Function and homeostasis of murine splenic dendritic cell subsets

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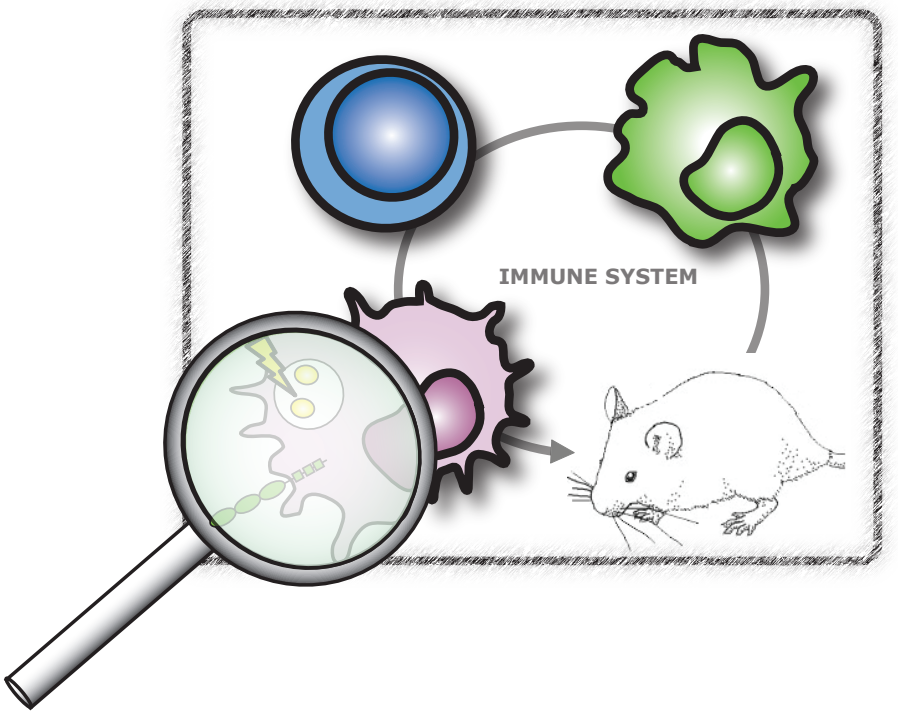
CHAPTER 1

GENERAL INTRODUCTION



1.1 INTRODUCTION

The immune system has evolved in all vertebrates as protection against pathogenic micro-organisms, thereby preventing us from a certain death by infections. The immune system can be divided into the innate immune system and the acquired or adaptive immune system, each with their specific network of cells to control pathogen invasion.

The innate immune system is an early and rapid defence mechanism against pathogens and is mainly dependent on the function of macrophages (Mφ), dendritic cells (DCs), granulocytes and natural killer (NK) cells. These cells are able to respond to pathogens with germ-line encoded pattern recognition receptors (PRRs). These PRRs include Toll-like receptors (TLRs)[1;2], C-type lectins[3;4], scavenger receptors and intracellular nucleotide-binding oligomerization domain (NOD)-like receptors[5]. PRRs recognize highly conserved and pathogen-specific PAMPs (pathogen-associated molecular patterns), ranging from proteins to nucleic acids.

Especially Mφ and granulocytes very efficiently internalize pathogens, which are then killed through the release of cytotoxic agents like reactive oxygen species (ROS). On the other hand, NK cells are important for preventing viral infections by killing virally infected host cells. The role of DCs will be discussed in detail in chapter 1.4.

The acquired immune system, formed by T cells and B cells, is able to elicit antigen (Ag)-specific immune responses and immunological memory. Due to this memory, the acquired immune system is able to launch Ag-specific, secondary immune responses to prevent illness during the second encounter of the same pathogen. T cells are divided into $CD8^+$ T cells and $CD4^+$ T cells that express unique T cell receptors (TCRs). TCRs recognize peptides presented by histocompatibility complex (MHC) class I or MHC class II molecules, respectively. T cells recognize Ag only when it is presented by self-MHC-molecules and this restriction results in a process of positive and negative selection during T cell development in the thymus. Since inappropriate T cell activation could possibly result in the development of autoimmune diseases, only the so-called professional antigen-presenting cells (APCs) are capable to instruct and regulate the activation of naïve T cells.

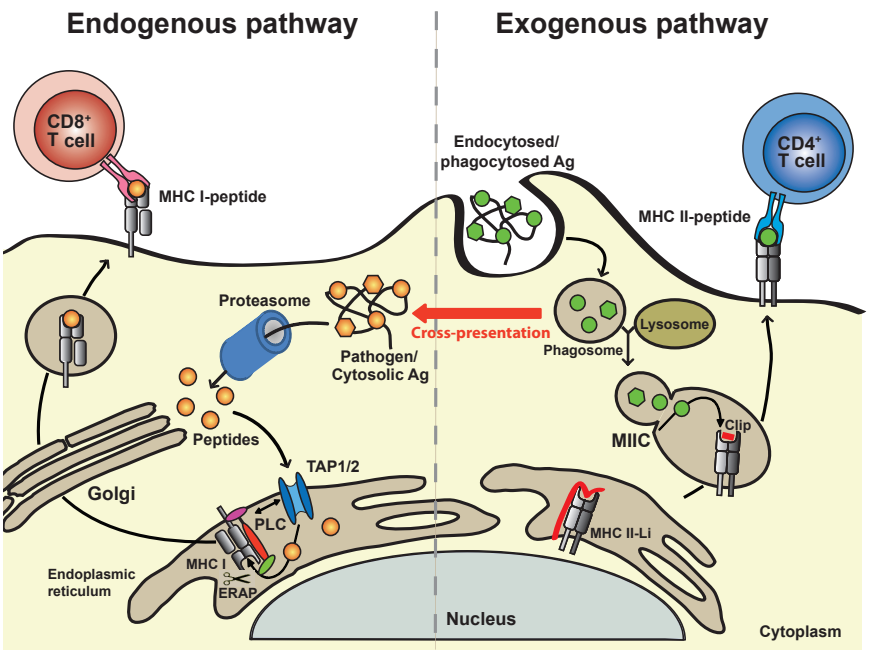
1.2 ANTIGEN PRESENTATION AND T CELL ACTIVATION

There are different routes for Ag-presentation, activating either $CD4^+$ T cells or $CD8^+$ T cells (Figure 1)[6;7]. APCs are able to take up extracellular Ags for presentation in MHC class II leading to $CD4^+$ T cell activation. After internalization, Ags are located in the phagosomes, which will fuse during maturation with early and late endosomes and lysosomes. The acidification machinery in these phago-lysosomes generates an optimal environment for cathepsins and other hydrolytic enzymes that are involved in Ag-degradation.

Intracellular peptides are generally presented in MHC class I molecules, an ability of almost all nucleated cells. MHC class I restricted Ags are derived from cytosolic proteins of both viral- and self-origin. Recognition of these Ag-MHC class I molecule complexes allows $CD8^+$ T cells to destruct virus-infected and cancer cells.

MHC class I is a heterodimer consisting of a single transmembrane heavy chain and a soluble β2 microglobulin light chain. The heavy chain has three polymorphic domains (α1-α3) and between the membrane distal domains α1 and α2 the peptide-binding groove is located, which binds peptides of typically 8-9 amino acids (aa) in length. For the generation of peptides in the MHC class I assembly route[8-10], the cellular protein-degradation pathway is used, meaning that Ags have to be processed by multicatalytic proteases, the proteasomes, that are normally regulating cytoplasmic protein turnover. In APCs, proteasomes are specially modified to facilitate MHC class I restricted Ag-processing by altering the cleavage pattern of proteins and promoting the generation of peptides. Proteasomes cleave ubiquitinated proteins into polypeptides consisting of 3-22 aa in length, with an average of 8-9 aa[11;12]. The peptides are then transported into the endoplasmatic reticulum (ER) by adenosine triphosphate (ATP)-dependent transporters associated with antigen processing (TAP1 and TAP2) (Figure 1). TAP is linked to the β2-microglobulin light chain of MHC class I by tapasin. Together with chaperones, protein disulfide isomerase, calnexin and/or calreticulin and ERp57, the peptide-loading complex (PLC) is formed, which facilitates the binding of peptides to the groove of the free MHC class I molecule[13;14].

Only peptides that bind with very high affinity can stabilize the MHC class I molecule. The TAP-transported peptides bind to MHC class I molecules with their C-terminus, but often contain at their N-terminus extra residues that do not fit into the binding-groove[15-17]. For optimal binding, the peptides have to be trimmed by ER amino-



|Figure 1| Loading and trafficking of MHC class I and II molecules.
Ag-processing and presentation can be divided into a endogenous and exogenous pathway. During endogenous Ag-presentation, intracellular proteins are degraded by proteasomes into peptides. These peptides are transported into the endoplasmatic reticulum (ER) by the transporter associated with antigen processing (TAP) complex. In the lumen of the ER, peptides are loaded onto available MHC class I molecules, a process that is facilitated by the peptide-loading complex (PLC). Once bound, peptides require further trimming by the ER-aminopeptidase I (ERAP). Peptide-loaded MHC class I molecules are transported via the Golgi complex to the cell surface where it can be recognized by $CD8^+$ T cells. Extracellular Ags are processed after internalization following the exogenous Ag-presentation pathway. During this process, phagosomes undergo maturation into phagolysosomes by fusing with lysosomes. Ags are degraded into peptides that are able to bind to MHC class II molecules. These MHC class II molecules are assembled in the ER and exist as dimers due to the specialized chaperone invariant chain (Li). This prevents binding of endogenous Ags. The MHC class II-Li complex is transported via the Golgi system into MHC class II containing compartments (MIIC), where also phagolysosomes will fuse. The Li is degraded into CLIP (class II-associated invariant-chain peptide). Antigenic peptides will replace CLIP and peptide loaded MHC class II molecules will be transported to the cell surface for interaction with $CD4^+$ T cells. Alternatively, DCs can also present extracellular Ags in the context of MHC class I to $CD8^+$ T cells during a process called cross-presentation. For this, Ags are diverted from the exogenous pathway into the conventional endogenous pathway. *[Adapted from Heath and Carbone 2001]*[22].

peptidase associated with Ag-processing (ERAAP) to generate final MHC class I-peptide complexes[18;19]. Once a peptide has bound, the PLC will dissociate and the loaded MHC class I molecule exits from the ER to the cell membrane and is now available for recognition by $CD8^+$ T cells.

1.3 CROSS-PRESENTATION

Direct MHC class I presentation by cells plays a crucial role in the removal of virally infected cells and tumor cells by $CD8^+$ T cells. However, for the initial activation of $CD8^+$ T cells, endogenous Ags need to be processed and presented by professional APCs, such as DCs. The need for APCs for activation of naïve T cells, implies that a virus could easily escape detection by the immune system by not infecting APCs, or by down regulating APC activation. To circumvent this, the immune system has developed an exogenous pathway for MHC class I loading that allows sampling from other cells. This so called cross-priming has first been described by Bevan in 1976 and is essential for activation of cytotoxic T cells (CTLs) towards non-replicating ligands or Ags that are not present in the APC itself, e.g. tissue specific viruses[20-23]. Cross-presentation seems to be a characteristic of mainly DCs[24] but also B-cells[25], endothelial cells[26;27], and Mφ[28-30] have been shown to exhibit cross-presentation capacities, although to a much lower extent. As described, cellular Ags are an obvious target for cross-presentation, but also many other types of Ags, varying from soluble Ags and immune complexes to intracellular bacteria and parasites, are shown to be cross-presented.

Cross-presentation seems to be completely dependent on TAP, as has been shown in bone marrow (BM)-derived cells[31;32], although a TAP independent pathway has also been described[33]. This TAP independent, vacuolar pathway involves proteolysis of endocytic Ags by cysteine proteases (e.g. cathepsins) and subsequent loading of recycled MHC class I molecules in this endosomal compartment[34]. It is, however, unlikely that endosomal digestion would result in the same peptide repertoire as proteasomal degradation. For the induction of protective immune responses, it is essential that the peptide repertoire produced by DCs is similar to that presented in MHC class I by infected cells. The relevance of this vacuolar pathway is therefore not clear, but is thought to play a role in cross-presentation by plasmacytoid DCs (pDCs)[35].

One of the major TAP dependent, proteasomal pathways requires egress of the internalized Ags into the cytosol where it can become a substrate for proteasome dependent peptide generation, and the delivery via TAP into the ER (phagosome-to-cytosol pathway)[36]. How exactly the delivery out of the phagosomes works is unclear. Phagosomes can also fuse directly with the ER, which provides MHC class I molecules, TAP, tapasin and other ER resident proteins. Ags are then transported into the cytosol for degradation by Sec-61 or by the ER associated degradation pathway (ERAD), a mechanism involved in the removal of misfolded

proteins from the ER[37]. Ags are subsequently transferred by TAP back into the phagosome (phagosome-to-cytosol-to-phagosome pathway) or into the ER (phagosome-to-ER pathway)[38-41].

Another pathway for cross-presentation is the leakage of proteins via endosomal pores or after rupture of the phagosomal membrane, which can be caused e.g. by infection with *C. neoformans*[42]. Intracellular peptides can also be transferred directly from the cytoplasm of a target cell into the cytoplasm of DCs through connexion 43-formed gap junctions[43].

Cross-presentation does not only induce the activation of $CD8^+$ T cells, but cross-presentation of cellular Ags by $CD8^+$ DCs has also been implicated in T cell tolerance[44;45].

1.4 DENDRITIC CELLS

DCs are phagocytes that have been shown to be the central players of the immune system[46]. They are the professional APCs of the body and are the only cell type able to induce primary immune responses by activating naïve T cells[46-48]. DCs are involved in innate recognition of pathogens resulting in the subsequent activation of T cells [reviewed by *Reiss e Sousa*[49]]. Therefore, DCs are considered to be bridges between the innate and acquired immune system.

Immature DCs are strategically positioned at locations that line the external environment like skin and mucosa, as sentinels constantly screening for incoming pathogens. DCs are equipped with a whole array of receptors (e.g. lectins, Fc- receptors and complement receptors) for recognition and internalization of Ag, allowing DCs to respond quickly to different pathogen related stimuli such as LPS or bacterial DNA. DCs use several pathways for Ag-capture. Immune-complexes, microbes, soluble Ags but also dying cells and tumor cells are taken up by (macro)-pinocytosis or receptor-mediated uptake.

DCs, although not all, show a biphasic life cycle, often referred to as the Langerhans cell (LC) paradigm. This means that after Ag-encounter or tissue damage, DCs mature into Ag-presenting cells. These APCs have lower Ag-uptake capacity but increased ability to activate T cells. During maturation, molecules that interact with T cells are up-regulated, e.g. several members of the B7-family (CD80/CD86, ICOS-L) and TNF-family (CD134, Ox40L, CD70). Mature DCs change their morphology and gain high cellular mobility. The up-regulation of members of the chemokine receptor family (e.g. CCR7) directs DCs to migrate towards secondary

lymphoid tissues where most T cells reside[50-52]. The transport of Ags to the lymph nodes (LN) is important for increasing the chances of interaction with naïve T cells.

DCs continuously migrate towards LN, also in steady state without any trigger for DC activation. These semi-mature DCs are important for immune-regulation and tolerance induction against self and environmental proteins. Mature DCs are immunogenic, whereas semi-mature DCs are tolerogenic, able to induce T regulatory (T_{reg}) cells.

By the release of large amounts of cytokines, DCs are able to direct and regulate the differentiation of $CD8^+$ T cells into CTLs, and of $CD4^+$ T cells towards T helper 1 (Th_1), Th_2, Th_{17} or T_{reg} cells. DCs not only induce differentiation and sustain the expansion of effector T cell functions, but are also required for the induction of immunological memory by these T cells. Next to their role in T cell proliferation[53;54], DCs are also shown to be involved in T cell dependent Ab production by B cells[55] and proliferation of NK cells[56;57].

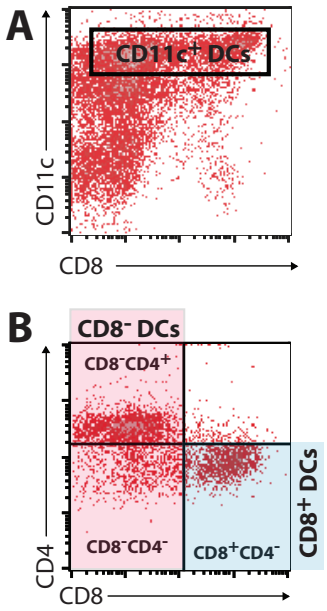
The ability of DCs to initiate immune responses distinguishes them from other phagocytes like Mφ and granulocytes[22;58]. DCs proved to be more than 100 times better at stimulating spleen cells in primary allogeneic mixed leukocyte reactions than Mφ[59]. It has been described that variations in phagosomal properties can impact Ag-presentation efficiencies[60]. The main function of Mφ is to destroy pathogens, rather than Ag-presentation[61]. Mφ therefore produce large amounts of reactive oxygen species (ROS), which together with proteases, are required for microbial killing. DCs are less efficient in the degradation and destruction of pathogens. They do not express the full array of proteases like Mφ do, and have specialized endocytic mechanisms to control protein degradation[62;63]. The more neutral pH and the limited proteolytic activity in DCs protects Ags from total degradation, which favors Ag-presentation. As a consequence, intact Ag was observed for prolonged time in specialized, MHC class II rich vesicles in DCs, representing an intracellular source of Ag for presentation.

1.5 DENDRITIC CELL SUBSETS

DCs form a heterogeneous population of cells[64]. Although all DCs can take Ags up and present them to T cells, different DC subsets can be identified based on distribution, phenotype and function [reviewed by *Shortman and Naik*[65]*, Villadangos and Schnorrer*[66]]. These DC subsets

are localized in different microenvironments and have a different life span. Due to their differences in PRR expression and responsiveness, DCs also differ in their capacity to produce certain cytokines and in their capacity to present Ags. These differences indicate that the distinct DC subsets have evolved to hold different functions in distinct niches in the immune system.

The first subdivision that can be made is between conventional DCs and non-conventional DCs (Table 1). Conventional DCs are defined by the expression of the integrin CD11c and MHC class II, although expression levels of these molecules are varying between one DC subset to another. Conventional DCs form the resident DC population in spleen, LN and thymus and are generated from precursors from the blood. These resident DCs can be further subdivided, mainly by the expression of the CD8αα homodimer and CD4[67] (Figure 2), but also by the expression of CD11b, SIRPα, DEC205 (CD205) and DCIR2. The spleen contains mainly $CD8^-DCIR2^+$ DCs (~70% of DCs) and a smaller population of $CD8^+DEC205^+$ DCs (see chapter 1.8 for detailed description of spleen histology and function).



|Figure 2| Three resident DC subsets in the mouse spleen.
Based on the expression of CD11c, CD8 and CD4, conventional DCs in the spleen can be divided into 3 subpopulations. **(A)** DCs can be purified from the spleen using CD11c-coated magnetic beads. When stained for CD11c and CD8, a main $CD8^-$ DC population and a smaller $CD8^+$ DC population can be identified. **(B)** The $CD8^-$ DC subset can be further subdivided into $CD8^-CD4^+$ and double negative $CD8^-CD4^-$ DCs by staining CD11c cells (gated cells in A) for CD4 and CD8.

The $CD8^-DCIR2^+$ DC population consists of $CD8^-CD4^+$ and double negative $CD8^-CD4^-$ DCs[68]. Since micro-array data showed that $CD8^-CD4^+$ and $CD8^-CD4^-$ DCs are quite similar in gene-expression[69], we refer throughout this thesis to the $CD4^+CD8^-$ and $CD4^-CD8^-$ DCs as a single $CD8^-$ DC type. Next to their phenotypical differences, conventional DC subsets are also found at different anatomical locations in the spleen and LN[70]. $CD8^+$ DCs are mainly found in T cell rich areas such as the periarteriolar lymphatic sheets (PALS) of the spleen and in paracortical regions of LNs[71-74]. Recently, co-staining with Langerin (CD207) also revealed $CD8^+$ DCs in the marginal zone (MZ)[75]. $CD8^-$ DCs

are located in the MZ and bridging channels of the spleen and in the subcapsular sinuses of LNs[76]. After stimulation with microbial products, DCs have been shown to migrate to the T cell areas of these organs[77;78].

Conventional tissue-derived DCs follow the lifecycle described by the LC paradigm and include LCs and interstitial DCs that populate all non-lymphoid tissues. LCs are characterized by the expression of the C-type lectin Langerin, CD1a and the cytoplasmic Birbeck granules, and are populating the epidermis in close proximity to the basal membrane. Interstitial DCs are localized throughout the body and include dermal DCs in the skin and DCs at mucosal sites. LCs and interstitial DCs act as

|Table 1| Splenic DC subsets; phenotype and function.

	Blood derived DCs		
	$CD8^+$ DCs	$CD8^-$ DCs	
		$CD8^-$ DCs	$CD8^-4^-$ DCs
Phenotype			
CD11c	+++	+++	+++
CD4	-	+	-
CD8	++	-	-
CD205	+	-	+/-
DCIR2	-	+	+
CD11b	+	++	++
Dectin-1	+	+	+
Langerin	+	-	-
CD1d	+	-	-
CD24	++	+	+
Birbeck granule	-	-	-
Sirpα	-	+	+
Localization	T cell zones of lymphoid organs and marginal zone	MZ and bridging channels of the spleen, subcapsular sinus of LN	MZ and bridging channels of the spleen, subcapsular sinus of LN
Function			
Ag processing and presentation	+++	+++	+++
Cross-presentation	+++	+	+
MHC class II presentation	+	+++	+++

+ indicates low level of expression and +++ signifies high levels of expression, - indicate lack of expression. Adapted from [*Pulendran 2004*][165] and [*Villadangos and Schnorrer 2007*][66]

sentinels for pathogens and migrate after Ag-encounter via the afferent lymph vessels towards local draining LNs. As a consequence, LCs can only be found in skin draining LN, while interstitial DCs can be found in all LNs. The spleen does not contain these tissue-derived DCs, since it is not connected to the lymphatic system.

Some other, non-conventional DC subsets, such as plasmacytoid DCs (pDCs)[79] or TNF/iNOS-producing (Tip)-DCs[65] can be identified both in mouse and man. These DC subsets are important for cytokine production and the mediation of innate immunity[80]. pDCs are determined by their $CD11c^{low}$, $B220^{hi}$, $CD45RA^{+}$ phenotype, and can be found in many tissues including the spleen. As response to various viral or bacterial infections, pDCs are the main producers of type I interferon (IFNs)[81-83]. This cytokine is important for blocking viral replication and for the activation of other DCs. Type I IFN production by pDCs has also been shown to be important for the induction of $CD8^{+}$ T cell memory[84]. The role of pDCs in Ag-uptake and Ag-presentation is unclear. Whereas some studies show a role for pDCs in Ag-specific T cell activation, others do not[85].

Under inflammatory conditions, e.g. during bacterial, viral, or parasitic infections, monocytes can differentiate into inflammatory DCs[65;86]. These so called Tip-DCs represent an 'emergency' source of DCs at the site of inflammation, however, their exact role in the induction and regulation of immunity is still unclear.

1.6 DENDRITIC CELL DEVELOPMENT

Since DCs are cells with a limited proliferative capacity and a relative short live span of about 10-14 days[87], there is high turnover of DCs in tissues. The complex development of DCs is still not fully understood. DCs originate from $CD34^{+}$ hematopoietic stem cells (HSC) in the BM[88;89] (Figure 3). These HSC differentiate into common myeloid precursors (CMP) and common lymphoid precursors (CLP) lacking 'lineage markers'[90]. Originally, it was thought that next to the generation of Mφ and granulocytes, also $CD8^{-}$ DCs were derived from CMPs. The myeloid origin of this DC subset was suggested by the ability to generate $CD8^{-}$ DCs after culturing BM in the presence of GM-CSF. $CD8^{+}$ DCs on the other hand were thought to develop from CLPs, in addition to T cells, B cells and NK cells. Now it is generally accepted that both CMP and CLP can give rise to all DC subsets found in mouse lymphoid organs[91-93]. CMP and CLP differentiate into macrophage-DC precursors or into pro-DCs. These pro-DCs, or common DC precursors, are a mixture of precursor cells

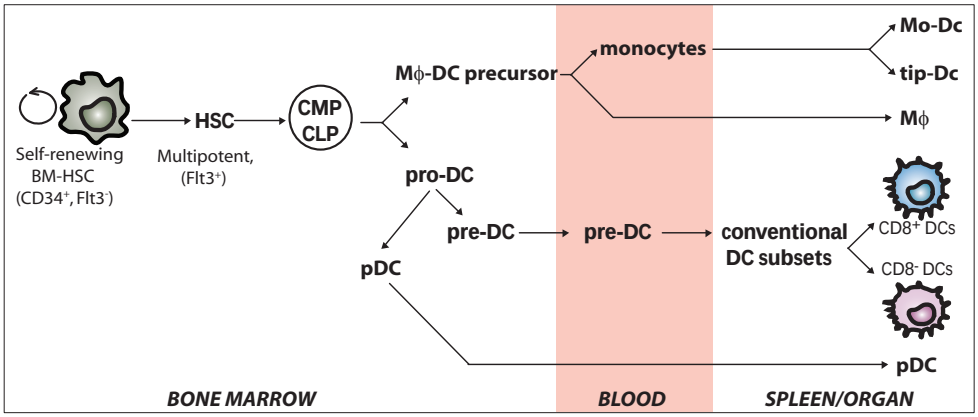
at various levels of differentiation not committed to a certain subset[88]. Commitment occurs at the pre-DC stage from which conventional DC subsets and pDCs originate.

To replenish the pool of DCs, pro-DCs continually migrate from the BM throughout the blood to repopulate peripheral organs, spleen and LNs as immature cells for final differentiation. Once differentiated, DC subsets are stable, not able to interconvert into other subsets. One report showed that $CD8^-$ DCs are able to differentiate into $CD8^+$ DCs[94], although these $CD8^+$ DCs could possibly have originated from direct precursor DCs not yet expressing CD8[95].

Although DC function in the immune system is studied extensively, the molecular basis of DC development and homeostasis is poorly understood. Nevertheless, studies with knockout mice revealed that there are several important factors for DC development, subset differentiation and DC homeostasis[65]. Some transcription factors are identified to be involved in $CD8^-$ DC homeostasis, like the NF-κB transcription family and the interferon regulatory factor (IRF) family.

The NF-κB family of transcription factors is ubiquitously expressed and is involved in the regulation of many genes. The subunits of this protein family, p50, p52, RelA (p65), RelB and c-REL form dimeric complexes that are located as inactive components in the cytosol. Activation induces the degradation of IκBs, the inhibitory proteins associated with these dimers, resulting in the translocation of the NF-κB subunits into the nucleus and subsequently the transcription of target genes. The involvement of the NF-κB superfamily in DC homeostasis is shown by mice deficient in one of the subunits. In mice lacking RelB, the $CD8^-$ DCs are strongly diminished in number[93;96;97] This indicates that RelB is essential for the development of $CD8^-$ DCs but not of $CD8^+$ DCs, while other NF-κB subunits are involved in the survival of more than one DC subset. Mice missing RelA and p50 have almost no DCs at all, probably due to an unresponsiveness to TRANCE and CD40L that control survival and IL12p40 production[98].

The IRF family regulates a large spectrum of DC functions during immune responses but is also involved in DC development[99-101]. DC subsets express different levels of IRF-4 and IRF-8. $CD8^+$ DCs and pDCs express high levels of IRF-8 but no IRF-4. The importance of IRF-8 in the development of these DC subsets is shown in mice lacking IRF-8, where both $CD8^+$ DCs and pDCs are largely absent[102]. On the other hand, IRF-4 is required for normal $CD8^-$ DC development[103]. Mice missing PU.1, an immune cell specific transcription factor of the Ets family which interact with IRF-8, do not have $CD8^-$ DCs[104].



|Figure 3| Splenic DCs develop from a common DC precursor.
$CD34^{+}Flt3^{-}$ hematopoietic stem cell (HSC) in bone marrow (BM) differentiate into $Flt3^{+}$ multipotent HSC with limited self-renewing capacities. During differentiation, the common myeloid precursors (CMP) and common lymphoid precursors (CLP) give rise to more committed progenitors, including the Mϕ-DC precursor and pro-DCs. Mϕ-DC precursors mainly develop into monocytes and Mϕ. Under inflammatory conditions, these monocytes can give rise to monocytes-derived DCs and TNF/iNOS-producing (Tip)-DCs. In the BM, pro-DCs divide and differentiate into pre-DCs and pDCs. Pre-DCs than migrate via the blood to the spleen and other lymphoid tissues, and en route pre-DCs differentiate into conventional $CD8^{+}$ and $CD8^{-}$ DC sublineages. The life span of these $CD8^{+}$ and $CD8^{-}$ DCs is short. During steady-state, the pool of splenic DCs is continuously replaced by new precursors recruited from the blood, or from an intrasplenic population of cells with low-level homeostatic self-renewal capacities.

Another group of molecules important for proper DC development are involved in the Notch signaling cascade. The canonical Notch-RBP-J signaling pathway is important for the survival of mainly $CD8^{-}$ DCs, as shown in mice where genetic inactivation of RBP-J resulted in reduced numbers of splenic DCs[105].

The transcription factor Id2 is crucial for the development of LCs and splenic $CD8^{+}$ DCs[106]. Ikaros is an important regulator for the specification of lymphoid lineage development. And although the effect is mainly on 'lymphoid' DCs, in $Ikaros^{-/-}$ animals both splenic $CD8^{-}$ and $CD8^{+}$ DCs are impaired in number[107;108]. For a more complete overview on transcription factors involved in DC homeostasis, see *Merad and Manz*[109].

There are several methods for *in vitro* generation of DCs. A standard method is to culture BM cells in the presence of GM-CSF. These GM-CSF generated BM-DCs are the most common DC type used in studies on mouse and human DC biology. However, GM-CSF generated DCs are not representing the conventional steady-state DC subsets in spleen and LN. GM-CSF preferentially expands 'myeloid' DCs, and moreover, they are described as *in vitro* equivalents of Tip-DCs. This indicates that although GM-CSF is dispensable for steady-state DC development (it is rather

working as a survival factor), it can play a role in DC differentiation during inflammation.

Another way to differentiate DCs from BM precursors is by culturing them with Fms-like tyrosine kinase 3 ligand (Flt3L). In contrast to GM-CSF, Flt3L is the main cytokine both sufficient and required for normal steady state development[110;111]. Flt3L has been shown to be a growth factor for early hematopoietic precursors in the BM[112-114] and induces the expansion and differentiation of all DCs, except LCs *in vivo* and *in vitro*[110;115-117]. Injection of Flt3L leads to massive expansion of DCs and pDCs[115;118], while Flt3L$^{-/-}$ mice have abrogated DC numbers[119]. Flt3L is produced *in vivo* by stromal cells and by activated T cells and is up-regulated during inflammation to ensure sufficient DC production[112]. BM-DCs produced by Flt3L cultures closely resemble the immature steady-state spleen DCs. Despite the lack of CD8 and CD4 expression, CD11c^{+}CD24^{+}CD11b^{low} and CD11c^{+}CD11b^{+}Sirpα^{+} DCs resemble CD8^{+} and CD8^{-} DCs, respectively, in function and phenotype[111]. Therefore, it is to be expected that this Flt3L culture system will replace the GM-CSF culture system as standard for *in vitro* DC studies in the near future.

1.7 HETEROGENEITY IN AG-PRESENTATION BETWEEN SPLENIC DC SUBSETS

As described above, splenic DC subsets differ in development, phenotype and localization. Also their capacity to present Ags and to activate CD4^{+} or CD8^{+} T cells is different. One of the differences between splenic DC subsets is their role in Th cell differentiation. CD8^{+} DCs induce Th_1 T cell responses by the production of large amounts of IL-12, while CD8^{-} DCs do not produce IL-12 and preferentially induce Th_2 responses[120-122]. Next to this, CD8^{+} DCs are more efficient than CD8^{-} DCs than in cross-presentation of cellular Ag[123], soluble Ag[124], latex bead associated Ag[125] and of Ag-coupled to Abs[76;126]. On the other hand, CD8^{-} DCs are more effective than CD8^{+} DCs at presenting exogenous Ags by MHC class II to CD4^{+} T cells for phagocytosed Ag[124;125] and DC targeted Ag[76].

What exactly causes these differences between the DC subsets? A simple explanation would be that differences in cross-presentation are caused by differential Ag-capture; for example, the specific cross-presentation of cellular Ags by CD8^{+} DCs was assumed to be caused by differential uptake of apoptotic cells. Clec9A, for example, is specifically expressed on CD8^{+} DCs and is involved in the recognition of apoptotic

cells[127;128]. Other studies, however, showed no differences in cellular uptake[125], and also the MHC class II presentation of cell-associated Ag, which are equal between $CD8^-$ and $CD8^+$ DCs [*JMM den Haan*, unpublished results] argue against such a differential Ag-uptake. But again, for other Ag-sources, differential Ag-uptake could play a role since multiple putative Ag-receptors, like the C-type lectins DEC205 and DCIR2, are differentially expressed among DC subsets.

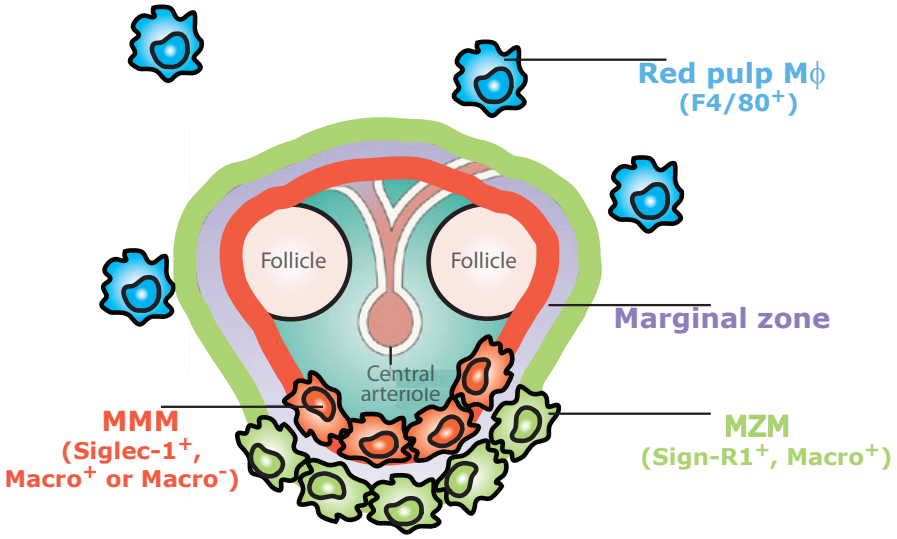
Research by Dudziak *et al.* showed a lower expression of MHC class I or class II components by the $CD8^-$ and $CD8^+$ DCs, respectively[76]. This however cannot completely explain the DC differences. First of all, all DCs express high levels of MHC class I and II, indicating that DCs are intrinsically able to generate class I and class II peptides. $CD8^+$ DCs are very important for MHC class II presentation of cellular Ag[129] whereas cross-presentation of immune-complexed Ags occurred by both DC subsets[130]. Also for endogenous Ag, no differences between DC subsets in MHC class I presentation are observed, indicating that it is not the expression of the MHC class I processing pathway that is insufficient, but rather the ability to process certain Ags that differs between DC subsets.

The efficiency of MHC class I or class II presentation is dependent on the routing of Ag. Differences in Ag-trafficking within the DCs will determine how much of the Ag is delivered into either pathway, and will also determine the degradation of Ags by regulating protease activity[131] and acidification of phagosomes[62]. For cross-presentation, Ag has to be transferred into the cytosol[132] and passaged through the ER[133]. These pathways seem to be constitutively active in $CD8^+$ DCs, while these are less efficient in $CD8^-$ DCs. However, intrinsic differences in cross-presentation of DC subsets can be overcome by particular modes of activation; $CD8^+$ DCs constitutively cross-present while $CD8^-$ DCs require activation by stimuli via $Fc\gamma R$[130], TCRs or T cell help[134]. However, more research is required for a more detailed description of DC subset function.

1.8 THE SPLEEN AS LYMPHOID ORGAN

In this thesis, many aspects of DC subsets within the spleen are described. For better understanding of DC functioning, knowledge about the architecture of the spleen and its function in the immune system is required.

The spleen is the largest lymphoid organ of the body. Histologically,



|Figure 4| Macrophage subsets in the spleen.
The white pulp of the spleen is composed of B cell follicles and T cell areas surrounding a central arteriole. Between the red and white pulp, the marginal zone (MZ) is located. In the MZ, two types of macrophages (Mϕ) are present. Marginal metallophilic macrophages (MMM) are located as a tight network in the inner part of the MZ near the white pulp. The MMM can be identified by the specific expression of Siglec-1. Marginal zone macrophages (MZM) express Sign-R1 and can be found in the outer MZ towards the red pulp. These two types of resident macrophages efficiently phagocytose dead cells, bacteria, and other foreign particles from the blood. In the red pulp, mainly F4/80 expressing red pulp Mϕ can be identified.

the spleen consists of red pulp, a venous sinusoidal system, and of white pulp. The white pulp is the lymphoid region within the spleen and is composed of B cell follicles and T cell areas surrounding a central arteriole. Between the red and white pulp, the MZ is located[135] and since the marginal sinuses are opening there, most of the arterial blood that enters the spleen is running through the MZ, where re-circulating lymphocytes can leave the blood. The MZ itself contains a large number of non-migrating, resident cells[136;137]. These resident cells, such as marginal metallophilic macrophages (MMM), marginal zone macrophages (MZM) and marginal zone B cells, are very potent phagocytic cells that are required for initial blood filtration and uptake of bacteria, red blood cells and other foreign particles from the blood[138]. The relative high frequency of phagocytosing cells indicates the role of the spleen as an important barrier against invading pathogens[139-143].

Of the splenic phagocytic cells, the DC subsets are already extensively described (chapter 1.5), but also Mφ form a very heterogeneous population of cells within the spleen. Splenic Mφ, differ in the expression of PRRs

and in the anatomical location in the spleen, indicating their diversity in immune recognition[144;145;146]. Mφ are involved in both innate and acquired immune responses, by Ag-unspecific phagocytosis and by acting as APC, respectively. Another important function of Mφ is the clearance of apoptotic bodies[147]. In this case, Mφ are not promoting immunity, but are involved in the maintenance of tolerance by down regulation of pro-inflammatory cytokines and the production of IL-10 and TGF-β[148]. One of the Mφ subpopulations in the spleen is the MZM, which is located at the outer border of the MZ (Figure 4). MZM express a whole array of pattern-recognition receptors (like scavenger-receptor A, MARCO and SignR1), but in contrast to other Mφ, MZM do not express MHC class II molecules[149]. It is thought that MZM are involved in clearance of blood-borne microorganisms and particulated Ag[150-152] and do not prime T cells but activate B cells instead[153;154]. Another splenic Mφ subset is the MMM. These cells are situated between the white pulp and the marginal sinus. MMM sample the blood[155] and might play an important role during (viral) infections[156], however the exact function of these MMM is still unknown. MMM express the sialic acid-binding immunoglobulin-like lectin-1 (Siglec-1, Sialoadhesion, CD169), an antigen (Ag) recognized by the monoclonal antibody MOMA-1[157;158;159]. Siglec-1 is a receptor thought to be involved in cell-cell interactions[160], in modulating T cell responses, and to serve as a phagocytotic receptor for sialyated pathogens[161]. Sialic acids are up-regulated by apoptotic cells[162], suggesting that MMM maybe are involved in the removal of apoptotic cells from the blood[163;164].

1.9 THESIS OUTLINE

Splenic DC subsets in the mouse spleen show functional and homeostatic differences. The scope of this research was to investigate these differences between DC subsets in more detail. The studies described in this thesis not only focus on Ag-presentation by $CD8^+$ and $CD8^-$ DCs, but also on questions concerning Ag-transfer between DCs and splenic Mφ, and questions concerning migration of DCs within the spleen were addressed.

In **chapter 2** of this thesis we studied the role of SIRPα in DC homeostasis. Mice deficient in functional SIRPα have reduced numbers of $CD8^-$ DCs in the spleen. We observed defective integrin mediated adhesion and migration in DCs with deficient SIRPα function and hypothesize that the decrease in $CD8^-$ DC numbers is due to inadequate migration towards the MZ and bridging channels of the spleen, which is

required for survival of $CD8^-$ DCs *in vivo*.

It has been previously shown that the $CD8^+$ DC subset specifically cross-present cell-associated antigens in the context of MHC class I to cytotoxic T cells. In **chapter 3** we determined whether other types of antigens, such as *Saccharomyces cerevisiae* encoded antigens, were also preferentially cross-presentated by this DC subset. Phagocytosis of *S. cerevisea* was mediated by recognition of yeast wall β-glucans by the dectin-1 receptor on both $CD8^+$ and $CD8^-$ DCs, resulting in presentation of yeast-derived antigen in MHC class II to $CD4^+$ T cells by both DC subsets. Surprisingly, cross-presentation of *S. cerevisea* was performed preferentially by the $CD8^-$ DC subset. In addition, IL-10, TNFα and IL-23 production in response to yeast was restricted to $CD8^-$ DCs, implicating that $CD8^+$ and $CD8^-$ DC subsets have different functions in immune responses depending on the type of Ag encountered.

Chapter 4 describes the role of phagosome acidification in Ag-presentation by DCs. We observed that Ag cross-presentation of several types of Ag was depending on the production of reactive oxygen species (ROS).

To further investigate the function of $CD8^+$ and $CD8^-$ DCs in the activation of T cells, we biochemically coupled OVA to antibodies specific for $CD8^+$ or $CD8^-$ DCs or for splenic macrophages. In **chapter 5**, we used these complexes to target DC subsets and macrophages *in vivo*. We observed that targeting MMMs resulted in cross-priming of $CD8^+$ T cells. This was due to specific transfer of Ags to $CD8^+$ DCs, indicating that different APCs can work together to induce effective immunity.

Finally, in **chapter 6**, the findings described in this thesis are summarized and discussed in the context of recent developments in DC research.

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