# The ontogeny and function of intestinal dendritic cells

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2014

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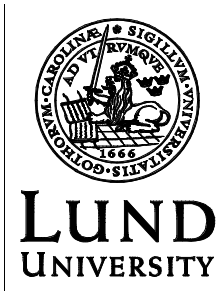


DOCTORAL DISSERTATION
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To be defended on Friday 11th of April, 9:00 in Belfragesalen, BMC D15, Sölvegatan 19, Lund

*Faculty opponent*

Professor Andrew MacDonald

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# The ontogeny and function of intestinal dendritic cells

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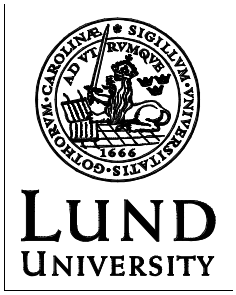

On the cover: Image of the structure of the human small intestinal mucosa revealing a $CD103^+$ subset of dendritic cells (yellow). The intestine is stained with antibodies to MHCII (green) and CD103 (red) and the cell nuclei were counterstained with DAPI (white). Image by Heli Uronen-Hansson ©

Lund University, Faculty of Medicine Doctoral Dissertation Series 2014:41
ISBN 987-91-87651-66-3
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2014

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**IRF4 transcription-factor-dependent CD103$^+$CD11b$^+$ dendritic cells drive mucosal T helper 17 cell differentiation****

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*In manuscript*

**Authors contributed equally*

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**Dendritic cell subsets in the lamina propria: Ontogeny and function**
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Persson EK, Jaensson E, Agace WW
*Immunobiology 2010 Sep-Oct;215(9-10):692-7*

**Authors contributed equally*

# Abbreviations

**DC** dendritic cell

**PAMP** pathogen associated molecular pattern

**DAMP** danger associated molecular pattern

**PRR** pattern recognition receptor

**TLR** toll like receptor

**NOD** nucleotide-binding oligomerization domain

**LN** lymph node

**APC** antigen presenting cell

**BCR** B cell receptor

**TCR** T cell receptor

**CD** cluster of differentiation

**MHC** major histocompatibility complex

**Th** T helper

**GM-CSF** granulocyte-macrophage colony-stimulating factor

**LPS** lipopolysaccharide

**PCA** principal component analysis

**MDP** macrophage and dendritic cell progenitor

**CDP** common DC progenitor

**CCR** chemokine receptor

**TF** transcription factor

**IRF** interferon regulatory factor

**Id2** inhibitor of DNA-binding 2

**Batf3** basic leucine zipper transcription factor, ATF-like 3

**IFN** interferon

**STAT** signal transducer and activator of transcription

**LT** lymphotoxin

**CLR** c-type lectin receptor

**NLR** nucleotide-binding oligomerization domain receptor

**SI** small intestine

**PP** Peyer's patches

**ILF** isolated lymphoid follicle

**GALT** gut-associated lymphoid follicles

**LP** lamina propria

**MLN** mestenteric lymph node

**HEV** high endothelial venules

**MZ** marginal zone

**EBI2** Epstein-Barr virus induced G-protein coupled receptor 2

**IL** interleukin

**Treg** regulatory T cell

**RA** retinoic acid

**FoxP3** forkhead box protein 3

**TGF** transforming growth factor

**IEL** intraepithelial lymphocyte

**FAE** follicle associated epithelium

**SED** subepithelial dome

**Ig** immunoglobulin

**LTi** lymphoid tissue inducer

**CP** cryptopatches

**SFB** segmented filamentous bacteria

**Myd88** myeloid differentiation primary response gene 88

**RORγT** retinoic acid receptor (RAR) –related orphan receptor gamma

**MadCAM-1** mucosal vascular addressin cell adhesion molecule 1

**CCL** chemokine ligand

**BM** bone marrow

**VAD** vitamin A deficient

**Zbtb46** zinc finger and BTB domain containing 46

**GFP** green fluorescent protein

# 1. The immune system

The immune system is a network of cells, tissues and mediators that defend organisms against potentially harmful bacteria, fungi, parasites and toxins to protect against disease. To exert this function, the immune system needs the ability to recognize foreign components but has to remain tolerant to components of self and to innocuous compounds. Failing to mount effective immune responses puts the organism at risk for disease while a breakdown in development of tolerance puts the organism at risk for developing immunity to self (autoimmunity) or chronic inflammation. The immune system of vertebrates is composed of two main branches: the innate and adaptive immune systems. Both depend on the ability to distinguish between self and non-self components but they differ in their means of pathogen recognition and in their ability to generate immunological memory.

## 1.1 Innate immunity

The more primitive innate immune system is found in all plants and animals and serves as a first line of defense to protect against invading pathogens. It is composed of several layers of increasing specificity of which physical barriers such as the skin of humans serve as a first layer of defense. However organisms cannot be sealed off completely from their environment and mucosal surfaces such as those in the lungs and in the gastrointestinal tract, where uptake of oxygen and nutrients occurs, are particularly vulnerable to infection. At such sites, innate immune mechanisms such as anti-microbial peptides may act as chemical barriers to protect the host. Should an invading pathogen breach these barriers, the cellular components of the innate immune system come into play.

The cellular players of the immune system are the white blood cells (leukocytes) that develop in the bone marrow from hematopoietic stem cells. Innate leukocytes include populations of monocytes, macrophages, granulocytes as well as dendritic cells (DCs). They can identify and eliminate pathogens, either by release of anti-microbial substances or by engulfing (phagocytosing) and then killing pathogens. Such innate effector responses develop very rapidly, within minutes to hours after initial recognition of the pathogen.

The recognition of pathogens by cells of the innate immune system depends on their ability to detect conserved structures on microbes, called pathogen-associated molecular patterns (PAMPs), using a limited numbers of pattern recognition receptors (PRRs). PRRs can occur in a secreted form, bound to the membrane of cells, or in intracellular compartments. They include various toll like receptors (TLRs) that bind conserved components of microbes as well as intracytoplasmic nucleotide-binding oligomerization domain (NOD) receptors that recognize components of bacterial peptidoglycan. Each PRRs has a specificity for a given PAMP but they can be expressed in various combinations by different cell populations.

Depending on cell type, activation of PRRs may result in various types of responses. Thus, PRR activation in macrophages typically leads to enhanced phagocytic activity whereas in granulocytes, the resulting response may be release of anti-microbial products that are contained within granules of those cells. Importantly, PRR activation may also lead to the release of inflammatory mediators such as cytokines and chemokines that can shape and strengthen the immune response by recruiting other immune cells to the site of infection, including cells of the adaptive arm of the immune system.

## 1.2 Adaptive immunity

The adaptive immune system evolved in early vertebrates and allows a more specific and efficient response to pathogens. Importantly, an adaptive immune response leads to the formation of immunological memory of the pathogen, and improved responses to subsequent encounters with the same pathogen. The adaptive immune system relies on the presentation of pathogen components (called antigens) by cells of the innate immune system, during a process called antigen presentation. Among the professional antigen presenting cells (APCs), which also include macrophages and certain B cells, DCs are the most specialized. In this regard, DCs may be considered to be part of both the innate and adaptive immune systems and indeed, provide critical links between the two.

The principal cells of the adaptive immune system are B cells and T cells that mature in the bone marrow and thymus, respectively. In contrast to the limited pattern recognition repertoire of the innate immune system, pattern recognition in B and T cells depends on a virtually infinite receptor diversity. This diversity is

generated by random recombination of DNA segments during the development of B and T cells resulting in each B or T cell expressing a receptor with unique specificity. As a result of the scarcity of any given receptor, adaptive immune responses develop more slowly than innate responses as B and T cells first need to locate their cognate antigen before they can initiate a response. To increase the likelihood of such encounters, initial stages of immune activation typically occur in lymph nodes, where immune cells, including APCs, are concentrated.

B cell receptors (BCRs) and T cell receptors (TCRs) differ in the ways they recognize antigen. BCRs are able to bind directly to native antigen. This binding leads to ingestion, breakdown and subsequent presentation of that antigen in the context of a major histocompatibility complex (MHC) molecule. The antigen-MHCII complex can be recognized by a matching T cell, which helps B cells to differentiate into so called plasma cells. Plasma cells specialize in secreting antibodies (soluble forms of the BCR) that can capture their cognate antigen and make it more accessible to cells of the innate immune system and limits the spread of infection. Production of antibodies is part of the so-called humoral arm of the adaptive immune system.

In contrast, T cells only recognize their cognate antigen in the context of presentation of MCH presentation by APCs. There are two distinct subsets of T cells, characterized by the expression of CD8 or CD4, which have different effector functions. $CD8^+$ T cells, which are cytotoxic, recognize antigens presented in the context of MHCI molecules and their main function is to kill cells that present their cognate antigen. In contrast, $CD4^+$ helper T cells recognize antigen in the context of MCHII molecules and mainly act by releasing cytokines that coordinate immune responses by acting on other cells. Thus, T cells constitute the cellular part of adaptive immunity.

In addition to TCR stimulation, $CD4^+$ T cells receive co-stimulatory signals and cytokines from APCs. Providing such signals, in addition to antigen presentation, is a key function of DCs. Further, depending on the type of signals they receive, $CD4^+$T helper (Th) cells can develop into different Th cell subsets with distinct functions. These processes will be discussed more in detail in the section that concerns DC function.

Importantly, the development of adaptive immunity also leads to immunological memory of the antigen initially encountered. This is achieved through the development of populations of memory B and T cells during the initial immune

response. These populations assure a quick response upon a second encounter with the same pathogen. Although immunological memory can vary in length of time, it is a clinically highly relevant mechanism that lies at the base of successful vaccinations.

# 2. Dendritic cells – Development and subsets

## 2.1 Conventional dendritic cells as a hematopoietic lineage

DCs were discovered just over 40 years ago when Ralph Steinman and his colleagues identified cells with "large stellate morphology" in preparations of adherent splenocytes [1]. Subsequent studies demonstrated a unique capacity of these cells to stimulate naïve T cells in mixed lymphocyte reactions [2], which suggested that they were uniquely suited to initiate and direct adaptive immune responses. For his discovery of the DC, and his recognition of the importance of DCs in adaptive immunity, Ralph Steinman was awarded the Nobel Prize in Physiology or Medicine in 2011. Following Steinman's initial findings, a variety of cell types were attributed the functional and phenotypic characteristics of DCs but a lack of markers uniquely expressed by conventional DCs (cDCs) made the distinction between cDCs and other mononuclear phagocytes challenging. The integrin CD11c, for example, has often been used as a marker for cDC identification however other cell types, including tissue macrophages, also express CD11c. Moreover, monocytes can develop many of the features of cDCs under inflammatory conditions. Such issues led to a long and considerable debate regarding the precursors-progeny relationship between cDCs and other mononuclear phagocyte populations [3]. To date no single surface markers exists that uniquely distinguishes conventional DCs (cDCs) from other immune cell populations.

More recently, systematic approaches [4] or genetic lineage tracing strategies [5] have led to considerable progress in our understanding of the developmental relationship between mononuclear phagocytes and identified novel markers for cDC identification. Using gene expression analysis, two such studies identified the transcription factor Zbtb46, which is selectively expressed by cDCs. The use of Zbtb46 green flourescent protein (GFP) reporter lines [6] or diphteria toxin receptor (DTR) ablation strategies [7] demonstrated that Zbtb46 is expressed by cDC restricted progenitors in the bone marrow and by cDCs in non-lymphoid as well as lymphoid tissues but is largely absent in other myeloid or lymphoid immune cell lineages, including pDCs and macrophages [6, 7]. Of note, however, Zbtb46 expression was not entirely exclusive to cDCs but was also observed in

monocytes cultured with GM-CSF, and in $CD206^+$ DCs that arose in mice from monocytes after LPS treatment [6]. Furthermore, expression of the c-type lectin DNGR-1 (CLEC9A) has been used to study cDCs ontogeny. In a study by Schraml *et al*., $lineage^-DNGR\text{-}1^+$ bone marrow progenitors gave rise specifically to cDCs and genetic tracking of DNGR-1 expression history marked cells previously ascribed to the cDC lineage, but not other leukocytes [5]. A recent effort by the Immunological Genome Consortium used a systematic approach to determine relationships between DC and macrophage populations through the use of principal-component analysis (PCA). The genetic relationships between more than 50 sorted macrophage and cDC subsets were examined leading to the identification of a molecular signature that distinguished tissue cDCs from macrophages [4]. Taken together, these studies help unravel the complexity of the mononuclear phagocyte system, provide useful tools for the functional study of cDCs, and support a view in which cDCs make up a unique lineage of hematopoietic cells, distinct from other mononuclear phagocytes.

## 2.1 Dendritic cell development in the bone marrow

DCs have a short life span and DC populations are continuously repopulated by progenitors that develop in the bone marrow from pluripotent hematopoietic stem cells through a series of intermediate precursors steps. The earliest progenitor thought to be committed to the mononuclear phagocyte lineage is the macrophage dendritic cell progenitor (MDP). When adoptively transferred, MDPs give rise to subsets of macrophages, monocytes and DCs, but not other myeloid lineages [8, 9]. The MDP progresses to give rise to a common DC progenitor (CDP) that retains potential only for cDC and pDC development [9-11]. Both MDPs and CDPs are lineage negative; express the Flt3L receptor (Flt3R) and intermediate levels of CX3CR1 however CDPs have lower expression of the stem cell factor receptor c-kit (CD117). CDPs progress into pre-cDCs, which are committed to the cDC lineage but have lost the potential to give rise to pDCs [9]. Phenotypically, pre-cDCs express CD11c but are MHC class II negative. Moreover, pre-cDCs express intermediate levels of SIRPα and CD11b and, similar to MDPs and CDPs, intermediate levels of CX3CR1. MDPs and CDPs are found only in the bone marrow whereas pre-DCs are also found in the blood and in tissues. In tissues, pre-cDCs complete their differentiation and give rise to all mature cDC subsets [9] **(Figure 1).**

Using clonal analysis, Naik et al. suggested some pre-existing commitment to either the cDC or pDC lineage within the CDP population [11]. On a similar note, Zbtb46 expression in CDP and pre-DC populations appears to identify a subset of cells that have lost pDC potential [6]. Also, adoptive transfer of $lin^{-}DNGR\text{-}1^{+}$ cells specifically gave rise to cDCs but not pDCs or other lineages, indicating that DNGR-1 is a useful marker of cDC-restricted progenitors [5]. In addition, it has been suggested that $CCR9^{-}$ immediate precursors of pDC (which are $CCR9^{+}$) can be transcriptionally reprogrammed to differentiate into cDC like cells by tissue-specific factors [12, 13] Thus, ontogenic relationships between CDPs, pre-cDCs and pDC progenitors remain to be fully established.

A recent study used barcoding technology to track the fate of single lymphoid-primed multipotent progenitor (LMPP) cells and determine their cellular outcome with regards to several cell types, including cDC subtypes. LMPP cells were transduced into partially irradiated mice and surprisingly, a marked proportion of LMPPs showed a distinct pattern of cellular output and some were dendritic cell biased. Thus, in contrast to the step-wise commitment model described above this study suggests that commitment to the cDC lineage (and to other immune cell lineages) may occur through processes that are initiated earlier on during hematopoiesis [14].

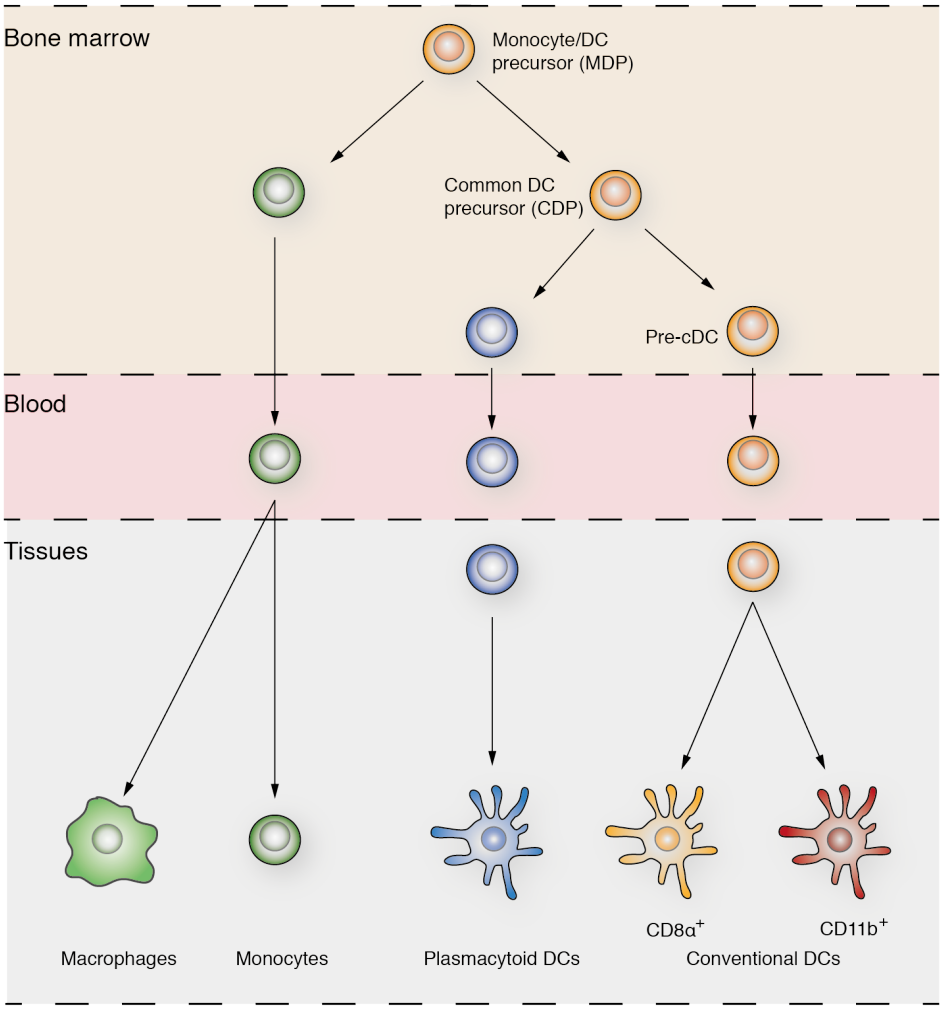


**Figure 1. The development of mononuclear phagocyte lineages.** cDCs, pDCs and monocytes/macrophages derive from bone marrow progenitors. MDPs give rise to CDPs and monocytes. CDPs differentiatie into pDCs or commited progenitors of cDCs (pre-cDC). Pre-cDCs, monocytes and pDCs transit through the blood and seed peripheral tissues. In tissues, pre-cDCs complete their differentiation into CD8α-like and $CD11b^+$ cDCs. Monocytes can migrate into tissues and differentiate into macrophages.

## 2.2 Development of dendritic cell subsets

### 2.3.1 CD8α-like conventional dendritic cells

Seminal observations that a subpopulation of thymic and splenic DCs expressed the marker CD8α provided the first examples of DC heterogeneity [15, 16]. Subsequent work demonstrated that $CD8\alpha^+$ DCs were superior at cross-presenting antigen to $CD8^+$ T cells [17, 18](discussed below), suggesting that $CD8\alpha^+$ and $CD8\alpha^-$ DCs (here termed $CD11b^+$ cDCs) were functionally distinct subsets. Numerous studies have since then cemented this view and further demonstrated that the development of $CD8\alpha^+$ and $CD11b^+$ cDCs depends on distinct transcription factor programs [19, 20].

CD8α-like cDCs include both the CD8α-expressing lymphoid resident cDCs found in spleen, lymph nodes and thymus and the $CD103^+CD11b^-$ DCs present in non-lymphoid organs, including the intestine [21]. Evidence that these cDC populations belong to a distinct cDCs lineage has come from studies demonstrating functional and phenotypic similarities, as well as a shared dependence on certain transcription factors [22-25]. Phenotypically, CD8α-like DCs are positive for the markers CD205, DNGR-1 (CLEC9A) and XCR1 but lack expression of CD11b and SIRPα [24-27]. Human counterparts of CD8α-like DCs lack CD8α expression but can be identified by the expression of BDCA3 (CD141) [28-30] and share, with mouse CD8α-like DCs the expression of DNGR-1 [27] and XCR1 [31, 32].

The development of CD8α-like cDCs depends on several key transcription factors. Mice that have a deficiency in the transcription factor interferon regulatory factor (IRF)8 have a complete loss of pDC and all CD8α-like cDCs, whereas $CD11b^+$ cDCs are still present [23, 33]. Accordingly, IRF8 is expressed at higher levels in mature CD8α-like cDCs than in $CD11b^+$ cDCs [23, 34]. In a competitive setting however, $CD11b^+$ DCs are reduced in numbers in IRF8 deficient mice [35] and IRF8 is also expressed in early DC progenitors. Hence, IRF8 deficient mice have reduced numbers of CDPs and an overproduction of granulocytes [35] implying that IRF8 is important for the development all cDC lineages. The mechanisms by which IRF8 endows specification in the cDC lineage remain to be fully elucidated but target genes include TFs Id2 and Bcl-6, which are known to regulate cDC development [35]. Inhibitor of DNA-binding (Id) 2 is expressed by all cDC

subsets with highest levels of expression in CD8α-like cDCs [23, 36] and Id2$^{-/-}$ mice lack lymph node resident CD8α$^{+}$ cDCs as well as non-lymphoid CD103$^{+}$CD11b$^{-}$ cDCs whereas CD11b$^{+}$ cDCs develop normally[23, 37]. Similar to IRF8 and Id2, Batf3 is required for the development of CD8α-like, but not CD11b$^{+}$ cDCs [22, 38]. Batf3 is thought to act at later stages of DC development and does not affect the development of CDPs or pre-cDCs [22]. Moreover, it has been demonstrated that molecular compensation by Batf can overcome the Batf3 requirement in CD8α-like cDCs during infection with an intracellular pathogen in response to IL-12 and interferon (IFN)-γ[39]. More recently, the induction of E4BP4 has been shown to be important for the development of CD8α$^{+}$ DCs. E4BP4-deficient CDPs had lower levels of BATF3 expression than control CDPs, and enforced expression of BATF3 in the mutant cells rescued CD8α$^{+}$ DC development *in vitro* [40].

Transcription factor requirements for human DC subsets have been less well studies but IRF8 may also be important early in the development of human DC as a patient with a loss of function mutation in the *Irf8* gene lacked monocytes, pDCs as well as all cDC subsets [41]. A role for Batf3 in the development of human BDCA3$^{+}$ DCs has also been implied by the observation that knockdown of BATF3 in human cord blood progenitor cells leads to impaired development of BDCA3$^{+}$ DCs in vitro [27].

### 2.3.2 CD11b$^{+}$ conventional dendritic cells

The identification of CD11b$^{+}$ cDCs as a unified cDC lineage proved more challenging than for CD8α-like cDCs, in large part due to phenotypical similarities between CD11b$^{+}$ cDC and monocyte-derived populations and the absence of a lineage marker for cDCs. In the spleen and in LNs, cDCs are relatively easily distinguished from macrophages by high expression of CD11c. In contrast, in non-lymphoid tissues the definition of CD11b$^{+}$ cDCs is complicated by the presence of macrophage populations that express high levels of CD11c and, similar to CD11b$^{+}$ cDCs, high levels of CD11b and SIRPα. Recently, the use of the high-affinity IgG receptor FcγR1 (CD64) as a lineage marker for monocytes-derived cells [42] has helped to resolve some of these issues and identified a previously unrecognized heterogeneity in CD11b$^{+}$ non-lymphoid DCs in e.g. the lung and intestine [43, 44]. Hence CD64$^{-}$CD103$^{-}$CD11b$^{+}$ cells in the lung [43] and CD64$^{-}$CD103$^{+}$CD11b$^{+}$ as well as CD64$^{-}$CD103$^{-}$CD11b$^{+}$ cells in the intestine [44]

are thought to represent cDCs, and are also $GFP^+$ in Zbtb46-GFP reporter mice [6]. In the spleen and in LNs, the majority of resident $CD8\alpha^-$ cDCs express CD4 and splenic cDCs are often divided into $CD8\alpha^+$, $CD4^+$ and $CD8\alpha^-$ $CD4^-$ double negative (DN) subsets. The DN population most likely contains progenitors of both $CD8\alpha^+$ and $CD4^+$ DCs hence $CD8\alpha^+$ and $CD11b^+$ may be a more useful terminology to describe splenic/LN resident cDC subsets.

The first transcription factor identified to regulate the development of $CD11b^+$ cDCs was the NF-κB family member RelB [45]. $CD8\alpha^-$ splenic and thymic DCs are considerably reduced in $RelB^{-/-}$ mice due a cell-intrinsic defect [45-47]. Similarly, development of $CD4^+$, but not $CD8\alpha^+$ DCs, is perturbed in mice lacking TRAF6 [47] or IRF2 [48]. Whether these factors contribute to the development of non-lymphoid $CD11b^+$ cDCs remains to be established. Similarly, mice with a genetic deletion in IRF4 indicated a role for this transcription in splenic $CD4^+$ DC development [49, 50]. Subsequently we, and others, have implicated a role for IRF4 also in the development of intestinal and lung $CD11b^+$ DCs (Paper II)[43].

Furthermore, it has been demonstrated that canonical Notch signaling is involved in the development of $CD11b^+$ DCs. Notch signaling is mediated by binding of ligands of the jagged or delta-like families to Notch receptors leading to cleavage of the intracellular domain of the transmembrane receptor. This domain is translocated to the nucleus, binds to TFs of the RBPJ family, which leads to activation of target genes [51]. Mice deficient in RBPJ have a 50% reduction in $CD11b^+$ splenic DCs [52-54] and DC specific deletion of the Notch2 receptor leads to a reduction in the number of $CD4^+$ splenic DC [53, 54]. The latter studies further demonstrated that $CD11b^+$ splenic DCs appear to comprise two subsets distinguished by the expression of the chemokine receptor CX3CR1 and the adhesion molecule ESAM (endothelial cell selective adhesion molecule) and that Notch2 signaling is specifically required for the development of $CX3CR1^{lo}ESAM^{hi}$ cells [53, 54]. The Notch ligand involved in directing $CD11b^+$ cDC development remain to be identified.

## 2.3 Extrinsic factors in dendritic cell development

The differentiation of cDCs is largely regulated by extrinsic factors and the cytokines Flt3 ligand (Flt3L) and GM-CSF are key regulators of DC commitment and homeostasis [55]. The receptor for Flt3L, Flt3R (CD135) is expressed on hematopoietic stem cells, is maintained on cDC progenitors and on mature cDCs but is lost in non-DC lineages [9, 56]. Mice that lack either Flt3L [57] or Flt3R [58] have strongly reduced numbers of cDCs whereas injection [59] or overexpression [59, 60] of Flt3L leads to a dramatic expansion of cDCs in both lymphoid and non-lymphoid tissues. Flt3L appears to function not only in the differentiation of cDCs, but also in the maintenance of peripheral DC homeostasis, by regulating DC proliferation [58]. The effects of Flt3L on cDC development are mediated through STAT3 (signal transducer and activator of transcription 3) and deletion of STAT3 causes a profound cDC deficiency through abrogated effects of Flt3L [61]. Flt3L can also be used to mimic DC development *in vitro* where it generates equivalents of $CD8a^+$ and $CD11b^+$ DCs from bone marrow cell suspensions [62].

The function of GM-CSF (Csf-2) in DC development was identified in *in vitro* studies and GM-CSF has been used extensively in *in vitro* culture systems to generate DCs and is commonly used to generate DCs-based vaccines for clinical use [63]. These *in vitro*-generated DCs however, are thought to derive from monocytes. GM-CSF signals through a heterodimeric receptor and mice deficient in GM-CSF or its receptors have very modest reductions in the number of splenic DCs and no effects on DC progenitors in the bone marrow [64, 65]. In contrast, GM-CSF appears to control the homeostasis of non-lymphoid DC populations and non-lymphoid $CD103^+CD11b^-$ cDC populations were reduced in GM-CSF receptor (*Csf2rb-/-Csf2rb2/-* mice) [65]. $CD11b^+$ non-lymphoid DCs were only modestly reduced however gating strategies suggest that these effects may have been underestimated due to the possible inclusion of $CD11b^+$ macrophages. Mechanistically, GM-CSF appears to maintain non-lymphoid homeostasis in part by promoting the cell survival [65]. Moreover, GM-CSF has been suggested to promote the acquisition of cross-presentation capacity (discussed below) [66] and in vivo *Csf2rb-/-Csf2rb2-/-* mice mount less efficient $CD8^+$ T cell responses to particulate antigen [65].

In addition to Flt3L and GM-CSF, lymphotoxin β signaling plays an important role of the homeostasis of splenic $CD11b^+$ cDCs. Deficiency in the tumour necrosis factor (TNF) receptor family members lymphotoxin β receptor (LTβR), or its ligand LTα1β2, leads to a reduction in the numbers of splenic $CD11b^+$ DCs in mice as a consequence of decreased proliferation [67-69]. Signaling is cell-intrinsic [53, 68], appears to be occur downstream of Notch signaling and to be required specifically for the $CD4^+ESAM^+$ compartment [53, 54]. B cells appear to be an important source of LTα1β2 as its overexpression in this cell compartment results in expansion of splenic $CD11b^+$ DCs [68]..

# 3. Functions of dendritic cells

## 3.1 Antigen recognition and uptake

As cDC precursors leave the bone marrow, they travel through the blood and home to tissues where they complete the final steps of their maturation. Immature cDCs constantly sample their environment through different PRRs. PRRs can be either membrane bound or cytoplasmic and are further classified according to ligand specificity, function and evolutionary history. In broad, membrane bound PRRs include TLRs and C-type lectin receptors (CLRs) whereas cytoplasmic PRRs include NOD-like receptors (NLRs) and RIG-1-like receptors (RLRs). As described in previous sections PRRs recognize PAMPs found on microbes. Moreover, they can detect damage-associated molecular patterns (DAMPs), which are components released by damaged cells. In most cases, ligand recognition by PRRs triggers intracellular signal transduction cascades leading to cDC maturation and release of signaling molecules (described below).

Once a cDCs has recognized foreign material, there are several mechanisms whereby this material can be taken up, including receptor mediated endocytosis, macropinocytosis and phagocytosis. Receptor-mediated endocytosis is mediated by the inward folding and budding of so-called clathrin-coated pits that form in the cells plasma membrane. The cytosolic molecule clathrin plays a major role in the formation of these pits and can concentrate receptors on the cell surface, leading to internalization of their respective ligands as the plasma membrane invaginates. Such receptors include Fc receptors, TLRs and c-type lectins such as the mannose receptor. Phagocytosis is a mechanism by which particulate matter can be taken up and involves the uptake of larger membrane areas than receptor-mediated endocytosis. Similarly, macropinocytosis involves invagination of the plasma cell membrane but captures extracellular fluids and its content in a non-specific manner [70]. DCs can also present antigen derived from within the cell itself. Such endogenous antigens include both self-antigens and antigens from intracellular pathogens.

## 3.2 Antigen processing and presentation

Exogenous antigens that have been taken up by the cell are targeted to a series of endocytic compartments in order to prepare them for degradation and presentation. In early and late endosomes, antigens are dissociated from their receptors. As endosomes fuse with vesicles from the Golgi network containing lysosomal acid hydrolyases, endosomes mature to from lysosomes, the principal hydrolytic cell compartment. Lysosomes digest material derived from exogenous sources (endocytosis and phagocytosis) as well as from materials derived from the process of autophagy, a catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through lysosomal degradation. Lysosomes contain an array of enzymes that can break down biological polymers. In lysosomes, peptides associate with MHCII molecules to form peptide:MHCII complexes. These complexes are transported to the cell surfaces for presentation of the antigen.

Endogenous antigens are mainly derived from degradation of endogenous proteins in the proteasome into peptides, which are transported to the endoplasmic reticulum, loaded on MHCI molecules and transported to the cell surface. cDCs can also present antigens derived from exogenous sources in peptide:MHCI complexes through a process known as cross-presentation. Cross-presentation is particularly important for mounting CD8 T cell responses to viruses that do not infect DCs. The cellular mechanisms underlying cross-presentation are not yet fully understood but involve transfer of antigens from endocytic to cytosolic compartments for proteolytic degradation [71, 72]. In addition, evidence suggests that endogenous antigens can be presented in the context of MHCII molecules via autophagy although these processes, and their contribution to adaptive immune responses, are incompletely understood [73].

In summary, cDCs present peptides derived from exogenous (and possibly endogenous) sources in peptide:MHCII complexes to $CD4^+$ T cells or from endogenous or exogenous sources in peptide:MHCI complexes to $CD8^+$ T cells. As previously mentioned, cDC subsets appear to differ in their ability to activate $CD4^+$ and $CD8^+$ T cells and CD8α-like cDCs have superior cross-presenting capacity [17, 18]. These differences have further been demonstrated in *in vivo* studies specifically targeting antigen to $CD8a^+$ or $CD11b^+$ cDCs via OVA conjugation to DEC205 and 33D1 antibodies, respectively. Thus, antigen delivery to $33D1^+$ ($CD11b^+$) cDCs preferentially resulted in MHCII-restricted antigen

presentation, whereas antigen deliver to DEC205$^{+}$ (CD8$\alpha^{+}$) cDCs induced MCHI-restricted antigen presentation with greater efficiency [26]. The underlying reasons for these differences are largely unknown but may include differences in antigen processing machinery between the two cDC subsets [26].

## 3.3 T cell activation and polarization by dendritic cells

### 3.3.1 Dendritic cells maturation and co-stimulation

The mechanisms described above are associated with the process known as DC maturation, which prepares DCs for their function as professional antigen presenting cells. Immature DCs are characterized by a high capacity for endocytosis and a lower T cell activation potential. They express relatively low levels of surface MHCII and instead MHCII molecules are abundant within lysosomal cell compartments where they can associate with ingested antigens. Adequate stimulus (e.g. TLR ligation) leads to immediate functional maturation of the DC, which is associated with increased transportation of MHC class II molecules from lysosomes to the plasma membrane.

Generally, the optimal activation of T cells requires two distinct signals from the antigen-presenting cell. The activation of the T cell receptor by presentation of antigen in the context of MHC molecules by DCs, is referred to as ”signal 1”. DC maturation is also associated with an upregulation of co-signaling molecules on the DC cell surface, which provide a second signal for T cells and determine the functional outcome of TCR signalling. T cell co-signalling receptors are either co-stimulatory or co-inhibitory and modulate TCR signaling in a positive or negative manner, respectively. Most T cell co-signalling receptors belong either to the immunoglobulin or the TNF receptor superfamilies [74]. One of the best characterized, and first described co-stimulatory receptors on T cells is CD28, which interacts with CD80 or CD86, bound on the membranes of DCs and provides a potent co-stimulatory signal to T cells [75]. However, during activation T cells integrate signals from diverse co-signaling pathways. How these are integrated to determine the functional outcome of the T cell is relatively poorly understood [74].

### 3.3.2 Migration and localization of dendritic cells

As previously mentioned, DCs are present in both lymphoid and non-lymphoid organs. Lymphoid resident DCs, which include DCs in the spleen, LNs, intestinal Peyer's patches (PPs) and thymus, derive from DC progenitors that have entered lymphoid structures via high endothelial venules (HEVs) or possibly in the case of the spleen, through marginal sinuses [9]. cDCs that derive from progenitors that have entered non-lymphoid tissue (via a so far unknown mechanism) migrate to associated LNs through afferent lymphatics following antigen encounter and maturation in their respective tissues.

In LNs and in the spleen, cDCs position to meet a T cell seeking its cognate peptide-MHC class II complex. Evidence suggests that cDC subsets localize to different areas of spleen and LNs. Thus, while in the spleen $CD8\alpha^+$ cDCs are localized primarily in the T cell zone, $CD11b^+$ cDCs are mainly located in an area of the white pulp known as the marginal zone (MZ) bridging channel [26, 76-78]. However, both subsets move into the T cell area in response to microbial stimulation [79, 80]. The migratory properties of cDC populations, and their positioning within LNs, are regulated by their responsiveness to chemotactic signals that remain largely uncharacterized. However while expression of CCR7 allows cDCs to move into T cell zones [81], recent studies suggest that $CD11b^+$ cDC localization to marginal zones depends in part on their expression of EBI2. Thus, mice lacking EBI2 expression had markedly reduced numbers of splenic and LN $CD11b^+$ cDCs. The remaining $CD11b^+$ were largely missing from marginal zones and were instead disturbed in the red pulp or in the T cell zone of the spleen. This defect could be rescued by treatment with LTβR agonistic antibody consistent with the possibility that in marginal zones, $CD11b^+$ cDCs receive LT signals that are required for their maintenance or proliferation [77].

### 3.3.3 Role of dendritic cells in $CD4^+$ T cell polarization

As previously mentioned, cDCs do not only provide antigen presentation and co-stimulation to naive T cells, but also polarizing cytokines that help direct the ensuing T cell response. The nature of those cytokines depends on the nature of the input signals that the cDCs receive at the time of initial microbe interaction. In this way, stimulation by a specific microbe results in effector T cells taking on a

phenotype that is best suited to drive a type of response most appropriate to combat that microbe. Importantly, distinct cDCs subsets may differ in their ability to drive the differentiation of distinct Th responses. Such differences can result from intrinsic differences in PRR expression. For example, CD8α-like cDCs specifically express TLR3, a PRR that recognizes double-stranded RNA carried by certain viruses [4]. In addition, it is possible that cDCs subsets differ in their intrinsic abilities to express and/or secrete certain cytokines.

***Effector Th cell development***

Effector T cell subsets are defined based on their cytokine production profiles and the differentiation of each is controlled by a distinct set of TFs. Currently, established subsets of effector $CD4^+$ T cells include Th1, Th2 and Th17 cells. Th1 cells are characterized by the production of IFNγ and develop in response to viral, bacterial and protozoan infections. Th1 differentiation involves activation of signal transducer of activated T cells (STAT)-1 and STAT4 by interferons and IL-12 [82]. These signals promote the expression of T-box transcription factor expressed in T cells (T-bet), the Th1 signature TF. Th2 cells develop in response to infections with parasitic helminthes and are characterized by production of cytokines IL-4, IL-5 and IL-13. However, Th2 cells are also associated with asthma and allergic responses. The TF specifying commitment to the Th2 lineage is GATA3 [83]. The Th17 subset was discovered more recently and is characterized by secretion of cytokines IL-17A, IL-17F and IL-22. Th17 cells play a major role in response to infection with extracellular bacteria and fungi and in barrier surface maintenance. However, they are also key drivers of inflammation associated with many autoimmune diseases. Cytokines that have been implicated in the development of Th17 cells include IL-6, IL-1β, IL-23, IL-21 and TGF-β [84]. In addition to Th1, Th2 and Th17 cells, additional Th cell subsets have recently been described, including Th9 [85] and Th22 cells [86]. Moreover, plasticity between states of Th cell differentiation is beginning to be recognized. The factors controlling transitions between Th subsets, and whether this reflects true plasticity or population heterogeneity, is currently under investigation [87].

***Regulatory T cell development***

In contrast the main function of regulatory T cells (Tregs), as the name suggests, is to prevent autoimmunity by regulating immune responses to self-antigens and to dampen effector T cell responses to limit immunopathology during infection. Tregs can be either natural (nTregs) or induced (iTregs). Natural Tregs are

characterized by their expression of the transcriptional repressor forkhead box protein 3 (FoxP3) and develop in the thymus as fully mature Tregs. In contrast $FoxP3^+$ iTregs are induced in the periphery from naïve T cells. An important factor in the development of iTregs is the cytokine TGF-β. Additionally, suppressor T cells that are $FoxP3^-$ but secrete the immunosuppressive cytokine IL-10 (Tr1 cells) can develop in the periphery and will be discussed further in the section concerning intestinal T cells.

# 4. The intestinal immune system

## 4.1 Structure and function of the intestine

The primary function of the intestine is to digest and absorb nutrients and minerals from food. Partly digested food material enters the proximal part of the small intestine (SI), the duodenum, from the stomach. In the duodenum, food is mixed with pancreatic juice containing digestive enzymes and bile, containing bile salts, which break it down further into protein, fats and carbohydrates. These are transmitted into the jejunum and ileum, which are the main sites of nutrient absorption. The last part of the intestine is the large intestine, or colon, which absorbs water from remaining food material, and finally passes indigestible waste material out of the body via the rectum.

The intestinal wall is made up of several distinct layers. The mucosa faces the intestinal lumen and comprises the epithelium and the underlying lamina propria, which is separated from luminal content by a single layer of epithelial cells. The luminal wall of the intestine is organized into villi, which are foldings of the intestinal wall that increase the surface area available for nutrient absorption. Intestinal epithelial cells derive from stem cells that reside in the intestinal crypts and differentiate into four principal cell types that make up the gut epithelium. Most epithelial cells are absorptive enterocytes responsible for taking up luminal nutrients. The apical side of enterocytes is covered by small extensions called microvilli that further increase the surface area of the intestinal wall. The epithelium also contains goblet cells, which secrete glycoproteins known as mucins. Mucins dissolve in water to form mucus, a viscous secretion that forms a biophysical barrier protecting the intestinal wall by dramatically reducing the bacterial load at the barrier between the epithelium and the lumen. Further, the epithelium contains paneth cells that are located in intestinal crypts and secrete anti-microbial peptides into the lumen [88]. Directly underneath the mucosa is the submucosa, a layer of loose connective tissues that supports the mucosa and joins it with the overlying muscular layers (the muscularis), responsible for gut movement such as peristalsis. Finally, enclosing the intestine is the serosa is a smooth membrane, which secretes a serous fluid and encloses the intestine.

## 4.2 The intestinal immune system

In the interest of nutrient absorption, the intestinal mucosa presents a huge surface area to the outside milieu. The epithelial barrier consists of a single cell layer and as a consequence, the intestines represent a major site of potential pathogen entry. In addition the intestinal immune system is continually exposed to antigens derived from foreign but innocuous food antigens and from a large number of resident intestinal microbes. In order to preserve the integrity of the mucosal barrier, and maintain intestinal homeostasis, the intestinal immune system relies on the ability to generate tolerogenic responses to innocuous antigen and at the same time possess mechanisms that allow effective immune responses to intestinal pathogens. The intestinal immune system has evolved numerous adaptations to cope with this challenging task. Still, dysregulated immune responses to commensal bacteria are thought to contribute to the development of inflammatory bowel diseases including Crohn's disease and Ulcerative colitis [89, 90] while a failure to tolerate harmless food antigens is associated with the development of food allergies and Celiac disease [91].

Intestinal immune compartments can be divided into inductive and effector sites. At inductive sites, adaptive immune responses to gut antigens are initiated and naïve T and B cell are primed. Inductive sites include Peyer's patches (PPs) and isolated lymphoid follicles (ILFs), which constitute the gut associated lymphoid tissues (GALTs), as well as the mesenteric lymph nodes (MLNs) that drain the intestinal mucosa via afferent lymphatics. Effector sites include the intestinal epithelium and the LP, where, lymphocytes, including B and T cells, primed at inductive sites carry out their function **(Figure 2)**. The phenotype and function of intestinal effector T cells will be further discussed in the section that concerns intestinal T cells.

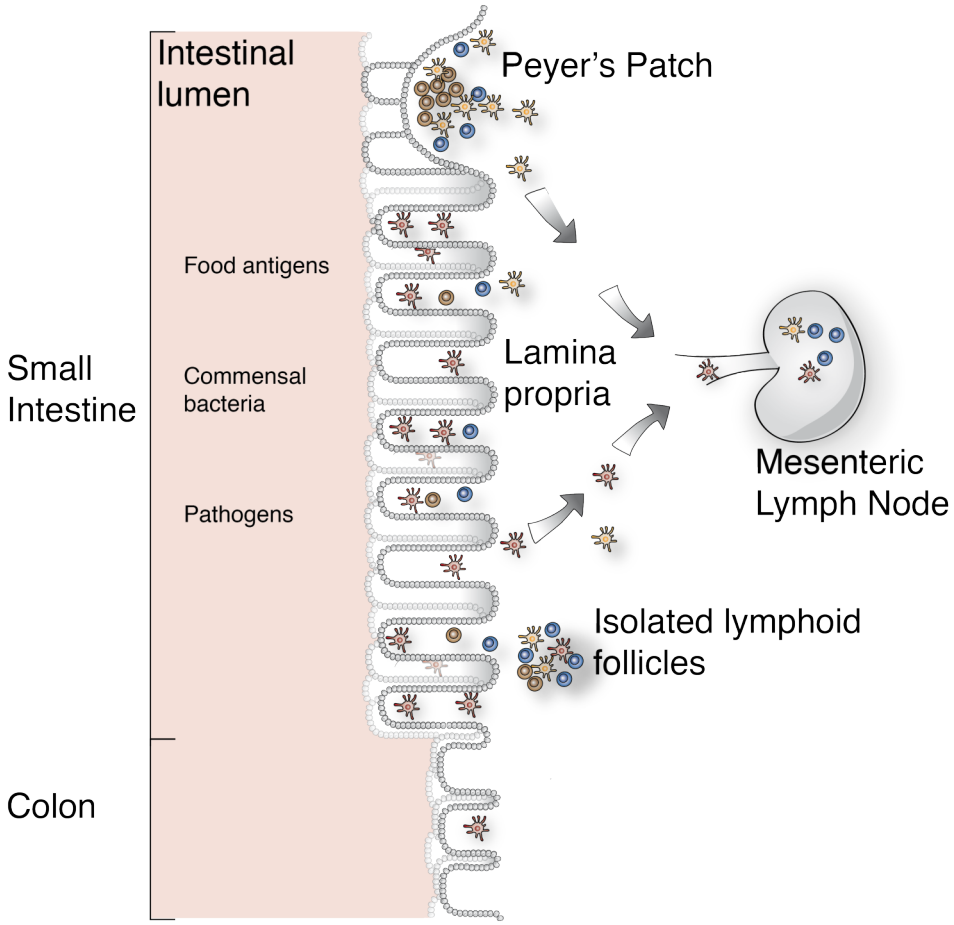


**Figure 2. Structure of the intestinal immune system.** The intestinal lamina propria (LP) is covered by a single layer of epithelium. T cells dominate in the epithelium and the underlying LP contains several cell subsets, including B and T cells, macrophages as well as conventional dendritic cells (cDCs). cDCs are also present in the GALT like Peyer's patches (PPs) and isolated lymphoid follicles (ILFs). The intestinal LP is drained by lymphatic vessels that connect the intesinte to mesenteric lymph nodes (MLNs). PPs, ILFs and MLNs make up the inductive sites of the intestinal immune system.

## 4.2 Inductive sites of the intestinal immune system

***Mesenteric lymph nodes***

MLNs are embedded within layers of the mesentery. Like other LNs, MLNs consist of an outer cortex and an inner medulla surrounded by a fibrous capsule. The cortex contains B cell follicles and T cells distributed in paracortical areas. The inner medulla is rich in macrophages and plasma cells. Lymph arriving from the intestine via afferent lymphatics drains into the subcapsular sinuses just beneath the capsule, continues into medullary sinuses and finally leaves the lymph node via efferent lymphatics. Eventually lymphocytes primed in MLNs can return to the blood stream and may home to intestinal effector sites such as the LP and IEL compartments. MLNs have a crucial role in the induction of mucosal immune responses and recognition of orally fed antigen occurs in the MLN within hours [92]. Moreover, MLNs have been implicated in the development of oral tolerance as mice lacking MLN (by surgical removal) are unable to generate tolerance to orally fed soluble antigen [93, 94].

***Peyer's patches***

PPs are aggregates of lymphoid tissue within the intestinal mucosa that, in humans, are largely confined to the ileum. They consist of several aggregated lymphoid follicles with distinct B and T cell zones. The epithelium covering PPs is called the follicle-associated epithelium (FAE) and contains specialized enterocytes called microfold (M) cells, which specialize in the uptake of particulate luminal antigen (discussed below). The area underneath the FAE is called the subepithelial dome (SED) and is rich in cDCs.

A striking feature of the intestinal immune system is the presence of a large number of $IgA^+$ plasma cells and PPs are important sites of IgA class switching. Secreted IgA limits bacterial association with the epithelium by sequestering bacteria in the lumen and is required to maintain gut homeostasis [95, 96].

***Isolated lymphoid follicles***

ILFs are lymphoid structures in the mucosa that, similar to Peyer's patches, are covered by FAE containing M cells. However, ILFs are much smaller than PPs and generally consist of a single B cell cluster surrounded by lymphoid tissue-inducer (LTi) cells, DCs and a smaller number of interspersed T cells [97, 98]. ILFs are thought to develop from even small lymphoid aggregates called

cryptopatches (CPs) that consist mainly of LTi cells surrounded by a few DCs [97]. The development of ILFs from CPs is a dynamic event that is dependent on LTi cells and requires signals from commensal microbes. Hence, in germ-free mice ILFs fail to develop properly [99]. It has been suggested that cDCs could play a role in ILF development by interpreting signals from intestinal microbes and in the recruitment of lymphocytes. Indeed, ILF cDCs produce the B-cell attracting chemokine CXCL13 and in CD11c-depleted mice, ILF formation regresses [100] however, a direct effect on B cells (which may express CD11c) was not excluded. Hence, the role of DCs in ILF development, as well as the ontogeny and phenotypes of ILF DCs, remains largely unexplored.

## 4.3 Intestinal T cells

### 4.3.1 T cells in the intestinal epithelium

Effector cells in the intestinal epithelium are called intraepithelial lymphocytes (IELs) and consist of a heterogenous population of T cells that are involved in host defence and maintenance of the epithelial barrier. The large majority of IELs are $CD8^+$. IELs are either "natural" or "induced" depending on mechanism of activation and on the type of antigen they recognize [101, 102]. Induced IELs are conventional T cells that are thought to have encountered their cognate antigen in LNs and subsequently migrated to the epithelium. They include a major population of $TCR\alpha\beta^+$ $CD8\alpha\beta^+$ and a minor population of $TCR\alpha\beta^+$ $CD4^+$ T cells that express a cytotoxic effector memory phenotype [103] and have an oligoclonal TCR repertoire [104]. $CD4^+$ T cells can encompass all Th subsets. Induced IEL numbers increase with increasing age of the individual [105]. In contrast, natural IELs are activated in the thymus where they are positively selected on self-antigens. Once selected in the thymus, they migrate directly to the epithelium [106, 107]. Natural IELs are either $CD8\alpha\alpha^+$ or $CD8\alpha\alpha^-$ and express $TCR\gamma\delta$ or $TCR\alpha\beta$ chains. As they do not depend on the recognition of exogenous antigen, they populate the gut early in life and maintain constant numbers over time [105].

### 4.3.2 T cells in the lamina propria

LP T cells are primarily conventional $CD4^+TCR\alpha\beta^+$ cells, with a small proportion of $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ cells. Similar to IELs, the large majority of LP T cells display

a previously activated or memory phenotype and are derived from naïve T cells primed and activated in inductive immune sites [108]. Under steady-state conditions, Th17, Th1 and FoxP3$^+$ regulatory T cells dominate the intestine and GALT. As Th2 cells are important for coordinating immune responses to parasitic helminthes, the relative scarcity of Th2 cells in the steady-state intestine likely reflects the absence of such parasites in specific pathogen-free mice [109]. The function of intestinal Th1 cells, in the steady state and in inflammation, as well as the factors driving their development, is currently unclear.

***Intestinal Th17 cells***

Compared to other tissues, Th17 cells are particularly enriched in the intestinal mucosa [110]. As such, in the field of mucosal immunology, Th17 cells have been a subject of particulate interest in recent years. The development of intestinal Th17 cells appears to be linked to the presence of intestinal microbiota. Thus, mice raised under germ-free conditions, as well as mice treated with an antibiotic cocktail, had decreased frequencies of Th17 cells in the SI compared to controls [110]. It should be noted, however, that a contradictory study found normal numbers of Th17 cells in the SI, and elevated numbers of Th17 cells in the colon of germ-free mice [111]. The induction of Th17 cells has also been coupled to the presence of specific microbial species. For example, colonization of the SI with the gram-positive segmented filamentous bacteria (SFB) was sufficient to increase the numbers of IL-17 and IL-22 producing cells in the SI LP [112]. Notably, mice deficient in the TLR signaling adaptor proteins Myd88 and TRIF had normal numbers of Th17 cells [110, 113] suggesting that under steady state conditions, Th17 cell development is TLR independent. Still, it has been reported that TLR9 deficient mice have reduced frequencies of Th17 cells in the SI LP [114] and moreover, a recent study found decreased numbers of RORγT$^+$ cells in Myd88 deficient mice [115]. The reasons for these contradictory findings are currently not clear.

The optimal development of intestinal Th17 requires expression of the TF RORγT [112]. Among the cytokines that are involved in Th17 development, TGF-β appears to be necessary for the development of intestinal Th17 cells as mice with a TGF-β1 deficiency or defective TGF-β activation, have fewer intestinal Th17 cells [110, 116]. Further studies have suggested a key role for IL-1β in intestinal Th17 development [115, 117]. Interestingly, two studies assessing the numbers of Th17 cells in the intestine of IL-6 deficient mice have reached different conclusions regarding the role of IL-6. Thus while Shaw et al found normal numbers of

RORγT cells, and normal IL-17 and IL-22 cytokine production in the intestine of IL-6 deficient mice [115], Hu et al. found reduced numbers of intestinal RORγT$^{+}$ cells in IL-6$^{-/-}$ mice [117]. In paper II of this thesis we address the role of intestinal cDC subsets in the generation of Th17 responses in the MLNs and demonstrate a key role for CD103$^{+}$CD11b$^{+}$ cDC derived IL-6 [34].

The cytokines produced by Th17 cells, notably IL-17A, IL-17F and IL-22, have been implicated in several aspects of intestinal immunity, both in steady state and under inflammatory conditions. Receptors for IL-17A, IL-17F and IL-22 are broadly expressed by intestinal epithelial cells [84] and IL-17A has been shown to stimulate epithelial secretion of G-CSF that drives the recruitment of neutrophils [118]. Moreover, IL-17A and IL-17F can promote β-defensin production by epithelial cells [119, 120] and IL-22 induced the expression of Reg family antimicrobial peptides from epithelial cells [121]. Thus, Th17 cells are likely involved in the control of pathogenic and commensal microbes. However Th17 cells have also been associated with intestinal inflammation and have been implicated in several models of inflammatory bowel disease [109]. Factors controlling their pathogenicity are currently under intense investigation.

***Intestinal regulatory T cells***

Conventional T cell in the intestine run the inherent risk of reacting towards antigens derived from the intestinal flora of from the diet. Indeed, when antigen-experienced T cells are adoptively transferred into immunodeficient hosts, they are capable of inducing intestinal inflammation [122]. Thus, to avoid adverse immune responses to gut antigens, the intestinal T cell pool contains a significant proportion of Tregs. Intestinal Treg include both natural (thymus-derived) Tregs and induced Tregs that are either FoxP3$^{+}$ or FoxP3$^{-}$ Tr1-type cells [123]. Intestinal Tr1 cells are characterized by abundant production of IL-10 and are abundant in the SI but rare in the colon [124, 125]. A substantial fraction of FoxP3$^{+}$ cells are also IL-10$^{+}$ [125]. Although natural and induced FoxP3$^{+}$ Tregs are phenotypically similar, the Ikaros family member Helios and the transmembrane protein neuropilin-1 have been suggested to be useful markers to permit segregation between the two [126-128].

The important regulatory function of Tregs is evident from the finding that FoxP3 deficient mice develop spontaneous and fatal lymphoproliferative disorder which can be overcome by adoptively transferring FoxP3$^{+}$ Tregs [129]. Furthermore, transfer of CD45RB$^{high}$ naïve T cell alone can induce colitis in immunodeficient

mice, whereas co-transfer of naïve T cells and CD25-expressing, antigen experienced, $CD45RB^{lo}$ cells (containing Tregs) can prevent disease [130]. Transfer of naïve T cells expressing a dominant-negative TGF-β receptor causes inflammation that cannot be suppressed by Tregs suggesting that TGF-β is an important Treg derived effector cytokine [123, 131]. In addition, the importance of IL-10 in regulating gut immune responses is evidenced by the fact IL-10 deficient mice develop spontaneous intestinal inflammation driven by components of the intestinal microbiota that are otherwise non-pathogenic [123]. Notably, spontaneous inflammation in $IL\text{-}10^{-/-}$ is dependent on $CD4^+$ T cells [132].

Factors that have been implicated in the development of $FoxP3^+$ iTregs and the possible role of cDCs in this process will be further discussed in subsequent chapters. In contrast the factors controlling the generation of IL-10 producing intestinal $CD4^+$ T cells are largely undetermined. Moreover, while $FoxP3^+$ iTregs have been suggested to be primed in MLNs and then undergo local expansion in the gut in response to IL-10 production by intestinal macrophages [133], it is currently not know if similar mechanisms generate and expand the population of intestinal $FoxP3^-$ $IL\text{-}10^+$ cells.

## 4.4 Homing of intestinal T cells

### 4.4.1 Basic mechanisms of immune cell homing to tissues

The entry of immune cells into tissues is mediated by the interaction between adhesion receptors on the surface of immune cells and their respective ligands on vascular endothelium. Effector cell subsets display tropism for different tissues, mediated by tissue-specific ligand expression and selective expression of adhesion receptors on immune cells. Cell migration into tissues is a well-characterized multi-step process mediated by a series of cellular interactions. The first step is mediated by low-affinity molecular interactions between selectins and their carbohydrate ligands, which facilitate the capture of lymphocytes that flow in the blood stream and allows them to slow down and roll along the vascular endothelium. During rolling, chemokines signal to chemokine receptors expressed on immune cells. This interaction leads to activation of intracellular pathways within the immune cells and rapid conformational changes of integrins. Integrins are heterodimers combining one α chain and one β chain, both of which exist in

multiple forms that bind to ligands of the immunoglobulin gene superfamily expressed on the endothelium. Binding of integrins with high ligand-affinity leads to firm adhesion and arrest of rolling cells. Finally, having firmly adhered to the endothelium, cells may migrate through or between endothelial cells into the respective tissue. Within tissues, immune cells can follow chemotactic gradients that guide them to distinct tissue compartments.

### 4.4.2 Homing of effector T cells to the small intestine

Several cellular adhesion receptors on T cells have been implicated in their homing to intestinal tissues, including the integrin $\alpha4\beta7$ and the chemokine receptor (CCR)9 [134, 135]. Their respective ligands MadCAM-1 (mucosal vascular addressin cell adhesion molecule 1) [136] and CCL25 are constitutively expressed on intestinal vascular endothelium [137] and allow gut-homing T cells to adhere firmly to the intestinal endothelium. The importance of these interactions has been demonstrated in adoptive transfer studies using T cells deficient in $\beta7$ chain [138] or CCR9 [139, 140]. Moreover, the relevance of these interaction have been demonstrated in studies using *in vivo* antibody blocking to CCL25 [141] and $\alpha4\beta7$ [142]. The generation of gut-tropic T cells *in vitro* and *in vivo* has been shown to depend on the presence of the signaling molecule retinoic acid (RA), which is derived from the metabolism of vitamin A [143, 144].

## 4.5 Intestinal dendritic cells

### 4.5.1 Intestinal dendritic cell subsets

cDCs are found throughout the intestinal LP, in PPs and ILFs as well as in MLNs. The characterization of cDCs in the intestine and in GALT has been the subject of intense investigation and controversy, particularly as the markers traditionally used to define DCs are expressed by intestinal macrophages. For example, $CD11c^{+}MHCII^{+}$ cells expressing high levels of the chemokine receptor CX3CR1 are abundant in the intestinal LP and were initially classified as DCs [44]. However, results from our study (Paper I), and studies from other groups clearly demonstrate that these cells are tissue-resident, monocyte-derived macrophages [145-149]. Moreover, cDCs can be distinguished by their lack of expression of the

high-affinity IgG receptor FcγR1, CD64 and all CX3CR1$^{high}$ cells (as well as a subset of monocyte-derived CX3CR1$^{int}$ cells) are CD64$^{+}$ [42, 149].

The majority of CD11c$^{+}$MHCII$^{+}$CD64$^{-}$ cDCs in the SI express the integrin CD103 (αE) [43, 44]. In the colon, the proportion of cDC expressin CD103 is lower than in the SI [44]. The ligand for CD103 is E-cadherin, which is present on the basolateral side of small intestinal epithelial cells. CD103 is also highly expressed by IELs and is thought to mediate their adhesion to epithelial cells as mice deficient in CD103 have reduced number of LP T cells and IELs [150] however the function of CD103 on intestinal cDCs is not known.

CD103$^{+}$ intestinal DCs can be divided into two main populations based on their expression of the integrin CD11b [146, 147]. The finding that CD103$^{+}$CD11b$^{-}$ cdDCs are markedly reduced in Id2 deficient mice [23, 146], which lack PPs [151], suggested that CD103$^{+}$CD11b$^{-}$ cDCs are located within these structures however the requirement for Id2 likely also reflect a cell-intrinsic defect in the development of CD8α-like DCs, including CD103$^{+}$CD11b$^{-}$ non-lymphoid cDCs [23]. Indeed, CD103$^{+}$CD11b$^{-}$ SI cDCs are present in the intestine of RORγT$^{-/-}$ mice [152], which lack ILFs and PPs [153], suggesting that they do not require GALT for their final differentiation. Nevertheless, CD103$^{+}$CD11b$^{-}$ cDCs are enriched in preparations of PPs of WT mice, where CD103$^{+}$CD11b$^{+}$ cDCs are relatively rare [146], suggesting that CD103$^{+}$CD11b$^{-}$ cDCs are mainly located in lymphoid structures of the intestine, whereas CD103$^{+}$CD11b$^{+}$ cDCs are mainly located in the LP. In addition to CD103$^{+}$ cDCs, the intestine contains a distinct population of CD11c$^{+}$MHCII$^{+}$CD64$^{-}$ cDCs that are CD103$^{-}$ and express heterogeneous levels of CD11b [43, 44]. Notably, CD103$^{-}$CD11b$^{-}$ SI cDCs are markedly reduced in RORγT$^{-/-}$ mice [152], suggesting that these reside in GALT. In Zbtb46-GFP reporter mice, all CD11c$^{+}$MHCII$^{+}$ cells that are CD103$^{+}$CD11b$^{-}$ or CD103$^{+}$CD11b$^{+}$ cells are uniformly GFP$^{+}$ and CD103$^{-}$CD11b$^{-}$ and CD103$^{-}$CD11b$^{+}$ cells also contained a substantial GFP$^{+}$ fraction. GFP$^{+}$ cells were also negative for the macrophage marker F4/80. These findings suggest that Zbtb46 is a useful marker of cDCs in intestinal tissues [54].

In contrast to the mouse, the study of human intestinal cDC populations is in its infancy. CD103$^{+}$ DC have been identified in the human SI LP by immunohistochemistry and in SI LP cell preparations by flow cytometry [154]. Moreover, CD103$^{+}$ DCs are present in the human MLNs [155]. In paper II we identify putative equivalents of mouse intestinal CD103$^{+}$CD11b$^{-}$ and CD103$^{+}$CD11b$^{+}$ cDC subsets. We assessed the expression of CD141 and DNGR1

which have been shown to mark human CD8α like DCs [27, 29-31] and show that human SI $CD103^+$ DCs can be split into two major subsets, a dominant population of $SIRP\alpha^+CD141^-$ DCs and a minor population of $SIRP\alpha^{lo/-}CD141^+$ DCs (the latter co-expressing DNGR1), which are the proposed human equivalents of mouse $CD103^+CD11b^+$ and $CD103^+CD11b^-$ cDCs, respectively. Recently, comparison of global gene expression in human and mouse intestinal cDC subsets has confirmed this suggestion [156].

### 4.5.2 Development of intestinal dendritic cells

***Precursors***

Adoptive transfers studies into diptheria toxin treated CD11c-DTR or WT mice have demonstrated that pre-cDCs give rise to $CD103^+CD11b^-$ and $CD103^+CD11b^+$ cDCs in the intestinal LP, whereas MDPs give rise to all DC and macrophage subsets. In contrast $Ly6C^{hi}$ monocytes give rise only to $CX3CR1^+$ LP cells [145, 146]. More recently, a subset of bone marrow lineage-$CD11c^+\alpha4\beta7^+B220^+CCR9^-$ cells (termed pre-μ(mucosal)DCs) was identified that preferentially gave rise to intestinal $CD103^+$ cDCs (primarily $CD11b^-$) when adoptively transferred into WT mice [157]. While this finding suggest the presence of a gut-homing DC progenitor, pre-μDCs also gave rise to splenic and lung DCs and moreover, preferentially to CD8α-like cDCs in all organs. The relative roles of DC progenitor subsets in the generation of intestinal cDCs thus remain unresolved. Furthermore, it remains to be established if pre-cDCs or pre-μDCs give rise to $CD103^-$ intestinal cDCs. In this regard, two reports demonstrated that adoptive transfer of $Ly6C^{high}$ monocytes gave rise exclusively to $F4/80^+CD64^+$ macrophages suggesting that $CD64^-$ mononuclear phagocytes are not monocyte-derived [42, 149].

***Transcription factors***

The first evidence that $CD103^+CD11b^-$ and $CD103^+CD11b^+$ intestinal cDCs depend on distinct TF programs came from two studies showing that $CD103^+CD11b^-$ are absent in the LP and PP of Batf3, Id2 and IRF8 deficient mice and thus belong the unified subset of CD8α-like DCs [22, 23]. $CD103^+CD11b^-$ LP cDCs also express the chemokine receptor XCR1 as well as the c-type lectin DNGR-1, recently shown to be prototypical markers of CD8α-like DCs [27]. In contrast, $CD103^+CD11b^+$ LP cDCs are unaffected by Batf3, Id2 or IRF8

deficiency [22, 23] and are negative for XCR1 and DNGR-1 [24, 25]. At the start of this thesis work, the TF requirements for $CD103^+CD11b^+$ intestinal cDCs were not known. Thus, we (Paper II) and others [43] have addressed this question and shown that mice lacking the TF IRF4 specifically in $CD11c^+$ cells, have significantly reduced numbers of intestinal and MLN $CD103^+CD11b^+$ cDCs. In addition, total numbers of splenic, lymph node resident and lung $CD11b^+$ cDCs were reduced (Paper II) [43] . Similarly, mice lacking Notch2 in $CD11c^+$ cells have reduced numbers of $CD103^+CD11b^+$ intestinal cDCs and, as previously described, reduced numbers of $CD4^+CD11b^+$ splenic cDCs expressing ESAM [53, 54]. Together these studies provide compelling evidence in favor of a developmental link between $CD11b^+$ cDC in various tissues. The TF requirements for $CD103^-$ intestinal cDCs are largely undetermined and most of the studies to date have not included CD64 in the analysis. However, our unpublished findings, similar to the findings of Schlitzer et al. [43], suggest that SI $CD103^-CD11b^+$ are found in normal numbers of IRF4 deficient mice. However, MLN migratory $CD103^-CD11b^+$ MLN cDC are partly affected by IRF4 deficiency (Paper II)[43].

Recently, Watchmaker *et al*. assessed the role of TFs Bcl6 and Blimp-1 in intestinal cDC development [156]. Bcl6 and Blimp-1 are transcriptional repressors that have counter-regulatory roles in the differentiation of effector T cell and B cells and were differentially expressed by $CD103^+CD11b^-$ and $CD103^+CD11b^+$ SI LP cDCs. Bcl6 was preferentially expressed by $CD103^+CD11b^-$ cDCs and these cells were lacking in the SI and MLNs of Bcl6 deficient mice. Bcl6 was also required for the development of lymphoid resident $CD8\alpha^+$ cDCs. In contrast Blimp-1 was expressed at higher levels in $CD103^+CD11b^+$ SI cDCs. In Blimp-1 deficient mice, $CD103^+CD11b^+$ SI cDCs were still present in the SI LP, however the ratio of $CD103^+CD11b^+$ over $CD103^+CD11b^-$ cDCs was decreased [156]. Thus, Bcl6 and Blimp-1 may have selective and opposing roles in the specification of intestinal cDCs subsets.

With regard to TF requirements for human intestinal cDC subsets, we have shown that $CD103^+SIRP\alpha^+$ cDCs, the counterparts of mouse $CD103^+CD11b^+$ cDCs, express higher levels of intracellular IRF4 protein than $CD103^+SIRP\alpha^-$ cDCs (Paper II). Recently a comparison of transcriptomes between mice and human intestinal cDC, identified several TFs that showed conserved differences between mouse and human DC subset. In agreement with our findings, IRF4 was expressed at higher levels in mouse $CD103^+CD11b^+$ versus $CD103^+CD11b^-$ cDCs and in human $CD103^+SIRP\alpha^+$ versus $CD103^+SIRP\alpha^-$ cDCs [156].

***Growth factors***

The cDC growth factor Flt3L is a crucial factor for the development of intestinal cDCs and mice lacking the Flt3 receptor have a marked reduction in the number of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ SI cDCs [43, 146]. Although this study did not assess the numbers of $CD103^-$ SI cDCs, it has been reported that SI $CD64^-$ are virtually absent in $FltL3^{-/-}$ mice implying that all intestinal cDC subsets depend on this growth factor [42]. In contrast, in WT:$Flt3R^{-/-}$ mixed BM chimeras, the number of $CD103^+CD11b^-$ and $CD103^+CD11b^+$, derived from $Flt3R^{-/-}$ BM were markedly reduced whereas $CD103^-CD11b^+$ SI LP were Flt3 independent and displayed the same chimerism as a control population [43]. Addition of exogenous Flt3L expands both $CD103^+$ SI cDC subsets but has a greater impact on $CD103^+CD11b^-$ cDCs (Paper II) [152]. It also expands $CD103^-CD11b^-$ and $CD103^-CD11b^+$ SI LP cDCs [152]. DC specific deletion of Pten, which negatively regulates PI3K-mTOR signaling induced by Flt3L, leads to increased numbers of intestinal $CD103^+CD11b^-$ cDCs [158]. Regarding the role of GM-CSF in intestinal cDC development, studies in *Csf2rb*$^{-/-}$*Csf2rb2*$^{-/-}$ (GM-CSFR) mice have demonstrated that $CD103^+CD11b^+$ SI cDCs are dependent on GM-CSF signaling to maintain normal numbers. Finally, $CD103^+CD11b^+$ SI cDC homeostasis appears to depend on lymphotoxin signaling. Thus, in mixed BM chimeras generated by the reconstitution of WT mice with WT:LTβR BM, $CD103^+CD11b^+$ cDCs derived from WT BM outcompeted those derived from LTβR BM [54].

### 4.5.3 Migration of intestinal dendritic cells to mesenteric lymph nodes

The migration of cDCs from the LP in the steady state is dependent on the chemokine recptor CCR7 [159-162] and $CD103^+$ cDCs are reduced in the MLNs but not the LP of CCR7 deficient mice [94, 160, 161]. In BrdU pulse-chase experiments, $CD103^+$ accumulate with delayed kinetics in the MLNs compared to the LP [155]. These observations provided indirect evidence that $CD103^+$ DCs represent a migratory cDC population in intestinal afferent lymphatics. Using confocal microscopy to study afferent lymph vessels *ex vivo* we have provided direct evidence that the large majority of $CD11c^+$ cells in the draining lymph are $CD103^+$ (Paper I). Subsequently, this finding was supported by a study in which thoracic duct lymph was cannulated from mice in which the MLNs had been surgically removed. DC subsets in the lymph were analyzed by FACS. 75-85% of

cDCs in the steady-state lymph were $CD103^+$ and the majority of the remaining $CD103^-$ DCs were $CD11b^+$ [152].

cDCs in mesenteric lymph nodes can be divided into migratory cDCs, that arrive from the intestinal LP via afferent lymphatics, and lymphoid tissue resident cDCs, which derive from circulating precursors that enter directly from the blood. Migratory and resident MLN cDCs can be distinguished on the basis of their MHCII expression. Thus whereas migratory cDCs are $CD11c^+MHC^{high}$, resident MLN cDCs are $CD11c^+MHCII^{int}$. $MHCII^{hi}$ MLN cDCs are largely $CD103^+$, with roughly equal numbers of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ cDCs. $MHCII^{hi}$ MLN cDCs also contain a minor population of $CD103^-$ cDCs, most of which are $CD11b^+$ (Paper II). The DC subset composition among $MHCII^{hi}$ MLN cDCs thus closely resembles that in the intestinal draining lymph [152]. $MHCII^{int}$ MLN cDCs, similar to splenic cDCs, can be divided into a $CD8\alpha^+$ and a $CD11b^+$ subset (Paper II).

### 4.5.4 Functions of intestinal dendritic cells

***Transport of antigen and initiation of adaptive immune responses***

Intestinal migratory cDCs are thought to play crucial roles in the initiation of adaptive immune responses to luminal antigen. $CCR7^{-/-}$ mice fail to mount T cell responses to orally delivered soluble antigen and to generate oral tolerance [94, 160]. Moreover, following administration of oral ovalbumin, $CD103^+$ but not $CD103^-$ MLN cDCs induce proliferation of ovalbumin-specific $CD4^+$ and $CD8^+$ T cells [155, 163], suggesting that migratory $CD103^+$ cDCs transport soluble antigen from LP to MLNs. Furthermore, there is evidence that migratory intestinal cDCs carry particulate luminal antigen to the MLN. Thus, after oral administration of *Salmonella*, bacteria were detected primarily with the $CD103^+CD11b^+$ MLN DCs [146]. Similarly, following administration of *Enterobacter cloacae*, a mouse commensal bacteria, or *Salmonella*, live bacteria were found to reside mainly within $CD11c^+CD11b^+$ cells in the MLNs [164].

As discussed previously, DC subsets differ in their ability to prime $CD4^+$ and $CD8^+$ T cells [26]. With regards to intestinal cDCs, *in vitro* experiments suggest that sorted $CD103^+CD11b^-$ cDCs are have an enhanced capacity to cross-present antigen to $CD8^+$ T cells, consistent with their developmental relationship with lymphoid tissue $CD8\alpha^+$ cDCs [152]. In vitro experiments using sorted cDC

subsets suggest that antigen presentation to $CD4^+$ T cells is a capacity of all migratory cDCs (Paper II)[152, 165] however, these studies did not address the roles of migratory cDC subsets *in vivo*. In paper III of this thesis we perform *ex vivo* studies to evaluate the role of migratory cDC subsets in $CD4^+$ T cell responses to orally administered antigen and further address the role of IRF4-dependent cDCs in the priming of $CD4^+$ T cell responses *in vivo* (Paper III).

***Induction of gut-homing receptors on T cells***

As discussed previously, the homing of T cells to the gut mucosa depends on their expression of CCR9 and α4β7, which are selectively induced on T cells in the GALT [134]. Moreover, RA is essential for gut-tropic T cell generation *in vitro* and in vivo [143, 144]. *In vitro* experiments demonstrated that, compared to splenic DCs, MLN DC have an enhanced capacity to generate induce CCR9 and α4β7 expression on CD8+ T cells [139]. Subsequently, intestinal DCs expressing CD103 were identified and shown to have an enhanced capacity to induce gut-homing molecules CCR9 and α4β7 on T cells *in vitro* [155, 160, 166]. This capacity of MLN $CD103^+$ cDCs to generate gut-homing T cells has been linked to their capacity to generate the vitamin A metabolite RA. Thus, MLN $CD103^+$ cDCs were shown to have an enhanced capacity to produce RA [155, 167]. Similarly, all migratory cDCs in lymph, including the $CD103^-$ cDCs have the ability to metabolize vitamin A and can induce CCR9 on T cells *in vitro* [152]. The role intestinal-derived cDCs in the generation of gut-homing T cells *in vivo*, however, has not been addressed. Hence, in Paper II and Paper III we assess the ability of mice lacking IRF4-dependent cDCs to induce expression of gut-homing receptors on $CD4^+$ T cells.

***Treg cell differentiation***

MLN is a site of enhanced iTreg differentiaton [163, 168]. Compared with other subsets, $CD103^+$ cDCs have an enhanced ability to generate iTreg cells from naïve T cells *in vitro*. Efficient *in vitro* conversion of iTregs can be accomplished in the presence of TGF-β and RA acts in synergy to enhance this conversion [169]. The ability of $CD103^+$ cDCs to enhance iTreg conversion can be blocked by inhibiting RA receptor signaling, indicating that it depends on the capacity of $CD103^+$ cDCs to produce RA [163, 168, 169].

Peripheral induction of iTregs *in vivo* depends on TGF-β [170]. TGF-β is highly expressed in the intestine as a latent protein complex and to be biologically active,

this complex needs to be activated [171]. DC-specific deletion of αvβ8, a TGF-β activating integrin, leads to reduced numbers of intestinal FoxP3$^+$ Tregs and systemic autoimmunity [172]. Further, antibody mediated blockade of TGF-β function abolishes the ability of CD103$^+$ to induce iTregs *in vitro* and CD103$^+$ MLN cDCs have an enhanced capacity to activate latent TGF-β compared to CD103$^-$ MLN cDCs. This ability was dependent on their expression of αvβ8 [173]. Although the relative contributions of CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ cDCs were not assessed in this study, our unpublished observations suggest that only CD103$^+$CD11b$^-$ cDCs express αvβ8. In this regard, however, mice deficient in the TR Baft3, which lack intestinal CD103$^+$CD11b$^-$ cDCs, have normal numbers of intestinal Tregs [22]. In a mouse model lacking both CD103$^+$ cDC subsets, it was recently demonstrated that although CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ cDCs are individually redundant for generating FoxP3$^+$ Tregs in the MLN, together they are required to imprint gut-homing receptors on Tregs in the MLN, thereby maintaining normal numbers of Tregs in the intestine [174]. In paper II and III, we have addressed the role of IRF4-dependent cDC in iTreg generation in the MLNs and in the maintenance of Tregs in the steady-state SI and colon.

***Th cell differentiation***

While intestinal CD103$^+$ cDCs were initially suggested to represent a "tolerogenic" cDC subset, particularly adept at generating regulatory T cell responses [163, 168], evidence now suggest that these cDCs are also important in driving the differentiation of effector Th cells. *In vitro* studies have indicated that while both CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ cDC subsets can drive Th1 polarization [175], CD103$^+$CD11b$^+$ cDCs are more effective in driving Th17 polarization (Paper II)[165, 175, 176]. Moreover, a recent study suggested that CD103$^-$ cDCs, isolated from intestinal draining lymph have an enhanced capacity to generate Th1 and Th17 cells *in vitro*, when compared to CD103$^+$ subsets [152]. In paper II of this thesis, we address the role of IRF4-dependent DCs in the generation of Th1 and Th17 cell generation *in vivo*.

### 4.5.5 Imprinting of intestinal dendritic cells

The phenotypic and functional properties of cDCs are determined not only be their ontogeny but also by imprinting signals that they receive locally within tissues. In the intestines, such signals are likely to be derived both from the host and from

microbial and dietary sources. As previously mentioned, an important characteristic of SI migratory cDCs, which appears to underlie many of their functional properties, is the ability to break down vitamin A to generate its metabolite RA. Evidence suggests that this ability is imprinted by environmental signals that cDCs receive within the intestinal tissue. In support of this idea, we have demonstrated that pre-cDCs in the BM, spleen and SI do not have vitamin A metabolizing activity [144]. Moreover, activity does not track with cDC lineage but varies depending on the tissue from which cells are isolated[177].

Several groups have demonstrated that the ability of SI and MLN $CD103^+$ cDCs to generate RA is markedly decreased in mice with Vitamin A deficiency (VAD mice) [144, 178-180] and that oral supplementation with vitamin A or RA can restore this ability [179, 180]. Moreover, addition of RA to *in vitro* cultures of BM-derived, peripheral LN or splenic DCs endowed them with an ability to metabolize Vitamin A. Thus, an important factor driving Vitamin A metabolizing ability in SI cDCs appears to be Vitamin A itself. *In vitro* experiments have suggested a direct role for RA in imprinting cDCs with this ability [144, 179, 180]. Further, using RA signaling reporter mice expressing luciferase under the control of RA responsive elements, it has been demonstrated that SI $CD103^+$ cDCs receive RA signals *in vivo* [144, 179] . Interestingly, in mice that are kept on a VAD diet for a period of four weeks, SI $CD103^+$ cDC have only a modestly reduced capacity to metabolize vitamin A activity, suggesting that dietary sources of Vitamin A alone do not explain the imprinting that occurs in the SI. As the liver constitutes an important storage site for Vitamin A metabolites, we have suggested that bile, which continually drains from the liver, may provide an additional source of RA to the SI [144]. Indeed, bile is able to induce Vitamin A metabolizing activity on BM derived DCs *in vitro* however this remains to be addressed *in vivo* [144]. Apart from RA, factors that have been implicated in the regulation of Vitamin A metabolizing activity in SI $CD103^+$ cDCs include TLR ligands as well as cytokines such as GM-CSF, IL-4 and IL-13 however the contribution of these factors *in vivo* during steady state or inflammatory conditions remain controversial [181].

## 4.6 Intestinal monocytes and macrophages

Intestinal macrophages represent the most abundant mononuclear phagocyte population in the intestines [182]. As previously mentioned, they are characterized by uniform expression the chemokine receptor CX3CR1 and are easily detected by flow cytometry in CX3CR1-GFP reporter mice [183]. Flow cytometric analyses have further demonstrated that intestinal macrophages uniformly express the IgG receptor FcγR1 (CD64) as well as high levels of MCHII, CD11b and the macrophage marker F4/80 (Paper I)[149].

Immunoflourescence studies of CX3CR1-GFP mice have revealed that intestinal macrophages in the LP are closely associated with the epithelial layer [183]. As such they are strategically located to gain access to luminal antigen and to provide instructions to effector immune cells in the LP. The role of intestinal macrophages in the uptake of luminal antigens will be discussed further in the "sampling of luminal antigen" section below. In addition intestinal macrophages are highly phagocytic cells and their ability to clear bacteria, apoptotic cells and other foreign materials is thought to contribute to the maintenance of intestinal homeostasis [149, 184]. Another characteristic feature of intestinal macrophages is their relative inability to produce pro-inflammatory cytokines in response to TLR stimulation [149]. Furthermore, intestinal macrophages could contribute to wound-healing processes in the intestine as they have been suggested to secrete factors supporting the renewal of epithelial cells [185]

We, and others, have shown that $CX3CR1^{high}$ macrophages do not migrate to MLNs (Paper I)[152]. Given that there are very few naive T cells in the intestine, intestinal macrophages are thus unlikely to be directly involved in the initiation of immune responses to intestinal antigens. Moreover, compared to intestinal cDCs, macrophages are poor at activating naïve T cells *in vitro* (Paper I). By contrast, however, macrophages could influence the functions of T cells locally in the LP. In this regard, they produce high levels of IL-10 [149] and this is thought to be important for the expansion and maintenance of $FoxP3^{+}$ T cells in the intestine. Moreover, their production of IL-1β has been suggested to support the development of Th17 cells locally in the intestinal LP [115].

Adoptive transfer studies have demonstrated that adoptively transferred $Ly6C^{hi}$ monocytes give rise to $CX3CR1^{high}$ intestinal macrophages [145, 146] through a

series of intermediate steps that involve the loss of Ly6C expression and the upregulation of F4/80 and CX3CR1 expression [149]. Their development from $Ly6C^{hi}$ monocytes is supported by the finding that in mixed BM chimeras, generated with WT and CCR2 deficient BM, $CX3CR1^{high}$ macrophages are almost exclusively derived from WT BM [42]. In contrast to cDCs, differentiation of intestinal macrophages does not depend on the growth factors GM-CSF and Flt3 (Paper I). Similar to other macrophages, however, they require CSF-1 signaling for their development. Thus, in the intestines of WT:$CSF1R^{-/-}$ mixed BM chimeric mice, macrophages are almost exclusively derived from WT BM [43]. Recently, studies of monocyte-derived cells in the intestine have shown that during inflammatory conditions, $Ly6C^{hi}$ monocytes take on a different fate. Thus, in different mouse models of colitis, a population of monocyte-derived, CCR2-dependent $CX3CR1^{int}$ cells expands dramatically. These cells have pro-inflammatory properties and are thought to contribute to the development of inflammation [42, 148, 149, 186]

## 4.7 Sampling of luminal antigen

***Role of epithelial cells***

The intestinal immune system constantly samples luminal antigen to survey its content, forming the basis for immune homeostasis. The uptake of antigen across the epithelial layer can occur via several distinct mechanisms and these mechanisms may differ depending on the site of sampling. Some types of antigen are capable of transcellular diffusion [187]. Such antigens could then be actively taken up by cells in the LP or reach the circulation [188]. In addition, luminal antigen can be taken up epithelial cells and administration of soluble antigen has been detected in intestinal epithelial cells *in situ* and *in vivo* [189, 190]. Further, epithelial cells express receptors that can participate in the controlled uptake of intestinal antigens, including the neonatal Fc receptor, has been shown to participate in the transcytosis of IgG-antigen complexes for delivery to LP DCs [191, 192]. Moreover goblet cells, which are epithelial cells that specialize in mucus production, have been suggested to participate in the transfer of luminal antigen to LP DCs [193].

***M cell dependent uptake***

As previously mentioned, the epithelium overlying GALT like PPs and ILFs differs in several ways from that overlying the LP. Importantly, FAE (follicle associated epithelium) contains specialized M cells, which actively transport antigen into underlying follicles [194]. M cells differ from normal enterocytes in that they lack microvilli, but instead have broader microfolds that give them their name. The mucus layer that covers the FAE is less thick than that covering the LP epithelium, which is thought to increase the accessibility of pathogens and other particulate antigens to M cells. M cells are highly phagocytic cells and release the content of their phagosomes into so called intraepithelial pockets where immune cells like cDCs can gain access to it [195]. Thus, the major function of M cells is to transfer luminal antigen for presentation by cDCs. Several pathogens however, including *Salmonella* and *Yersinia*, exploit M cells as a way to penetrate the intestinal epithelium and use molecules expressed on the apical surfaces of M cells as invasion receptors [196-198].

***Role of dendritic cells and macrophages***

In addition to epithelial cells, DCs and macrophages have been implicated in direct sampling of luminal antigen. Initial studies using *in vitro* cultured monolayers suggested that $CD11c^{+}$ cells extend dendrites between epithelial cells and sample luminal bacteria [199]. Subsequently, transepithelial dendrites (TEDs) were associated with $CX3CR1^{+}$ cells and their presence appeared to be dependent on their expression of this chemokine receptor [183]. Although described as DCs in these initial studies, subsequent work has shown that these CX3CR1+ cells are in fact macrophages (Paper I)[200]. Functional assessment of TEDs demonstrated that microbial stimulants like *Salmonella, Aspergillus* and certain TLR ligands [183, 201, 202] could induce the formation of TEDs [183]. On the other hand, uptake of non-invasive *Aspergillus* was unaffected in CX3CR1 deficient mice [201]. In summary, the role TEDs in the uptake of luminal antigen remains controversial.

Two recent publications used multiphoton microscopy to identify antigen-sampling cells in the intestine of $CD11c^{+/YFP}$ x $CXCR1^{+/GFP}$ mice [193, 203]. This dual reporter system permitted the simultaneous identification of cells that were interpreted as intestinal cDCs ($CX3CR1^{-}CD11c^{+}$) and macrophages ($CX3CR1^{+}CD11c^{+}$) based on their flourescence. McDole *et al* detected the presence of goblet cell-associated antigen passages (GAPs) that appeared to pass

soluble antigen directly to underlying cDCs. This finding contrasts with our (paper I) and others finding that, after intra-luminal injection, fluorescently labeled OVA is primarily taken up by $CX3CR1^+$ macrophages and not cDCs (Paper I) [190, 203]. In the study by Farache et al, a population of $CD103^+$ DCs was visualized within the epithelial layer. Such intraepithelial $CD103^+$ cDCs increased in response to luminal administration of *Salmonella* and extended dendrites between epithelial cells to sample the bacteria [203]. A recent publication suggested a division of labour between intestinal macrophages and dendritic cells in the uptake and presentation of soluble luminal antigen. Thus, while $CX3CR1^+$ macrophages primarily took up soluble antigen, the authors suggest that they transfer this antigen to $CD103^+$ cDCs via gap junctions [190]. In conclusion, further studies will be required to clarify the mechanisms by which intestinal cDCs and macrophages acquire soluble and particulate antigen from the gut lumen.

## 4.8 Oral tolerance

As a consequence of the high load of innocuous antigens derived from food and commensal microbes in the intestine, mechanisms that allow the intestinal immune system to tolerate these are crucial to maintain homeostasis in the gut. Oral tolerance is a phenomenon by which oral administration of innocuous antigen leads to a state of local and systemic immunological unresponsiveness to subsequent administration of the same antigen [204]. The mechanisms underlying oral tolerance are multi-factoral and incompletely understood but have been proposed to include absence of T cells with correct specificity (deletion), lack of sufficient activation signals for T cells (anergy) [204] as well as induction of regulatory T cell populations [133].

The induction of oral tolerance appears to occur primarily in the MLNs and mice lacking MLN (by surgical removal) are unable to generate systemic non-responsiveness to orally fed soluble antigen [93, 94]. In contrast, oral tolerance can be induced in mice lacking PPs [93, 205, 206]. Further, T cell homing to the gut is required for oral tolerance as absence of CCR9 and $\alpha4\beta7$ on T cells, or lack of Madcam-1 expression in mice, results in impaired DTH responses [133, 207]. Moreover oral tolerance appears to require the conversion of naïve T cells into $FoxP3^+$ iTregs [133, 208] and expansion of $FoxP3^+$ cells in the LP [133]. In this regard, it was demonstrated that in mice lacking CX3CR1, intestinal $CX3CR1^+$ macrophages have impaired IL-10 production, impaired accumulation of $FoxP3^+$ T

cells in the LP and impaired oral tolerance [133]. A recent suggested that the impaired oral tolerance induction in CX3CR1 deficient mice results from a reduced ability of macrophages to take up soluble luminal antigen [190]. In contrast, our results suggest that the ability of $CX3CR1^{+}$ macrophages to take up soluble luminal antigen is unimpaired in CX3CR1 deficient mice (Paper I).

Intestinal DCs are required to initiate T cell responses to luminal antigen and as such, are thought to contribute to the establishment of oral tolerance [209]. Indeed, expansion of cDCs *in vivo* by injection of Flt3L was shown to lead to increased oral tolerance [210]. Moreover, oral tolerance [94] and proliferation of antigen-specific T cells in the MLN is abrogated in mice lacking the chemokine receptor CCR7 [94, 160], suggesting that DC migration from LP to MLNs (see intestinal DC migration section) is a prerequisite for oral tolerance induction [94]. Further, intestinal $CD103^{+}$ DCs display an enhanced capacity to generate $FoxP3^{+}$ Tregs *in vitro* compared to LP and MLN $CD103^{-}$ DC subsets [163, 168] however whether this contributes to oral tolerance *in vivo* has not been addressed. In paper III of this thesis we address the role of IRF4-dependent cDCs in the establishment of oral tolerance.

# 5. Aims of this thesis

The overall aim of this thesis work was to study the development and function of dendritic cells subsets in the intestine and MLNs.

Specifically, the aims of the thesis were:

- To assess the development and compare the functions of intestinal CX3CR1$^{-}$ expressing cells and CD103$^{+}$ DCs

- To assess the lineage relationships between intestinal CD103$^{+}$CD11b$^{+}$ and other DC lineages and to assess their functions in intestinal immune responses

- To assess the function of IRF4 transcription factor dependent DCs in T cell responses to innocuous luminal antigen and in the development of oral tolerance

# 6. Summary of papers I-III

***Paper I: Intestinal CD103$^+$, but not CX3CR1$^+$ antigen sampling cells migrate in lymph and serve classical dendritic cell functions***

Prior to the start of this work, DCs expressing CD103 had been identified in the intestinal LP and in the MLNs that, through their ability to metabolize Vitamin A, were found to have an enhanced capacity to induce gut-homing receptors and generate FoxP3$^+$ T cells *in vitro* [163, 168]. Separate studies, using mice expressing GFP under control of the CX3CR1 promotor, had identified a major subset of mononuclear cells in the intestinal LP expressing high levels of CX3CR1 that, based on their expression of CD11c, were considered DCs [183]. These CX3CR1$^+$ cells appeared to be capable of extending dendrites across the epithelial layer to sample luminal antigen and as such, were thought play important roles in the initiation of intestinal adaptive immune responses [183]. In this paper we assessed the relationship between CD103$^+$ DCs and CX3CR1$^+$ with regards to ontogeny and function, using CX3CR1-GFP reporter mice.

Phenotypic analysis of LP DCs confirmed that while CX3CR1$^+$ cells expressed the DCs markers CD11c and MHCII, they did not express CD103. CX3CR1$^+$ cells expressed lower levels of co-stimulatory molecules CD40, CD80 and CD86 than CD103$^+$ DCs. Further; CX3CR1$^+$ cells stained positive for CD11b and F4/80. These results confirmed that CD103$^+$ DCs and CX3CR1 cells represent distinct LP populations and further, strongly suggested that the latter were macrophages and not DCs. We also identified a mononuclear phagocyte population expressing intermediate levels of CX3CR1 that, based on phenotypical heterogeneity, appeared to consist of several cell subsets.

To assess the turnover rate of the described populations, we performed BrdU pulse-chase experiments. These experiments demonstrated that CX3CR1$^+$ cells had a much slower rate of BrdU incorporation when compared to CD103$^+$ DCs, indicative of a slower turnover rate *in vivo*. We also compared the *in vivo* responses of these cell populations to Flt3L and GM-CSF overexpression. Whereas CD103$^+$ DCs expanded efficiently in response to both of these growth factors, CX3CR1$^+$ cells failed to responds to either.

Previous evidence had suggested that CD103$^+$ DCs could migrate from the LP to MLNs [155] however the migratory capacities of CX3CR1$^+$ cells were not known.

Here, using multiphoton confocal microscopy, we observed that afferent lymphatic vessels draining the intestine contained $CD11c^+$ cells, but none of these were $CX3CR1^+$. Further, FACS staining of lymph collected from afferent vessels showed that the large majority of lymph $CD11c^+$ cells were $CD103^+$, both during steady state conditions and after TLR stimulation. These results demonstrated that $CD103^+$ but not $CX3CR1^+$ cells migrate to MLNs and strongly suggested that CX3CR1+ cells do not participate in the initiation of adaptive immune responses in MLNs.

Furthermore, we assessed the capacities of $CD103^+$ and $CX3CR1^+$ cells to take up soluble luminal antigen, to initiate T cell responses and to imprint gut-homing receptors on T cells. We found that whereas administered soluble antigen was more efficiently taken up by $CX3CR1^+$ cells than by $CD103^+$ DCs, $CD103^+$ DCs were superior at inducing proliferation of T cells both *in vitro* and *in vivo*. $CD103^+$ DCs also had an enhanced capacity to metabolize vitamin A and were more efficient at inducing expression of gut-homing molecule CCR9 on T cells *in vitro*.

Collectively, these results established that $CD103^+$ DCs and $CX3CR1^+$ cells are distinct populations of mononuclear phagocytes that derive from distinct precursors and provide evidence of a division of labor between the two with regards to adaptive intestinal immune responses.

***Paper II: IRF4 transcription-factor-dependent $CD103^+CD11b^+$ dendritic cells drive mucosal T helper 17 cell differentiation***

In this paper we assessed the ontogeny and *in vivo* functions of intestinal $CD103^+CD11b^+$ cDCs. We (Paper I), and others [146] had previously recognized that SI $CD103^+$ DCs were heterogeneous for the expression of CD11b and $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs appeared to differ in terms of their localization, function and development. Thus, while $CD103^+CD11b^-$ DCs were the dominant population in the colon and in PPs, $CD103^+CD11b^+$ DCs dominated in the LP (Paper I) [146]. Further, characterization of DCs in mice lacking Id2, IRF8 and Batf3 had shown that the development of $CD103^+CD11b^-$, but not $CD103^+CD11b^+$ DCs depended on these TFs however the TF requirements for $CD103^+CD11b^+$ DCs were unknown. Further, while a number of studies had started to address the functions of intestinal DC subsets *in vitro*, their roles in intestinal immune responses *in vivo* were not known.

Whereas $CD103^+CD11b^-$ DCs are commonly found in peripheral tissues, and during the course of this work were shown to represent a unified DC subsets together with lymphoid $CD8\alpha^+$ DCs [22, 23], DCs expressing both CD103 and CD11b are not found in significant numbers anywhere else than in the small intestine. We hypothesized that they could be developmentally related to splenic $CD4^+$ DCs (that also express $CD11b^+$) and phenotypic comparison confirmed that SI $CD103^+CD11b^+$ DCs and splenic $CD4^+$ DCs share several phenotypic characteristics, including expression of SIRPα and the c-type lectin dendritic cell inhibitory receptor (DCIR)2.

The TF IRF4 had previously been shown to be a requirement for the development of splenic $CD4^+$ DCs [49, 50]. We confirmed that both splenic $CD4^+$ DCs and SI $CD103^+CD11b^+$ DCs express high levels of IRF4 mRNA and protein and as a result, we wanted to determine if IRF4 expression was also necessary for the development of SI $CD103^+CD11b^+$ DCs. For this purpose, $Irf4^{fl/fl}$ mice were crossed to *CD11c-cre* mice to deplete Irf4 specifically in DCs. Analysis of these mice confirmed a reduction in splenic $CD4^+$ DCs and revealed a significant reduction in the number of SI $CD103^+CD11b^+$ DCs. Further, $CD103^+CD11b^+$ DCs were almost completely missing from the MLNs. These results identified an important role for IRF4 in the homeostasis of $CD103^+CD11b^+$ DCs in vivo and suggested that they are developmentally related to $CD4^+$ splenic DCs.

Further, we demonstrated that IRF4-dependent DCs play an important role in the generation and maintenance of the intestinal Th17 cell compartment. In mice lacking IRF4 in DCs, Th17 cells in the SI and in the colon were reduced by approximately 50% and adoptive transfer experiments with antigen-specific T cells showed that IRF4-dependent DCs were important for Th17 cell differentiation at the level of the MLNs. Mechanistically, *in vitro* and *in vivo* experiments suggested that the capacity of these DCs to induce Th17 differentiation was linked to their capacity to produce the Th17 polarizing cytokine IL-6.

In this study, we also identified putative equivalents to IRF4-dependent DCs in the human LP. These studies led to the identification of two $CD103^+$ DC subsets in the human ileum; a dominant population of $SIRP\alpha^{high}CD141^-$ DCs and a minor population of $SIRP\alpha^{lo}CD141^+$ DCs. The former expressed intracellular IRF4 and likely represents the human equivalent of mouse $CD103^+CD11b^+$ SI DCs, whereas the latter likely belongs to the branch of human Batf3-dependent DCs [28].

Taken together, these results show that the development of SI $CD103^+CD11b^+$ DCs depends on IRF4 and provide evidence that IRF4-dependent DCs play key roles in the generation of the intestinal Th17 compartment.

***Paper III: IRF4-dependent dendritic cells regulate $CD4^+$ T cell responses to soluble oral antigens***

In this manuscript we address the role of IRF4-dependent DCs in the regulation of $CD4^+$ T cell responses to orally administered innocuous antigen *in vivo*, specifically with regards to iTreg induction, oral tolerance and gut-homing receptor induction.

Previous *ex vivo* studies had suggested that $CD103^+$ migratory DCs are important for the priming of $CD4^+$ T cell responses to orally administered soluble antigen [155, 163] however differential roles of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ subsets, and the role of $CD103^-CD11b^+$ DCs, had not been addressed. Here we show that $CD103^+CD11b^+$ and $CD103^-CD11b^+$ but not $CD103^+CD11b^-$ intestinal migratory DCs appear to play key roles in initiating $CD4^+$ T cell responses to orally administered antigen. Surprisingly, the priming of antigen-specific T cells in the MLNs *in vivo* was not affected in mice lacking IRF4-dependent DCs, despite the almost complete absence of $CD103^+CD11b^+$ at that site. Further experiments suggested that in mice lacking IRF-dependent DCs, CD103-CD11b+ migratory DCs were mainly responsible for the priming of $CD4^+$ T cells.

$CD103^+$ intestinal migratory DCs display an enhanced capacity to generate iTregs from naïve precursors *in vitro* [163, 168]. However we, and others, had previously demonstrated that mice lacking IRF4-dependent DCs have normal numbers of $FoxP3^+$ Tregs in the SI and colon (Paper II) [43]. Here we show that the generation of iTregs in MLNs does not require the presence of IRF4-dependent DCs. In contrast, IRF4-dependent DCs appear to regulate the development of $IL\text{-}10^+$ T cells as a higher fraction of T cells in mice lacking IRF-dependent DCs expressed IL-10. Further, we assess the establishment of oral tolerance and show that it is not impaired in IRF4 deficient mice.

Earlier *in vitro* studies had also demonstrated that $CD103^+$ MLN DCs have an enhanced capacity to induce CCR9 and $\alpha4\beta7$ expression on T cells. Previously, (Paper II) we had shown that after intraperitoneal immunization, antigen-specific T cells primed in the MLNs of IRF4 deficient mice expressed comparable amounts of CCR9 and $\alpha4\beta7$, compared to control mice. Here we show that IRF4-dependent

DCs are not required for the induction of gut-homing receptors in response to orally administered antigen. In contrast, levels of CCR9 were maintained on higher levels in mice lacking IRF4-dependent DCs.

Collectively, these results point out several alterations in the phenotype of $CD4^+$ T cells following their priming in the MLN of mice lacking IRF4-dependent DCs after oral antigen administration. Future studies should address the mechanisms underlying these changes.

# 7. Discussion and outstanding questions

The studies included in this thesis broaden our understanding of the ontogeny and in vivo functions of intestinal cDCs. In this section I will discuss our results in light of recent findings and highlight current outstanding questions.

During the course of this thesis work, our understanding of the cDC lineages and their development has evolved substantially as developmental requirements for cDC subsets have been determined. Moreover, the distinction between cDCs and monocyte-derived cells has improved as systematic, functional and genetic approaches have clarified ontogenic relationships between mononuclear phagocyte populations. The work presented in this thesis, together with the work of others, has contributed to our current understanding of mononuclear phagocyte heterogeneity in the intestine. According to current views, intestinal monocytes and monocyte-derived macrophages are characterized by the expression of CD64 and express intermediate (monocytes) or high (macrophages) levels of CX3CR1. In contrast cDCs are $CD64^-$ and most of them express CD103. However we have now also come to appreciate that there are $CD103^-$ intestinal cDCs. Most $CD103^-$ cDCs are phenotypically similar to $CD103^+CD11b^+$ cDCs but whereas $CD103^+CD11b^+$ cDCs are $CX3CR1^-$, $CD103^-$ cDCs are $CX3CR1^{int}$. In retrospect, the $CD103^-CX3CR1^{int}$ cells that we identified in Paper I are thus likely to contain both $CD103^-$ cDCs and $CX3CR1^{int}$ monocytes that may represent intermediates in the development of intestinal macrophages. It has also become clear, during the course of our studies, that $CD103^+CD11b^-$ and $CD103^+CD11b^+$ cDCs have distinct ontogonies. Thus, while others have demonstrated that $CD103^+CD11b^-$ cDCs belong to the CD8a-like cDC lineage, we, and others have shown that $CD103^+CD11b^+$ cDCs belong to the $CD11b^+$ cDC lineage.

Adoptive transfer studies have demonstrated that both $CD103^+CD11b^-$ and $CD103^+CD11b^+$ intestinal cDC are derived from pre-cDCs. That pre-cDCs also give rise to $CD103^-$ cDCs remains to be formally demonstrated, however given their phenotypical and functional properties, this appears to be a reasonable assumption. Moreover, the relationship between $CD103^-CD11b^+$ and other cDC subsets is currently not known. Phenotypically, these cells resemble $CD103^+CD11b^+$ cDCs and it is possible that they belong to the same cDC lineage. Future studies, including global transcriptional analysis of all cDC subsets, could help to resolve this question.

According to current models of cDC development, pre-cDCs develop in the bone marrow, circulate in the blood and home to tissues where they complete the final steps of their maturation. It has been suggested that CD24 expression on pre-cDCs can segregate precursors of $CD8\alpha^+$ and $CD8\alpha^-$ cDCs suggesting that, within the pool of cDC progenitors, there are cells which are committed to CD8α and CD11b-like cDCs, respectively [211]. In the intestine, cDC subsets localize to different areas and whereas $CD103^+CD11b^-$ cDCs predominate in PPs, $CD103^+CD11b^+$ cDCs are mostly located in the LP. In light of this, if would be of interest to determine if distinct progenitors preferentially localize to different parts of the intestine or, alternatively, if cDC lineage commitment is determined by local imprinting factors in the intestine.

In paper II, we show that the development of $CD103^+CD11b^+$ cDCs depends on the transcription factors IRF4. IRF4 is a lymphoid-specific TF that belongs to the IRF family of TFs. In contrast to several others members of the IRF family, is not activated by interferons, but by diverse mitogenic stimuli. Among IRF family members, it is most closely related to IRF8. IRF4 and IRF8 are critical mediators of several aspects in immune cell differentiation [212]. Both IRF4 and IRF8 recognize IFN-stimulated response elements (ISRE) and can form complexes with PU.1 and bind as heterodimers to Ets-IRF Composite Elements (EICEs). Chromatin immunoprecipitation followed by sequencing (ChIP-seq) in *in vitro* generated DCs showed that both IRF4 and IRF8 can bind to EICEs [213]. Moreover, IRF4 and IRF8 in DCs can interact with AP-1 sites at gene promoters. AP-1 sites were adjacent to ISRE and these sites were termed AP-1-IRF Composite Element (AICE). A recent study showed that IRF4 and IRF8 in *in vitro* generated DCs bind to both EICE and AICE composite motifs through interactions with PU.1 and BATF, respectively [214]. The importance of these interactions in isolated primary cDC populations, including those in the intestine, remains to be addressed. Our data have suggested that mechanistically, IRF4 appears to regulate $CD103^+CD11b^+$ cDC numbers by sustaining the survival (Paper II). Although we did not address how IRF4 prevented apoptosis in these cells, it is interesting to note that in T cells, IRF4 regulates the expression of anti-apoptotic molecule Bcl-2 [213]. Which factors regulate the differential expression of IRF4 and IRF8 in cDC subsets remains to be established.

Interestingly, using gene expression and ChIP-Seq analyses, Vander Lugt *et al.* demonstrated the presence of an IRF4-dependent regulatory module that programs DC for MHCII antigen presentation. In accordance, both PU.1-IRF4 and BATF-

IRF4 complexes were shown to assemble at the MHCII transactivator *Ciita* locus and mice with IRF4-deficient DCs had major defects in the ability to prime systemic Th responses [214]. Similarly, Pan et al using ChIP with anti-IRF4 antibody, were recently able to detect association of IRF4 with Ciita promoters in BM derived DCs [215]. In accordance, our data, presented in paper III, demonstrates that after intraperitoneal immunization with OVA, OT-II cell responses are markedly reduced in the spleen and MLN of mice lacking IRF4-dependent DCs. These findings suggest that it might be of interest to assess *Ciita* expression also in primary DC from IRF4 deficient and IRF4 sufficient mice. In sharp contrast to $CD4^+$ T cell responses after intraperitoneal antigen administration, $CD4^+$ T cell responses were not reduced after oral OVA administration (Paper III). *Ex vivo* studies suggested that the dominant subset priming OT-II cells in IRF4-deficient mice after oral OVA administration are $CD103^-CD11b^+$ migratory cDCs. This suggests that the MHCII presentation capacity of $CD103^-CD11b^+$ migratory cDCs is not entirely defective in IRF4 deficient mice and moreover, that these cells are numerically sufficient to prime OT-II cells in IRF4 deficient mice.

In paper II, we demonstrate that $CD103^+CD11b^+$ cDCs are important for the induction of Th17 in the MLNs via production of IL-6. The importance of $CD103^+CD11b^+$ cDCs in maintaining the intestinal Th17 compartment has been confirmed by two recent studies [43, 174]. Moreover, mice lacking MHCII expression only in $CD103^+CD11b^+$ intestinal cDCs, have normal frequencies of Th17 cells in the LP compared to controls, suggesting that Th17 development in the LP depends on the presence of $CD103^+CD11b^+$ cDCS but does not require their cognate interaction with $CD4^+$ T cells [174].

Interestingly, recent studies have demonstrated that IRF4-dependent cDCs have important roles in the priming of Th2 cells in response to cutaneous antigen challenge [216, 217]. Further, mice having IRF4 specifically deleted in $CD11c^+$ cells have a defect in Th2 type lung inflammation and Th2 differentiation was dependent on IRF4 expression in DCs [218]. The specific roles of intestinal cDC subsets in Th2 differentiation have not been addressed but these findings suggest that the role of IRF4-dependent intestinal cDCs in responses to intestinal Th2 polarizing pathogens merits investigation.

Our findings have challenged the view that $CD103^+$ migratory intestinal cDCs are inherently tolerogenic and shown that they are also capable of inducing effector T cell responses. The microbial-derived signals that may imprint intestinal cDCs

with the ability to generate Th17 cells, other effector T cells as well as regulatory T cells are by large unknown. Furthermore, bacterial-derived metabolites have been shown to influence several aspects of the intestinal immune responses [219] The role of intestinal cDCs in this regard represents an interesting avenue for future research.

# Acknowledgements

I would like to thank everyone who contributed in one way or another to this thesis.

First of all, thank you **Bill**, for taking me on as a PhD student and for introducing me to the wonderful world of DC subsets. I have really learnt a lot from you. Your ambition, enthusiasm and attention to detail have been, and continue to be very inspiring. Thank you for always finding the time to discuss science, for always being open to new ideas, and for encouraging me to finish sentences…one day I will get there.

I would also like to thank present and previous members of the WA, MSF and BJL groups for great times in- and outside the lab. When I eventually leave I will miss you all (and I trust you to take responsibility for keeping the stainless steel tables in great condition!). **Madde**, **Karin**, **Kasia S** and **Petra** – vi har delat nästan hela doktoranduppleveIsen och den hade inte varit densamma utan er! **Kasia L** – thank you for your positive attitude and for many good laughs. **Knut** – thank you for your kindness, patience and for figuring out where the GFP came from. **Aymeric** and **Thorsten** – thank you for being great workmates and for keeping the lab very organized! **Daniel** – tack för all uppmuntran, skön humor och bra låtar – fortsätt att skicka! **Heli** – tack för din omtanke, vänlighet och humor! **Elin** – tack för att du tog hand om mig när jag var ny på labbet. **Monika** – thanks for great times and for teaching me some of that german efficiency. **Kerstin**, **Mimoza**, **Lieneke** and **Vasileios** – it's been great getting to know you!

Särskilt tack till **Ansa** för bokstavligt talat tusentals PCR typningar!

Tack **Gudrun** för hjälp med administrativa frågor.

Thank you all past and present co-workers at the **Immunology section** for contributing to a great atmosphere and the important friday "fika".

Thank you colloborators and fellow gut DC nerds in Glasgow, **Allan** and **Charlie**, for interesting discussions and great times in the lab and at conferences!

Tack också till familj och vänner.

**Isabel**, **Fredde** och lille **Jacob** – tack för alla gånger ni ställt upp för mig och Isak, bättre vänner kan man inte hoppas på. Hoppas på fler trevliga semestrar framöver!

Särskilt tack till **Pappa** och **Diana, Kitte** och **Agnes** samt familjen **Pettersson**/**Gustavsson** – ni sätter guldkant på tillvaron utanför labbet! **Mamma** och **Matilda**, utan er hade det inte varit möjligt. Tack för att ni alltid ställer upp, även med extremt kort varsel (när den berömda FACS:en krånglar).

Sist men inte minst, tack till världens finaste **Isak** för att du har varit så tålmodig när jag har jobbat med ”karucellerna” och med den här avhandlingen. Älskar dig mysis!

*Emma*

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