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Murine spleen contains a diversity of myeloid and dendritic cells distinct in antigen presenting function

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Abstract

The spleen contains multiple subsets of myeloid and dendritic cells (DC). DC are important antigen presenting cells (APC) which induce and control the adaptive immune response. They are cells specialized for antigen capture, processing and presentation to naïve T cells. However, DC are a heterogeneous population and each subset differs subtly in phenotype, function and location. Similarly, myeloid cell subsets can be distinguished which can also play an important role in the regulation of immunity. This review aims to characterize splenic subsets of DC and myeloid cells to better understand their individual roles in the immune response.

Keywords: dendritic cell • myeloid cell • antigen presentation

Introduction

The spleen is a secondary lymphoid organ specialized for filtering blood-borne antigens and removal of old or damaged erythrocytes. It comprises two morphologically and functionally distinct compartments (Fig. 1). The red pulp comprises an extensive network of cords containing venous sinuses that act as filters to trap old or damage erythrocytes that are phagocytosed by red pulp macrophages [1]. The white pulp is involved mainly with initiation of immune responses against blood-borne antigens and pathogens. It comprises three regions: the T cell zone or periarteriolar sheath (PALS), B cell follicles, and the marginal zone [2]. The PALS is further divided into inner PALS comprising mainly $CD4^+$ T cells, some $CD8^+$ T cells, interdigitating DC and migrating B cells. The outer PALS contains macrophages [3]. B cell follicles are continuous with PALS and comprise B cells, $CD4^+$ T cells and follicular DC which have a distinct mesenchymal origin [3]. The marginal zone is strategically situated at the interface of the red pulp and PALS for screening blood-borne antigens and pathogens. It contains a large reservoir of resident cells that participate in mounting an adaptive response against blood-borne antigens. Several common subsets of DC in spleen have been well characterized, along with a number of distinct macrophage/monocyte cell types. Other DC subsets are less well known. The functional importance of all subsets of DC and macrophages/monocytes in spleen is still under investigation in terms of their comparative roles in antigen presentation.

Common DC subsets in murine spleen

While DC are the most efficient APC in the immune system, with unique ability to activate naïve T cells, they are closely aligned with myeloid cell types which can also function as APC. Multiple subsets have been identified in both humans and mice [4, 5]. However, DC

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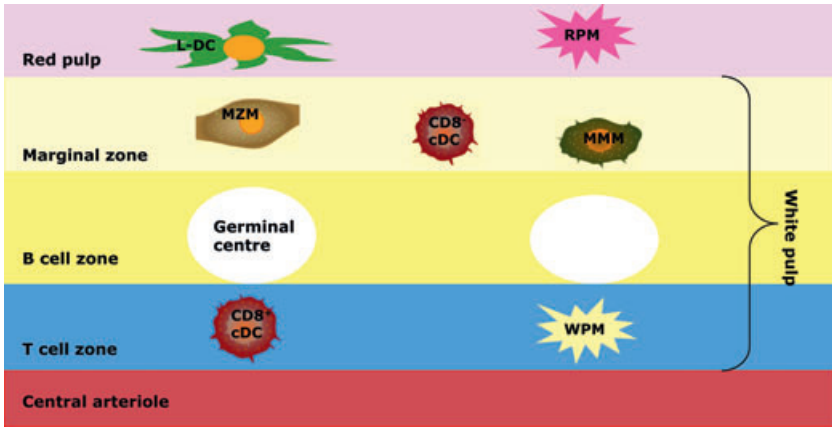

Fig. 1 Compartmentalization of antigen presenting cells (APC) within splenic red and white pulp regions. Both $CD8^+$cDC and white pulp macrophages (WPM) lie in the T cell zone, whereas marginal-zone metallophilic macrophages (MMM), marginal-zone macrophages (MZM) and $CD8^-$ cDC lie within the marginal zone. Red pulp macrophages (RMP) are localized in the red pulp. The tentative location of L-DC within the red pulp region is based on experimental verification of high accessibility for blood-borne antigen compared with other DC subsets.

are unique in that they are capable of antigen uptake, processing and presentation to naïve T cells. They are a heterogeneous class of cells with subtypes differing in tissue location, migratory pathway, cell surface marker expression, immunological function and dependence on infections or inflammatory stimuli for their generation [4]. They are widely distributed throughout the body and distinct subsets have been described in spleen, mucosa, intestine and epidermal tissue [4]. Some DC have been classified as migratory and these appear to survey the environment by constant uptake of tissue antigens. In the presence of pathogen-related 'danger signals', they mature and migrate to lymph nodes (LN) to present antigens to T cells [6]. In contrast, lymphoid tissue-resident DC do not migrate, but take up and present incoming antigen to T cells [6].

While the first observation of murine spleen DC was reported by Steinman in 1973 [7], tools to isolate and study the population in detail were not available until 1982 when the first DC-specific monoclonal antibody was isolated [7, 8]. Now with multiple antibodies and high-speed flow cytometry, the isolation and characterization of splenic DC is a common procedure. Conventional DC (cDC) represent the main DC subset in spleen and have been further classified into $CD8\alpha^+$ and $CD8\alpha^-$ subsets. $CD8\alpha^+$ cDC are phenotypically distinct as $CD11c^+CD11b^-CD8\alpha^+MHCII^+B220^-$ cells, whereas $CD8\alpha^-$ cDC are $CD11c^+CD11b^+CD8^-MHCII^+B220^-$ cells [9] (Table 1). These subsets differ in immune function, including cytokine production and ability to cross-present antigen [10]. The mechanism of cross-presentation is considered in a later section on

Table 1 The phenotype and T cell activation capacity of dendritic and myeloid subsets in murine spleen

Subset	Phenotype	T cell activation*		
		Th	Tc	Tc:CP
$CD8^+$ cDC	$CD11b^-CD11c^{hi}MHCII^+CD8^+Ly6C^-Ly6G^-$	++	+++	+++
$CD8^-$ cDC	$CD11b^{lo}CD11c^{hi}MHCII^+CD8^-Ly6C^-Ly6G^-$	+++	+++	+
pDC	$CD11b^-CD11c^+MHCII^{lo}CD8^{+/-}Ly6C^{hi}Ly6G^-$	+	+	+
Tip-DC	$CD11b^{hi}CD11c^{inter}MHCII^+MAC\text{-}3^+$	++	++	–
L-DC	$CD11b^{hi}CD11c^{lo}MHCII^-CD8^-Ly6C^-Ly6G^-$	–	+	+++
Mo-DC	$CD11b^{hi}CD172\alpha^+Ly6C^-Ly6G^-F4/80^+CD24^+CD115^-DC\text{-}SIGN^+$	+++	+++	+++
Inflammatory monocytes	$CD11b^{hi}CD11c^-MHCII^-CD8^-Ly6C^{hi}Ly6G^-F4/80^+CD115^+DC\text{-}SIGN^-$	–	–	–
Macrophages	$CD11b^{hi}CD11c^-MHCII^-CD8^-Ly6C^{lo}Ly6G^-F4/80^+$	–	+	+
Neutrophils	$CD11b^{hi}CD11c^-MHCII^-CD8^-Ly6C^+Ly6G^+F4/80^-$	–	+	+

*Th, helper T cell; Tc, cytotoxic T cell; Tc:CP, Tc cross-primed.

the role of DC in antigen presentation. It has been proposed that $CD8\alpha^+$ cDC play a role in the maintenance of tolerance to self-antigens, consistent with their close proximity to T cells in the resting state, and their notable capability for cross-presentation [11, 12]. $CD8\alpha^+$ cDC are also the predominant producer of IL-12, important for $CD8^+$ T cell proliferation [4]. In addition, they have recently been described as a major producer of IFN-λ in response Toll-like receptor (TLR) 3 stimulation with double-stranded RNA [13]. In contrast, $CD8\alpha^-$ cDC have weaker cross-priming ability and are mainly localized in the marginal zone of spleen [11]. Upon stimulation with lipopolysaccharides, $CD8\alpha^-$ cDC migrate to T cell areas and secrete inflammatory chemokines [11]. The primary role of $CD8\alpha^-$ cDC, however, still remains unclear [5].

Another common DC subset in spleen is the plasmacytoid DC (pDC). Their history dates back to the first study on interferon and its role in viral interference [14]. Numerous groups over 50 years have investigated and identified a subset of cells, specialized for production of interferon in viral infection [15]. However, over time each group named the subset differently, *e.g.* natural IFN producing cells [16], plasmacytoid T cells [17], plasmacytoid monocytes [18] and DC2 [19]. It was only in 1999 that it was determined that natural interferon producing cells were found to a DC subset, namely the pDC [18, 20]. Plasmacytoid DC exist as a plasmacytoid pre-DC (p-pre-DC) in the steady-state. They are relatively long-lived, circulating cells, which produce high levels of type I interferon after stimulation with viral or other microbial agents [21]. Plasmacytoid DC like cDC have been described to arise from common dendritic progenitors (CDP) [22]. Inflammatory stimuli initiate the conversion of p-pre-DC into pDC, and the production of type I interferons which enhance the function of NK cells, B cells, T cells and DC during antiviral responses. Subsequently, pDC differentiate to give the $CD8^+CD205^-$ DC subset, distinct from $CD8^+$ cDC that also regulate T cell function [23]. Plasmacytoid DC are mainly distinguished by their $CD11c^{lo}CD11b^-B220^+$ phenotype and production of type I interferons in response to TLR7 and TLR9 signalling [6, 24] (Table 1).

DC represent a loosely defined class of cells either detectable in spleen, or derived *in vitro* from myeloid precursors. Monocyte-derived DC (mo-DC) represent a distinct subset derived from monocytes in the presence of inflammatory factors like GM-CSF and TNF-α [25, 26]. They can be induced by *in vitro* culture of monocytes, and have also been demonstrated *in vivo* when inflammatory states lead to the exit of monocytes into tissue from blood with induction of DC properties [25–27]. Until recently it has been difficult to distinguish mo-DC from cDC *in vivo* using the common markers of DC. They have a distinct phenotype as $CD11b^+CD11c^{lo}$ $F4/80^{lo}CD172\alpha^+$MHC-II$^+$Gr1$^-$CD115$^-$ cells (Table 1), distinguishing them from both cDC and macrophages/monocytes. Recently DC-SIGN/CD209 was identified as a specific marker of *in vivo* and *in vitro* derived mo-DC [28]. They also demonstrate comparable antigen presenting function to cDC, being capable of cross-presentation to $CD8^+$ T cells [29]. However, mo-DC only develop under inflammatory conditions, commonly by LPS stimulation *in vitro*, or by *in vitro* culture of myeloid precursors or monocytes with inflammatory factors like GM-CSF and TNF-α.

TNF/iNOS-producing DC (TipDC) can also be identified in spleen. Inflammatory stimuli appear to recruit circulating $CD115^+$Ly6-$C^{hi}CCR2^+$ inflammatory monocytes from blood into spleen and lymph nodes where they differentiate to give TipDC [30, 31]. TipDC can be distinguished from steady-state cDC on the basis of their unique phenotype as $CD11b^{hi}CD11c^{inter}$MAC-3^{hi}MHCII$^+$ cells [32] (Table 1). In general, TipDC secrete high levels of TNF-α and nitric oxide to induce clearance of microbes from tissues.

Regulatory DC (DCreg) are also characterized by a common myeloid $CD11c^{-/lo}CD11b^+$MHCII$^{-/lo}$ phenotype with ability to suppress $CD4^+$ T cell activity [33]. Some DCreg have also been found to induce the differentiation of T helper $(h)_1$ cells into regulatory T cells (Treg) through production of IL-10 and TNF-β [34]. Subsequently, Tregs can suppress $CD4^+$ T cell proliferation through direct contact [34]. The *in vivo* counterpart of DCregs has been reported [35, 36], although these cells did not induce the formation of Tregs. Instead, suppression of $CD4^+$ T cells was mediated by DCreg secretion of high levels of nitric oxide (NO). Indeed, the DCreg cell type is still poorly defined and likely represents a heterogeneous class of cells, some of which derive by further differentiation of mature DC.

Common macrophage/monocyte subsets in murine spleen

Monocytes are phagocytic cells of the myeloid lineage present in several organs including bone marrow (BM), blood and spleen. During inflammation, they are recruited into bloodstream and tissues. Monocytes develop in BM from macrophage/dendritic cell progenitors (MDP) [37], and continuously migrate into blood by a CX_3CL1-driven mechanism [30]. When blood monocytes enter tissues they terminally differentiate to give macrophages. Monocyte-derived DC may also develop in lymphoid sites under inflammation induced by pathogen invasion [38]. Monocytes are distinct from granulocytes by their $F4/80^{lo}CD115^+$ phenotype and from lymphocytes by $CD11b^{hi}CD11c^-$ expression [30]. In general, two subsets of monocytes can be identified in blood: the classical $CD115^+$Ly6C^{hi} inflammatory monocyte and the non-classical $CD115^+$Ly6C^{lo} resident monocyte [30]. Inflammatory monocytes home to sites of infection where they differentiate to give TipDC [39], whereas resident monocytes home to non-inflammatory sites and are thought to be precursors of macrophages resident in steady-state normal tissues like liver and spleen [39]. The current model is that monocytes either terminally differentiate to give macrophages, or become TipDC upon entry into tissues. However, Swirski and coworkers recently identified a reservoir of undifferentiated monocytes resident in spleen which resemble classical blood monocytes phenotypically, morphologically and transcriptomically [40]. Upon inflammation, both these and resident monocytes were mobilized from spleen to sites of inflammation. Classical monocytes from spleen were involved in clearing damaged tissues, while resident monocytes promoted wound healing [40]. In addition to this, spleen was able to deploy large numbers of monocytes to a site of injury faster than BM [40]. Hence, the deployment of

a reservoir of splenic monocytes has been hypothesized as a mechanism facilitating a faster immune response.

Four subsets of macrophages have been described in spleen. Two of these are located within the marginal zone; namely, marginal-zone metallophilic macrophages (MMM) and marginal-zone macrophages (MZM) [3]. MMM can be visualized by staining with the MOMA-1 monoclonal antibody, and localize near the PALS and the B cell follicles [3]. While the functional potential of MMM has not been completely elucidated, they appear to concentrate blood-borne pathogens in the marginal zone *via* SIGLEC1 that interacts with sialic-acid residues on pathogens [3]. Subsequently, clearance of pathogens occurs through SIGLEC- and LPS-mediated phagocytosis. The MZM are located closer to the red pulp and are characterized by SIGNR1 expression [3]. MZM express a number of pattern recognition receptors like TLR, type 1 scavenger receptor, MARCO and the C-type lectin, SIGNR1, all of which play an important role in the clearance of microorganism [41]. SIGNR1 efficiently binds polysaccharide antigen on microorganisms like *Mycobacterium tuberculosis* [41], and can also bind antigens expressed on viruses [42], so emphasizing a role for MZM in viral clearance. In contrast, MARCO expressed by MMM mainly binds antigen presented on pathogenic bacteria like *Escherichia coli* and *Staphylococcus aureus* [43]. The tangible body macrophages within the splenic white pulp can be distinguished from other macrophages by expression of CD68 ($CD11b^{-}F4/80^{-}CD68^{+}$) [44] (Table 1), and their involvement in the phagocytosis of apoptotic B cells generated during germinal centre reactions. The red pulp macrophages have distinct F4/80 marker expression ($CD11b^{+}F4/80^{+}CD68^{+}$), distinguishing them from MMM, MZM and tangible body macrophages, all of which are $F4/80^{-}$ [44] (Table 1). Red pulp macrophages are involved in the clearance of old or damaged RBC and the recycling of heme groups.

Delineation of DC progenitors

Haematopoiesis occurs in BM where haematopoietic stem cells (HSC) give rise to multipotent progenitors (MPP), which in turn generate a range of lineage progenitor termed CLP (common lymphoid progenitors) and CMP (common myeloid progenitors). CLP give rise to a range of cells including NK (natural killer) cells, and pro-T and pro-B cells that develop to give T and B cells respectively. The CMP is thought to be a direct precursor of MDP [45] that in turn generate CDP [37, 46–48]. Recent studies claims that MDP generate cDC and monocytes which in turn develop to give macrophages in tissues. The downstream CDP exclusively generates cDC and pDC [37, 47]. However, there is still some uncertainty over the relationship between these subsets, and particularly between CMP and MDP with CDP. MDP are a progenitor of cDC as they can replenish splenic DC without formation of a monocytic intermediate [47]. These experiments utilized BM chimaeras generated by reconstitution of lethally irradiated wild-type mice with BM from CD11c-diphtheria toxin (DTx) mice. Irradiation depleted MDP from the host, whereas DTx treatment depleted macrophages and DC from donor BM. However, it is still questionable whether the replenishment of splenic DC occurred from MDP in donor BM or from host monocytes under the inflammatory state.

While monocytes and p-pre-DC exit BM and enter blood as mature cells, cDC leave BM and enter blood as pre-cDC precursors that further differentiate and divide in spleen and other lymphoid tissues [49]. There is also evidence that $CD11c^{+}MHCII^{-}Ly6C^{+}B220^{+}$ precursors exist in BM, which can give rise to pDC on adoptive transfer [45]. The $B220^{-}$ variant of this $CD11c^{+}MHCII^{-}Ly6C^{+}$ precursor also gives rise to $CD8\alpha^{-}$ cDC [38]. In blood, distinct precursors for cDC and pDC have been described as $CD11c^{inter}CD11b^{+}B220^{-}$ and $CD11c^{lo}CD11b^{-}B220^{hi}$ cells respectively [46]. More recently, an immediate precursor of cDC (pre-cDC) with a $CD11c^{+}CD43^{+}SIRP\text{-}\alpha^{+}$ phenotype and restricted cDC lineage potential was identified in spleen, blood and BM [37, 49]. Pre-cDC are clearly distinct from monocytes based on phenotype, morphology and immune function as $CD11c^{inter}CD11b^{lo}F4/80^{lo}SIRP\text{-}\alpha^{inter}$ cells, while monocytes are clearly distinct as $CD11c^{-}CD11b^{hi}F4/80^{hi}SIRP\text{-}\alpha^{inter}$ cells [49]. Pre-cDC also differ from monocytes in that the latter can differentiate in response to M-CSF in culture [49]. Plasmacytoid DC develop from $CD11c^{lo}B220^{+}$ circulating immediate precursors (p-pre-DC) in blood that lodge in spleen under inflammatory conditions to produce pDC [46].

Role of DC in antigen presentation

Dendritic cells constantly endocytose antigens from the environment for presentation to T cells. Exogenous antigens are phagocytosed or pinocytosed by DC, processed and presented on MHC-II to $CD4^{+}$ T cells. Lysosome-containing proteolytic enzymes fuse with phagosomes carrying exogenous antigens to cleave antigen into peptides. Newly synthesized MHC-II molecules are delivered by vesicular transport to phagolysosomes containing peptides. Peptides are then loaded on to MHC-II molecules for delivery to the cell membrane and subsequent presentation to $CD4^{+}$ T cells [50]. Activated $CD4^{+}$ T cells differentiate to become effector T cells that produce cytokines and activate monocytes to control infection. By contrast, antigens produced endogenously by infected cells, or defective ribosomal products, are tagged with ubiquitin for destruction in the cytoplasm [51]. Ubiqitinated proteins are then proteolytically cleaved into peptides. The transporter associated with antigen processing (TAP) selectively allows peptides to enter the endoplasmic reticulum (ER) where they are loaded on to MHC-I molecules [50]. The MHC-I peptide complex is then transported to the cell membrane within vesicles for antigen presentation to $CD8^{+}$ cytotoxic T cells.

Some DC subsets can 'cross-present' exogenous antigens acquired from dead tumour cells or virally infected cells on to MHC-I molecules for presentation to $CD8^{+}$ T cells [51, 52]. Cross-presentation is a function largely restricted to $CD8\alpha^{+}$ cDC [53–56]. The exact mechanism by which exogenous antigens gain access to newly synthesized MHC-I molecules remains under investigation. One model involves ER-phagosome fusion. According to this model, the presence of ER-derived components in phagosomes allows the ER dislocon (a protein transporter), to participate in cross-presenta-

tion at the phagosomal membrane by allowing translocation of peptides from phagosomes into the cytosol [57]. Another model involves peptides leaking from phagosomes into cytoplasm, or peptides escaping into cytoplasm through rupture of the phagosomal membrane, and becoming loaded on to MHC class I molecules outside the ER [58]. While other cell types like neutrophils [59] and monocytes/macrophages [60, 61] are thought to function in cross-presentation, these earlier reports remain unsubstantiated. Indeed, definitive information on antigen presenting function is very dependent on clear subset definition and isolation of pure subsets of cells.

In vitro culture systems for generation of dendritic and myeloid cells

Since DC are a rare cell type, *in vitro* culture systems that generate large numbers of DC have therefore been popular for studies of function. Recently, two culture systems were described for generation of DC with distinct function [62]. The first utilizes a cytokine cocktail of granulocyte macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α and interleukin (IL)-4 which stimulates myeloid precursors or monocytes to give mo-DC [62, 63]. The second utilizes Flt3L, a cytokine which stimulates proliferation and differentiation of BM-derived pre-DC to give cDC and pDC [62, 64, 65]. Indeed IL-4 alone can induce blood monocytes to differentiate into immature DC, and these cells have very similar phenotype to GM-CSF/IL-4 derived immature DC [66]. The GM-CSF/IL-4 culture system is an attractive model for generating large numbers of DC. However, it produces specifically mo-DC which express CD11c, MHC-II and other properties reflective of DC generated under inflammatory conditions. They do not reflect steady-state cDC or immature DC. Evidence that GM-CSF levels are low in resting mice but increase dramatically during inflammation is consistent with cDC and pDC being present in normal levels in spleens of *GM-CSF*$^{-/-}$ mice [67].

FMS-related tyrosine kinase 3 ligand (Flt3-L) is a known proliferative factor for DC [68], and Flt3-L supplemented BM cultures have been shown to support production of both pDC and cDC [69]. Flt3-L has also been described as a growth factor for haematopoietic progenitors and mobilizes haematopoietic progenitors and stem cells *in vivo* [70]. *Flt3-L*$^{-/-}$ mutant mice demonstrate a lower DC number in spleen, thymus and lymph nodes, emphasizing the importance of Flt3L in DC development [71]. Recently, Naik *et al.* [2005] showed that *in vitro* grown Flt3L-induced DC resemble with the *in vivo* subsets of CD8α^+ cDC and CD8α^- cDC in spleen. Phenotypes of CD24^{hi}CD11b^{lo}CD172α^{lo} and CD24^{lo}CD11b^{hi}CD172α^{hi} correspond with CD8α^+ cDC and CD8α^- cDC present in murine spleen [49, 65].

The Flt3L culture system contrasts with the GM-CSF/IL4 culture system in that it generates steady-state DC instead of inflammatory DC. Indeed, GM-CSF-induced DC and Flt3L-induced DC appear to derive from distinct precursors [62]. When the CD11b^{hi} Ly6C$^+$Ly6G$^-$ monocyte population in BM was cultured with GM-CSF, this gave rise to CD11b^{hi}CD11c^{inter}Mac-3$^+$MHC-II$^+$ DC, identical with TipDC [39], and no DC production was induced upon culture with Flt3-L. Inflammatory monocytes in BM are precursors of GM-CSF-induced DC, and GM-CSF-induced DC may be the *in vitro* counterpart of TipDC. TipDC [32, 72] and mo-DC [26, 39] populations have been shown to exist *in vivo*, but their full phenotype and function are not yet fully characterized. The conditions under which they form, and their specific role in immunity compared with cDC and pDC are yet to be distinguished.

As with DC, macrophages are present in low numbers *in vivo*, which hampers *in vivo* studies and cell isolation. Macrophages can be readily isolated from peritoneal cavity or lung by lavage. A culture system was developed for increased cell production. This system is very similar to the DC culture system, although different cytokines are used to induce cell production. Macrophages differentiate from BM progenitors upon addition of macrophage colony-stimulating factor (M-CSF). Bone marrow-derived macrophages have been deemed naïve, reflecting steady-state macrophages, and these are phenotypically distinct from described subsets of inflammatory monocytes which have the phenotype CD11b$^+$CD11c$^-$MHC-II$^-$Ly6C^{hi}Ly6G$^-$F4/80$^+$ [30, 39].

Long-term spleen cultures also produce dendritic-like cells

A distinct dendritic-like cell type has been described in this laboratory which is produced *in vitro* in murine splenic long-term cultures (LTC) [73]. These 'LTC-DC' develop from spleen-derived progenitors maintained within murine splenic stromal cultures [74]. Splenic stroma maintained within LTC supports the continuous, but restricted development of only this cell type without addition of cytokines like GM-CSF, TNF-α, IL-4 or Flt3L. The continuous production of cells within LTC suggests the presence of self-renewing progenitor or stem cells within the stromal layer [75]. Recent studies have confirmed that progenitors are maintained in LTC by endothelial-like cells unique to spleen [76]. Haematopoietic cells form foci of proliferating cells above the stroma, and continuously shed dendritic-like cells into the medium from where they can be collected for study.

Non-adherent cells collected from LTC comprise a minor population of small precursors/progenitors and a major population of large cells that resemble immature splenic DC [77]. The non-adherent small cells also represent a heterogeneous population of cells expressing markers of haematopoietic progenitors like cKit, Sca-1 and Thy-1, with subsets of cells expressing CD11b, CD11c, CD117 and CD80, along with weak endocytic ability [77]. Indeed LTC-DC production was achieved when the sorted small cell population was cultured over a splenic stromal cell line STX3, derived from a LTC that had lost haematopoietic cells with passage over time and hence demonstrating the presence of self-renewing progenitors within the small cell population [75]. In contrast, the large non-adherent LTC-DC produced reflect a subset of immature myeloid splenic DC on the basis of cell surface phenotype as CD11c^{lo}CD11b^{hi}MHC-II$^-$CD8α^-MHCI$^+$CD80$^+$CD86$^+$ cells [77]. The absence of MHC-II or CD40 expression is a characteristic of these cells, also consistent with their

derivation independently of pathogens or inflammatory cytokines. In this respect, LTC-DC differ from MHC-II$^+$CD40$^+$ mature DC generated from monocytes cultured in GM-CSF and IL-4 [62, 63], or cDC and pDC derived from BM precursors cultured with Flt3L [62, 64, 65].

LTC-DC have now been shown to have cross-priming capability [68]. When compared with freshly isolated CD11c$^+$ cells from spleen, LTC-DC are superior in activating CD8$^+$ T cells in an antigen-specific manner [68]. LTC-DC do not (or only weakly) activate CD4$^+$ T cells, consistent with their MHC-II$^{-/lo}$ phenotype. *In vitro* derived LTC-DC are highly efficient in cross-presentation, yet they have a distinct marker expression and function which distinguishes them from other DC subsets described in spleen, or derived by cytokine-induced culture of DC precursors [68].

Recently, an *in vivo* equivalent subset to LTC-DC, namely 'L-DC', was identified in murine spleen based on its common phenotype, its cross-priming capability and its inability to activate CD4$^+$ T cells [68]. L-DC are large cells, which express the same CD11c^{lo} CD11b^{hi}CD8$^-$MHC-II$^-$ phenotype as LTC-DC (Table 1). The *in vitro* cross-priming capability of L-DC was found to be comparable with that of CD8$^+$cDC [4, 68]. Like CD8$^+$cDC, cross-presentation increases after LPS activation [68]. Evidence of an *in vivo* cell type equivalent to LTC-DC gives some physiological relevance to *in vitro* derived LTC-DC and L-DC. The possibility that spleen contains an endogenously derived APC with function specific to the spleen environment with exposure to blood-borne pathogens is under further consideration.

Preliminary studies have also shown that L-DC are more poised than other cDC or myeloid subsets in spleen for uptake of antigens in blood. Hence, they have been tentatively located in the red pulp region of spleen as shown in Figure 1. *In vivo* antigen priming prior to isolation of APC subsets, followed by *in vitro* assays of T cell activation, indicated that L-DC were superior to CD8α^+ cDC and monocytes in uptake and presentation of intravenous antigen [68]. L-DC have also been shown capable of cross-priming CD8$^+$ T cells in adoptive transfer experiments [68], and may therefore represent an important cross-presenting subset in spleen, especially under inflammatory conditions. To date, they have been identified as cells within the CD11b^{hi}CD11c^{lo}CD8α^-MHC-II$^-$FSC^{hi} splenic subset, which encompasses several subsets including those differing in expression of Ly6C. Further characterization of L-DC is therefore underway, using a broad range of dendritic, haematopoietic and myeloid markers to more definitively characterize the L-DC phenotype and its relationship with known splenic DC and myeloid cell subsets.

Localization and functional role of dendritic and myeloid subsets in spleen

Our hypothesis is that L-DC represent a spleen-endogenous, steady-state DC. It will be imperative to review the splenic location of L-DC in relation to other DC and myeloid cell types. In spleen, CD8α^- cDC are strategically located within the marginal zone where they endocytose antigen in blood carried from the terminating capillaries [3] (Fig. 1). In contrast, CD8α^+ cDC are located within the PALS in direct contact with T cells [3] (Fig. 1) consistent with their cross-priming function [4]. Others have shown that CD8α^+ cDC also internalize apoptotic cells *in vivo* [78, 79]. These CD8α^+ cDC could also be derived from CD8α^- cDC that have up-regulated CD8α expression upon migration into PALS. On the basic of phenotype, L-DC are thought to localize within the red pulp region in close proximity with blood vessels, based on their superior ability to endocytose blood-borne antigen for presentation to CD8$^+$ T cells (Fig. 1).

While DC subsets within splenic lymphoid compartments have been defined in terms of function and location, the complete pathway for antigen uptake and presentation by DC still remains unclear. It is not clear if CD8α^- cDC in the marginal zone endocytose blood-borne antigen then transfer it to CD8α^+ cDC in the PALS for effector T cell activation, or whether CD8α^- cDC directly cross-prime T cells in the white pulp [80]. Another possible explanation is that CD8α^+ cDC phagocytose dead/dying antigen-loaded CD8α^- cDC and then migrate to the PALS. The antigen collection and presentation pathway in spleen still remains an enigma, and any role for L-DC in antigen handling in spleen is equally enigmatic.

Granulocytes in spleen are mainly localized in red pulp, but some exist in transition within the marginal zone as blood-borne cells flowing through marginal zone into red pulp [81]. In spleen, the most common granulocyte is the neutrophil [81]. They are key components of the inflammatory response, recruiting APC, transporting antigen and controlling T cell expansion and differentiation [59]. They can be distinguished from other leucocytes based on their phenotype as CD11b^{hi}CD11c$^-$Ly6G$^+$7/4$^+$F4/80$^-$ MHCII$^-$ cells [59, 82] (Table 1). Recently, it was shown that neutrophils from inflammatory peritoneal exudates could cross-prime CD8$^+$ T cells both *in vivo* and *in vitro* [59, 83]. These studies demonstrated cross-priming capability under inflammatory conditions which may not be reflective of steady-state neutrophils in spleen [84]. The L-DC subset has already been investigated in terms of its relationship with monocytes, macrophages and granulocytes. Absence of the marker Ly6G, clearly distinguishes them from granulocytes. Absence of macrophage markers like MAC-3, MOMA-1 and SIGNR1 distinguishes them from splenic macrophages (data in preparation). L-DC are clearly a distinct subset of myeloid dendritic-like cells worthy of further investigation.

Conclusion

Spleen contains a diversity of DC and myeloid types which are both phenotypically and functionally distinct. In addition to the well-defined subsets, there are several smaller and less well-known subsets. The current system of identifying subsets based on cell surface marker phenotype obtained using flow cytometry has limitations, and their function as an APC is another important consideration in terms of lineage origin. The main cDC and myeloid subsets are replenished by precursors from bone marrow that seed into the spleen. However, spleen also produces an endogenously derived APC subset distinct from previously described splenic DC. This L-DC subset is a functionally distinct APC which participates in induction of cytotoxic T cells but not helper T cell responses. L-DC are thought to participate in

immune responses against blood-borne antigen based on their superior capability to endocytose and cross-present antigen by comparison with cDC.

L-DC phenotype reflects a myeloid dendritic-like cell based on CD11b expression. It is clearly distinct from mo-DC in that it lacks MHC-II expression. In addition, L-DC exist in the steady-state, whereas mo-DC require addition inflammatory conditions and stimuli such as LPS to develop. Indeed future studies will be required to determine the lineage of L-DC in relation with other myeloid and DC subsets.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

References

1. **Adema GJ.** Dendritic cells from bench to bedside and back. *Immunol Lett*. 2009; 122: 135–7.
2. **Cesta MF.** Normal structure, function, and histology of the spleen. *Toxicol Pathol*. 2006; 34: 455–65.
3. **Mebius RE, Kraal G.** Structure and function of the spleen. *Nat Rev Immunol*. 2005; 5: 606–16.
4. **Heath WR, Belz GT, Behrens GMN, *et al.*** Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev*. 2004; 199: 9–26.
5. **Naik SH.** Demystifying the development of dendritic cell subtypes, a little. *Immunol Cell Biol*. 2008; 86: 439–52.
6. **Shortman K, Naik SH.** Steady-state and inflammatory dendritic-cell development. *Nat Rev*. 2007; 7: 19–30.
7. **Steinman RM, Cohn ZA.** Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973; 137: 1142–62.
8. **Nussenzweig MC, Steinman RM, Witmer MD, *et al.*** A monoclonal antibody specific for mouse dendritic cells. *Proc Natl Acad Sci USA*. 1982; 79: 161–5.
9. **Vremec D, Pooley J, Hochrein H, *et al.*** CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol*. 2000; 164: 2978–86.
10. **Hochrein H, Shortman K, Vremec D, *et al.*** Differential production of IL-12, IFN-α, and IFN-β by mouse dendritic cell subsets. *J Immunol*. 2001; 166: 5448–55.
11. **De Smedt T, Pajak B, Muraille E, *et al.*** Regulation of dendritic cell numbers and maturation by lipopolysaccharide *in vivo*. *J Exp Med*. 1996; 184: 1413–24.
12. **Steinman RM, Pack M, Inaba K.** Dendritic cells in the T-cell areas of lymphoid organs. *Immunol Rev*. 1997; 156: 25–37.
13. **Lauterbach H, Bathke B, Gilles S, *et al.*** Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *J Exp Med*. 2010; 207: 2703–17.
14. **Isaacs A, Lindenmann J.** Virus interference. 1. The interferon. *Proc R Soc Biol*. 1957; 147: 258–67.
15. **Fitzgerald-Bocarsly P, Dai J, Singh S.** Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine Growth Factor Rev*. 2008; 19: 3–19.
16. **Ronnblom L, Ramstedt U, Alm GV.** Properties of human natural interferon-producing cells stimulated by tumor cell lines. *Eur J Immunol*. 1983; 13: 471–6.
17. **Facchetti F, De Wolf-Peeters C, Mason DY, *et al.*** Plasmacytoid T cells Immunohistochemical evidence for their monocyte/macrophage origin. *Am J Path*. 1988; 133: 15–21.
18. **Cella M, Jarrossay D, Facchetti F, *et al.*** Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med*. 1999; 5: 919–23.
19. **Grouard G, Rissoan MC, Filgueira L, *et al.*** The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med*. 1997; 185: 1101–11.
20. **Siegal FP, Kadowaki N, Shodell M, *et al.*** The nature of the principal Type 1 interferon-producing cells in human blood. *Science*. 1999; 284: 1835–7.
21. **Pelayo R, Hirose J, Huang J, *et al.*** Derivation of 2 categories of plasmacytoid dendritic cells in murine bone marrow. *Blood*. 2005; 105: 4407–15.
22. **Onai N, Obata-Onai A, Schmid MA, *et al.*** Identification of clonogenic common Flt3 +M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol*. 2007; 8: 1207–16.
23. **O'Keeffe M, Hochrein H, Vremec D, *et al.*** Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8- dendritic cells only after microbial stimulus. *J Exp Med*. 2002; 196: 1307–19.
24. **Diebold SS, Montoya M, Unger H, *et al.*** Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature*. 2003; 424: 324–8.
25. **Inaba K, Steinman RM, Pack MW, *et al.*** Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med*. 1992; 175: 1157–67.
26. **Randolph GJ, Inaba K, Robbiani DF, *et al.*** Differentiation of phagocytic monocytes into lymph node dendritic cells *in vivo*. *Immunity*. 1999; 11: 753–61.
27. **Randolph GJ, Beaulieu S, Lebecque S, *et al.*** Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science*. 1998; 282: 480–3.
28. **Cheong C, Matos I, Choi JH, *et al.*** Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209+ dendritic cells for immune T cell areas. *Cell*. 2010; 143: 416–29.
29. **von Euw EM, Barrio MM, Furman D, *et al.*** Monocyte-derived dendritic cells loaded with a mixture of apoptotic/necrotic melanoma cells efficiently cross-present gp100 and MART-1 antigens to specific CD8+ T lymphocytes. *J Transl Med*. 2007; 5. doi:10.1186/1479-5876-5-19.
30. **Geissmann F, Auffray C, Palframan R, *et al.*** Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. *Immunol Cell Biol*. 2008; 86: 398–408.
31. **Leon B, Lopez-Bravo M, Ardavin C.** Monocytes-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity*. 2007; 26: 519–31.
32. **Serbina NV, Salazar-Mather TP, Biron CA, *et al.*** TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity*. 2003; 19: 59–70.

33. **Steinbrink K, Wolfl M, Jonuleit H, *et al.*** Induction of Tolerance by IL-10-Treated Dendritic Cells. *J Immunol*. 1997; 159: 4772–80.
34. **Wakkach A, Fournier N, Brun V, *et al.*** Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity*. 2003; 18: 605–17.
35. **Tang H, Guo Z, Zhang M, *et al.*** Endothelial stroma programs hematopoietic stem cells to differentiate into regulatory dendritic cells through IL-10. *Blood*. 2006; 108: 1189–97.
36. **Zhang M, Tang H, Guo Z, *et al.*** Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. *Nat Immunol*. 2004; 5: 1124–33.
37. **Liu K, Victora GD, Schwickert TA, *et al.*** In vivo analysis of dendritic cell development and homeostasis. *Science*. 2009; 324: 392–7.
38. **Diao J, Winter E, Cantin C, *et al.*** In situ replication of immediate dendritic cell (DC) precursors contributes to conventional DC homeostasis in lymphoid tissue. *J Immunol*. 2006; 176: 7196–206.
39. **Geissmann F, Jung S, Littman DR.** Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003; 19: 71–82.
40. **Swirski FK, Nahrendorf M, Etzrodt M, *et al.*** Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009; 325: 612–6.
41. **Kang YS, Yamazaki S, Iyoda T, *et al.*** SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran. *Int Immunol*. 2003; 15: 177–86.
42. **Oehen S, Odermatt B, Karrer U, *et al.*** Marginal zone macrophages and immune responses against viruses. *J Immunol*. 2002; 169: 1453–8.
43. **Elomaa O, Kangas M, Sahlberg C, *et al.*** Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell*. 1995; 80: 603–9.
44. **Noel G, Guo X, Wang Q, *et al.*** Postburn monocytes are the major producers of TNF-alpha in the heterogeneous splenic macrophage population. *Shock*. 2007; 27: 312–9.
45. **Diao J, Winter E, Chen W, *et al.*** Characterization of distinct conventional and plasmacytoid dendritic cell-committed precursors in murine bone marrow. *J Immunol*. 2004; 173: 1826–33.
46. **Saunders D, Lucas K, Ismaili J, *et al.*** Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. *J Exp Med*. 1996; 184: 2185–96.
47. **Varol C, Landsman L, Fogg DK, *et al.*** Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med*. 2007; 204: 171–80.
48. **Fogg DK, Sibon C, Miled C, *et al.*** A clonogenic bone harrow progenitor specific for macrophages and dendritic cells. *Science*. 2006; 311: 83–7.
49. **Naik SH, Metcalf D, van Nieuwenhuijze A, *et al.*** Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol*. 2006; 7: 663–71.
50. **Groothuis TA, Neefjes J.** The many roads to cross-presentation. *J Exp Med*. 2005; 202: 1313–8.
51. **Sigal LJ, Crotty S, Andino R, *et al.*** Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature*. 1999; 398: 77–80.
52. **Huang AYC, Bruce AT, Pardoll DM, *et al.*** In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter. *Immunity*. 1996; 4: 349–55.
53. **Den Haan JMM, Lehar SM, Bevan MJ.** CD8 + but not CD8- dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med*. 2000; 192: 1685–95.
54. **Dudziak D, Kamphorst AO, Heidkamp GF, *et al.*** Differential antigen processing by dendritic cell subsets in vivo. *Science*. 2007; 315: 107–11.
55. **Pooley JL, Heath WR, Shortman K.** Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. *J Immunol*. 2001; 166: 5327–30.
56. **Schulz O, Reis E, Sousa C.** Cross-presentation of cell-associated antigens by CD8+ dendritic cells is attributable to their ability to internalize dead cells. *Immunology*. 2002; 107: 183–9.
57. **Gagnon E, Duclos S, Rondeau C, *et al.*** Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell*. 2002; 110: 119–31.
58. **Vyas JM, Van der Veen AG, Ploegh HL.** The known unknowns of antigen processing and presentation. *Nat Rev Immunol*. 2008; 8: 607–18.
59. **Beauvillain C, Delneste Y, Scotet M, *et al.*** Neutrophils efficiently cross-prime naive T cells in vivo. *Blood*. 2007; 110: 2965–73.
60. **Ramirez MC, Sigal LJ.** Macrophages and dendritic cells use the cytosolic pathway to rapidly cross-present antigen from live, vaccinia-infected cells. *J Immunol*. 2002; 169: 6733–42.
61. **Randolph GJ, Jakubzick C, Qu C.** Antigen presentation by monocytes and monocyte-derived cells. *Curr Opin Immunol*. 2008; 20: 52–60.
62. **Xu Y, Zhan Y, Lew AM, *et al.*** Differential Development of murine dendritic cells by GM-CSF *versus* Flt3 ligand has implications for inflammation and trafficking. *J Immunol*. 2007; 179: 7577–84.
63. **Sallusto F, Lanzavecchia A.** Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J Exp Med*. 1994; 179: 1109–18.
64. **Brawand P, Fitzpatrick DR, Greenfield BW, *et al.*** Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol*. 2002; 169: 6711–9.
65. **Naik SH, Proietto AI, Wilson NS, *et al.*** Cutting Edge: generation of Splenic CD8+ and CD8- Dendritic Cell Equivalents in Fms-Like Tyrosine Kinase 3 Ligand Bone Marrow Cultures. *J Immunol*. 2005; 174: 6592–7.
66. **Roy KC, Bandyopadhyay G, Rakshit S, *et al.*** IL-4 alone without the involvement of GM-CSF transforms human peripheral blood monocytes to aCD1adim, CD83+ myeloid dendritic cell subset. *J Cell Sci*. 2004; 117: 3435–45.
67. **Vremec D, Lieschke GJ, Dunn AR, *et al.*** The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. *Eur J Immunol*. 1997; 27: 40–4.
68. **Tan JKH, O'Neill HC.** Haematopoietic stem cells in spleen have distinct differentiative potential for antigen presenting cells. *J Cell Mol Med*. 2010; 14: 2144–50.
69. **Brasel K, De Smedt T, Smith JL, *et al.*** Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood*. 2000; 96: 3029–39.
70. **Merad M, Manz MG.** Dendritic cell homeostasis. *Blood*. 2009; 113: 3418–27.
71. **McKenna HJ, Stocking KL, Miller RE, *et al.*** Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood*. 2000; 95: 3489–97.
72. **Sunderkötter C, Nikolic T, Dillon MJ, *et al.*** Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004; 172: 4410–7.
73. **O'Neill HC, Wilson HL, Quah B, *et al.*** Dendritic cell development in long-term

spleen stromal cultures. *Stem Cells*. 2004; 22: 475–86.

74. **Wilson HL, Ni K, O'Neill HC.** Identification of progenitor cells in long-term spleen stromal cultures that produce immature dendritic cells. *Proc Natl Acad Sci USA*. 2000; 97: 4784–9.
75. **Wilson H, O'Neill HC.** Dynamics of dendritic cell development from precursors maintained in stroma-dependent long-term cultures. *Immunol Cell Biol*. 2004; 81: 144–51.
76. **Despars G, O'Neill HC.** Splenic endothelial cells support development of dendritic cells from bone marrow. *Stem Cells*. 2006; 24: 1496–504.
77. **Quah B, Ni K, O'Neill HC.** *In vitro* hematopoiesis produces a distinct class of immature dendritic cells from spleen progenitors with limited T cell stimulation capacity. *Int Immunol*. 2004; 16: 567–77.
78. **Iyoda T, Shimoyama S, Liu K, *et al.*** The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J Exp Med*. 2002; 195: 1289–302.
79. **Morelli AE, Larregina AT, Shufesky WJ, *et al.*** Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood*. 2003; 101: 611–20.
80. **Belz GT, Smith CM, Kleinert L, *et al.*** Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci USA*. 2004; 101: 8670–5.
81. **Nolte MA, T Hoen ENM, Van Stijn A, *et al.*** Isolation of the intact white pulp. Quantitative and qualitative analysis of the cellular composition of the splenic compartments. *Eur J Immunol*. 2000; 30: 626–34.
82. **Abadie V, Badell E, Douillard P, *et al.*** Neutrophils rapidly migrate *via* lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood*. 2005; 106: 1843–50.
83. **Tvinnereim AR, Hamilton SE, Harty JT.** Neutrophil involvement in cross-priming CD8+ T cell responses to bacterial antigens. *J Immunol*. 2004; 173: 1994–2002.
84. **Witmer-Pack MD, Hughes DA, Schuler G, *et al.*** Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *J Cell Sci*. 1993; 104: 1021–9.