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Phenotype and function of intestinal CD103+ dendritic cells

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Phenotype and function of intestinal CD103$^+$ dendritic cells

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This thesis will be defended on Friday the 8th of April at 9:00am
in Belfragesalen, BMC D15, Klinikgatan 32, Lund

Supervisor: Professor William W. Agace
Faculty opponent: Professor Brian L. Kelsall

On the cover: Immunohistochemistry stainings of: Intestinal villi; part of an intestinal Peyers patch; solitary intestinal lymphoid tissue; intestinal Peyers patch and villus, intestinal CD103+ DC.

On the back: Immunohistochemistry stainings of: Intestinal Peyers patch and villi; part of an mesenteric lymph node, intestinal villi transversely sectioned; CD103+ DC in mesenteric lymph node; intestinal villi.

Front and back immunohistochemistry stainings by Elin Jaensson-Gyllenbäck.

Till mamma och pappa

CONTENTS

PAPERS INCLUDED IN THIS THESIS

Paper I

Small intestinal CD103$^+$ dendritic cells display unique functional properties that are conserved between mice and humans

Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW.

J Exp Med. 2008 Sep 1;205(9):2139-49.

Paper II

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

Schulz O*, Jaensson E*, Persson EK, Liu X, Worbs T, Agace WW, Pabst O.

J Exp Med. 2009 Dec 21;206(13):3101-14.

Paper III

Bile retinoids imprint intestinal CD103$^+$ dendritic cells with the ability to generate gut-tropic T cells

Jaensson-Gyllenbäck E*, Kotarsky K*, Zapata F, Persson EK, Gundersen TE, Blomhoff R, Agace WW.

Mucosal Immunology, doi: 10.1038/mi.2010.91

* Authors contributed equally

REVIEW & PAPERS NOT INCLUDED IN THIS THESIS

The diverse ontogeny and function of murine small intestinal dendritic cell/macrophage subsets

Persson EK, <u>Jaensson E</u>, Agace WW.

Immunobiology. 2010 Sep-Oct;215(9-10):692-7.

Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8$^+$ T cells

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IL-10-dependent partial refractoriness to Toll-like receptor stimulation modulates gut mucosal dendritic cell function

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The survival of effector/memory CD4 cells within the gut lamina propria requires OX40 and CD30

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* Authors contributed equally

ABBREVIATIONS

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
APC	antigen presenting cell
ATP	adenosine 5'-triphosphate
Batf	basic leucine zipper transcription factor ATF like
BMDC	bone marrow derived DC
BrdU	bromodeoxyuridine
CCL	CC Chemokine ligand
CCR	CC Chemokine receptor
cDC	classical or conventional DC
CDP	common DC precursor
CFSE	carboxyfluorescein succinimidyl ester
CXCR	C-X-C chemokine Receptor
DC	dendritic cell
DSS	dextran sulfate sodium
FAE	follicle-associated epithelium
Flt3	FMS-like tyrosine kinase 3
FoxP3	forkhead box P3
GALT	gut-associated lymphoid tissue
GFP	green flourescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
HEV	high endothelial venule
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
ICAM	Intercellular adhesion molecule
Id	inhibitor of differentiation/ DNA binding protein
IEL	intestinal epithelial lymphocyte
IFN	interferon
IL	interleukin
ILF	isolated lymphoid follicle
iNOS	inducible-nitric-oxide-synthase
IRF	IFN regulatory protein
KO	knock out
LDLN	lung draining lymph node
LFA	lymphocyte function-associated antigen
LN	lymph node
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
LRAT	lecitin:retinol acyl transferase
M cell	microfold cell
MadCAM	mucosal addressin adhesion molecule
M-CSF	macrophage colony-stimulating factor
MDP	macrophage and DC precursor
MHC	major histocompability complex
MLN	mesenteric lymph node
MoDC	monocyte derived dendritic cell
MyD	myeloid differentiation primary-response gene
NFKB	nuclear factor KB
NOD	nucleotide-binding oligomerization domain
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
pDC	plasmacytoid DC
PLN	peripheral lymph node
PNAd	peripheral node addressin

PP	peyer's patch
PRR	pattern recognition receptors
RA	retinoic acid
RALDH	retinaldehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response elements
RBP	retinol binding protein
RXR	retinoid X receptor
RXRE	RXR elements
SDR	short-chain dehydrogenase/reductase
SED	subepithelial dome
T reg	regulatory T cell
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TipDC	TNF–inducible NO synthase (iNOS)–producing DC
TLR	toll-like receptor
TNF	tumor necrosis factor
VAD	vitamin A deficient

1. THE IMMUNE SYSTEM – AN INTRODUCTION

The immune system is a biological system of cells, proteins and organs that protect against diseases by identifying and eliminating pathogens such as bacteria, virus, fungi and parasites. However, while protecting against pathogens it must also remain tolerant to non-hazardous substances including food components or the body's own proteins.

The immune system can be divided into two categories; the innate and the adaptive immune system, which both function by first detecting the pathogen followed by a response.

1.1 Innate immunity

The innate immune system is composed of cells and soluble mediators that act as a first line of defense to ultimately kill or render pathogens harmless. The innate immune system is also important for activation and attraction of different immune cells, including cells of the adaptive immune system. Components of the innate immune system are found in most organisms including insects and fungi. Cellular components of the innate immune system include monocytes, macrophages, dendritic cells (DCs), neutrophils and eosinophils.

The innate immune system is activated very fast; recognition is immediate and responses occur within a few minutes to a few hours. The system is based on recognition of structures or patterns found on and common to most pathogens and microbes called pathogen-associated molecular patterns (PAMPs). PAMPs bind to pattern recognition receptors (PRRs) present in or on cells. PRRs include Toll like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors.

TLRs are a family of at least 13 receptors that are found on the surface of cells and intracellularly [1]. TLRs bind to a variety of pathogenic components, commonly of bacterial or viral origin, and each TLR has different specificity. For example: TLR4

binds to lipopolysaccharide (LPS), TLR5 to flagellin, and TLR9 to bacterial CpG DNA [2]. Activation of TLRs often lead to synthesis and secretion of cytokines and activation of the host defense, most commonly through activation of myeloid differentiation primary-response gene 88 (MyD88) and nuclear factor KB (NFKB) [3]. NOD receptors are intracytoplasmic receptors that recognize components of bacterial peptidoglycan [1]. Activation of NODs often leads to production of inflammatory molecules via activation of NFKB [1].

Cells that express PRRs include macrophages and DCs and epithelial cells in the small intestine. Interactions between PAMPs and PRRs may result in phagocytosis of the pathogen containing the PAMP and release of toxic products that kill the phagocytosed pathogen. Binding of PAMPs to PRRs may also result in activation of cells, including macrophages, which then can release cytokines, chemokines and inflammatory mediators that activate and attract other cells of the immune system to the site. Hence, PRRs, which are expressed on and in many cell types, can mediate several different functions, including phagocytosis and attraction of immune cells.

1.2 Adaptive immunity

The adaptive immune system evolved in early vertebrates and recognizes specific antigens rather than generic patterns. The response is slower than that of the innate immune system, but if the adaptive immune system encounters a pathogen for the second time, the response, called immunological memory, is rapid and effective. The key events in adaptive immunity are antigen detection and presentation by antigen presenting cells (APCs, i.e. macrophages and DCs) followed by activation and response by T cells and B cells. Hence macrophages and DCs may be considered to be part of both the innate and adaptive immunity.

APCs are found in most tissues and organs and constantly sample the surrounding environment for microorganisms and antigens. Once antigens have been taken up they are processed and displayed on the surface for recognition by T cells by use of major histocompatibility molecules (MHCI or MHC II) on the surface of APCs.

T cells and B cells are generated in the thymus and bone marrow, respectively. Their differentiation is tightly regulated and results in T cells and B cells each with a specific receptor recognizing a particular antigen; T cell receptors (TCR) or B cell receptors (BCR) respectively. Consequently, each cell is only able to bind to one specific pre-determined molecular configuration. The specificity of T cells and B cells depends on recombination of DNA segments that are subsequently transcribed and translated into TCRs and BCRs. Activated B cells either become plasma cells or memory cells. Plasma cells produce antibodies that aid in the destruction of pathogens. Memory cells are long-living B cells that assure a quick response upon re-infection with the same pathogen. Activated T cells can take on a range of effector functions including, but not limited to, release mediators that aid in activation of B cells, activation of innate immune cells (CD4 T cells) and direct killing of infected or tumor cells (CD8 T cells).

1.2.1 Activation of T cells

The initial activation of naïve T cells generally occurs in the T cell zone of lymph nodes (LNs) or the spleen [4, 5]. Here, interaction of MHCI:peptide or MHCII:peptide complexes on APCs with matching TCR molecules on T cell leads to activation and clonal expansion of T cells. Costimulatory molecules and soluble mediators secreted from the APCs during this interaction play a fundamental role in determining the phenotype and functionality of the activated T cells.

1.2.2 CD4 T cell subsets

Activated CD4 T cells can differentiate into for example T-helper (Th) subsets or regulatory T cells (T regs). Th subsets include Th1, Th2, Th17 and T follicular helper cells (Thf). Th1 cells mainly secrete interleukin (IL)-2 and the macrophage activating cytokine interferon (IFN)γ and mediate responses against intracellular pathogens. Th2 cells mainly secrete IL-4, IL-5, IL-10 and IL-13 and mediate host defense against extracellular parasites including helmiths. Th2 cells also have a critical role in allergy and asthma [6]. Th17 cells mediate responses against extracellular bacteria/fungi and are most likely involved in many autoimmune diseases [6]. Thf cells aid in the generation of B cells into plasma cells that produce antibodies [7].

T regs can be divided into 'natural' T regs that acquire forkhead box P3 (FoxP3) expression and regulatory properties during their development in the thymus and 'induced' T regs, which acquire regulatory properties (and in some cases FoxP3 expression) upon activation [8, 9]. Induced T regs include Foxp3$^+$ T regs and FoxP3$^-$ IL-10 secreting T regulatory (Tr)1 cells [8, 10]. In general, T regs are important for tolerance to self-antigens, down-regulation of immune responses and in prevention of autoimmunity [6, 11-13].

2. DENDRITIC CELLS AND MACROPHAGES

DCs and macrophages are positioned at the interface between innate and adaptive immunity, with critical abilities to initiate and influence antigen-specific immune responses. Both macrophages and DCs are effective at taking up soluble and particulate antigens. Macrophages through phagocytosis and the use of an array of degradative enzymes and reactive oxygen species play a primary role in killing of pathogens, but can also contribute to wound repair by promoting the production of extracellular matrix or via production of anti-inflammatory cytokines [14, 15]. Macrophages may also have regulatory functions, mediated by for example production of transforming growth factor (TGF)β or IL-10 [15]. DCs are considered to be of central importance in the initiation of adaptive immune responses [16, 17]. In this regard, depletion of DCs (CD11c+ cells) using a diphtheria toxin-based system abrogated priming of antigen specific CD8 T cells in response to cell-associated antigens [18], strongly reduced the early CD4 T cell response to *Mycobacterium tuberculosis* [19] and abrogated the activation of antigen specific CD4 T cells after oral or nasal immunization [20]. Further, in contrast to macrophages, DCs are able to migrate via afferent lymphatics to draining LNs and are very efficient at priming naïve T cells *in vitro*.

Except for these functional differences, DCs and macrophages also differ in expression of cell surface receptors; DCs typically express high levels of MHCII and CD11c while macrophages typically express lower levels of these markers along with higher levels of CD14, CD11b and F4/80. However, a large number of macrophage subpopulations, based on surface expression and function can be identified, and the heterogeneity and plasticity of these cells is enormous [21, 22]. In addition, as will be discussed later, categorization of cells into either DCs or macrophages is not always clear, especially when it comes to cells located in non-lymphoid tissues such as lung and intestine [23].

2.1 Ontogeny of dendritic cells and macrophages

DCs are generated in the bone marrow from a hematopoietic stem cell via a series of progenitor stages, see *figure 1*. In the mouse, the first characterized progenitor is the macrophage and DC precursor (MDP). The MDP, which can be phenotypically defined as Lin$^-$c-kit^{hi}CD115$^+$CX3CR1$^+$Flt3$^+$, has the capacity to develop into lymphoid tissue DCs, plasmacytoid DCs (pDCs) and monocytes [24, 25]. MDPs progress into common DC precursors (CDP), which are proliferating cells that have lost the potential to become monocytes but have the ability to become either pre-DCs or pDCs [26, 27]. Pre-DCs are found in bone marrow, blood, and spleen and are the progenitors of lymphoid resident and splenic DCs, including CD8α^+ and CD8α^- DCs [26, 27], and most likely also of CD103$^+$ intestinal DCs, as discussed later [25, 26, 28].

Tissue macrophages derive from circulating monocytes [26, 29]. Monocytes are, similarly to DCs, derived form MDPs. From the MDP, two monocyte subsets are generated in the mouse, the Gr1$^+$/Ly6c^{hi}CCR2^{hi}CX3CR1^{low} ('classical' or 'inflammatory') monocytes and the Gr1^{low}/Ly6c^{low}CCR2$^-$CX3CR1^{hi} ('non-classical', 'resident' or 'patrolling') monocytes [26, 30, 31], see *figure 1*. Ly6c^{hi} monocytes give rise to CX3CR1$^+$ intestinal cells and during inflammation TNF–inducible NO synthase (iNOS)–producing DCs (TipDCs) and inflammatory macrophages while Ly6c^{low} monocytes give rise to tissue macrophages including alveolar macrophages and have been suggested to patrol blood vasculature [26, 30, 32, 33].

Generation of DCs and monocytes in the bone marrow is dependent on hematopoietic growth factors. In short, generation of monocytes requires macrophage colony-stimulating factor receptor (M-CSFR; also called CD115, encoded by *Csf1r*) [34] whereas generation of cDCs and pDCs depends on fms-related tyrosine kinase 3 receptor (Flt3) [26, 35]. Flt3 is highly expressed in CDPs and pre-DCs [27, 36, 37]. The granulocyte-macrophage colony-stimulating factor (GM-CSF) is also involved in the generation of DCs and monocytes, especially in

the generation of inflammatory monocyte-derived DCs and tissue DCs including those in skin and intestine [28, 38].

In addition, the expansion, homeostasis and acquiring of functional properties of DCs and monocytes/macrophages may be influenced by the local environment in LNs, spleen and tissues. For example, DCs populations may be expanded *in situ* via Flt3 dependent division [27, 39].
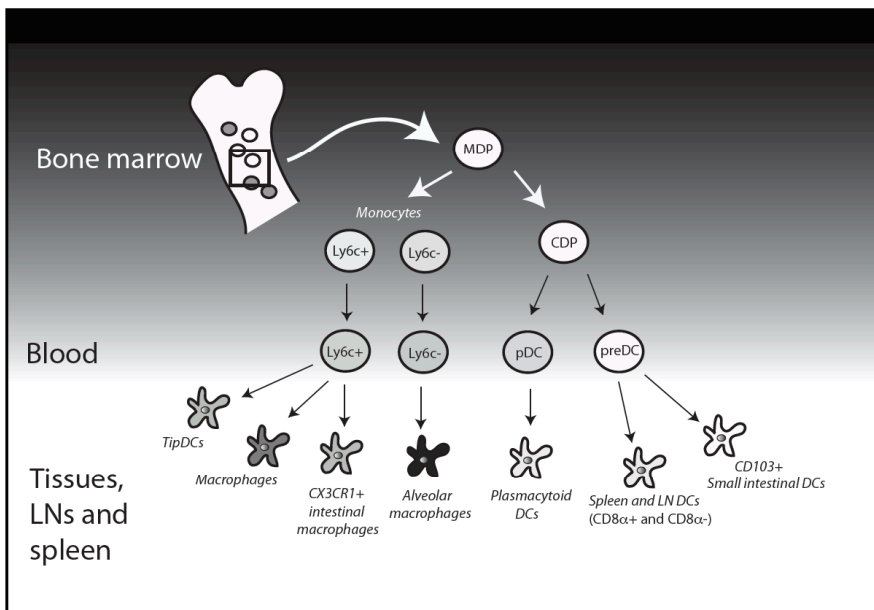
Figure 1. **Ontogeny of murine dendritic cells and macrophages.** In the bone marrow, MDPs give rise to both CDP, which is the common DC precursor, and monocytic precursors. CDPs further progress into either pre-DCs or plasmacytoid DCs (pDCs). Pre-DCs which are found not only in the bone marrow but also in blood and spleen, are the progenitors of resident spleen and LN DCs including CD8α^+ and CD8α^- DCs as well as CD103$^+$ intestinal DCs. Monocyte precursors are divided into Gr1$^+$/Ly6c^{hi}CCR2^{hi}CX3CR1^{low} ('Ly6c$^+$') and GR1^{low}/Ly6c^{low}CCR2$^-$CX3CR1^{hi} ('Ly6c$^-$') monocytes. Ly6c$^+$ monocytes give rise to macrophages (including CX3CR1$^+$ intestinal macrophages) and TipDCs. Ly6c$^-$ monocytes give rise to for example alveolar macrophages Adapted from Geissman *et al*, 2010[26] .

2.2 Classification of dendritic cell subsets

DCs can be classified into pDCs, lymphoid resident DCs or migratory DCs. To distinguish lymphoid resident and migratory DCs from pDCs, the term conventional or classical DC (cDC) is commonly used.

2.2.1 Lymphoid resident dendritic cells

Lymphoid resident DCs include DCs in the spleen, LNs, Peyer's patches (PPs) of the small intestine and thymic DCs. These DCs are often categorized into subpopulations based on expression of cell surface markers. Two major subsets, common to many murine LNs and the spleen are the $CD8\alpha^+CD11b^-$ and the $CD8\alpha^+CD11b^-$ subpopulations. In addition to these two major subpopulations, LNs contain populations of DCs deriving from the tissue that the LN in question drains. Hence, skin-draining LNs will contain migratory DCs from the skin and mesenteric LNs (MLNs) will contain migratory DCs from the intestinal lamina propria and possibly PPs.

$CD8\alpha^+CD11b^-$ DCs are primarily found in T cell rich areas of LNs and spleen while $CD8\alpha^-CD11b^+$ DCs are found in marginal zones of the spleen, the sub capsular sinus of LNs, and the subepithelial dome of PPs [40, 41]. Functionally, both $CD8\alpha^+CD11b^-$ and $CD8\alpha^+CD11b^-$ subpopulations of DC subsets have been studied in great detail and play multiple roles in T cell priming and differentiation. However, from a simplified view it appears that $CD8\alpha^+CD11b^-$ DCs are more prone to induce Th1 responses [40], are generally good at cross-presenting antigens to CD8 T cells [41, 42], and are involved in uptake of dying cells [43]. In contrast $CD8\alpha^-CD11b^+$ DCs appear more efficient at priming CD4 T cells [41] and to induce Th2 responses [40].

While both $CD8\alpha^+CD11b^-$ and $CD8\alpha^-CD11b^+$ derive from pre-DCs, their development requires different sets of transcription factors. Thus, development of $CD8\alpha^+CD11b^-$ DCs is dependent on basic leucine zipper transcription factor ATF

like (Batf)3, IFN regulatory protein (IRF)8 and inhibitor of differentiation/ DNA binding protein (Id)2 [26, 44-47] while the development of CD8α⁻CD11b⁺ DCs is strongly influenced by transcription factors IRF2, IRF4 and v-rel reticulo-endotheliosis viral oncogene homolog B (RelB) [26, 48].

2.2.2 Migratory dendritic cells

Migratory DCs are found in peripheral tissues of the body, including the skin, lung, and intestine. These DCs constantly sample peripheral tissues for foreign antigen and travel via afferent lymphatic vessels to draining LNs [49]. Migratory populations in the skin include Langerhans cells as well as two dermal DC populations that can be distinguished based on their expression of langerin and αE integrin CD103 [50-53]. While in the lung, the primary migratory population has been proposed to be the CD103⁺CD11b^low DC subpopulation [54-56], at the onset of these thesis studies, the identity of the main migratory DC population of the small intestinal lamina propria was not clear, although CD103⁺ DCs had been suggested [57].

2.2.3 Plasmacytoid dendritic cells

Plasmacytoid DCs are a minor population of DCs that are present in blood and LNs and in tissues during viral infections or autoimmunity [58, 59]. Murine plasmacytoid DCs express B220 and intermediate levels of CD11c, can express MHCII and co stimulatory molecules after activation, but do not express CD11b [58]. Plasmacytoid DCs primarily respond to virus and CpG oligonucleotides and produce high amounts of IFN-α and -β after activation, which act to block viral replication and activate other parts of the immune system [58, 60]. However, non-activated pDCs may also have a role in peripheral tolerance and have been observed to convert T cells into T reg cells *in vitro* [58]. Development of pDCs is dependent on the basic helix-loop-helix transcription factor E2-2 and IRF8 and strongly influenced by Flt3 ligand [58, 61]. Although without doubt important in shaping immune responses [58, 62], the pDCs are not the focus of this thesis and will not be further discussed.

2.3 Monocyte derived dendritic cells

Monocytes may, particularly in the setting of inflammation, become monocyte-derived DCs (MoDCs) that have DC like phenotype (CD11c^{+}MHCII^{hi}, high levels of costimulatory molecules) and/or DC like functions (ex. ability to stimulate naive T cells or crosspresent antigens to T cells) [22, 30, 63, 64]. The differentiation of monocytes to DCs may be shaped by inflammatory cues in the local environment [22]. Inflammatory cues may also alter the composition of DC and monocyte derived subsets in tissues and LNs via recruitment of monocyte derived subsets [64, 65, 66]. While murine *in vitro* MoDCs were described already in the 70's [67] it was not until recently that the *in vivo* relevance of these cells in infection and antigen presentation was established [22, 64, 68]. In this regard, a recent report by Cheong *et al* described the development and recruitment of murine DC-SIGN[+] MoDCs to peripheral LNs after challenge with gram-negative bacteria. Notably these MoDCs were shown not only to express CD11c, CD40, CD80 and CD86 and high levels of MHCII (markers associated with DCs) but critically also to present and crosspresent antigens to T cells and to localize to the T cell area of LNs [64]. Hence, these MoDCs showed several features normally attributed to DCs rather macrophages. Another population of MoDCs in the mouse are TipDCs which are derived from the 'classical' or 'inflammatory' monocyte subset. TipDCs are selectively recruited to inflamed tissues [69-71] and have been reported to play a role in the immune defense against bacterial infections including *Listeria monocytogenes* and *Salmonella* Typhimurium [69, 71]. Further examples of monocyte-derived populations in inflammation are the populations of E-cadherin^{+}CD103^{+} cells derived from predominantly Gr1^{+} monocytes as well as the TLR2^{+}CCR2^{+}Gr1^{+} macrophages and CX3CR1[low/int] mononuclear phagocytes all of which are increased in the colon of colitic mice [65, 66, 72]. However, the interrelationships between these recently described subpopulations of recruited monocyte derived cells require further investigation.

2.4 Dendritic cells in uptake, processing and presentation of antigen

According to current dogma, immature DCs take up antigen in tissues and subsequently migrate via afferent lymphatic vessels to the draining LN where they as mature DCs present the antigen to responding T cells.

2.4.1 Antigen uptake

Antigens originating from outside the cell, called exogenous antigens [73], can be taken up by several mechanisms including receptor mediated endocytosis, macropinocytosis, and phagocytosis [74]. Receptor mediated endocytosis generally involves inward budding of clathrin-coated vesicles and is mediated by specific receptors binding to the substances or molecules being internalized [74]. These receptors include DEC-205, Fc-receptors and mannose receptors [75, 76]. Phagocytosis generally involves polymerization of actin and is, similar to receptor mediated endocytosis, mediated by receptors, including Fc- and complement-receptors [77].

2.4.2 Antigen processing and presentation

After uptake, exogenous antigens are targeted to endocytic compartments where they are processed into peptides in organelles called lysosomes. In lysosomes, peptides then associate with MHCII molecules to form MHCII:peptide complexes, which are transported to the plasma membrane and presented on the surface of APCs. Antigens can also be derived from within the cell itself and are then called endogenous antigens [73]. These include both self-antigens and antigens from intracellular pathogens. Endogenous antigens are mainly degraded into peptides by the proteasome in the cytoplasm. These peptides can then be transported into the rough endoplasmic reticulum where they can associate with MHCI molecules. The resulting MHCI:peptide complexes are then presented on the surface of cells.

DCs can also present exogenous derived antigens in MHCI:peptide complexes via a mechanism known as cross presentation [78, 79]. Cross presentation may be of particulate importance for initiation of MHCI mediated CD8 T cell responses to viruses that do not infect APCs. While exact mechanisms and relative contribution of different mechanisms in cross presentation are still poorly understood, cross presented exogenous antigens may be taken up by receptor mediated endocytosis, pinocytosis or phagocytosis and are thought to reach the cytosol where they may either be processed in a proteasome-dependent manner or in endocytic compartments [79]. Further, endogenous antigens may be delivered to MHCII molecules, and possibly MHCI molecules, via autophagy, an autophagosome mediated degradation process of intracellular contents [78, 80]. However, the relative contribution of autophagy on the MHCII and MHCI peptide repertoire and the effector immune responses resulting from autophagy are not well understood [78, 80, 81]. In summary, DCs, via uptake of exogenous and processing of endogenous and exogenous antigens, present peptides on MHCII or MHCI to CD4 and CD8 T cells respectively.

3. T CELL HOMING

Entry of T cells into LNs and peripheral tissues is mediated by the interaction of adhesion receptors expressed on the T cell surface with their respective ligands expressed the vascular endothelium in lymphoid and extra-lymphoid tissues. Thus the migratory potential of any given T cell subset is determined by the set of adhesion receptors it expresses and the expression profile of adhesion receptor ligands on any given vascular endothelium [82, 83].

3.1 Homing in general

Lymphocyte interactions with vascular endothelium can be divided into four stages each of which is mediated by interactions of molecules on the lymphocyte and corresponding ligands on vascular endothelium [82-84].

Step one - tethering/rolling: This first step is primarily mediated by selectins, including including L-selectin (expressed on leukocytes) and P- and E-selectin (expressed on vascular endothelium) which form transient low-affinity interactions with their carbohydrate ligands expressed on the vascular endothelium or leukocytes respectively. The binding of selectins to these carbohydrate ligands is dependent on specific carbohydrate structures, including sialyl Lewis X or 6-sulfo sialyl Lewis X [85, 86]. Generation of these structures is mediated by several enzymes, including fucosyltransferases and sialyltransferases [86]. In this regard, mice double deficient in fucosyltransferases IV and VII show reduced homing of transferred wt lymphocytes to both MLNs and peripheral LNs (PLNs) [87]. Proteins that are scaffolds for the carbohydrate ligands include CD34, CD44, and P-selectin glycoprotein ligand (PSGL)1 [86]. Thus, transient low-affinity interactions between selectins and their carbohydrate ligands on glycoproteins allow lymphocytes to slow down and roll along the vascular endothelium.

Step two - activation: During the rolling, chemokines (which are chemotactic cytokines) signal through chemokine receptors expressed on the rolling lymphocyte. Chemokines comprise of a large family of structurally related proteins that are divided into four major groups based on the spacing of the two first conserved cysteines in their N-terminus: CXCL, CCL, (X)CL and CX3CL chemokines. Chemokine receptors belong to the family of 7-transmembrane G-protein coupled receptors and can be divided, based on the ligands they bind, into CXC, CC, (X)C, and CX3C receptors [88, 89]. While the CCL and CX3CL subgroups consist of several members there is only one member of the CX3CL family (fractalikne) and two members of the (X)CL family (lymphotactin α and β) [89]. Of note, most chemokine receptors bind multiple chemokines within a specific group, and some chemokines can bind multiple receptors.

Interaction of chemokines with their chemokine receptors leads to binding of intracellular heterotrimeric G proteins to the receptor, resulting in dissociation of α and βγ subunits of the G protein and subsequent activation of many intracellular pathways which in turn can lead to restructuring of the cytoskeleton, granule release, and modulation of gene transcription [88, 90]. While there are over twenty different G protein α-subgroups, divided into four families ($G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i/o}$), G-proteins of the $G\alpha_i$ family couple to chemokines [90]. In regards to lymphocyte adhesion, interactions of chemokines with chemokine receptors generates signals that lead to rapid changes in integrin conformation (integrin activation) via a process known as "inside-out" signaling. Interactions of chemokines with chemokine receptors can also lead to integrin clustering. These changes result in increased integrin affinity and avidity for its endothelial ligands and are prerequisites for the subsequent firm adhesion and arrest of the rolling cells [84, 91].

Of note, negatively charged glycosaminoglycans on the endothelial surface are thought to play an important role in presenting chemokines to rolling lymphocytes and may protect chemokines from proteolytic cleavage as well as contribute to the establishment of chemokine gradients [92]. In this regard, chemokines with

mutations in their glycosaminoglycan binding domain are unable to mediate cell migration into the peritoneum of mice [93] and mice in which *Ext1*, an enzyme critical for the synthesis of the glycosaminoglycan heparan sulfate, has been conditionally inactivated displayed a decrease in the homing of labeled wt lymphocytes to secondary lymphoid organs including PLN and MLN [94]. Thus, binding of chemokines, presented by the vasular endothelium, to chemokine receptors on lymphocytes, lead to in activation of integrins, which in turn allows for firm adhesion and arrest of the lymphocytes.

Step three - firm adhesion: In this step, integrins expressed on lymphocytes firmly bind to ligands on the endothelial wall. Integrins are heterodimeric proteins made up of an α and a β chain that play key roles in regulating cell-cell and cell-matrix interactions [95]. While there are at least 18 different α and 8 different β subunits that together pair up to make at least 24 different integrin $\alpha\beta$ heterdimers [91], it is primarily members of the $\beta1$ and $\beta2$ integrin families that regulate leukocyte adhesive events with vascular endothelium [84, 91]. In this regard, *in vivo* blocking of $\beta2$ integrin by administration of a neutralizing antibody led to reduced adherence of leucocytes to venular endothelium and reduced accumulation of leukocytes in inflamed skin [96]. Further, humans with leukocyte adhesion deficiency (LAD)I caused by mutations in *Itgb2* leading to decreased or abolished expression of the integrin $\beta2$ chain, show frequent infections and inability to clear pathogens, which at least in part is thought to be caused by impaired homing of leukocytes [97, 98].

Cellular ligands of integrins are often members of the Immunoglobulin (Ig) superfamily and include inter-cellular adhesion molecule (ICAM)-1 and ICAM-2. Of note, binding of activated integrins to their ligands can induce "outside-in" signaling which is thought to stabilize the firm adhesion of integrins to their ligands [84].

Step four - transmigration After firmly adhering to the vascular endothelium, leucocytes may migrate through or between endothelial cells, and through the underlying basement membrane, into the tissue. This step is mediated by several different groups of molecules and interactions including ICAM-1 binding to integrin αMβ2 (also called macrophage antigen-1; Mac-1) or integrin αLβ2 (also called lymphocyte function-associated antigen-1; LFA-1) as well as junctional adhesion molecules (JAMs), binding to integrin αLβ2 or JAM-A [84].

3.2 Homing of naïve T cells

Adhesion receptors expressed by naïve T cells allow them to entry into secondary lymphoid organs while they are generally not able to enter peripheral tissue. Entry into secondary lymphoid organs occurs across specialized vascular endothelial areas termed high endothelial venules (HEVs) [99]. First, interactions between L-selectin expressed by T cells and carbohydrates on peripheral node addressin (PNAd) expressed by HEVs mediates rolling of the T cells. Next, CC-chemokine ligand (CCL)21 expressed by high endothelial cells and to some extent CCL19, expressed by perivascular cells and transported to the luminal surface of HEVs, bind CC chemokine receptor (CCR)7 on naïve T cells leading to activation of integrin αLβ2. Interaction of integrin αLβ2 with ICAM-1 and ICAM-2 then leads to firm adhesion of the lymphocyte to the HEV surface. However, several other adhesion molecules and integrins have also been suggested to play roles in T cell entry into LNs [99]. For example, rolling and firm adhesion of lymphocytes on MLN and PP HEV can be mediated via mucosal addressin adhesion molecule (MadCAM)-1 interactions with L-selectin (rolling) or integrin α4β7 (rolling and firm adhesion) [99-103]. Thus T cells deficient in β7 integrin, while capable of gaining entry into peripheral lymph nodes, fail to enter into PPs [104].

3.3 Effector T cell migration to peripheral tissues

Homing of effector T cell subsets to different peripheral tissues is mediated by differential expression of homing receptor ligands in vascular beds. For example, MadCAM-1 is constitutively expressed on vascular endothelial cells in the intestine but not skin [83, 100, 105, 106] and the chemokine CCL25 (also called TECK; thymus expressed chemokine), is constitutively and selectively expressed by epithelial cells in the small intestine [107-110] and is present on the surface of small intestinal endothelial cells [109, 111]. In contrast, endothelial cells in inflamed skin express E- and P-selectin, the chemokines CCL17, CCL27 and vascular cell adhesion protein (VCAM)-1 [112, 113]. As will be discussed below for the small intestine, such selective homing receptor ligand expression leads to the recruitment of distinct subsets of effector cells from the circulating effector T cell pool.

3.3.1 Effector T cell homing to the small intestine

As mentioned above, CCL25 and MadCAM-1 are constitutively expressed on vascular endothelial cells within the intestine. The *in vivo* importance of CCL25 and MadCAM-1, and their ligands in mediating lymphocyte recruitment to the intestinal mucosa has been shown by adoptive transfer studies where T cells deficient in CCR9 [114, 115] or integrin $\beta7$ chain [116] were impaired in their ability to home to the small intestinal mucosa or small and large intestinal mucosa respectively. Experiments where the interaction between CCR9 and $\alpha4\beta7$ and their ligands was blocked with neutralizing antibodies to CCL25 or $\alpha4\beta7$ have further shown the *in vivo* relevance of CCL25 and $\alpha4\beta7$ in lymphocyte recruitment to the small intestine [117, 118]. In addition, studies using competitive adoptive transfer experiments of wt and CCR9 deficient CD8 T cells have suggested that CCR9 plays a more prominent role in regulating T cell migration to the proximal small intestine [119]. Taken together, CCR9 and $\alpha4\beta7$, via their ligands CCL25 and MadCAM-1 respectively, are considered to play important roles for lymphocyte homing to the intestine.

3.4 Modulation of lymphocyte homing for the treatment of inflammatory bowel disease.

Inflammatory bowel disease is characterized by chronic destructive inflammation in the gastrointestinal tract with increased recruitment of leucocytes [11, 120] and is linked to inappropriate immune responses to commensal bacteria [11, 120, 121]. IBD can be divided into Crohn's disease and Ulcerative colitis [120-122]. Crohn's disease affects the entire gastrointestinal tract but particularly the colon and the distal parts of the small intestine. The inflammation is often discontinuous (i.e. normal areas are interspersed between involved areas), affects all layers of the intestine and shows marked infiltration CD4 T cells and macrophages. Ulcerative colitis is restricted to the colon and is often continuous and confined to the mucosa and submucosa and can be characterized by infiltration of T cells and neutrophils [120-122].

Given that T cells are thought to be involved in the pathogenesis of IBD, it has been proposed that blocking pathogenic T cell migration to the intestine may be of therapeutic benefit in IBD patients. This approach is particularly attractive as targeting specific molecules involved in T cell migration to the intestinal mucosa is likely to prevent inflammation in a tissue specific manner and may thus have less side effects compared to more broad anti-inflammatory therapies. Based on the roles for CCR9 and $\alpha 4\beta 7$ in the homing of T cells to the small intestine as discussed above, both molecules have been targeted for drug design and are currently in human trials for the treatment of IBD. An orally-available small molecule CCR9-antagonist (GSK-1605786; CCX-282; Traficet-EN) has been developed for the treatment of small bowel Crohn's disease and has in recent Phase II/III clinical trials been found to maintain remission in moderate to severe Crohn's disease patients [123-126] and a humanized monoclonal antibody to the $\alpha 4\beta 7$ integrin (MLN-02; Vedolizumab), which is currently in Phase III studies, has been found to be more efficient that placebo in inducing remission in patients with active Ulcerative colitis or Crohn's disease [127-130].

However, one potential drawback with anti-adhesion molecule therapy is that the molecules that are blocked to prevent T cell migration in inflammation also mediate immunosurveillance in tissues and hence, blocking of their function may potentially predispose patients to infection. One example of this is the humanized monoclonal antibody to the α4 integrin (Natalizumab) that binds to the α4 subunit of the integrins α4β1 (involved in recruitment of T cells to the brain) and α4β7 (involved in recruitment to the gut mucosa) and inhibits the α4-mediated adhesion of T cells to endothelial ligands [131, 132]. While Natalizumab has been found to be effective in reducing relapse rates for patients with Multiple Sclerosis or Crohn's disease [131-133] it has also been associated with progressive multifocal leukoencephalopathy [134, 135], a fatal viral disease of the brain caused by Polyomavirus JC [136]. Thus, the targets used for anti-adhestion adverse therapies need to be thoughtfully selected and the adverse effects of these therapies involving targeting of specific molecules implied in T cell migration need to be closely monitored.

4. OVERVIEW OF THE INTESTINAL IMMUNE SYSTEM

4.1 Structure of the intestine

The intestine is divided into small intestine, comprising of the duodenum, jejunum and ileum, and the large intestine, comprising of the cecum, colon and rectum. In the proximal part of the intestine (duodenum), partly digested food is mixed with bile, mucus and digestive enzymes and broken down into proteins, fats and carbohydrates. This process continues along the length of the small intestine with nutrient absorption taking place primarily in the jejunum and ileum

Macroscopically, the intestine is composed of several layers, which from inside the lumen outwards are the mucosa, submucosa, muscularis and serosa. The mucosa is composed of a lamina propria layer and an overlying single layered epithelium and is organized into villous and crypt structures see *figure 2*. Generally, the villi are tall thin structures in the proximal small intestine and decrease in length down the small intestine. Separating the epithelial layer from the lamina propria is a layer of extracellular matrix, called the basal lamina.

The epithelial layer consists mainly of absorptive enterocytes but also enteroendocrine cells, mucus-secreting goblet cells and paneth cells that produce antimicrobial peptides [137, 138]. The apical (facing the lumen) surface of each enterocyte is covered by small extensions termed microvilli, which increase the surface area of the intestinal mucosa for nutrient absorption and the exchange of water and electrolytes. Each of the cell types of the epithelium (i.e. enterocytes, enteroendocrine cells, goblet cells and paneth cells) originates from stem cells that reside in the intestinal crypts [139, 140]. In these intestinal crypts, proliferation of stem cells generates daughter cells that divide and subsequently reach the junction between the crypt and the villus where they start to differentiate into their specific cell fates. The proliferation of crypt cells is balanced by apoptosis and shedding of cells at the tip of the villus. In this way, the epithelial sheet is continually moving upward and epithelial cells are replaced every 3-5 days [139, 141, 142].

The lamina propria is comprised of loose connective tissue that contains stromal cells, including fibroblast and myofibroblasts, smooth muscle cells, nerve fibers, blood capillaries and lymphatic vessels [138, 143].

4.2 Immune compartments of the intestinal mucosa

The immune compartments of the intestinal mucosa can be divided into inductive sites and effector sites. Inductive sites are sites of naïve T and B cell priming and where initiation of adaptive immune responses occur while effector sites are where effector cells exert their action [144]. Inductive sites of the intestinal mucosa includes PPs and isolated lymphoid follicles (ILFs), which both are structures of the organized gut-associated lymphoid tissue, (GALT), and the MLN which drains the intestinal mucosa. Effector sites consist of the intestinal epithelium and lamina propria [138, 144, 145], see *figure 2*.

4.2.1 Inductive sites

Mesenteric lymph nodes
MLNs are located within the mesentery and are connected to the small intestine via afferent lymphatic vessels. Each MLN consists of an inner medulla and an outer cortex, surrounded by a fibrous capsule. Macrophages and plasma cells preferentially reside in the medulla, while the cortex is home to T cells and B cell follicles. Intestinal antigens enter the MLN via afferent lymphatics, either in soluble form or associated with DCs [138]. Soluble antigen may reach LN resident DCs in T cells zones via a conduit system built up by reticular fibers [146].

Dendritic cells in mesenteric lymph nodes: DCs in the MLNs, like other LNs, are either migratory or resident/non-migratory [11, 147, 148]. Migratory DCs enter the MLN via afferent lymphatics, which drains from the intestinal lamina propria and possibly PPs [49, 138] while resident/non-migratory DCs enter the MLN via the blood, through HEVs [26].

The main subpopulations of DCs in the MLNs, including both migratory and non–migratory are the $CD11c^{hi}CD11b^+CD8\alpha^-$, the $CD11c^{hi}CD11b^-CD8\alpha^+$ and the $CD11c^{hi}CD11b^-CD8\alpha^-$ subpopulations [11]. However, DCs in MLN may instead be subdivided based on expression of the αE integrin CD103. $CD103^+$ DCs will be further discussed in the *CD103$^+$ dendritic cells* section below.
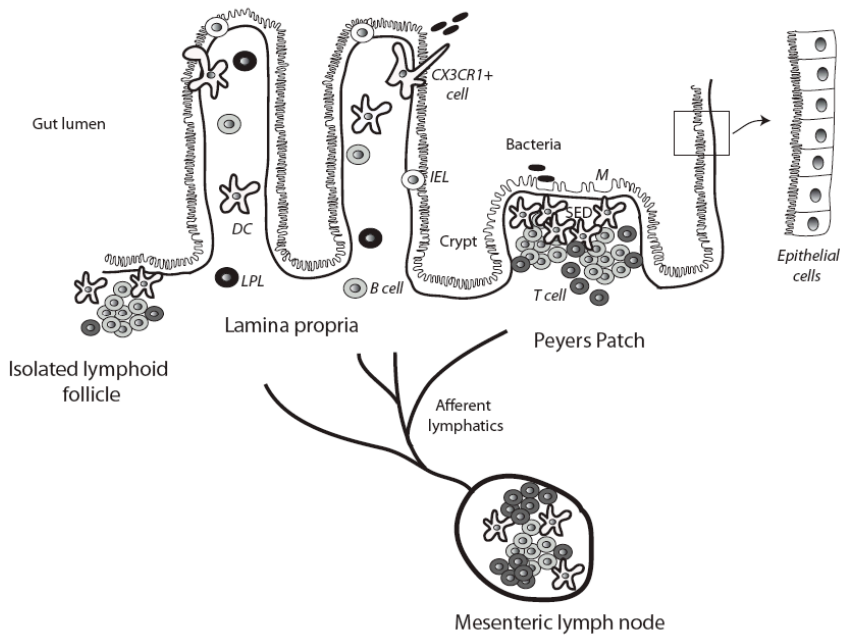
Figure 2. **Structure of the small intestinal immune system.** The small intestinal lamina propria is covered by a single layer of epithelium with intraepithelial lymphocytes (IELs) interspersed within the epithelium. While T cells dominate within the IEL compartment several immune cell subsets, including B cells, lamina propria lymphocytes (LPLs), dendritic cells (DCs) and macrophages reside in the underlying lamina propria. The intestinal lamina propria is drained by lymphatic vessels that connect the intestine to the mesenteric lymph node (MLN). The mesenteric lymph nodes (MLNs) together with Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) make up the inductive sites of the small intestinal immune system. PPs are covered by a follicle associated epithelium (FAE) containing microfold (M) cells that are important for antigen uptake. Under the M cells is the sub-epithelial dome (SED) where many DCs are positioned. PPs also contain B cell follicles and T cells. ILFs are also covered by a FAE containing M cells with DCs, B cells and T cells positioned underneath.

Peyers patches

PPs are lymphoid aggregates observed as thickenings of the small intestinal mucosa. There are approximately 10 PPs per mouse small intestine and over one hundred per human intestine [149]. PPs are composed of several large B cell follicles and interfollicular T cell zones [138]. The epithelium covering the PP is termed follicle–associated epithelium (FAE) and contains microfold (M) cells that are specialized in taking up particulate antigens [138, 150] as discussed in the *Uptake of intestinal antigens* section below. The FAE also contains DCs and macrophages [138]. The area underneath the FAE is called the subepithelial dome (SED) and contains several DCs.

Dendritic cells in Peyers patches: In the PPs, DCs can acquire antigens directly from the intestinal lumen or via M cells [150, 151]. The DCs may then prime T cells within the FAE/SED or the T cell–rich interfollicular regions IFR of PPs or possibly also, after migration, in MLNs [49, 138, 152-154]. As outlined below, multiple DC subsets exist in the PPs and the DC subset presenting the antigen likely plays an important role in determining the outcome of the ensuing adaptive immune response. DC subsets that have been described in murine PPs include $CD11c^{hi}CD11b^+CD8\alpha^-$ DCs, $CD11c^{hi}CD11b^-CD8\alpha^-$ (double negative) DCs, $CD11c^{hi}CD11b^-CD8\alpha^+$ DCs and epithelial-associated DCs [147, 153-155].

$CD11c^{hi}CD11b^+CD8\alpha^-$ DCs are mainly localized to the sub-epithelial dome of PPs while $CD11c^{hi}CD11b^-CD8\alpha^+$ DCs are localized within the interfollicular region and $CD11c^{hi}CD11b^-CD8\alpha^-$ (double negative) DCs are localized both to the sub-epithelial dome and the interfollicular region [147, 153-155]. The epithelial-associated DCs consist of at least two populations of DCs with close association to the follicle associated epithelium covering PPs. The first population is the $CD8\alpha^-CD11b^-$ DCs that express intracellular MHCII and are located above the basement membrane in the FAE covering the PP [155]. The second population is the $CCR6^+CX3CR1^-$ DCs that are either $CD11b^+CD8\alpha^-$ or $CD11b^-CD8\alpha^-$. These cells are localized to the subepithelial dome but can move into the FAE upon infection where they may

activate CD4 T cells [153, 154]. Thus, many different DC subsets are found in the PPs, many of which have specialized functions.

Isolated lymphoid follicles

ILFs are found both in small intestine and colon [156]. They are, similarly to PPs, covered by a follicle-associated epithelium (FAE) with M cells [157] and contain B cell follicles. However, ILFs are generally smaller than PPs and lack discrete T cell zones [158]. Recent findings suggest that ILF develop from small structures called cryptopatches (CP) [159]. CPs are small aggregates found at the base of the intestinal crypts that contain mainly Lin⁻c-kit⁺IL-7Rα⁺ cells but also CD11c⁺ DCs, while devoid of B cells [160, 161]. While CPs develop in germfree mice, ILFs are dependent on microbial flora for their development [160].

4.2.2 Effector sites

Intestinal epithelium

Effector cells in the epithelial layer are termed intraepithelial lymphocytes (IELs) and are almost exclusively CD8 T cells. IELs can be further divided into type a and type b IELs based on expression of CD8αβ *vs*. CD8αα [162]. Type a IELs include TCRαβCD8αβ and a minor population of TCRαβCD4 T cells that are MHC restricted, derive from conventional TCRαβ T cells and have cytolytic effector functions [162-165]. Type b IELs lack expression of CD8β but express the CD8αα homodimer and are either TCRαβ or TCRγδ T cells and contain large numbers of self-reactive T cells and can respond to antigens not associated to classical MHC molecules [162, 164, 165]. While the functions of CD8αα IELs are not completely understood, they appear to play a role in tissue homeostasis [166] including turnover and growth of intestinal epithelial cells [167, 168]. CD8αα IELs are also thought play a role in tolerance [166]. In this regard, adoptive transfer of intestinal TCRαβCD8αα could prevent the subsequent induction of colitis in a model where transfer of CD4⁺CD45RBʰⁱ T cells into severe combined immunodeficiency (SCID) recipient mice give rise to intestinal inflammation [169].

Intestinal lamina propria

Effector cells in the lamina propria include lamina propria lymphocytes (LPLs) of both T cell and B cell lineage. The T cells can be divided into two major populations, a major TCRαβCD4 and a minor TCRαβCD8αβ population [164]. LPLs resemble the IEL type a cells as they have effector/memory phenotype, indicating prior activation and/or exposure to antigen, and are believed to derive from naïve conventional T cells that have entered the intestinal mucosa following activation in secondary lymphoid organs [164, 170].

The TCRαβCD4 lamina propria T cell population includes Th1 cells, Th2 cells, Th17 cells and T regs [9, 164, 171]. Th17 cells are particularly abundant in the small intestinal lamina propria, compared to other tissues [171] and the number of Th17 cells, but not total CD4 T cells, is severely decreased in germ-free mice. In particular, segmented filamentous bacteria (SFB), which are gram-positive commensal microbiota, have been suggested to induce Th17 cells via a mechanism that may involve serum amyloid A (an acute-phase response protein) acting on DCs [172]. Further, adenosine 5'-triphosphate (ATP) which can be derived from commensal bacteria [173] could, upon injection into germ-free mice increase the numbers of IL-17-producing CD4 T cells in the intestine and addition of a non-hydrolysable ATP analogue to CD11c$^+$ lamina propria cells increased the expression levels of *Il17a* and *Il17F* in cocultured CD4 T cells [174] suggesting that bacterial ATP may drive generation of intestinal Th17 cells. Thus, the induction of intestinal IL-17 secreting cells may be controlled, at least in part, by commensal bacteria.

Intestinal T regs include both natural and induced T regs [8, 9] and have been suggested to play roles in both oral tolerance and prevention of intestinal inflammation such as colitis [175, 176]. However, both the generation and intestinal function of these regulatory subsets *in vivo* is in many cases not clear. Nonetheless, strains of *Clostridium* bacteria may expand T regs of the large intestine via induction of TGFβ from intestinal epithelial cells [177] indicating that the intestinal flora may modulate both intestinal T regs and Th17 cells. Still, the number of T regs in the

small intestine of germ-free mice is not decreased relative to 'conventional' mice [171, 178] and the generation of small intestinal T regs may therefore be independent of the intestinal flora. Hence different mechanisms may lead to generation or expansion of T regs in the small *vs.* large intestine.

4.3 Uptake of intestinal antigens

Several cell populations have been described to participate in antigen uptake from the intestinal lumen including M cells, intestinal DCs/macrophages and intestinal epithelial cells.

4.3.1 Intestinal M cell-dependent antigen uptake

M cells have very short (mouse) or lack (human) surface microvilli [179] and have a mucus layer that is less thick than the layer covering intestinal epithelial cells [138]. Together these characteristics are thought to increase the accessibility of pathogens and particular antigens to M cells. The surface of M cells also contains carbohydrate structures that likely contribute to bacterial binding and uptake. Indeed several pathogens, including species of *E. coli*, *Salmonella*, *Shigella* and *Yersinina*, have been observed to specifically bind and/or invade M cells [179-183]. In some cases specific receptors that mediate such binding have been identified. For example, β1 integrins may mediate attachment of *Yersinia tuberculosis* [184, 185]. These specific features of M cells, together with the ability of M cells to phagocytose, endocytose and pinocytose bacteria and particular materials [150, 179, 186] provide M cells with the ability to act as an entry point for a large number of intestinal antigens.

After uptake, antigens can be transcytosed from the luminal surface of the M cell to the basolateral membrane. Here, at the basolateral side of M cells, invaginations called pockets are observed. These pockets may function to reduce the distance that antigens have to travel within M cells [179]. At the basolateral membrane antigens can be passed on to DCs [150, 179]. In summary, one of the major functions of M cells is to take up luminal antigen for presentation by DCs.

4.3.2 Uptake of intestinal antigen by dendritic cells/macrophages

M-cell independent bacterial uptake in the small intestine has been particularly associated with cells that have transepithelial dendrites and express the fractalkine receptor CX3CR1. In an initial study by Rescigno *et al*, DCs were shown to infiltrate *in vitro* cultured epithelial monolayers and to take up *Salmonella* Typhimurium via transepithelial dendrites [187]. Further, upon injection of *Salmonella* into ligated loops of intestine, CD11c^{+} cells were observed to extend dendrites, which came in contact with *Salmonella* bacteria, into the lumen of the intestine [187] indicating that transepithelial dendrites may form *in vivo*. Subsequent studies using CX3CR1[GFP/WT] mice (mice with targeted deletion of CX3CR1 with GFP insertion) then demonstrated the presence of, what the authors called, DCs in the lamina propria that expressed CX3CR1 and penetrated the epithelial layer to gain direct access to antigens in the gut lumen [188]. In addition, further studies have demonstrated the presence of *Salmonella* in GFP^{+} cells of CX3CR1[GFP/WT] mice after oral infection with *Salmonella* Typhimurium [189]. Together, these findings indicate that CX3CR1^{+} intestinal cells play a role in uptake of luminal microbes.

In further studies, CX3CR1[GFP/GFP] mice, which lack expression of CX3CR1, had reduced uptake of both *E. coli* and invasive and mutant non-invasive *Salmonella* Typhimurium compared to CX3CR1[GFP/WT] and wild-type control mice [188] suggesting that uptake of bacteria by CX3CR1^{+} cells is dependent on the CX3CR1 protein. Further, CX3CR1[GFP/GFP] mice were, in the same study, reported to lack transepithelial dendrites in terminal ileum, indicating a role for the CX3CR1 protein also in formation of transepithelial dendrites in CX3CR1^{+} cells [188]. However, a subsequent study observed transepithelial dendrities in proximal ileum of CX3CR1[GFP/GFP] mice [190] and hence the role of the CX3CR1 protein in the formation of transepithelial dendrites is still unclear.

Nonetheless, the number of transepithelial dendrites appear to increase following oral challenge with invasive [188, 190] or non-invasive strains of bacteria [190, 191] and by administration of the TLR ligand LPS while treatment with antibiotics has

been reported to decrease the number of transepithelial dendrites [190]. In this regard, the mechanism for the induction of transepithelial dendrites has been indicated to involve epithelial MyD88 dependent signaling via TLRs [190]. Hence, formation of transepithelial dendrites is likely regulated by the presence of intestinal bacteria and/or TLR signaling.

While the use of CX3CR1[GFP/WT] mice have demonstrated the presence of transepithelial dendrites in CX3CR1^{+} cells in distal parts of the small intestine [188], it is still unclear whether transepithelial dendrites are restricted to CX3CR1^{+} cells. Thus, when presence of transepithelial dendrites was examined using CD11c[GFP/WT] or MHCII[GFP/WT] mice, transepithelial dendrites were found throughout the jejunum with a slight decrease in frequency in terminal ileum [190]. Further, after *Salmonella* Typhimurium infection, bacteria were observed in GFP-negative phagocyte cells in CX3CR1[GFP/GFP] mice [188] and in CX3CR1^{-}CD103^{+}CD11b^{+} DCs [28]. Although these studies may indicate that bacteria can be transferred from CX3CR1^{+} cells to CX3CR1^{-} cells, it cannot be excluded that CX3CR1^{-} cells also can form transepithelial dendrites. Even so, transepithelial dendrites were absent in the BALB/c mouse strain [191] and thus the relative contribution of transepithelial dendrites in antigen uptake in the intestinal mucosa remains a source of debate.

4.3.3 Alternative routes of uptake of intestinal antigens

Alternative routes of antigen uptake include uptake by epithelial cells, transepithelial diffusion and direct access of antigens to the intestine lamina propria in areas of epithelial damage.

Epithelial uptake
Epithelial cells, positioned at the interface between the gut lumen and the intestinal tissue have been suggested to contribute to the uptake of intestinal antigens including soluble proteins and food antigens. Antigen uptake by epithelial cells may be mediated via invagination of the epithelial membrane or pinocytosis [192, 193]. To this end, uptake of orally administered fluorescent OVA has been observed in

intestinal epithelial cells *in situ* [194]. Further, receptors that aid in uptake of antigens are present on the surface of epithelial cells and include the neonatal Fc receptor (FcRn) and DEC-205 [195-198]. Antigens taken up by epithelial cells likely become available to DCs and macrophages upon epithelial apoptosis [199, 200]. In addition, vesicles containing MHCII molecules and antigens, called exosomes, are secreted by epithelial cells [201]. While the function(s) of these exosomes are not clear they have been hypothesized to provide liver cells with peptides derived from intestinal antigens [201, 202].

Diffusion

Antigens may also be freely diffusing through the epithelial barrier [203]. Antigens that enter the intestinal mucosa by diffusion could subsequently be taken up by intestinal lamina propria cells or reach the circulation [204]. As discussed below, soluble antigen, reaching peripheral blood may result in systemic tolerance [205, 206].

4.4 Migration of intestinal dendritic cells to MLN

DCs continually migrate from the small intestine to the MLN, even in the absence of infection or inflammation [207, 208]. This steady state migration is thought to play a role in presentation of antigen derived from apoptotic intestinal epithelial cells and in the generation of oral tolerance [199, 200, 209]. Migration of tissue DCs, including lamina propria DCs, to LNs is dependent on CCR7 expression on DCs [199, 210-214], and ligands CCL19 and CCL21 constitutively expressed on afferent lymphatic endothelium (CCL21) and in LNs (CCL19, CCL21) [210, 212]. Migration of DCs from the lamina propria to the MLNs is increased by inflammatory stimuli, such as lipopolysaccharide (LPS) or TLR7/8 agonists [210, 215-217] and may, in the case of TLR7/8 agonists be mediated by TNFa secreted by pDCs [216]. At the start of these studies Johansson-Lindbom *et al* had noted that CD103^{+} DCs were markedly reduced in CCR7 KO mice [211], indicating that CD103^{+} MLN DCs represented a lamina propria derived migratory population. However direct evidence that CD103^{+} DCs migrated in the lymph, and whether additional subsets of lamina propria derived CD11c^{+} cells migrated to the MLN in the steady state or after adjuvant administration remained unclear.

4.5 Oral tolerance

The intestinal mucosal tissue presents an enormous surface area to the outside milieu and is continually exposed to foreign antigens in the food we eat and commensal microbiota. In addition, the intestinal mucosa is also a major site of exposure to potential pathogens [218]. Consequently the intestinal immune system must possess mechanisms that generate effective immune response against pathogenic invasion while at the same time control the immune responses to commensals and food antigens [175].

Oral tolerance can be defined as 'specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen by the oral

route' [219]. While the mechanisms underlying oral tolerance are multifactorial and not fully elucidated, oral tolerance has been suggested to depend on absence of T cells with the correct specificity (deletion), lack of sufficient activation signals (anergy) and induction of CD4 T regs (active suppression) [204, 219]. There is evidence to suggest that the mechanism of induction of oral tolerance depends on the dose of the antigen, where a low dose may induce expansion of T regs and/or production of TGFβ, IL-4 and IL-10 [220-222] while a high dose may generate depletion or anergy of antigen specific cells [223, 224] although this is still an issue under debate.

Induction of oral tolerance has in many studies been proposed to involve antigen-specific T cell proliferation within PPs and MLNs [204, 225, 226] but also within the spleen and PLNs [204, 227]. Further, as OVA after oral administration can be rapidly detected in the blood [204, 205, 228-230] and systemic tolerance can be induced by injection of large quantities of non-cell-bound antigen into the portal vein via cells resident in the liver [231], tolerance may be suggested to also involve liver-resident cells.

Still, oral tolerance can be induced in mice lacking PPs [232-234] while mice lacking MLNs are incapable of generating systemic non-responsiveness to ingested antigens [209, 233, 234]. Further, although divided antigen-specific T cells are present in spleen and PLNs after feeding of oral antigen, administration of oral antigen with subsequent block of re-circulation of lymphocytes (by administration of the drug FTY720), led to proliferation of antigen-specific T cells in MLN while no divided antigen specific T cells were detected in PLNs or spleen [209]. In consequence, divided T cells detected in PLNs and spleen following oral feeding of antigen in the absence of FTY720 likely originate from re-circulating T cells activated in the MLN. Hence, oral tolerance has been suggested to be induced in MLNs rather than in PPs, PLNs or the spleen [235].

4.5.1 Dendritic cells and oral tolerance

In vivo expansion of DCs via administration of Flt3 ligand can result in enhanced oral tolerance [226, 236] and subcutaneous transfer of antigen loaded lamina propria DCs into mice with adoptively transferred T cells can result in reduced delayed type hypersensitivity (DTH) after challenge with antigen and adjuvant compared to transfer of antigen loaded spleen DCs [237]. Thus, DCs, and in particular intestinal DCs, appear to play a role in oral tolerance. In addition, oral tolerance cannot be induced in CCR7 KO mice and proliferation of antigen-specific T cells in the MLN in response to oral antigen is abrogated in CCR7 KO mice suggesting that induction of oral tolerance depends on DCs migrating from the small intestinal lamina propria to the MLN [209, 211, 235]. Furthermore, intestinal derived DCs have in many studies been suggested to play a role in induction of anergy and in the generation of T regs (both inducible FoxP3$^+$ T regs and IL-10 producing FoxP3$^-$ Tr1 cells) *in vitro* [238-244]. Thus, intestinal DCs, as opposed to non-intestinal DCs, are believed to be particularly adapt at generating tolerogenic T cell responses.

4.6 Macrophage and dendritic cell subsets in the small intestinal lamina propria

Based on different functional and phenotypic properties, several subsets of DCs and macrophages in the small intestinal lamina propria have been identified [49, 147, 245, 246]. However, different research groups have used different combinations of antibodies to phenotype these cells and hence the interrelationship and potential overlap of the identified subpopulations is in need of further investigation. In addition, the subdivision of DCs and macrophages in the small intestine is not always straight forward as the expression of many traditional DC and macrophage markers differs from that of DCs and macrophages in the spleen and lymphoid organs as well as between different non-lymphoid organs. For example, the classical DC marker CD11c is expressed on tissue macrophages while the classical macrophage markers CD11b and F4/80 can be expressed on intestinal DCs [23, 247].

In recent years, several small intestinal lamina propria DC (or macrophage) subsets have, based on different phenotypic definitions, been described and include CD103^{+}, CX3CR1^{+}, CD11b^{+}, TLR5^{+} and TipDC populations [11, 147, 245, 247-250]. However, at the onset of this thesis two major DC/macrophage subsets in the small intestinal lamina propria were described; CD103^{+} DCs and CX3CR1^{+} cells [188, 190, 191, 211, 251]. Still, the ontogeny of intestinal CD103^{+} DCs and CX3CR1^{+} cells and the relationship between CD103^{+} DCs and CX3CR1^{+} cells (for example if these were overlapping subsets of cells) was, at the beginning of these thesis studies, not clear.

4.6.1 CD103^{+} dendritic cells

CD103, the αE chain of the αEβ7 integrin, is expressed on T cells associated with epithelial surfaces including all IELs, a subset of LPLs, and a subset of CD4^{+}CD25^{+} T regs [252-254]. The ligand for CD103 on intestinal T cells is E-cadherin, present on the basolateral side (facing away from the lumen) of small intestinal epithelial cells and interactions between CD103 and E-cadherin have been proposed to mediate the adhesion of T cells to epithelial cells [253, 255, 256]. On this note, mice lacking CD103 have a reduction in the number of intestinal T cells, both in the lamina propria and in the epithelium [257].

CD103 is also expressed on DCs in LNs and tissues, including the intestine, while it has limited expression on DCs in spleen [54, 211, 251, 258, 259]. In the MLN and colon of mice, about 30-50% of the DCs express CD103 while the majority of small intestinal lamina propria DCs have been reported to express CD103 [211, 251].

Intestinal CD103^{+}CD11b^{+} vs. CD103^{+}CD11b^{-} dendritic cells
Small intestinal lamina propria CD103^{+} DCs can be divided into CD11b^{+} and CD11b^{-} DCs [28, 260]. As Id2 KO mice, which lack PPs and ILFs, almost completely lack small intestinal CD103^{+}CD11b^{-} DCs while the CD103^{+}CD11b^{+} population is not affected, and DCs isolated from PPs primarily are of CD103^{+}CD11b^{-} subtype [28], CD103^{+}CD11b^{-} DCs have been suggested to primarily

reside in PPs and ILFs [28] (but possibly also in the lamina propria [199]) while CD103$^+$CD11b$^+$ DCs are found primarily in the lamina propria [28, 199].

While the absence of CD103$^+$CD11b$^-$ DCs in the Id2 KO mice could be due to lack of PP and ILF structures, recent studies of *Id2*$^{-/-}$:wt bone marrow chimeras which have a selective reduction of intestinal CD103$^+$ CD11b$^-$ [260] have demonstrated that Id2 also is important in the generation of intestinal CD103$^+$ CD11b$^-$ DCs. In contrast, generation of CD103$^+$CD11b$^+$ DCs was not dependent on Id2 [260]. Further, mice deficient in transcription factors Batf3 [261], and IRF8 [260, 261] also show a dramatic reduction of small intestinal CD103$^+$CD11b$^-$ but not CD103$^+$CD11b$^+$ DCs and hence, intestinal CD103$^+$CD11b$^+$ and CD103$^+$CD11b$^-$ DC subpopulations appear to have distinct transcription factor requirements for their development. Still, transcriptional factors specifically required for the development of CD103$^+$CD11b$^+$ small intestinal DCs remain to be determined.

Functional properties of CD103$^+$ DCs

The ability of lymphocytes from MLN to preferentially localize to the small intestine was observed over 30 years ago [262, 263] and it has since been recognized that T cells activated in MLNs express the gut homing receptors α4β7 and CCR9 and preferentially home to the intestine while T cells activated in PLNs express P-selectin ligand and preferentially home to the skin [118, 264, 265]. Hence, expression of homing receptors and the ability of T cells to preferentially localize to specific tissues is thought to be obtained upon activation in the LN and to be influenced by the LN environment. In particular, *in vitro* studies have implicated a role for MLN and PP DCs in the generation of gut tropic T cells, and a role for PLN DCs in the generation of skin tropic T cells [114, 118, 266-270]. Further, studies by Johansson-Lindbom *et al* and Annacker *et al* have demonstrated that CD103$^+$ MLN DCs are more efficient than CD103$^-$ MLN DCs in inducing the gut homing receptor CCR9 on responding T cells *in vitro* [211, 251]. Thus, CD103$^+$ MLN DCs appear particular able to generate gut tropic T cells.

The *in vitro* generation of T cells expressing CCR9 has been demonstrated to involve the Vitamin A metabolite retinoic acid (RA) [271]. In this regard, *aldh1a2*, the gene encoding RALDH2, which is critical in the conversion of the Vitamin A metabolite retinal to RA [272], has been observed to be expressed in higher levels in CD103$^+$ MLN DCs compared to CD103$^-$ MLN DCs [242, 273] while only weakly or not at all in PLN and spleen DCs [242, 271]. Further, MLN DCs were able to induce higher RA receptor (RAR) signals in responding T cells *in vivo* compared to splenic DCs [274] and *in vitro* generation of gut tropic T cells induced by MLN DCs can be blocked by RAR antagonists [271, 274]. Thus the generation of gut tropic T cells by CD103$^+$ DCs appears to be linked to their ability to metabolize Vitamin A and dependent on their ability to induce RA signaling in T cells. However, the mechanisms whereby CD103$^+$ MLN DCs are selectively imprinted with the ability to metabolize Vitamin A and induce expression of gut homing receptors on responding T cells were at the start of this thesis unknown.

Of note, MLN and lamina propria CD103$^+$ DCs are also more efficient at converting Foxp3$^-$ CD4 T cells to FoxP3$^+$ T regs *in vitro* compared to their CD103$^-$ counterparts [240, 242]. This conversion likely involves RA [240-244, 275, 276], which is thought to enhance TGFβ mediated differentiation of T regs [240-242, 244, 275]. Thus RA likely plays a central role both in the generation of gut tropic T cells and the generation of FoxP3$^+$ T regs.

4.6.2 CX3CR1$^+$ cells

Intestinal lamina propria cells that express the fractalkine receptor CX3CR1 were initially identified by Niess and co-workers using CX3CR1$^{GFP/WT}$ mice [188]. These CX3CR1$^+$ cells were shown to express CD11c and CD11b have, because of expression of CD11c, up until very recently been considered to represent a population of DCs. However as will be discussed later, studies in thesis, as well as studies by others have demonstrated that they are closely related to tissue resident macrophages [25, 28, 247]. CX3CR1$^+$ cells are also present in the in small intestinal muscularis and/or serosa, and in PPs, MLNs and colon [28, 188, 277].

Functional properties of CX3CR1+ cells

CX3CR1+ cells have been proposed to play a key role in the sampling of luminal antigens as previously described. In addition, a population of small intestinal and colonic CD11c+CD11b-MHCII+CX3CR1+ cells was recently described to rapidly (within 90 minutes after bacterial challenge) migrate into the intestinal lumen in response to *Salmonella* and suggested to play a role in clearance of bacteria [278]. In addition, small intestinal CD11b+F4/80+ (mainly CX3CR1+) macrophages [275] and colonic CX3CR1^{hi} (but not CX3CR1^{low}) cells [72] have been associated with IL-10 production and small intestinal CX3CR1+ cells or colonic CD11c+CD11b+F4/80+ (likely CX3CR1+) cells have been implicated in IL-10-mediated expansion of FoxP3+ cells in oral tolerance [279] and IL-10 dependent FoxP3+ T reg-mediated suppression of colitis [280] respectively. Meanwhile, intestinal CX3CR1+CD70+ DCs [174] and colonic CX3CR1+ cells [277] have been implicated in generation of Th17 cells. Accordingly, the functional properties of intestinal CX3CR1+ cells appear to be diverse and could be hypothesized to be a reflection of the presence of several functionally different subpopulations and/or inflammatory conditions. Whether CX3CR1+ cells are able to induce expression of gut homing receptors on responding T cells was at the onset of these thesis studies not clear.

5. VITAMIN A METABOLISM

Vitamin A and its metabolites are essential in many aspects of biology, including embryonic development, reproduction and maintenance of epithelial surfaces [272]. Disruption of many of the genes in Vitamin A or retinol metabolism pathways are associated with embryonic lethality or death at birth [281]. For example, mice with a mutation in the retinol dehydrogenase RDH10 gene that disrupts the ability of the enzyme to oxidize retinol to retinal, die by approximately embryonic day (E)13.0 [282] and mice lacking retinal dehydrogenase 2 (RALDH2 KO mice) die between E 9.5 and 10.5 [283].

5.1 Uptake and storage of Vitamin A

Mammals are not able synthesize Vitamin A which therefore must be obtained from the diet. Dietary Vitamin A is primarily taken up in the small intestine [272, 284, 285] where it after conversion from retinyl esters or β-carotene to retinol is taken up by enterocytes [272], see *figure 3*. The conversion from retinyl esters to retinol is mediated by pancreatic triglyceride lipase and phospholipase B. The conversion of β-carotene to retinol is mediated by β,β-carotene oxygenases which generate retinal and the conversion of retinal to retinol is thought to be mediated by intestinal retinal reductases [272]. In enterocytes, retinol is bound to cellular retinol-binding protein type II (CRBP-II) and is re-esterified to retinyl esters by acyl-CoA:retinol acyltransferases (ARATs), including lecitin:retinol acyl transferase (LRAT) and diacylglycerol acyltransferase (DGAT)1 [286, 287]. Retinyl esters are then packaged into structures called chylomicrons. [272]. Chylomicrons are lipoprotein complexes composed of triaglycerol, phospholipids, free and esterified cholesterol and apolipoprotein B that in addition to retinyl esters also carry cholesteryl esters and small quantities of retinol [272, 288]. Chylomicrons are released by enterocytes into the lymph from which they reach the circulation where they are modified into chylomicron remnants. Chylomicron remants are formed by hydrolysis of triacylglycerol and exchange of apolipoproteins. Chylomicron remnants are

subsequently cleared by hepatocytes in the liver and to some extent by cells in other organs including the bone marrow, spleen and kidneys and adipose and muscle tissue [288]. In hepatocytes, retinyl esters are converted to retinol for subsequent secretion, or stored [272]. Storage takes place in perisinusoidal stellate cells. These stellate cells contain up to 80% of the total retinol in the body of mammals, mainly stored in the form of retinyl esters [272]. In addition to release by intestinal enterocytes of chylomicrons, these cells can also release retinol directly into the circulation [288].
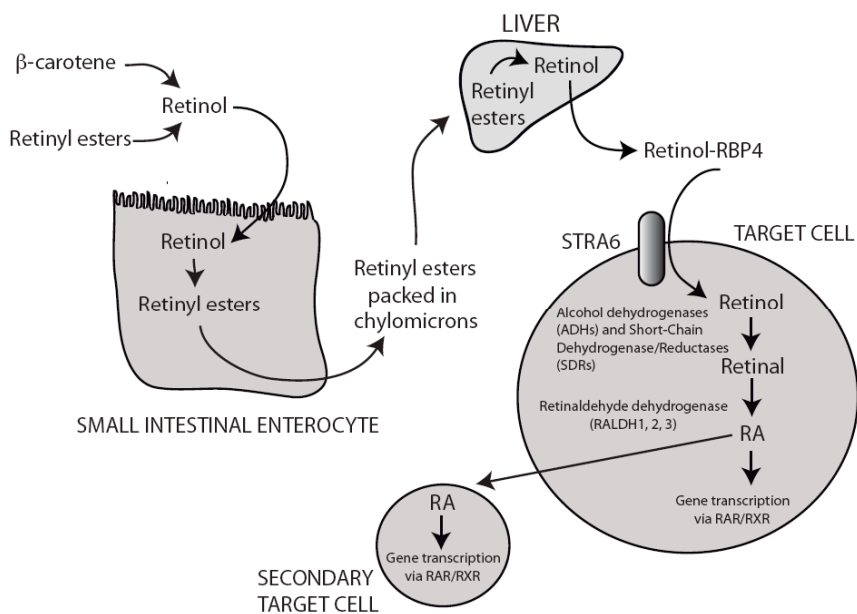
Figure 3. **Vitamin A metabolism.** Dietary Vitamin A, in the form of either β-carotene of retinyl esters is converted to retinol in the gut lumen and subsequently taken up by small intestinal enterocytes. After re-conversion to retinyl esters, Vitamin A derivates are packaged into chylomicrons which are cleared by liver hepatocytes. In the liver, retinyl esters are either stored or converted to retinol, and released in complex with RBP4. Released retinol-RBP4 complexes can be bound by STRA6 and retinol can subsequently be delivered to the target cell. In cells, retinol can be oxidized to retinal by alcohol dehydrogenases (ADHs) and short-chain dehydrogenase/reductases (SDRs) and then further oxidized to retinoic acid by retinal dehydrogenases (RALDH1, 2, 3). The resulting RA can either act on gene transcription in the target cell via retinoic acid receptors (RARs and RXRs) or cross the cell membrane to act in a paracrine manner.

5.2 Liver secretion, cellular uptake and metabolism of retinol

Retinol, bound to RBP4, is secreted from the liver into the blood [272]. The concentration of retinol in plasma is around 2µM [272]. Retinol-RBP4 is then taken up by targets cells, presumably via surface receptors [272]. One such receptor called STRA6 was recently identified [289]. STRA6 uncouples retinol from RBP4 and transports retinol across the cell membrane [289]. In the target cell, retinol can then, in a reversible reaction, be oxidized to retinal by cytosolic alcohol dehydrogenases (ADHs) and by members of the membrane-bound short-chain dehydrogenase/ reductase (SDR) family [272, 289]. Retinal is then, in an irreversible reaction, further oxidized into RA by retinal dehydrogenases (RALDH 1-4) [272, 289]. ADHs and SDRs are expressed in most cells including DCs while the expression of RALDHs is specific to particular cells and tissues [271, 272]. For example, as previously mentioned, CD103^{+} MLN DCs express higher levels of *aldh1a2* (the gene encoding RALDH2) than CD103^{-} MLN DCs [242, 273] while CD103^{+} (and CD103^{-}) MLN DCs have low to no expression of the genes encoding RALDH1 and RALDH3 [273]. The gene encoding RALDH4 appears to mainly be expressed in the liver and kidney of mice [272, 290].

5.3 Retinoic acid signaling

Inside cells, the major function of RA is assumed to be activation of transcription factors. In this regard, all-trans-retinoic acid (ATRA) binds to retinoic acid receptors RARs (consisting of RARα, β, γ), which are ligand-dependent transcription factors. RARs function as heterodimers, bound to retinoid X receptors, RXRs (consisting of RXRα, β, γ) [291]. In general, RARs and RXRs contain a conserved DNA-binding site, a ligand-binding site, a dimerization domain, transactivation domains and several phosphorylation sites.

RAR/RXR heterodimers bind to response elements in the promoter of their target genes [291]. Many of these response elements are direct repeats (DR) of the consensus sequence (A/G)G(G/T)TCA that are separated by two (DR2 elements) or five (DR5 elements) nucleotides or one (DR1 elements) nucleotide respectively [272, 291]. However, RAR/RXR heterodimers may also bind degenerate DRs (containing multiple mismatches) or 'half sites' (non-repeated consensus sequences) [292, 293]. In the absence of ligand, RAR/RXR complexes are bound to response elements but are in association with corepressors that repress transcription. In the presence of ligand, the corepressors dissociate, and instead coactivators and subsequently RNA polymerase and general transcription factors are recruited [291, 294]. However, RAR may also form heterodimeric complexes with nuclear factors other than RXR, including estrogen receptor and GATA2 [295, 296].

6. AIMS OF THIS THESIS

The aim of this thesis was to study the macrophage and DC populations present in the small intestinal lamina propria and the draining MLNs with the goal of understanding their interrelationships and their different functions with a focus on T cell activation and generation of gut homing T cells.

The specific aims of this thesis were:

- To assess the phenotype, turnover and function of intestinal CD103$^+$ DCs and to investigate whether CD103$^+$ DCs are present in human

- Phenotypic and functional assessment of small intestinal lamina propria CX3CR1$^+$ cells and their relation to CD103$^+$ DCs

- To dissect the mechanism(s) imprinting CD103$^+$ DCs with the ability to generate gut homing T cells

7. SUMMARY AND KEY CONCLUSIONS OF PAPERS I-III

Paper I: Small intestinal CD103$^+$ dendritic cells display unique functional properties that are conserved between mice and humans.

In this paper, we showed that CD103$^+$ DCs are present in several different tissues and LNs and that in general, CD103$^+$ DCs are phenotypically more similar to each other than to their CD103$^-$ counterparts. We further showed that when co-cultured with T cells, CD103$^+$ DCs from small intestine and MLNs more efficiently induce CCR9 on, and RA signaling in, responding T cells compared to CD103$^-$ DCs from small intestine and MLN or CD103$^+$ and CD103$^-$ DCs from colon, lung or lung draining LNs (LDLNs).

We also observed rapid labeling of CD103$^+$ DCs in BrdU pulse-chase experiments, absence of CD103$^+$Ki67$^+$ small intestinal DCs and that the majority of DCs in the small intestine 6 days after intestinal graft surgery were derived from the host rather than the donor. Together these observations suggest that small intestinal lamina propria CD103$^+$ DCs are unlikely to derive from CD103$^-$ DCs or tissue resident precursors, but instead derive from circulating blood precursors.

We also showed that CD103$^+$ MLN DCs were likely derived from small intestinal tissue while CD103$^-$ MLN DCs were likely to be derived from precursors in the blood. Further, we showed that CD103$^+$ MLN DCs were more efficient at presenting orally derived antigen *ex vivo* to both CD4 and CD8 T cells compared to CD103$^-$ MLN DCs and thus we suggested that CD103$^+$ MLN DCs likely represent the major DC population in MLN inducing T cell responses to soluble oral antigens.

In addition, we showed that CD103$^+$ DCs are present in healthy human MLNs and MLNs of small bowel Crohn's patients. Of note, these CD103$^+$ DCs, as opposed to their CD103$^-$ counterparts, were able to induce expression of CCR9 on responding T cells in a RA-dependent manner, suggesting that the selective ability of CD103$^+$ MLN DCs to induce CCR9 is maintained in IBD.
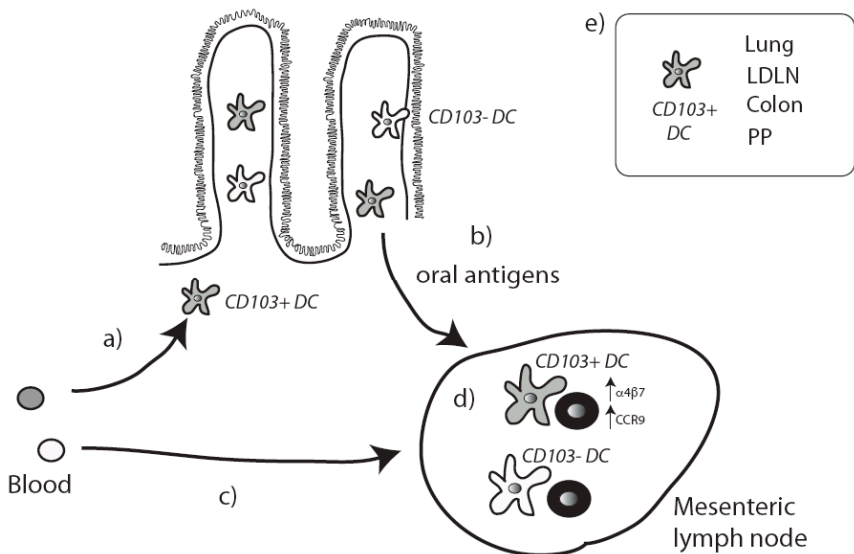
Figure 4. **Schematic summary of paper I**. **a**) Small intestinal CD103⁺ DCs are generated from a blood
bourne precursor, and not from CD103⁻ cells or local proliferation. **b)** After administration of oral antigen
CD103⁺ DCs in the MLN are able to activate T cells, presumably due to their ability to migrate v
lymphatic vessels to the MLN. **c)** CD103⁻ DCs in the MLN likely derive from blood rather than intestin
tissue. **d)** CD103⁺ but not CD103⁻ DCs from the MLN (and the small intestine) are able to generate T cel
expressing the gut homing receptors CCR9 and α4β7. **e)** CD103⁺ DCs were also found in lung, colon ar
PPs. These CD103⁺ DCs outside the MLN and intestine did not efficiently induce CCR9 on responding
cells.

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

In this paper we compared CD103$^+$ DCs and CX3CR1$^+$ cell populations in the small intestinal lamina propria. Here, CX3CR1$^+$ cells expressed the classical dendritic cell markers CD11c and MHCII, but lacked expression of CD103 and had lower expression of the co-stimulatory molecules CD40, CD80 and CD86 compared to CD103$^+$ DCs. CX3CR1$^+$ cells also expressed the classical macrophage markers CD11b and F4/80. Thus, phenotypically, CX3CR1$^+$ cells expressed markers typical for both macrophages and dendritic cells.

To assess the relationship between CD103$^+$ DCs and CX3CR1$^+$ small intestinal lamina propria cells we examined their *in vivo* responses to GM-CSF and Flt3 ligand. Here, CX3CR1$^+$ cell populations had poor responses to GM-CSF and Flt3 ligand in comparison to CD103$^+$ DCs. Further, CX3CR1$^+$ cell populations had slower turnover rate than CD103$^+$ DCs as determined by BrdU pulse chase experiments, which together with the *in vivo* responses to GM-CSF and Flt3 ligand indicated that these two populations had different ontogeny.

We also assessed the ability of CD103$^+$ DCs and CX3CR1$^+$ cells to migrate via the lymphatics to the draining MLNs and their ability to stimulate naïve T cells, traits that are closely associated with the definition of DCs. Here, we observed that CD103$^+$ DCs but not CX3CR1$^+$ cells were present in lymphatic vessels draining the small intestine and that CX3CR1$^+$ cells were less efficient than CD103$^+$ DCs at stimulating T cell proliferation, both *in vivo* and *in vitro*. We also showed that CX3CR1$^+$ cells were less efficient at inducing expression of the gut homing receptor CCR9 on responding T cells *in vitro*.

Together, these results establish that CX3CR1$^+$ cells and CD103$^+$ DCs are distinct populations of intestinal cells, with the former resembling macrophages rather than DCs.
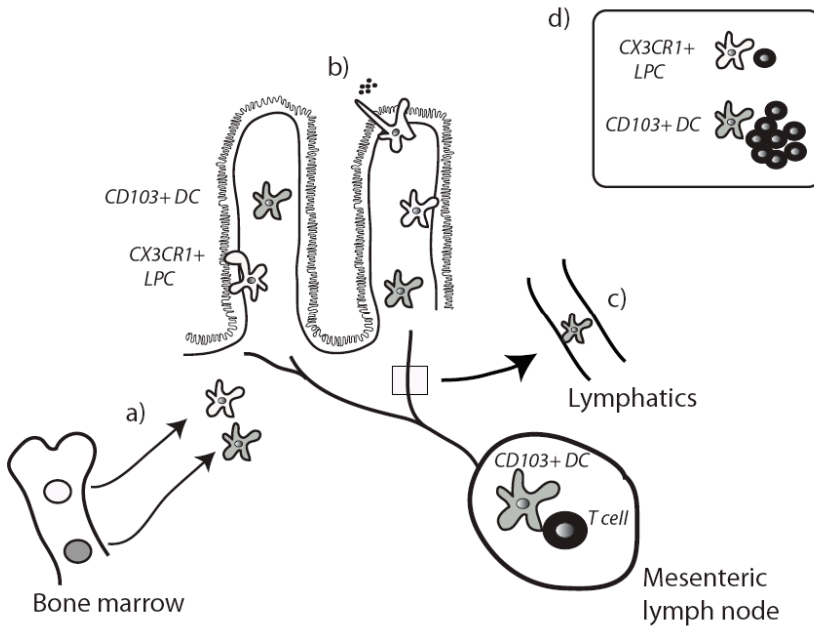
Figure 5. **Schematic summary of paper II. a)** CD103^{+} DCs and CX3CR1^{+}/CD103^{-} small intestinal lamina propria cells (LPCs) are likely to have different ontogeny. **b)** CX3CR1^{+}/CD103^{-} LPCs are more efficient at taking up intestinal antigen than CD103^{+} DCs. **c)** CD103^{+} DCs, but not CD103^{-}/CX3CR1^{+} LPCs were found in the lymphatic vessels draining the small intestine. Hence, CX3CR1^{+} cells appear to be a small intestinal resident population, while CD103^{+} DCs appear to be migratory. Presence of CD103^{+} DCs in lymph was dependent on CCR7 and hence migration of intestinal CD103^{+} DCs is likely dependent on CCR7. **d)** Intestinal CD103^{+} DCs are more efficient than CX3CR1^{+}/CD103^{-} LPCs at inducing T cell proliferation.

Paper III: Bile retinoids imprint intestinal CD103^{+} dendritic cells with the ability to generate gut-tropic T cells

In this paper, we investigated the mechanism by which CD103^{+} DCs are imprinted with their ability to convert retinol to RA, implicated in the generation of gut homing T cells. Here our initial observation was that intestinal CD103^{+} DCs expressed low levels of the retinol-converting enzyme *aldh1a2* and had low aldehyde dehydrogenase (ALDH) activity in Vitamin A deficient (VAD) mice while CD103^{+} DCs isolated from mice that did not receive Vitamin A continually from the diet but had normal systemic levels of retinol had normal *aldh1a2* expression and ALDH activity. These observations suggested that RA was important for the imprinting of DCs with the ability to metabolize retinol to RA and indicated that dietary retinoids were dispensable for this imprinting and led us to investigate the role of retinoids in imprinting CD103^{+} DCs.

Further, we observed that small intestinal tissue contained higher levels of retinol than colonic tissue, and that CD103^{+} DCs isolated from the small intestine constitutively received higher RA signals compared CD103^{+} DCs isolated form the colon. Further, BMDCs could be imprinted to express *alh1a2* and induce expression of CCR9 on responding T cells by addition of RA *in vitro*, and intrarectal administration of RA could increase ALDH activity in colonic CD103^{+} DCs *in vivo*. Together these results suggest that RA may play a direct role in imprinting DCs and that local levels of RA may mediate imprinting of CD103^{+} DCs *in vivo*.

Further we observed that even in the absence of dietary Vitamin A intake, CD103^{+} small intestinal DCs received higher RA signals than colonic CD103^{+} DCs, indicating that in the absence of continual retinoid intake, the supply of retinoids to the small intestines may be maintained, leading us to investigate non-dietary sources of intestinal retinoids. In these studies we observed that bile contained retinol at a level exceeding that in serum and that bile could induce ALDH activity in BMDCs and imprint them with the ability to induce CCR9 expression on responding T cells.

Critically, both the induction of ALDH and the ability to generate CCR9 expression on responding T cells was dependent on RA signaling. Taken together, these results suggest that small intestinal CD103$^+$ DCs may be imprinted with the ability to metabolize Vitamin A directly by RA and that this process is mediated, at least in part, by retinoids in bile.

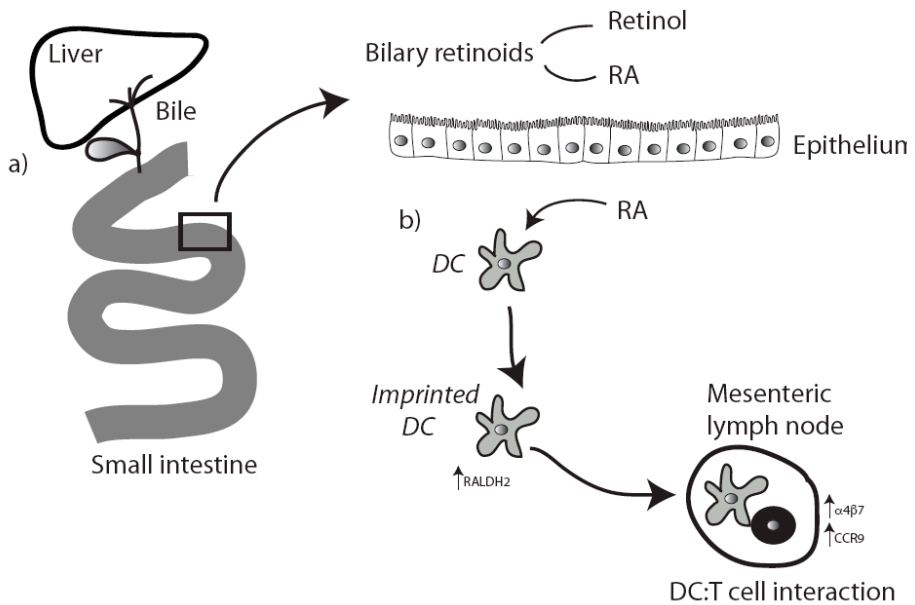*Figure 6.* **Schematic summary of paper III. a)** Bile, which drains from the liver, contains retinoids th will selectively reach the small intestine. However, retinols are also derived from the diet. **b)** In the sm intestine, retinoids will act on DCs and imprint them with the ability to metabolize retinol which in tu will allow them to generate T cells that express the gut homing receptors CCR9 and α4β7.

8. DISCUSSION OF THIS THESIS

In the following sections I will discuss selected topics related to the original papers of this thesis.

8.1 Definition of DC/macrophage subsets in small intestinal lamina propria

Classification of cells into either DCs or macrophages is, as described in the introduction, based on both phenotypic and functional properties [148, 297]. In short, DCs have been associated with migration from peripheral tissue to LNs, antigen presentation, localization to T cell zones, stimulation of naïve T cells and the expression of MHCII and CD11c while macrophages traditionally have been associated with phagocytosis, killing of microbes, and expression of CD14, F4/80 and CD11b [148, 298]. However, as we are beginning to understand the diversity and complexity of DC and macrophage subsets in not only lymphoid but also non-lymphoid tissues, an increasing functional and phenotypic overlap between DCs and macrophages is observed [23, 298, 299]. Hence, the definition of DC and macrophage subsets in the small intestinal lamina propria is a quite complicated topic.

At the start of these thesis studies, intestinal DCs were defined as CD11c$^+$MHCII$^+$ cells and anything that fell into this definition was generally considered a DC. Consequently, in **paper I** we described, based on CD11c, MHCII, and CD103 expression, two populations of intestinal DCs; CD103$^+$ and CD103$^-$. In retrospect, based on our subsequent studies (**paper II**) and those of others [25, 28], it has become clear that monocyte-derived cells in intestinal lamina propria can express both CD11c and MHCII. In particular, studies using CX3CR1$^{GFP/WT}$ mice have demonstrated that the vast majority of MHCII$^+$CD11c$^+$CD103$^-$ small intestinal lamina propria cells are CX3CR1$^+$ cells that express intermediate or high levels of GFP [25, 28], *figure 7*.

As will be discussed below, we now believe that CX3CR1^{hi} cells represent tissue resident macrophages while the functions and ontogeny of CX3CR1^{int} cells, at this point, is less clear.
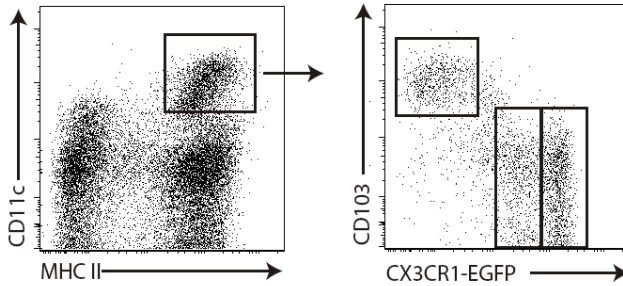
Figure 7. **Small intestinal CD11c$^+$MHCII$^+$ cells consist of mainly CD103$^+$ or CX3CR1$^+$ cells.** Representative dot plots of small intestinal lamina propria cells. CX3CR1$^+$ cells can be further divided into two populations; CX3CR1^{int} and CX3CR1^{hi} based on the flourecence intensity of GFP. See also *figure* 9. Cells are pre-gated on FSC/SSC, PI (to exclude dead cells), and CD45.

8.1.1 Origin of small intestinal CD103$^+$ DCs vs. CX3CR1$^+$/CD103$^-$cells

To investigate the origin(s) of CD103$^+$ DCs *vs*. CX3CR1^{hi} and CX3CR1^{int} populations (**paper II**) we investigated the response of these three populations to *in vivo* administered GM-CSF and Flt3 ligand. In these experiments, we observed that CX3CR1^{hi} cells had poor responses to GM-CSF and Flt3 ligand in comparison to CD103$^+$ DCs.

In related studies, Bogunovic *et al* observed that Flt3 KO mice had impaired development of CD103$^+$CD11b$^+$ and CD103$^+$ CD11b$^-$ small intestinal lamina propria DCs but not CD103$^-$CD11b$^+$ (mainly CX3CR1^{hi}) lamina propria cells [28]. Further, in the same study, bone marrow chimeras reconstituted with mixtures of wt:*Flt3$^{-/-}$* progenitors had decreased percentage of intestinal CD103$^+$CD11b$^+$ and CD103$^+$ CD11b$^-$ DCs, and to some extent decreased percentage of CD103$^-$CD11b$^+$ cells, derived from Flt3 deficient precursors relative to their wt counterparts [28]. Similarly, Varol *et al* used bone marrow chimeras reconstituted with mixtures of

wt:*Flt3*^{-/-} progenitors and noted decreased numbers of CD11c$^+$CD11b$^-$ colonic lamina propria cells (which represent a proportion of the CD103$^+$ DCs) in the Flt3 deficient population relative to wt CD11c$^+$CD11b$^-$ cells. In the same study by Varol *et al*, *in vivo* injection of Flt3 ligand resulted in a relative increase of the CD11c$^+$CD11b$^-$ population in colonic and ileal lamina propria compared to CD11c$^+$CD11b$^+$ cells (which are a mix of CX3CR1$^+$ and CD103$^+$ cells) [25]. Thus, intestinal lamina propria CD103$^+$ DCs can be expanded by Flt3 ligand *in vivo* and appear dependent on Flt3 for their development while intestinal lamina propria CX3CR1$^+$ cells are not expanded to the same extent as CD103$^+$ DCs in response to Flt3 ligand and appear not to require Flt3 for their development.

Related studies on the role of GM-CSF have, similar to our findings, shown that CD103$^+$ DC subpopulations can respond to GM-CSF and appear to require GM-CSF for their development while the responsiveness of the CX3CR1$^+$ population to GM-CSF may be less clear. In this regard, Varol *et al* reported that colonic and ileal CD11c$^+$CD11b$^+$ cells (which are a mix of CX3CR1^{int}, CX3CR1^{hi} and CD103$^+$ cells) respond to GM-CSF and that bone marrow chimeras reconstituted with mixtures of wt:*Csf2a*^{-/-} progenitors had decreased numbers of CD11c$^+$CD11b$^+$ colonic lamina propria cells in the GM-CSF deficient population relative to their wt counterpart [25]. Meanwhile, Bogunovic *et al* reported that expression of the receptor for GM-CSF is not required for the differentiation of small intestinal lamina propria CD103$^-$ DCs (mainly CX3CR1^{hi}), but for the differentiation of both CD11b$^+$ and CD11b$^-$ CD103$^+$ DCs and that bone marrow chimeras reconstituted with mixtures of wt:*Csf2a*^{-/-} progenitors had decreased percentage of CD103$^+$ DCs but not CD103$^-$ small intestinal lamina propria cells in the GM-CSF KO population relative to wt CD103$^+$ and CD103$^-$ populations respectively [28]. Thus, although in the studies by Varol *et al* the CD11c$^+$CD11b$^+$ population which contains CX3CR1$^+$ cells responds to GM-CSF, our results (**paper II**) and those of Bogunovic *et al* suggest that CX3CR1^{hi} cells do not respond to GM-CSF and hence it is probable that the responsiveness of the CD11c$^+$CD11b$^+$ population in the Varol *et al* study is due to CD103$^+$ DCs and CX3CR1^{int} cells within this population.

Of note, Bogunovic *et al* also reported that M-CSF was required for the development of CD103^{-}CD11b^{+} (mainly CX3CR1^{hi}) small intestinal lamina propria cells [28]. Taken together, the above studies indicate that CD103^{+} DCs and CX3CR1^{hi} lamina propria cells have differential requirement for growth factors, where CD103^{+} DCs appear to require Flt3 and GM-CSF and CX3CR1^{hi} cells appear to require M-CSF and GM-CSF. In turn, the differential growth factor requirements indicate that CD103^{+} DCs and CX3CR1^{hi} lamina propria cells have different origin.

More direct indications that CD103^{+} DCs and CX3CR1^{hi} cells have different origin came from studies where adoptively transferred pre-DCs into DC-ablated mice gave rise to CD103^{+} DC subsets in the small intestine [28] and CX3CR1^{-}CD11c^{hi} (likely CD103^{+}) DCs in the colon but not CD103^{-}CD11b^{+}/CX3CR1^{hi} lamina propria cells [25, 28]. In contrast, adoptively transferred Ly6C^{hi} monocytes into DC-ablated mice generated ileal and colonic intestinal lamina propria CD11b^{+}CX3CR1^{+}CD103^{-} cells but not CD11c^{+}CD11b^{-} cells (which represent a subpopulation of CD103^{+} DCs) [25] or small intestinal lamina propria CD103^{-}CD11b^{+} cells but not CD103^{+} DCs [28]. Together these results suggest that intestinal CD103^{+} DCs derive from pre-DCs while CX3CR1^{hi} cells derive from Ly6C^{hi} monocytes.

8.1.2 Turnover of small intestinal CD103^{+} DCs vs. CX3CR1^{+}/CD103^{-} cells

In studies using BrdU pulse chase we investigated the interrelationship of CD103^{+} *vs*. CD103^{-}/CX3CR1^{+} cells in terms of turnover rates (**paper I, II**). In these studies, BrdU^{+}CD103^{+} DCs had a more rapid appearance than BrdU^{+}CD103^{-}CD11c^{+}MHCII^{+} cells (**paper I**) or BrdU^{+}CX3CR1^{int} and BrdU^{+}CX3CR1^{hi} cells (**paper II**). Hence CD103^{+} DCs appear to have a higher turnover rate than CD103^{-}/CX3CR1^{+} cells. In agreement with this, using parabiotic mice, Bogunovic *et al* have reported that the percentage of donor derived DCs was higher in the intestinal lamina propria CD103^{+} DC population than in the CD103^{-}CD11b^{+} population of cells [28] suggesting that in the lamina propria, the turnover rate of CD103^{+} DCs is higher than the turnover rate of CD103^{-}CD11b^{+} cells.

In addition, utilizing small intestinal tissue transplants, we observed that already 6 days after surgery, almost all CD11c$^+$MHCII^{hi} cells (many of which probably were CD103$^+$ DCs) in the villi of the transplanted intestine were of host origin and thus CD103$^+$ DCs appear not only to have a fast turnover, but also likely derive from circulating blood-precursors, rather than tissue-resident precursors (**paper I**). Meanwhile, lamina propria CX3CR1$^+$ cells were not reduced in the villi of the transplanted intestine compared to intestinal tissue of control CX3CR1$^{+/GFP}$ mice (**paper II**) and are thus likely derived from tissue-resident precursors.

Further, in **paper I**, we also assessed local cell proliferation of small intestinal CD103$^+$ DCs and CD103$^-$CD11c$^+$MHCII$^+$ cells (primarily CX3CR1$^+$, *see figure 7*) by staining for Ki67, a marker that correlates with active cell proliferation. In these experiments, a small proportion of intestinal CD103$^-$CD11c$^+$MHCII$^+$ cells, but virtually no CD103$^+$ DCs, stained positive for Ki67. On this note, Varol *et al* observed that when green and red fluorescent monocytes (which are precursors of CX3CR1$^+$ cells [25, 28]) were co-transferred into DC-depleted recipients, ileal villi were populated exclusively by green or red lamina propria cells organized in clusters. In addition, Varol *et al* also observed that intestinal CD11c$^+$CD11b$^+$ cells (which contain CX3CR1$^+$ cells) derived from CFSE-labeled monocytes, gradually lost CFSE intensity after their differentiation into lamina propria CD11c$^+$CD11b$^+$ cells in DC-depleted mice [25]. Together these results may indicate that at least a proportion of intestinal CX3CR1$^+$ cells undergo local proliferation. Based on these studies one could propose a simplified model where CD103$^+$ DCs rapidly turn over and are replaced by blood derived precursors, while CX3CR1$^+$ cells (or subpopulations thereof) may have slower turnover rate, limited recruitment of their precursors and may be maintained by local expansion. However, one should bear in mind that the experimental conditions in some of these studies (such as intestinal surgery and DC-ablation) may lead to results which deviate from the physiological steady state turnover of intestinal DC and macrophage subsets and thus refined experimental strategies will likely be needed to elucidate the physiological steady state turnover of these cells.

8.1.3 Migration of small intestinal CD103$^+$ DCs vs. CX3CR1$^+$/CD103$^-$ cells

In **paper I**, we show, using BrdU labeling, that BrdU$^+$CD103$^+$ DCs have a delayed appearance in the MLN compared to BrdU$^+$CD103$^-$ MLN DCs. This, together with studies in CCR7 KO mice demonstrating selective reduction of CD103$^+$ DCs in the MLN [28, 209, 211] strongly suggested that CD103$^+$ MLN DCs derive primarily from the small intestinal lamina propria. To provide direct proof that CD103$^+$ DCs migrated in afferent lymph (**paper II**) we examined lymphatic vessels draining the small intestine. Here, CD11c$^+$MHCII$^+$ cells were readily identified in lymphatic vessels and their numbers increased dramatically after oral administration of TLR7/8 agonist. Importantly, almost all of the CD11c$^+$MHCII$^+$ cells isolated after oral administration of TLR7/8 agonist expressed CD103, demonstrating that CD103$^+$ DCs are the major migratory population in the intestine. In contrast to CD103$^+$ DCs, we failed to observe CX3CR1^{hi} cells in afferent lymph, indicating that CX3CR1^{hi} cells are a non-migratory cell population. The non-migratory property of CX3CR1^{hi} cells is supported by studies of *LysM-Cre x ROSA26-floxstop-floxGFP* mice, where monocytes (which are the precursors of CX3CR1$^+$ cells [25, 28] and express LysM [300]) acquire irreversible expression of eGFP. In these mice, 90% of the CD103$^-$ CD11b$^+$ small intestinal lamina propria cells were GFP$^+$, whereas in MLN only 20% of the CD103$^-$CD11b$^+$ cells were GFP$^+$ [28] and thus CD103$^-$CD11b$^+$ MLN cells are unlikely to be derived from CD103$^-$CD11b$^+$ lamina propria cells. Further, we and Bogunovic *et al* [28] have failed to observe CX3CR1^{hi} cells in the MLN, see *figure 8*. Taken together, these studies strongly suggest that small intestinal lamina propria CX3CR1^{hi}/CD103$^-$CD11b$^+$ cells do not migrate the MLN. However, these studies do not rule out that inflammatory conditions exist where the CX3CR1$^+$ cells are able to migrate to the MLN.

In terms of migration it is also of interest to note that upon oral infection with *Salmonella* Typhimurium, lamina propria CD103$^+$CD11b$^+$ DCs have been indicated in migration to the MLN [28, 301]. In addition, small intestinal CD11c^{hi}CD11b^{hi}TLR5$^+$ DCs which express CD103 [248] and respond to the TLR5

ligand flagellin [302] have been suggested to play a role in TLR5 dependent transport of *Salmonella* from the intestine to the MLN [302]. Thus, a specific role for CD103^{+}CD11b^{+} DCs in migration to the MLN in infection may be suggested. Still, further studies are needed to understand the migration patterns of different small intestinal DC and macrophage subpopulations in intestinal inflammation.
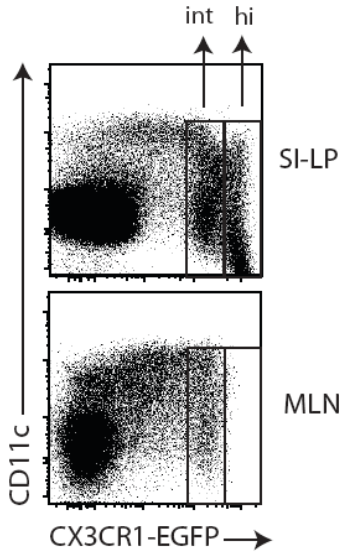
Figure 8. **Lack of CX3CR1^{hi} cells in the MLN**. Representative dot plots of small intestinal lamina propria and MLN populations of CX3CR1^{+} cells in CX3CR1-GFP mice. The small intestinal lamina propria contains two populations of CX3CR1^{+} cells, one "hi" population and one "int" population. MLN contains onty the "int" population. Cells are pre-gated on FSC/SSC, PI (to exclude dead cells), and CD45.

While it is currently unknown what controls the migration of CD103^{+} DCs in steady state and why CX3CR1^{hi} cells do not migrate, we have observed that small intestinal CD103^{+} DCs, but not CX3CR1^{+} cells, upregulated CCR7 after *in vitro* stimulation with LPS (**paper II**). Similarly, small intestinal lamina propria CD103^{+}CD11b^{+} DCs have been reported to express higher levels of CCR7 than CD103^{-}CD11b^{+} lamina propria cells (which to a large part consist of CX3CR1^{+} cells) [28] suggesting that the ability to express CCR7 and hence the ability to migrate to the MLN in a CCR7-dependent fashion, may differ between small intestinal CD103^{+} DCs and CX3CR1^{+} cells.

8.2 Vitamin A and the imprinting of intestinal CD103$^+$ DCs

It has been hypothesized that DCs are imprinted with the ability to metabolize retinol and generate tissue tropic T cell subsets by signals they receive in their local tissue environment [303, 304]. In this regard, we have demonstrated that CD103$^+$ DCs isolated from the small intestinal lamina propria have the ability to induce CCR9 on responding T cells (**paper I**), which together with the higher expression of *aldh1a2* and higher ALDH activity in CD103$^+$ small intestinal lamina DCs (**paper II**) compared to CD103$^-$/CX3CR1$^+$ small intestinal lamina propria cells suggested that small intestinal CD103$^+$ DCs are imprinted with the ability to metabolize retinol already in the intestine or that this activity was present on CD103$^+$ DC precursors prior to their entry into the intestine. In this regard, several studies have implicated local intestinal epithelial cells in imprinting CD103$^+$ DCs in the small intestine, [113, 305-307]. For example, upregulation of *aldh1a2* has been observed after co-culture of BMDCs with an epithelial cell line [306]. In addition, antigen-loaded BMDCs when co-cultured with small intestinal epithelial cells were able to induce α4β7 and CCR9 expression on responding T cells by soluble factor(s) [305] although it from this study was unclear if the soluble factor(s) were acting on the BMDCs or on the T cells directly, as supernatant from small intestinal epithelial cell cultures directly added to T cells stimulated with α-CD3 and α-CD28 induced expression of homing receptors the T cells. Thus, although epithelial cells may contribute to the local imprinting of intestinal DCs, the mechanism(s) and contribution of epithelial cells are not clear. In addition to epithelial contact and epithelial derived factors, TLR2 ligands [308], IL-4 [309, 310], IL-13 [309], PPARγ agonists [311], GM-CSF [309] and RA [306, 309, 312] were during these thesis studies implicated in imprinting retinol metabolizing activity in DCs. However the role of many of these factors in *in vivo* imprinting of small intestinal CD103$^+$ DCs remains largely unclear.

8.2.1 Imprinting of CD103⁺ DCs by retinoic acid

In VAD mice, we observed that both small intestinal and MLN CD103⁺ DCs, while present in similar numbers as in control mice, expressed lower levels of the retinol-metabolizing enzyme *aldh1a2* and had reduced ALDH activity (**paper III**). These results are in agreement with a study by Yokota *et al* showing that total CD11c⁺ MLN cells from VAD mice have reduced *aldh1a2* expression and ALDH activity [309], and a very recent study by Molenaar *et al* demonstrating reduced ALDH activity and *aldh1a2* expression in CD103⁺ MLN DCs isolated from VAD mice [312]. Thus, Vitamin A appears to be critically involved in imprinting small intestinal CD103⁺ DCs with retinol metabolizing activity. In addition, we and others have demonstrated that *in vitro*, that RA enhances *aldh1a2* mRNA expression [306, 309, 312, 313] (**paper III**) RALDH2 protein expression [313] and ALDH activity in BMDCs [313] (**paper III**) and imprints these cells with an enhanced ability to generate CCR9⁺ T cells [313] (**paper III**) indicating that RA may directly imprint DCs.

In addition, in the studies of **paper III** we observed higher levels of retinol in the small intestine than in other tissues, (except the liver but including the colon) along with higher RAR signals in small intestinal CD103⁺ DCs compared to colonic CD103⁺ DCs suggesting a local role for Vitamin A in imprinting intestinal CD103⁺ DCs. In agreement with a role for RA in imprinting CD103⁺ DCs but in disagreement with imprinting occurring locally in the intestine, Feng *et al* have very recently suggested that RA produced locally in the bone marrow imprints DCs with retinol metabolizing activity and directs these DCs in a CCR9-dependent fashion to the small intestine [313]. This hypothesis was based on the observations that (i) ALDH activity was found in bone marrow cells, (ii) addition of RA to BMDCs increased the levels of *aldh1a2* and CCR9 expression in BMDCs and (iii) RA-treated BMDCs were more efficient that non-RA-treated BMDCs in *in vitro* generation of FoxP3⁺ T regs and induction of CCR9 on responding T cells [313]. However, we have been unable to observe a reduction in total numbers of CD103⁺ DCs in the small intestine of VAD (**paper III**) or CCR9 KO mice (K.K.,

unpublished observation) compared to control and wt mice respectively and have further showed that pre-DCs (which, as described above, are the precursors of small intestinal CD103$^+$ DCs [25, 28]) in the bone marrow and intestinal lamina propria lack ALDH activity (**paper III**) arguing against RA- or CCR9-mediated recruitment of imprinted pre-DCs to the lamina propria. Instead, our results suggest that RA imprints CD103$^+$ DCs with the ability to metabolize retinol locally in the small intestine.

Of note, while we currently believe that the signal(s) that imprint CD103$^+$ DCs are delivered locally in the small intestine, there are several suggestions that the local environment in MLNs also influences generation of gut tropic T cells. In this regard, lymph node resident stroma cells expressing *aldh1a1*, *aldh1a2* and *aldh1a3* have been suggested to play a role in the generation of T cells expressing α4β7 and CCR9 [273, 314]. As retinol is found in high levels in the MLN (**paper III**), conversion of retinol to RA by stroma cells in the MLN may participate in the generation of gut homing T cells. Interestingly, similar to the role of Vitamin A in imprinting of intestinal DCs with the ability to metabolize retinol [309, 312, 313] (**paper III**), *aldh1a2* expression in MLN stroma cells was recently shown to be decreased in VAD mice compared to control mice [312] and hence imprinting of MLN stroma cells may, similar to the imprinting of intestinal CD103$^+$ DCs, be dependent on Vitamin A. However, further studies are required to fully understand the relevance of MLN-derived factors in the generation of gut homing T cells.

8.2.2 Imprinting in proximal vs. distal small intestine

In **paper III**, we also investigated if DCs, based on differential localization within the intestine (i.e. proximal *vs*. distal) were differentially imprinted with the ability to metabolize Vitamin A. The rationale for this was based on the observations that (i) in rats, Vitamin A is primarily absorbed in the proximal, compared to the distal part of the small intestine [315] and (ii) homing of T cells to the small intestinal epithelium is more dependent on CCR9 in the proximal parts of the intestine

compared to distal parts [316]. Based on this one might expect that proximal DCs are imprinted to a higher degree compared to distal DCs. However, we do not see this pattern in our examination of ALDH activity of CD103$^+$ intestinal DCs (**paper III**). One possible explanation for this might be that TLR signaling, which has been indicated to play a role in induction of *aldh1a2* [308, 313] may be more available in the distal part of the intestine where the highest number of commensals are present [317]. Hence, increased TLR signaling could compensate for the lower uptake of Vitamin A. However, recent analysis of ALDH activity in CD103$^+$ and CD103$^-$ DCs in MLN of germfree as well as MyD88 KO, Trif KO and MyD88/Trif double KO mice have demonstrated that TLR signaling and/or bacteria do not play a major role in imprinting DCs [312, 318] although the role of TLR signaling and/or bacteria in imprinting of DCs in proximal *vs.* distal small intestine has not, to my knowledge, been assessed. However, other compensatory mechanisms may exist. Of course, it is also possible that the differential uptake of Vitamin A is not a property of murine intestine or that the difference in uptake is not large enough to be biologically significant.

8.3 Intestinal CD103$^+$ DCs vs. CD103$^+$ DCs in other tissues

CD103$^+$ DCs are not only found in the small intestine and MLN [211, 251] but also in colon, dermis of the skin, PLNs, lung, LDLNs and spleen [50-52, 55, 251, 319]. In our studies for **paper I** we identified CD103$^+$ DCs in MLN and small intestine as well as in the lung, PPs, colon, spleen and all LNs investigated, including LDLN, inguinal, axillary, brachial, lumbar and caudal LNs (**paper I** and E. J-G. unpublished observations) and hence CD103$^+$ DCs are not restricted to the gastrointestinal tract but rather found in many tissues and LNs as well as the spleen, although the percent of DCs expressing CD103 relative to other DC populations at these sites varies; for example, in the spleen CD103 is expressed on only a small population (5-10%) of DCs while in LDLN and MLN CD103 is expressed by about 25 and 40% of the DCs respectively (**paper I**).

8.3.1 CD103$^+$ DCs in intestine, lung and dermis

In contrast to small intestinal CD103$^+$ DCs that contain populations of both CD11b$^+$ and CD11b$^-$ cells, CD103$^+$ DCs in the lung and dermis of the skin are primarily CD11b$^{low/-}$ [25, 28, 52, 54, 319-321]. Interestingly, pulmonary and dermal CD103$^+$ DCs share several phenotypic and functional similarities to small intestinal CD103$^+$ DCs.

For example, similar to intestinal CD103$^+$ DCs, lung and dermal CD103$^+$ DCs are migratory [51, 52, 54-56]. CD103$^+$ DCs isolated from colon [277], small intestine [322] or lung [323] also appear to express lower levels of TLRs compared to CX3CR1$^+$ cells from the colon or CD103$^-$ DCs from lung or small intestine or respectively.

Further, similar to small intestinal CD103$^+$ DCs [25, 28] dermal and pulmonary CD103$^+$CD11b$^{low/-}$ DCs appear to require Flt3 for their development [260]. In addition, development of lung, dermal and small intestinal CD103$^+$CD11b$^-$ DCs is dependent on transcription factors Batf3 [261], Id2 [28, 260] and IRF8 [260, 261] indicating that these CD103$^+$CD11b$^-$ non-lymphoid populations are developmentally related. Further CD103$^+$ DCs in lung and skin draining LNs appear to be highly efficient at cross-presenting tissue derived antigen to CD8 T cells [55, 324, 325], similar to CD103$^+$ DCs from MLNs (**paper I**). Notably, as mentioned earlier, LN and splenic CD8α^+ DCs are also dependent on Batf3, IRF8 and Id2 [26, 44-47] and have been associated with cross-presentation of antigens [41, 42] suggesting that non-lymphoid CD103$^+$CD11b$^-$ DCs and lymphoid organ resident CD8α^+ cDCs may be both developmentally and functionally related. It will thus be of interest to further investigate functional activity (ex. cross-presentation of antigens, migration, response to TLR stimulation) of small intestinal CD103$^+$CD11b$^+$ DCs compared to CD103$^+$CD11b$^-$ DCs.

8.3.2 Vitamin A and imprinting of DCs outside the gut

In addition to the populations of $CD103^+ALDH^+$ DCs in small intestinal lamina propria and MLN (**paper II, III**), $CD103^+$ DCs (and to some extent $MHCII^+CD11c^+CD103^-$ cells) in the lung and colon also contained a small percentage of $ALDH^+$ cells, (*figure 9* and E J-G. unpublished observation) [318]. Further, Guilliams *et al* recently reported that dermal $CD103^-CD11b^{hi}$ DCs and corresponding $CD103^-CD11b^+$ PLN DCs have ALDH activity exceeding that of dermal $CD103^+$ DCs. Of note, the same authors also reported that $CD103^-CD11b^+$ PLNs DCs appear to be able to mediate generation of T regs *in vitro* in a process dependent on RA-signaling [318] suggesting that retinol metabolizing activity in DCs may be linked to the generation of T regs in both MLNs and PLNs. Still, how these $ALDH^+CD103^-$ PLN DCs are imprinted with retinol metabolizing activity is not known. However, as we in preliminary studies have observed that the percentage of $ALDH^+CD103^+$ DCs in the colon and lung, similar to the percentage of $ALDH^+$ $CD103^+$ DCs in small intestine and MLN is decreased in VAD mice (*figure 9*), it may be hypothesized that Vitamin A plays a role also outside the intestine in imprinting DCs with retinol metabolizing activity and in generating DCs with the ability to induce $FoxP3^+$ T regs.

Taken together, studies of $CD103^+$ DCs from small intestine, MLN, lung, LDLNs, dermis and LNs draining the skin may suggest that $CD103^+$ DCs in general share developmental (ex. requirement for Flt3) phenotypical (ex. low TLR expression) and functional (ex. cross presentation of antigen) properties while other properties (ex. generation of T regs) appear to be distributed among both $CD103^+$ and $CD103^-$ DCs.
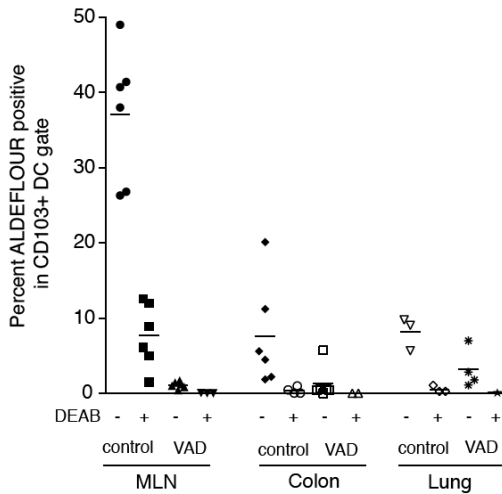
Figure 9. **ALDH+ cells are present in MLN, colon and lung.** Flow cytometric analysis of CD103+ DCs from MLN, colon and lung in control and VAD mice using ALDEFLOUR to assay ALDH activity. Each dot is representative of one mouse. DEAB: diethylaminobenzaldehyde, an ALDH inhibitor.

8.4 Concluding remarks and outstanding questions

During the course of my thesis studies, a few concepts have become increasingly evident to me. First, classification and division into subsets is a problematical but often necessary exercise in studies of DCs and macrophages. While classification may be based on development, phenotype, function or localization where each approach has its own advantages and limitations, the 'right way' to classify recently described populations is an area certainly open for discussion [40, 326, 327]. In particular, different research groups quite often have their own preferred ways to classify their cells of interest and hence comparison of different studies are sometimes complicated and confusing. This has been exceptionally apparent in the classification of DC and macrophage subsets in the small intestine as previously discussed. Still, classification into subsets is often a requirement in order to detect developmental and functional differences of cell populations and may be essential in therapeutic inhibition of autoimmune diseases, effective anti-tumor therapies or in generation of novel vaccines where specific modulation of immune responses, rather than effects on the immune response on a global level, are desired.

Secondly, it has become increasingly clear that homeostasis in the intestine is influenced by the balance between different DC or macrophage subsets that have different specialized functions that together help maintain gut homeostasis. In this regard, CD11c-depleated mice reconstituted with Ly6c^{hi} monocytes (predominantly leading to generation of CD11b^{+}CX3CR1^{+} macrophages) have been reported to be more susceptible to experimental dextran sulfate sodium (DSS)-induced colitis than mice that were not CD11c-depleted, persistently CD11c-depleted, or transiently CD11c-depleted [25], indicating that the balance between intestinal CD103^{+} DCs and CX3CR1^{+} cells may play a role in maintaining intestinal homeostasis. Further, it has recently been suggested that induction of oral tolerance relies on cooperation between CD103^{+} DCs and CX3CR1^{+} cells, where the two populations mediate discrete and sequential steps [280]. Thus, several different DC or macrophage subsets may, in a synchronized fashion, cooperate to generate appropriate immune responses and maintain gut homeostasis.

In addition, it has become very clear to me that interactions between environmental cues and immune and non-immune cells and shape the outcome of immune responses. Thus, although we have just recently begun to understand the complex interactions of local factors and cellular responses in the intestine, it appears that environmental cues, including RA, TLRs or even specific commensal bacteria, as well cell-derived factors including GM-CSF production by intestinal CD11c^{-} F4/80^{+}cells critically can influence the outcome of T cell stimulation by DCs or the presence of specific subsets of T cells in the intestine [11, 113, 172, 176, 177, 304, 309, 328-330]. Together, the combination of environmental and cell-derived cues in combination with the maturation status of DCs may generate an exponential number of ways that DCs can be modulated, and in my mind, the resulting properties of DCs are hence likely to be the sum of all inputs rather than a to be the result of a single specific signal.

It has also been very apparent that by answering one question, several further questions arise. Thus, while the studies that make up this thesis have answered some

questions regarding the main migratory small intestinal DC population, the interrelationships between small intestinal CD103$^+$ DCs and CX3CR1$^+$ cells and the mechanisms behind the imprinting of CD103$^+$ DCs with the ability to metabolize Vitamin A, many questions remain. Some of the outstanding questions related to these thesis studies are briefly discussed below.

8.4.1 Key outstanding questions

Intestinal antigen handling

Although we and others have suggested a role for CX3CR1$^+$ cells in the uptake of intestinal antigens [188-191] (**paper II**) and a role for CD103$^+$ DCs in migration and presentation of intestinal antigens to T cells in the MLNs [28, 209, 211] (**paper I, II**) there is currently a gap in the knowledge of how intestinal antigens reach migratory CD103$^+$ DCs. In this regard, Bogunovic *et al* have reported that 24h after infection with *Salmonella* Typhimurium, both CD103$^+$CD11b$^+$ and CD103$^-$CD11b$^+$ small intestinal DC subsets contained intracellular *Salmonella* [28] while Hapfelmeier *et al* have observed that bacteria were passed on from a CD11c$^+$CX3CR1$^+$ population to a CD11b$^+$CD11c$^-$CX3CR1$^-$ population during the course of cecal infection with non-invasive *Salmonella*, although the identity of this CD11c$^-$ population remains unclear [189]. While these results may indicate that antigens taken up by CX3CR1$^+$ cells are transferred to populations in the lamina propria, including CD103$^+$ DCs, they may also indicate that CD103$^+$ DCs directly or indirectly, via non-CX3CR1 cells, obtain access to intestinal antigens. In this regard, a population of DCs present in rat mesenteric lymph have been reported to contain apoptotic material [200] and murine MLN DCs expressing β7 (an integrin chain that can pair up with αE i.e CD103) have been observed to contain cellular debris and fragmented DNA, suggested to originate from apoptotic cells [199]. Thus it may be hypothesized that intestinal CD103$^+$ DCs obtain access to intestinal antigens via apoptotic cells containing intestinal antigens. However, further studies of bacterial and soluble antigen uptake will be needed to clarify this issue of intestinal antigen handling.

CD103⁺ DCs in intestinal inflammation and infection

Remarkably little is known about CD103$^+$ DCs as well as other intestinal DC/macrophage subsets and their functions and relative roles in intestinal inflammation and infection. For example, we do not know if intestinal CD103$^+$ DCs are capable of generating effector responses in inflammation or infection or if/how Vitamin A metabolizing enzymes are regulated in different intestinal DC and macrophage subsets in inflammation or infection. However, our studies in **paper I** of CD103$^+$ DCs from MLN of small bowel Crohn's patients indicate that CD103$^+$ DCs are present in the MLN in patients with intestinal inflammation and that human CD103$^+$ MLN DCs retain the ability to induce CCR9 expression on responding T cells in inflammation. Further, a recent study of intestinal immune responses in colitic mice has demonstrated that CD103$^+$ DCs isolated from MLN of colitic mice had lower *aldh1a2* expression and ALDH activity than CD103$^+$ DCs isolated from MLN of control mice [331]. CD103$^+$ DCs isolated from MLN of colitic mice were also slightly less efficient at generating FoxP3$^+$ T regs but more efficient at generating IFNγ and IL-17 producing T cells *in vitro* compared to CD103$^+$ DCs isolated from MLN of control mice [331], while the ability of CD103$^+$ DCs to generate gut tropic T cells was not assessed. Thus, CD103$^+$ MLN DCs in colitic mice may be suggested to mediate effector, rather than regulatory responses. However, further studies of the phenotype and function of intestinal CD103$^+$ DCs are needed to elucidate the role of these DCs in inflammation and infection.

CD103⁺ DCs in human intestine and MLNs

The phenotype or function of human intestinal and MLN DC subpopulations has not been extensively studied. However, in **Paper I,** we reported the presence of both CD103$^+$ and CD103$^-$ Lineage$^-$HLA-DR$^+$CD11c$^+$ DCs in MLN of humans. Of note, the percentage of DCs expressing the maturation markers CD40 and CD80 was higher in the CD103$^+$ subpopulation than the CD103$^-$ subpopulation and CD103$^+$ DCs were significantly better at inducing CCR9 on responding human T cells compared to CD103$^-$ DCs. Similarly, Iliev *et al* have reported the presence of CD103$^+$ and CD103$^-$ Lineage$^-$HLA-DR$^+$CD11c$^+$ DCs in MLN. Notably, in the study

by Iliev *et al*, CD103$^+$ DCs expressed higher levels of CCR7 and were more efficient at inducing regulatory T cells compared to their CD103$^-$ counterparts [307]. Hence, human and murine CD103$^+$ DCs share some phenotypic and functional properties. CD103$^+$ DCs are also present in the human small intestine [332] although the phenotype and function of these CD103$^+$ DCs and the relationship to murine intestinal CD103$^+$ DC subsets clearly warrants further investigation.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunsystem består av ett nätverk av olika sorters celler och molekyler, vars uppgift är att försvara oss mot patogener (sjukdomsalstrande organismer) som till exempel virus och bakterier, medan det samtidigt måste tolerera proteiner och ämnen som finns naturligt i vår kropp. Immunsystemet kan delas upp i två delar; det medfödda (eller ospecifika) och det adaptiva (eller specifika) försvaret. Det medfödda immunförsvaret består av proteiner och celler som känner igen strukturer som är gemensamma för många patogener. Det adaptiva immunförsvaret består främst av celler vilka specifikt kan känna igen olika delar av patogener.

En typ av celler som ingår i det adaptiva immunförsvaret är T-celler. Varje T cell har en unik förmåga att känna igen ett visst ämne (så kallat antigen) som finns i till exempel bakterier och virus. För att T-celler ska känna igen ett visst antigen krävs att antigenet visas upp av antigen-presenterande celler, vilka utgörs av dendritceller och makrofager. Dessa dendritceller och makrofager kan ta upp proteiner från sin omgivning, bryta ner dem och sedan visa upp delar av de nerbrutna proteinerna (antigenen) på sin yta. När en T cell hittar en antigen-presenterande cell som visar upp det antigen som T-cellen i fråga känner igen så börjar den T-cellen dela sig. Detta sker framför allt i lymfnoder och ger upphov till många aktiverade T-celler, som nu är kapabla till att döda infekterade celler eller till att aktivera, reglera eller locka till sig andra celler som ingår i immunförsvaret. Aktiveringen av T-cellerna, men också T-cellernas förmåga att efter aktivering rekryteras till olika vävnader, till exempel tarm, hud eller lungor (där T-cellerna till exempel kan döda infekterade celler) styrs av antigen-presenterande celler, främst dendritceller. Rekryteringen av aktiverade T-celler till olika vävnader möjliggörs av olika molekyler som uttrycks på ytan av aktiverade T-celler samt molekyler som uttrycks specifikt i olika vävnader.

I denna avhandling vi studerat vilka olika populationer av antigen-presenterande celler det finns i tunntarmen hos möss samt hur dendritceller från tunntarm kan styra T-celler till att rekryteras till tunntarmen. Eftersom tunntarmen och dess slemhinna oavbrutet utsätts för ämnen och strukturer via mat och tarmbakterier är det viktigt att immun-reaktionerna här är väl styrda. Defekter i styrningen av immunförsvaret i tarmen kan

bland annat ge upphov till okontrollerad rekrytering av T-celler vilket kan ses vid inflammatoriska tarmsjukdomar, som till exempel Crohns sjukdom.

I artiklarna som ingår i den här avhandlingen har vi visat att det i tunntarmen i möss finns två stora populationer av antigen presenterande celler. Dessa celler kan identifieras genom att de har olika protein på sin yta, nämligen CD103 och CX3CR1. De celler som har CD103 på sin yta kan vandra via lymfatiska kärl från tunntarmen till de närliggande mesenteriska lymfnoderna. De är också effektiva i att aktivera T-celler vilka då samtidigt kan styras till att uttrycka ett protein som kallas CCR9. CCR9 är sedan tidigare känt för att medverka till att T-celler rekryteras till tunntarmen. De celler som har CX3CR1 på sin yta kan däremot inte vandra till de mesenteriska lymfnoderna. De är dessutom mindre effektiva i att aktivera T-celler och kan inte heller styra T-celler till att uttrycka CCR9. Å andra sidan verkar celler som har CX3CR1 på sin yta vara bättre på att ta upp proteiner från sin omgivning jämfört med dendritceller som uttrycker CD103. Alltså har celler med CD103 eller CX3CR1 på sin yta olika funktioner.

Vi har dessutom studerat mekanismer som ligger till grund för hur dendritceller med CD103 på sin yta kan styra T-celler så att de uttrycker CCR9. I dessa studier har vi kommit fram till att Vitamin A kan påverka dendritceller som har CD103 på sin yta så att dessa ökar sitt uttryck av ett enzym som kallas RALDH2. Detta enzym är kritiskt för att celler ska bilda Vitamin A-syra, vilket sedan tidigare har visat sig vara viktigt för att T-celler ska kunna styras till att uttrycka CCR9 och därmed rekryteras till tarmen. Därmed är Vitamin A i tunntarmen viktigt för att dendritceller ska kunna rekrytera T-celler till just tunntarmen.

Vi har också i våra studier funnit att dendritceller med CD103 på sin yta finns i mesenteriska lymfnoder i människor samt att dessa dendritceller, liksom dendritceller i möss som har CD103 på sin yta, kan styra T-celler till att uttrycka CCR9.

Genom dessa studier har vi lärt oss lite mer om hur tarmslemhinnors immunförsvar fungerar, vilket i förlängningen kan bidra till att utveckla mediciner och behandlingar mot inflammatoriska sjukdomar i tarm och andra slemhinnor, men även till att utveckla vacciner.

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