficient for CD47 develop all DC subsets, but with a reduced frequency of $CD11b^+$ splenic cDCs [92]. In addition, reports had shown that the cDC subset expressing CD11b had an impaired ability to migrate in skin-afferent lymph[92,93] . Hence, if $CD11b^+$ cDCs migration in intestinal lymph was also reduced in these animals, this could help us elucidate the function of this subset in immune responses towards orally introduced antigens. Unfortunately, we were not able to cannulate the $CD47^{-/-}$ mice and could therefore not directly assess the DC subsets that migrate in intestinal lymph. The reason for this was that the $CD47^{-/-}$ mice available to us were bred onto a BALB/c background. We discovered a major anatomical difference between C57Bl/6 and BALB/c, in which the latter have an obstructed thoracic duct, thus severely diminishing the success rate of cannulations. Nevertheless, the influence on immune responses initiated after oral antigen delivery of the ubiquitously expressed CD47 had not previously been studied. In addition, this knockout strain allowed us to study the frequency of $CD11b^+$ DCs in intestinal tissues. Thus, $CD47^{-/-}$ mice served as an interesting model to explore intestinal immune responses.

Mice deficient in CD47 harbour an evident reduction of $CD11b^+$ gut-derived DCs in MLN. $CD47^{-/-}$ DCs show normal CCR7 expression, and no defect in migration towards CCL19 in mice on a BALB/c background. Interestingly, this differs from mice on C57Bl/6 background, which display impaired migration towards CCL19, measured by the same technique[92,93]. Notably, the $CD47^{-/-}$ mice used in our study were of BALB/c genotype. Thus, the reduced frequencies of $CD11b^+$ DCs we detected in MLNs of $CD47^{-/-}$ is not due to differential chemokine receptor expression, but rather to the expression of CD47. In contrast to the situation in MLN of CD47 deficient mice, unaltered frequencies of $CD11b^+$ DCs in PP were observed, particularly in relation to $CD11b^-/CD8^+$ DCs. It has been shown that PP, but not

MLN or ILFs, are the major site for generation of antigen-specific IgA following oral immunization with adjuvants[267]. This is further supported by the observation that CCR6$^{-/-}$ mice, that selectively lack CD11b^{+} DC in SED of PP, showed impaired IgA responses following oral immunization with CT[236]. In addition, the frequency of CD11b^{+} cDCs increased in spleens of BMCh constituted with WT BM, with a similar trend in MLN, but the BMCh mice did not regain the capacity to produce IgA following oral immunization with CT. This strongly suggests that the defect in the production of antigen-specific intestinal IgA following oral immunization of CD47$^{-/-}$ mice is not caused by the reduced frequencies of CD11b, but rather is due to the CD47 deficiency in radioresistant or non-hematopoietic cells.

Oral tolerance is dependent on DC carriage of antigen to MLN. This has been shown both in CCR7$^{-/-}$ mice that have reduced DC migration from the intestine and in small bowel transplants lacking intestinal afferent lymph. In both settings, oral tolerance cannot be generated[246]. DCs, in particular CD11b^{+} DCs, within both CD103^{+} and CD103^{+} populations, were reduced in the CD47$^{-/-}$ mice compared to WT mice. Moreover, impaired T cell activation was detected in CD47$^{-/-}$ mice following oral immunization. In addition, our preliminary results show equivalent frequencies of Foxp3^{+} cells in CD47-deficient mice and WT mice. Thus, despite reduced cellularity in GALT, lower frequency of CD11b^{+} DCs, and impaired proliferation of CD4^{+} T cells in MLN, oral tolerance is maintained in CD47$^{-/-}$ mice. This suggests that the reduced numbers of DCs - either CD11b^{+} or CD11b^{-} - that reach the MLN, are still sufficient to induce oral tolerance. Dependence on DCs, at least with regard to antibody generation, has been shown to be affected by the dose of antigen administered. Alternatively cDCs may not be required. The results presented in paper I clearly show that other APCs can activate CD4+ T cells when antigen is not limiting. However, induction of oral tolerance in CD47-deficient mice was - if anything - stronger than WT, with a ten fold lower antigen dose required to induce tolerance.

Our study of the oral immune response in CD47$^{-/-}$ mice has uncovered several interesting findings. However, our initial intent, which was to study the requirement for CD11b^{+} DCs, was made difficult by some of these obesrvations. In addition, the reduction in CD11b^{+} DCs was only partial in GALT. Today, more refined animal models are generated allowing the possibility to study different DC populations more specifically. The Cre-Lox system has recently been engineered with floxed *irf4* (a gene important for the generation of CD4^{+} DCs) combined with the Cre enzyme under the promoter of CD11c (CD11c-Cre). The resultant CD11c-cre.Irf4$^{fl/-}$ mice display a significantly reduced frequency of CD11b^{+} DCs in both MLN and LP[268]. Mice with a DC deletion in Irf4 display reduced numbers of intestinal IL-17-secreting T cells and fail to support the differentiation of IL-17 secreting T cells in MLN following immunization[268]. These mice could serve as a good model to study the requirement for CD11b^{+} DCs in oral tolerance induction, as well as for the generation of antibody responses following oral immunizations. An additional model to study the involvement of CD11b^{+} cDCs, CD11c-creNotch2-receptor deficient mice, was recently described. [239,269]. These mice also lack CD11b^{+} cDCs in spleen and intestinal lamina propria. Both models, would probably provide interesting strategies with which to study CD11b^{+} cDC-dependence in orally induced immune responses.

The role of CD47 in immune responses induced by oral CT

We uncovered that CD47$^{-/-}$ mice, specifically in GALT, had reduced numbers of leukocytes and that the expansion of antigen-specific T cells in these tissues was impaired following oral immunization. The reduced cell numbers were not restricted to a single cell type, but

represented a general reduction. Furthermore, we showed that $CD47^{-/-}$ mice have a reduced antigen-specific intestinal antibody response following immunization with CT. This could be due to the decreased proliferation of $CD4^+$ T cells seen within MLN and PP in these mice following oral administration of antigen, or to the general reduction in intestinal leukocytes. However, while antigen-specific intestinal antibodies were reduced, total IgA levels were maintained. The question is then: why are total intestinal IgA responses not affected? A possible explanation could be that the total IgA levels that we detect in our IgA measurements constitute mostly IgA not requiring T cell help and that this is possibly generated in situ, as reported by [270]. ILFs have been shown to play a role in IgA production, possibly independently of T cells [271]. ILFs have been suggested to be influenced by leukocyte migration. For example $CCR7^{-/-}$ mice, that display impaired migration to MLN and reduced intestinal antibody responses, have enlarged ILFs[18]. Hence in CD47-deficient mice, in which the migration of leukocytes is impaired, enlarged ILFs could potentially compensate for the loss of cells in other GALT structures and increase output of total IgA from ILFs. There may also be other homeostatic mechanisms in place ensuring that once a certain level of IgA is reached, feedback mechanisms regulate the production. Hence, although the number of IgA secreting cells in the intestine is reduced two–fold, these are sufficient to maintain the required threshold.

Another explanation for the reduced intestinal antibody response in $CD47^{-/-}$ mice, could be a defective migration of leukocytes. In particular, extravasation from blood vessels and subsequent homing of IgA plasma cells to the intestine, could be impaired. The consensus regarding migration of antibody-secreting cells is that pre-plasma cells migrate from PP via lymph and TD back to the blood. They then cross endothelial blood vessels to home to intestinal tissue[272]. This is consistent with fewer intestinal antibody-secreting cells detected by ELISPOT. Phosphorylation of ITIMs in the cytoplasmatic tail of CD172a is essential for the function of CD172a. Phosphorylation of CD172a on endothelial cells requires not only CD47, but also integrin-mediated adhesion. Thus, the transmigration of leukocytes across endothelium could be affected, even with CD47-competent leukocytes in a mouse with CD47-deficient endothelium [88]. Therefore, with an endothelium deficient in CD47, leukocytes expressing CD47 may still have a reduced capacity for integrin-mediated transmigration, as phosphorylation of CD172 may be important for transmigration. This could be a possible explanation for our observation of continued low levels of antigen-specific intestinal antibodies following immunization of BMCh in which the hematopoietic compartment is CD47-competent. In addition, memory B and T cells, and plasma cells producing serum antibodies, reside in BM and do not depend on endothelial transmigration. This is in line with our findings, as the level of serum antibody remains normal but with a decrease specifically in intestinal antibody responses,. Neutrophils have been shown to signal via CD47, as treatment with antibodies against CD47 delayed their migration over collagen-coated filters *in vitro*[273]. In addition, transfer of CD47-competent antigen-pulsed BM-DCs to CD47-deficient mice induced similar proliferation of $CD4^+$ T cells as WT controls[93]. This suggests that CD47 is required on DCs, and not on the endothelium, to regulate DC trafficking across lymphatic vessels and endothelial barriers *in vivo* [93].

Signaling pathways involved in the oral adjuvant effect of CT

We have determined that cDCs are essential for oral immune responses adjuvanted by CT, when antigen is limiting. In addition, the role of different cDCs subsets following oral immunization with this adjuvant was also addressed. Finally, we assessed the signaling pathways involved in the activation of intestinal DCs and the adjuvant effect of oral CT.

MyD88-deficient mice fed OVA and CT generated normal levels of antigen-specific intestinal IgA antibodies, as well as serum IgG. This is consistent with increased levels of CD86 by gut-derived DCs after oral immunisation of these KO mice. The Myd88-independent pathway employed by TLR-signaling through TRIF was also addressed using double mutant MyD88/IFNαR$^{-/-}$ mice, as activation of TRIF leads to release of type I interferons. Antibody generation was not impaired in the double KO, nor was intestinal DC activation by oral CT, as measured by increased level of CD86. Hence TLR signaling is not required for the oral adjuvant activity of CT. This is in agreement with transcutaneous immunization, in which MyD88/TRIF double-mutant mice maintain the adjuvant effect of CT [224]. However, in mice deficient in IFN-αR, the CTL response was impaired, showing that TRIF-independent type I interferon production was required. Whether the differential requirement of IFN-αR for the adjuvant effect of CT is due to CD4^{+} T cell activation or CD8^{+} T cell activation, or due to routes of immunization, remains to be determined. IFN-αR signaling in DCs and non-hematopoetic cells has also been reported to be required for efficient CD4^{+}CXCR5^{+} Tfh development and the generation of high affinity antibodies after i.p. immunisation with antigen and LPS or polyI:C. Thus, IFN-αR signaling is involved in the generation of antigen-specific antibodies using parenteral immunisations with TLR-ligands. That type I interferons may play a more limited role in oral immune responses is suggested by the observation that type III IFN and not type I IFN are of major importance for protection against rotavirus which exhibits strong intestinal epithelial cell tropism[274,275]. Taken together, signaling pathways important for oral CT adjuvanticity does not involve TLR pathways.

Canonical inflammasome signaling involves caspase 1 activation. Caspase 1 and caspase 11 are too close genomically to be separated by recombination, and so the caspase 1$^{-/-}$ mice which we used to investigate the role of the inflammasome signaling pathway for oral adjuvant activity of CT were, in fact, Casp1/11$^{-/-}$[141]. Gut-derived DCs in caspase1/11$^{-/-}$ failed to upregulate CD86 after oral CT administration. In addition, the antigen-specific intestinal IgA titer was significantly impaired. Caspase 1 activates IL-1β and IL-18 from inactive precursors. Thus, activation of cDCs in mice deficient in the receptors for IL-1β and IL-18 were assessed following oral immunization with CT. In contrast to caspase1/11$^{-/-}$ mice, gut-derived DCs in both IL1R$^{-/-}$ and IL-18R$^{-/-}$ displayed efficient activation after oral CT. In agreement with these results, both IL-1R and IL-18R employ MyD88 for intracellular signaling. Our results, thus suggests that CT induces the caspase 1/11 signaling pathways that are important for cDC activation and antigen-specific IgA production, but that are independent of IL-1β and IL-18. Recent reports have shown that inflammasome activation can lead to the release of inflammatory mediators independently of IL-1β and IL-18[276]. During the last few years, several routes by which the assembly of inflammasomes can lead to caspase 1 activation have been described. The route described by von Moltke et al is also utilized by CT, if this secretion of inflammatory mediators is coupled to adjuvanticity remains to be discovered. Interestingly, the contribution of release of inflammatory mediators to the diarrheal response induce by CT has been known for a long time[277]. GM1$^{-/-}$ mice, which are unable to respond to CT, display an even lower anti-OVA IgA titer after OVA and CT immunisation. Additionally, serum IgG titers to OVA in Casp1/11$^{-/-}$, were similar to that of WT mice. Thus, this would suggest that CT may induce antibody responses, at least in the intestine, via caspase-activating inflammasomes, even though cDCs migrating from the intestine are not efficiently activated. This may indeed reflect that DCs migrating to MLN are not the major DC population involved in the induction of intestinal IgA responses, but that this capacity lies within cDC populations elsewhere, for example, PP and/or ILF. Alternatively, although co-stimulation is needed for effective T cell activation by DCs, the level of CD86 expressed by migrating DCs per se may not be directly linked to IgA responses following oral immunisation. For example, we have

consistently observed lower levels of CD86 expression by DCs in PP than in MLN under homeostatic conditions and, more importantly, the upregulation of CD86 was not as pronounced after oral immunisation with CT.

Interestingly, both Casp1/11 and $CD47^{-/-}$ display reduced antigen-specific intestinal antibodies while maintaining the level of antigen-specific antibodies in serum. This suggests differential mechanisms for local and systemic antibody responses. Separate inductive sites may be responsible for local versus systemic antibody generation. However, in paper II we confirm that MLN are dispensable for both local and systemic antigen responses following oral CT immunisation, suggesting that PP are the major inductive site. A recent study has shown that Th17 cells in PP are important for the generation of T cell dependent IgA[278]. Furthermore, IL-1 has been shown to be important for the generation of Th17 cells, recently suggested as important for the mucosal adjuvant effect of inhaled CT[279]. Macrophages exposed to CT also show increased release of IL-1β, possibly by caspase-1 activation[280]. However, in preliminary experiments we have observed that $IL\text{-}1R^{-/-}$ mice generate similar titers of antigen-specific antibodies as WT mice following oral immunization with CT. This suggests that if Th17 cells are important for the induction of IgA following oral immunisation, they can be generated independently of IL-1.

The adjuvant effect of CT in relation to its enzymatic activity has been studied in by site-directed mutagenesis of the toxin. In some reports, the enzymatic activity was reduced, but with retained adjuvant activity[281-283], whereas others have shown that the non-toxic derivates lose both adjuvant effect and enzymatic activity[284,285]. Thus, it is still unclear how the adjuvant effect of CT, and of other enterotoxins, is linked to their enzymatic activity. Recent results from our group have demonstrated that GM1, the receptor for CT, needs to be expressed by DCs in order for oral adjuvant activity[265]. In this study mixed BMCh, based on CD11c-DTR and $GM1^{-/-}$ mice, were used. CD11c-DTR mice have limitations for use in the study of antibody responses after multiple immunizations because DTx also affects plasma cells[243] and GC B cells[286]. In CD11c-Cre mice, Cre expression in B cells is less (5%, compared to >95% for cDCs)[287]. We therefore conducted experiments with CD11c-creGsaflox mice to specifically study the role of $G_s\alpha$ activity within cDCs. CD11c-Gsa DC did not respond to CT by up-regulation of CD86, neither *in vitro* nor *in vivo*. Furthermore, CD11c-Gsa mice were unable to generate antigen-specific antibody-production following oral immunization with CT. CD11c is expressed by cells other than cDCs in the gut. $CX3CR1^{hi}CD103^{-}$ macrophages express CD11c, but these cells are non-migratory during homeostasis, and even after administration of TLR7/8L, a highly potent inducer of migration[101], and are poor inducers of T cell priming[101,102]. Therefore, it is unlikely that the concomitant deletion of these cells can explain the loss of responses in these mice. This strongly suggests that the oral adjuvant activity of CT *in vivo* is directly dependent on Gsα signaling in cDCs and their subsequent activation.

Several mutants of enterotoxins have been suggested to function as oral adjuvants without having enzymatic activity. This has been determined by in vitro assays or by showing a loss of diarrheal responses in vivo. However, we clearly show that intact $G_s\alpha$-signaling, specifically in cDCs, is essential for the oral adjuvant activity of CT. It would therefore be of great interest to test whether these mutants of enterotoxins maintain their oral adjuvant activity in the CD11c-Gsa mice. If they lose their activity, then this would suggest that although the level of enzymatic activity is insufficient to induce a diarrheal response in epithelial cells, it is still sufficient and required for adjuvanticity.

Conclusion

Few mucosal vaccines are available on the market today, most likely because of the low number of available mucosal adjuvants. However, the most promising oral adjuvants in clinical trials today are based on detoxified enterotoxins. In order to improve the functionality of such mucosal adjuvant, greater knowledge of their mechanism of action is pivotal. DCs are important in sensing the microenvironment and serve to link innate and adaptive immunity and were, therefore, the focus of this study. Our results show that cDCs are required for the activation of antigen-specific T cells and the generation of antigen specific antibodies when using limiting doses of antigen and CT as an adjuvant. In addition, we show in vivo that intact Gsa-expression, specifically in cDCs, is essential for the oral adjuvant activity of CT in vivo. We found $CD11b^{-}CD8^{+}$ cDCs to be dispensable for the generation of antigen-specific intestinal antibodies after oral immunization with OVA and CT. The importance of CD47, expressed by non-hematopoietic cells for efficient generation of intestinal IgA following immunization with CT was established. Finally, the oral adjuvanticity of CT was shown to be independent of classical TLR-signaling, but Caspase 1/11 activity is involved in generation of intestinal IgA but serum IgG following oral immunization with CT.

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