Chapter 1

Delineation of Niches which Support Hematopoiesis

Hong Kiat Lim and Helen C. O'Neill

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/58715

1. Introduction

Hematopoiesis is the process of blood cell formation from self-renewing, multipotent hematopoietic stem cells (HSC) crucial for blood homeostasis of all living organisms [1]. This developmental process has two distinct phases, primitive and definitive hematopoiesis. In mice, the primitive phase of hematopoiesis occurs in the yolk sac at embryonic (E) day 7.5 [2-5]. It results in the production of primitive hematopoietic cells including mainly large, nucleated erythroblasts, some megakaryocytes, and primitive macrophages needed for embryonic growth [2-5]. The definitive phase of hematopoiesis in mice begins in the extra-embryonic yolk sac at E8.25 [2, 6]. Development in this phase is a hierarchical process, with two main lymphoid and myeloid lineages forming from which all blood cells develop. The lymphoid lineage comprises T and B lymphocytes and natural killer cells that arise from a common lymphoid progenitor (CLP). In contrast, the myeloid lineage comprises granulocytes (neutrophils, eosinophils, mast cells, and basophils), monocytes, erythrocytes and dendritic cells (DC), all developing from a common myeloid progenitor (CMP). The multipotent HSC resides at the apex of this hematopoietic hierarchy [1, 7].

Most of our present knowledge on hematopoiesis stems from decades of research on bone marrow, in part due to the clinical relevance of bone marrow as the main site for hematopoiesis in adults. However, there is cumulating evidence to suggest that the spleen can adopt extramedullary hematopoiesis in both the steady-state, and during disease and inflammation. While very little is understood about extramedullary hematopoiesis, there portends to be huge potential for regenerative medicine, if the hematopoietic capacity of spleen can be harnessed. For example, the hematopoietic output of spleen could be experimentally enhanced on bone marrow transplantation using either *in vivo* or *in vitro* means. In this report, information on hematopoiesis in bone marrow is reviewed and the current understanding of extramedullary hematopoiesis in spleen is considered.

2. Hematopoietic Stem Cells

HSC are multipotent adult stem cells, capable of differentiating to give all mature blood cell types, while still maintaining a pool of stem cells due to their unique ability to self-renew [8]. Definitive hematopoiesis occurs at E8.5 and is detected experimentally at E10.5 in the aorta-gonad-mesonephros region of the embryo [6, 9-11]. At E11.5, HSC appear in fetal liver where they exhibit high proliferative and differentiative potential with cell number expansion [12]. HSC appear in fetal spleen at E13-14 and undergo proliferation and differentiation to form mature blood cells [13]. At E17.5, HSC then proceed to bone marrow, the major hematopoietic site in adults where they are maintained during postnatal life [14].

HSC in murine bone marrow represent a heterogeneous population characterised phenotypically by the absence of lineage (Lin)-specific markers and high expression of Sca-1, also known as lymphocyte antigen 6A (Ly6A) and the c-Kit tyrosine kinase receptor (c-Kit). Within the $Lin^-Sca\text{-}1^+c\text{-}Kit^+$cell subset, HSC can be further delineated as $CD150^+CD34^-Flt3^-CD244^-CD48^-$ long-term reconstituting HSC (LT-HSC) and $Flt3^-CD34^+$short-term reconstituting HSC (ST-HSC) [15-17]. Functional studies involving adoptive transfer of HSC to reconstitute the hematopoietic system of lethally irradiated mice, remains the gold standard assay to distinguish ST-HSC from LT-HSC [18]. While LT-HSC are able to sustain reconstitution of the hematopoietic system over more than 25 weeks, and even a lifetime [19], ST-HSC provide short-term reconstitution for only ~6 weeks [17].

3. The fate of HSC

HSC can adopt several fates throughout the lifetime of an organism, including quiescence, dormancy, self-renewal and differentiation. Quiescence refers to a state of inactivity, whereby most HSC are in the G_0/G_1 phase of cell cycle and are not dividing [20]. It is believed that keeping HSC in the quiescent state is necessary for long-term maintenance of the HSC compartment, in part due to reduced stress associated with cellular respiration and DNA replication [20]. Signalling involving the TGF-β/Smad pathway has been implicated in the maintenance of quiescence in HSC [21], and the addition of TGF-β to *in vitro* cultures of LT-HSC can inhibit cell proliferation. The state which involves HSC in long-term quiescence is called dormancy [22]. Self-renewal is the process by which HSC undergo symmetrical or asymmetrical cell division to produce one or more daughter stem cells [23]. This process leads to expansion of stem cell numbers during development, and restores the stem cell pool after injury [23]. Self-renewal, along with quiescence, prevents the depletion of stem cells. Differentiation then involves specialisation of multipotent HSC to give mature blood cells, and is needed to replenish the hematopoietic system since most mature hematopoietic cell types are short-lived.

While the hematopoietic system has a high daily rate of cell turnover, most HSC are quiescent and divide very rarely. This raises the perplexing question of how the hematopoietic system can produce such large numbers of mature blood cells from slowly dividing HSC. The answer

lies in the balance between the various HSC fates of quiescence, dormancy, self-renewal and differentiation, such that tight regulation occurs between these fates to ensure that HSC are both maintained for the life of the animal, and sufficient mature blood cells are produced to meet the demands of their development. The importance of this balance is highlighted in disease states such as leukaemia, which result from abnormal HSC development.

4. Niches for HSC

In the 1970s, Schofield introduced the concept of the HSC 'niche' after observing that once HSC were removed from the bone marrow microenvironment, they quickly lost the capacity to self-renew and to reconstitute the hematopoietic system. The HSC 'niche' involves a microenvironment comprising non-hematopoietic stromal cells, extracellular matrix and soluble regulatory factors that regulate the different fates of HSC. To date, three stromal cell types have been found to contribute to the HSC niche, namely endosteal, vascular, and perivascular cells [24-26]. However, it is still not known to what extent these distinct niches are truly independent of each other, and the extent of hematopoietic support contributed by each cell type is not clear.

The endosteal niche comprises a heterogeneous group of osteoblastic cells residing close to the endosteal lining of trabecular bone where they interact with HSC. The role of osteoblastic cells in HSC maintenance has been demonstrated in studies which vary the number of osteoblastic cells experimentally [27]. In experimental mouse models, it was shown that expression of a constitutively active form of parathyroid hormone (PTH) or the PTH-related protein receptor (PPR) important for calcium regulation, under control of the type 1 collagen α1 promoter, gave a marked increase not only in number of osteoblastic cells but also in HSC [27]. Osteoblastic cells maintain and regulate HSC through secretion of cytokines like angiopoietin-1 (ANGPT1), thrombopoietin (THPO) and osteopontin (SPP1), which bind to cell surface receptors on HSC, namely endothelial-specific receptor tyrosine kinase (TEK), myeloproliferative leukemia virus oncogene (MPL), and either CD44 or integrins including $\alpha_4\beta_1$, $\alpha_5\beta_1$,$\alpha_9\beta_1$, and $\alpha_4\beta_7$ [28-30]. A role for THPO and ANGPT1 in HSC maintenance was demonstrated in studies which showed a reduction in the number of HSC in the bone marrow of *Thpo*$^{-/-}$ and *Angpt*$^{-/-}$ mutant mice [28-30]. Similarly, *Spp1*$^{-/-}$ mice showed a marked increase in the number of HSC cycling, consistent with osteopontin (SPP1) being a negative regulator of HSC proliferation [30]. In addition, osteoblastic cells express Jagged 1, which leads to the inhibition of HSC differentiation, and the enhancement of HSC self-renewal when engaged with Notch receptors on HSC [27].

There is increasing evidence now that HSC niches are located in the vicinity of blood vessels, and in close proximity with sinusoids in bone marrow, so allowing rapid mobilisation of HSC into the bloodstream after administration of granulocyte colony-stimulating factor (G-CSF) [16]. Vascular niches also play an important role during embryogenesis since HSC self-renew and differentiate at a stage of foetal development when bone marrow cavities are not yet formed [31]. Endothelial cells in the vascular niche express cell surface molecules that allow HSC and immune cells to move between the bone marrow and the periphery [32]. Vascular

niches also rely on the organisation of sinusoids or capillaries similar to fenestrated endothelium. When hematopoietic cells enter tissues, the slow blood flow in the larger sinusoids allows blood-borne cells to interact with sinusoidal endothelial cells via adhesion molecules, resulting in movement of cells through the sinus wall and into the extravascular space within bone marrow [33-35]. Sinusoidal endothelial cells express adhesion molecules like E-selectin and vascular cell adhesion molecule 1 (VCAM1) [32]. The importance of vascular niches for hematopoiesis was demonstrated by conditionally deleting the signalling protein vascular endothelial growth factor receptor 2 (VEGFR2) in adult mice [36]. This resulted in an inability of sinusoidal endothelial cells to develop after irradiation of mice, so preventing reconstitution of the hematopoietic system [36]. In addition, sinusoidal endothelial cells express gp130, a cytokine receptor essential for HSC self-renewal [37]. The importance of endothelial cells expressing gp130 in hematopoiesis was also demonstrated in reconstitution studies in mice where bone marrow cells from *gp130*$^{-/-}$mice could reconstitute the hematopoietic system of irradiated wild-type mice, while wild-type bone marrow cells were unable to reconstitute the hematopoietic system of *gp130*$^{-/-}$mice [38].

Recently, three populations of perivascular reticular cells expressing high levels of CXCL12, were identified as important niche elements for HSC in bone marrow. They have been described as CXCL12-abundant reticular (CAR) cells [39], nestin-GFP^{+}mesenchymal stem cells [40] and leptin receptor^{+}stromal cells [41]. CAR cells were characterised as bipotent adipo-osteogenic progenitors, which develop around sinusoids [39, 42]. By conditionally ablating CAR cells using transgenic mice with the diphtheria toxin receptor gene inserted into the *cxcl12* locus, CAR cells were shown to promote HSC proliferation, while maintaining them in an undifferentiated state [42]. The absence of CAR cells resulted in a reduction in HSC and progenitors, and of early myeloid differentiation of HSC.

Perivascular niches are also described by nestin^{+}mesenchymal stem cells situated near the larger blood vessels in BM [40]. These cells were identified in close association with HSC by their nestin expression. When nestin^{+}mesenchymal stem cells were conditionally ablated from mice, the frequency of HSC decreased, indicating the importance of these cells as a perivascular niche element in bone marrow [40]. Leptin receptor^{+}stromal cells have also been identified as perivascular cells surrounding sinusoids. This subset expresses high levels of CXCL12 and stem cell factor (SCF), and may overlap with other previously described CAR cell subsets. All three described perivascular subsets are an important source of SCF [41], a cytokine that binds to the c-Kit tyrosine kinase receptor expressed on hematopoietic stem/progenitor cells [43]. A recent study demonstrated depletion of the HSC pool in *Scf*$^{-/-}$mice, highlighting the importance of SCF as well as perivascular cells in HSC maintenance [41].

5. Spleen as a hematopoietic organ

The spleen is the largest blood-filtering organ. It also contains structures which support lymphoid cell development and function, and in this respect resembles a lymph node. The spleen is central to both hematopoiesis and immunity. Anatomically, it is composed of red

pulp which is responsible for blood cell removal, and white pulp, which houses T and B lymphocytes in discrete regions [44]. Red and white pulp of murine spleen are separated by a marginal zone comprising marginal zone macrophages, B cells, and DC, and a marginal sinus where the smallest arterial branches terminate [44]. The white pulp is further divided into T cell zones and B cell follicles, with germinal centres containing B cells [44]. While murine and human spleens are largely similar in terms of their architecture, there are some notable differences [44]. Unlike murine spleen, the marginal sinus is not present in the human spleen, although human spleen does contain a perifollicular region, external to the marginal zone in the red pulp, which is absent in the murine spleen.

5.1. Development of murine spleen

Spleen organogenesis involves formation of the splanchnic mesodermal plate from lateral plate mesoderm at E9.5 [45, 46]. The splanchnic mesodermal plate comprises epithelial-like cells which are arranged as an organized plate of cells at the right and left side of the foregut [45]. The initial condensation of mesenchyme within the dorsal pancreatic mesenchyme occurs adjacent to the splanchnic mesodermal plate at E10.5 [46]. By E10.5, there is progressive loss of thickness on the right side of splanchnic mesodermal plate, causing it to be thicker at the left side [46]. In addition, the cells constituting the splenic condensing mesenchyme also acquire the splenic cell fate [45]. At the same time, leftward growth of pancreatic mesenchyme and spleen anlagen occur [45]. By E11.5, the spleen anlage proper is formed laterally to the developing stomach [45, 47].

While the exact cellular requirements governing development of spleen remain poorly understood, it is widely known that the interaction between the non-hematopoietic $CD45^-VCAM1^+ICAM1^+LT\beta R^+$lymphoid tissue organiser (LTo) cells and hematopoietic $CD45^+CD3^-CD4^+cKit^+LT^+$lymphoid tissue inducer (LTi) cells is crucial for the development of secondary lymphoid organs such as lymph nodes and Peyer's patches [48]. This is due to lymphotoxin $LT\alpha\beta$ signaling, which occurs as a result of binding between lymphotoxin-α1β2 ($Lt\alpha_1\beta_2$) expressed on LTi cells and the lymphotoxin-β receptor ($LT\beta R$) on LTo cells [49]. Engagement of $Lt\alpha_1\beta_2$ and $LT\beta R$ induces the activation of two NFκB pathways which promote mesenchymal and endothelial cell differentiation [50]. In addition, increased expression of adhesion molecules like VCAM1, intracellular adhesion molecule 1 (ICAM1) and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1), and homeostatic chemokines such as CXCL13, CCL19 and CCL21, ensues as part of the differentiation program of mesenchymal cells [51]. These adhesion molecules and chemokines attract and retain additional hematopoietic cells at the formation site.

The importance of $Lt\alpha_1\beta_2$ signaling in secondary lymphoid organ development was first documented after the initial observation that lymph nodes and Peyer's patches failed to develop in $LT\alpha^{-/-}$mice [52]. Identification of what are currently known as LTi cells began with the search for the $LT\alpha\beta$-expressing cell subset which was first found in developing mesenteric lymph nodes [53]. These $LT\alpha\beta$-expressing cells were later characterised as $CD45^+CD3^-CD4^+$cells. Insight into the role of $LT\alpha\beta$-expressing $CD45^+CD3^-CD4^+$cells in secondary lymphoid organ development was gained with the generation of two mutant mice that lacked

$CD45^+CD3^-CD4^+$cells [54, 55]. A detailed analysis of these two mutant mice revealed an absence of lymph node and Peyer's patch development. However, the architecture of the T and B cell areas in spleen was unaffected [55, 56], suggesting the existence of multiple cellular subsets, which provide instructions to the splenic stromal cells.

5.2. The red pulp regulates erythrocyte turnover

The red pulp region has a very specialised function as a blood filter. This region contains many splenic cords formed by red pulp fibroblasts and reticular fibres where blood first enters via the afferent splenic artery [44]. Blood then enters venous sinuses lined by endothelium with a discontinuous structure [44, 57]. Slits formed in the endothelium of the sinuses allow the passage of most blood cells but not aged or damaged platelets and erythrocytes. These are phagocytosed by red pulp macrophages in the cords [44, 58].

In addition to the filtration of blood, the red pulp is involved in recycling iron. Phagocytosis of aged erythrocytes by splenic macrophages results in release of hem from hemoglobin, which is converted into biliverdin and ferrous iron [44, 59]. The resultant iron is either stored or released as ferritins from cells. Plasmablasts and plasma cells are also present in the red pulp for antibody production [44].

5.3. The white pulp facilitates T and B lymphoid responses

The white pulp of spleen contains the T cell zones and B cell follicles. In T cell zones, fibroblastic reticular cells encircle the central arterioles and form a network that connects the T cell zones to the marginal zone [60, 61]. This network serves to guide T cells in their migration from marginal zone to T cell zones [60, 61]. It is in the T cell zone where T cells interact with DC and passing B cells. In contrast, B cell follicles comprise activated B cells undergoing clonal expansion, isotype switching and somatic hypermutation. They also house specialised antigen handling cells called follicular dendritic cells, which form the architectural framework of the follicles, and are involved in retaining and presenting antigens to activated B cells in the germinal centre.

5.4. Multiple subsets of DC exist in spleen

Dendritic cells are specialised antigen presenting cells capable of inducing an adaptive immune response [62]. They do so by acquiring, processing and presenting antigens on major histocompatibility complex (MHC) molecules to naïve T cells. While all DC have capacity for antigen acquisition, processing and presentation, they are heterogeneous and their subtypes differ in location, migratory pathway, cell surface marker expression and immunological function [62]. The definition of a DC is therefore not straightforward, although most DC are phenotypically marked by high expression of CD11c and MHC-II [63].

The commonly described DC subsets in murine spleen include conventional DC (cDC), plasmacytoid DC (pDC), regulatory DC (DCreg) and monocyte-derived DC (mo-DC) [64, 65]. Conventional DC represent steady-state, mature DC with $CD11c^{hi}MHC\text{-}II^{hi}$ marker expression that can be further classified into $CD8\alpha^+$and $CD8\alpha^-$subsets [65]. $CD8\alpha^+$cDC are phenotypically

distinguishable as CD11c^{+}CD11b^{-}CD8α^{+}MHC-II^{+}B220^{-}cells, while CD8α^{-}cDC are CD11c^{+}CD11b^{+}CD8α^{-}MHC-II^{+}B220^{-}cells [65, 66]. Plasmacytoid DC exist as less mature DC precursors in the steady-state, producing natural type I interferon (IFN-α) upon viral exposure. They can be distinguished from other DC subsets by expression of Ly6C, B220, and low expression level of the CD11c marker [65, 67]. Under certain inflammatory conditions, monocytes which are phagocytic myeloid cells, are induced to differentiate into mo-DC for antigen presentation [68]. These cells can be distinguished from other DC by CD64 (FcR) expression.

5.5. Lineage origin of splenic DC

With high heterogeneity within the DC lineage, an important question is whether each subset is developmentally distinct. Early studies provided direct evidence for a myeloid origin of DC following reconstitution of cDC and pDC in the spleen and thymus when mouse bone marrow-derived CMP were transplanted into irradiated recipients [69]. It is now well established that all leukocytes originate from bone marrow-derived HSC. In the early stages of hematopoiesis, successive commitment steps result in the divergence of lymphoid and myeloid lineages, generating CLP and CMP [70]. The CLP give rise to B, T and natural killer cells, whilst CMP develop into macrophage/DC progenitors (MDP), identified as Lin^{-}CX3CR1^{+}CD11b^{-}CD115^{+}cKit^{+}CD135^{+}cells [70]. These subsequently give rise to either common DC progenitors (CDP) or two monocyte subsets distinguishable as Ly-6C^{+}and Ly-6C^{-}cells [64, 71]. In contrast to the developmental flexibility of MDP in terms of ability to produce cDC, pDC and monocytes, CDP are restricted to producing cDC and pDC [71]. It has also been shown that adoptive transfer of either CMP or MDP can give rise to CDP and monocytes, indicating that CDP are downstream of CMP or MDP [70].

While monocytes and pDC have been found to exit bone marrow as mature cells, cDC leave as immature precursors that further differentiate and mature within lymphoid organs [71]. In mouse blood, two populations of DC precursors have been described as CD11c^{int}CD11b^{+}CD45RA^{-}and CD11c^{lo}CD11b^{-}CD45RA^{hi} cells that differentiate to give mature CD8α^{-}cDC in the presence of TNF-α and GM-CSF, and IFN-α producing pDC in the presence of GM-CSF and CpG [72]. CD8α^{-}cDC are able to stimulate T cells to produce IL-2 in response to microbial stimuli, while pDC weakly stimulate T cells by producing large quantities of natural type I interferon [69]. A recent study reported the identification of the most immediate cDC precursors (pre-DC) in spleen with a CD11c^{int}CD45RA^{lo}CD43^{int}SIRP-α^{int}CD4^{-}CD8α^{-}phenotype [73]. Progenitors in bone marrow give rise to pre-DC which enter the spleen where they differentiate further to give cDC and p-pre-DC.

6. Extramedullary hematopoiesis in spleen

The spleen can support hematopoiesis, and this finding is based on early evidence documenting the recovery of mice lethally irradiated while the spleen was shielded [74]. This process is termed extramedullary hematopoiesis, since it occurs in sites other than the bone medullary cavity. Extramedullary hematopoiesis can be viewed as an active or a passive process. While

extramedullary hematopoiesis occurs as an active natural process during fetal development and also during infection, passive extramedullary hematopoiesis occurs due to impairment of hematopoiesis in the bone marrow [75]. Occurrence of an active process is evidenced by studies, showing that the low number of hematopoietic stem/progenitor cells present in murine spleen in the steady-state increases quickly following inflammation [76, 77]. The presence of hematopoietic stem/progenitor cells in the steady-state is however not restricted to murine spleen as spleens of pigs, baboons and humans was also found to retain a low number of hematopoietic stem/progenitor cells under steady-state conditions [78]. Moreover, in cell tracing experiments, spleen cells derived from both neonatal and adult mice were able to provide hematopoietic reconstitution of lethally irradiated host mice following adoptive transfer [79, 80]. These findings confirm that spleen can adopt a role in extramedullary hematopoiesis, at least during times of stress or inflammation. Evidence, which suggests a role for spleen in hematopoiesis, also raises the possibility of a splenic HSC niche that supports the maintenance of HSC in the resting state.

6.1. Splenic stromal cell network

Thus far, HSC niches in spleen have not been well investigated, although at least six distinct stromal cell types have been mapped to different regions of the spleen. These include $gp38^{+}$fibroblastic reticular cells in the T cell zones, $CD35^{+}$follicular dendritic cells in the B cell follicles, $MAdCAM1^{+}$marginal reticular cells in the marginal zone, and red pulp fibroblasts, lymphatic endothelial cells and vascular endothelial cells in the red pulp [81].

Fibroblastic reticular cells are mesenchymal cells, which along with reticular fibres and fibrous extracellular matrix bundles are required for the formation of a reticular network that serves as a scaffolding for the three-dimensional structure of secondary lymphoid organs [82]. In addition to creating a network in the T cell areas of spleen, which serves to guide T lymphocytes in their migration from marginal zone to the T cell zones [60, 61], fibroblastic reticular cells are also involved in creating a conduit system that selectively allows molecules of low molecular mass such as chemokines and antigens to enter the T cell zone [82-84]. Large molecules are trapped in the cortical sinuses by subcapsular sinus macrophages [82-84]. Such a conduit system offers an opportunity for different types of signals to be delivered in secondary lymphoid organs for optimisation of immunity to different pathogens [84].

In addition to their structural role, fibroblastic reticular cells are also involved in regulation of the immune response. They secrete homeostatic chemokines like CCL21, CCL19 and CXCL12 which attract naïve T cells expressing the chemokine receptors CCR7 and CXCR4 [85]. Fibroblastic reticular cells also enhance the survival of naïve T cells in the steady-state by producing interleukin-7. This is important in increasing the probability that naïve T cells meet cognate antigen presented on antigen presenting cells. A recent study revealed that fibroblastic reticular cells stimulate the activation of cytotoxic T cells via alarmin IL-33 secretion during viral infection [86].

Marginal reticular cells represent a unique subset of stromal cells located in the marginal zone of the spleen. Despite expressing many common stromal cell surface markers like ER-TR7, desmin, laminin, VCAM1, and MAdCAM1, marginal reticular cells appear to also specifically

express RANKL, the receptor activator of NF-κB ligand, and secrete CXCL13 [87]. While RANKL has been found to be essential for lymph node development [88], its function in spleen remains elusive. These stromal cells form a conduit system which can capture and deliver antigens to the B cell follicles [87].

In the red pulp region, red pulp fibroblasts form the splenic cords necessary for filtration of blood [81]. They also control splenic blood flow and assist in removal of dead or dying red blood cells [81]. Additionally, red pulp fibroblasts are involved in the localisation of different cells in the red pulp of spleen through expression of the cell adhesion molecule ICAM1 that binds to lymphocyte function-associated antigen 1 (LFA1), a heterodimeric receptor protein found on lymphocytes [89, 90]. For instance, ICAM1-LFA1 interaction between red pulp fibroblasts and plasma cells results in movement of plasma cells into the red pulp region for secretion of antibodies into the circulation [91].

7. Evidence that splenic stroma supports hematopoiesis

Evidence suggesting that the spleen contains stromal cells, which support *in vitro* hematopoiesis has been previously reported by this lab [92-95]. In particular, it was demonstrated that the stromal monolayer forming in long-term cultures (LTC) of 8-day old murine spleen supports the maintenance of small progenitors and the production of a distinct class of large, immature DC, coined 'LTC-DC'. These can be produced continuously in LTC for years in the absence of added growth factors and cytokines [92-95]. When the small progenitors maintained in LTC were specifically sorted and co-cultured over STX3 stroma, large immature dendritic-like cells were produced [96]. STX3 stroma had been isolated as a stromal cell line from a splenic LTC which had ceased production of dendritic-like cells after multiple passages, apparently due to the loss of progenitors [97]. Interestingly, similar dendritic-like cells, termed 'L-DC' were also produced when STX3 was overlaid with lineage-negative (Lin^-) cells derived from bone marrow [98]. L-DC, like LTC-DC, are large cells which express CD11b and CD11c but not MHC-II and CD8α[99, 100]. These cells are highly efficient in endocytosis and cross presentation of antigen for $CD8^+$T cell activation, particularly after exposure to lipopolysaccharide [99, 100].

In order to better understand the cellular composition of the heterogeneous STX3 stroma, the line was cloned to form 102 splenic stromal lines [98]. These include the 5G3 clone, which supports *in vitro* hematopoiesis, and the 3B5 clone, which is a non-supporter. The 5G3 clone supports production of 'L-DC' from hematopoietic progenitors in a highly reproducible and contact-dependent manner, similar to the parent line, STX3 [94, 99]. Transcriptome analysis of 5G3 stroma has revealed high expression of genes including *Sca-1*, *Vcam1*, *Pdgfrα/β* and *CXCL12*, which are associated with perivascular reticular cells described in the bone marrow (data in preparation). In addition, 5G3 was shown to have osteogenic but not adipogenic differentiative capacity (data in preparation). Such evidence raises the hypothesis that spleen contains a unique perivascular niche comprising mesenchymal stromal cells resembling osteoprogenitors that supports extramedullary hematopoiesis.

The highly reproducible nature of DC production in splenic LTC and in stromal co-cultures, suggests that a potential equivalent process may exist *in vivo*. Already there is some evidence for an *in vivo* equivalent L-DC subset in spleen, which was originally termed 'IVL-DC' [101]. However, the *in vivo* equivalent stromal supporter cell line has not yet been identified. Moreover, since DC production in splenic stromal co-cultures can be maintained for a long time, it seems likely that the progenitors, which are maintained in cultures, are self-renewing. One hypothesis is that the particular splenic stromal cells maintain self-renewing progenitors through close contact. To test this hypothesis, various progenitor subsets from bone marrow and spleen were sorted and tested for their capacity to seed 5G3 stroma with production of L-DC. 5G3 stromal co-cultures were established with MDP, CDP, ST-HSC or LT-HSC. Since no L-DC production was observed in co-cultures overlaid with MDP and CDP, it was evident that L-DC did not derive from the same progenitors which gave rise to cDC, pDC and monocytes [102]. When the $Flt3^{-}c\text{-}Kit^{+}Lin^{-}Sca\text{-}1^{+}$subset of LT-HSC from bone marrow, and the $Flt3^{+}c\text{-}Kit^{+}Lin^{-}Sca\text{-}1^{+}$subset of ST-HSC were overlaid on 5G3 stroma, L-DC production was clearly supported. In some co-cultures, contact between non-adherent cells and stroma was prevented using a Transwell membrane [92]. L-DC production occurred for up to 35 days from overlaid HSC, but only in co-cultures where overlaid cells maintained contact with 5G3 stroma [92]. The development of L-DC is therefore dependent on the interaction between primitive HSC and the competent 5G3 stroma. The progenitor of L-DC therefore appears to be a subset of HSC, some cells of which express Flt3 and resemble ST-HSC and the less primitive multipotent progenitors (MPP) [92, 102].

Indeed, it has become clear that L-DC progenitors *in vivo* reflect HSC endogenous to spleen, and that the process of L-DC development reflects extramedullary hematopoiesis. L-DC progenitors first appear in murine spleen at E18.5, while progenitors of cDC appear after 4 days [103]. This raises the possibility that hematopoietic progenitors in spleen are laid down during ontogeny, and that hematopoiesis in spleen and the differentiation of dendritic-like 'L-DC' occurs as an active process, independent of inflammatory signaling. One model is that the splenic stromal microenvironment supports the restricted differentiation of endogenous progenitors to give antigen presenting cells unique to the spleen microenvironment. Indeed, studies to date on the *in vivo* tissue distribution of cells equivalent to L-DC, confirm that L-DC reflect a novel subset which is limited in its tissue distribution to spleen [100]. Such a model does not discount the possibility that spleen can also act as a site for extramedullary hematopoiesis under inflammatory conditions.

While it is known that hematopoiesis can be driven by inflammation *in vivo* [104], it is important to consider whether *in vitro* hematopoiesis in 5G3 cocultures involves the same mechanism. Whether the same stromal microenvironment contributes to both steady-state and inflammatory processes remains to be determined. This laboratory has studied the role of toll-like receptor signaling in the production of L-DC in *in vitro* co-cultures involving 5G3 stroma. Toll-like receptor binding of pathogen components triggers an inflammatory response. When co-cultures were established with bone marrow progenitors derived from mutant mice lacking the adapter proteins MyD88 and TRIF crucial for toll-like receptor signaling, theywere found to be producers of L-DC [92]. This suggests that L-DC production must occur independently

of toll-like receptor signaling and inflammation. L-DC production therefore appears to reflect an active process of extramedullary hematopoiesis, dependent on primitive HSC endogenous to spleen.

8. Conclusion

While spleen has been traditionally viewed as an organ of immunity, recent evidence has shed light on its involvement in hematopoiesis. In this report, we have advanced the existence of niches for hematopoiesis in spleen, comprising a perivascular reticular cell type that supports extramedullary hematopoiesis from primitive HSC. Previous evidence in mouse and humans suggests that perivascular reticular cells reflect mesenchymal stem/progenitor cells located in close proximity with endothelial cells associated with the vasculature. Since the perivascular cells in bone marrow which constitute the HSC niche surround the sinusoids, it is quite possible that a similar type of cell exists in spleen, particularly in the red pulp region, where copious numbers of sinusoids are found. Moreover, transcriptome analysis of spleen-derived 5G3 stroma has revealed a close genetic resemblance between 5G3 stroma and perivascular cells in the bone marrow (data in preparation). Also consistent with this is the osteogenic capacity of 5G3 cells, suggesting the presence of mesenchymal osteoprogenitors in spleen with ability to support *in vitro* hematopoiesis.

The hypothesis that spleen contains perivascular cells as niches for hematopoiesis is not without functional precedence. Perivascular reticular cells present in the red pulp of spleen could provide a niche for maintenance of HSC in the steady-state. Furthermore, restricted hematopoiesis leading to development of only L-DC raises the possibility that the spleen microenvironment may support the production of tissue-specific antigen presenting cells. Their location in red pulp could reflect a specific role in monitoring blood-borne antigens and interacting with migrating lymphoid cells.

The lack of understanding of spleen as a hematopoietic organ has limited clinical application to date. A history of work from this lab has however considered spleen as a site for extramedullary hematopoiesis. Since spleen is a secondary site for hematopoiesis, it offers potential for reengineering niches to increase hematopoietic cell production. For example, if these unique stromal cells could be isolated and used to expand HSC *in vitro*, or provided as an ectopic niche *in vivo* for the same purpose, then the potential exists to enhance hematopoiesis during HSC transplantation. Regeneration or expansion of these niches could represent future therapy for patients undergoing myeloablative treatment, involution of lymphoid tissue with ageing, or HSC transplantation.

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia, and the Australian Research Council.

Author details

Hong Kiat Lim and Helen C. O'Neill*

*Address all correspondence to: Helen.ONeill@anu.edu.au

Research School of Biology, Australian National University, Canberra, Australia

References

[1] Seita, J. and I.L. Weissman, *Hematopoietic stem cell: self-renewal versus differentiation.* Wiley Interdiscip Rev Syst Biol Med, 2010. 2(6): p. 640-53.

[2] Hirschi, K.K., *Hemogenic endothelium during development and beyond.* Blood, 2012. 119(21): p. 4823-7.

[3] Ueno, H. and I.L. Weissman, *Clonal Analysis of Mouse Development Reveals a Polyclonal Origin for Yolk Sac Blood Islands.* Developmental Cell, 2006. 11(4): p. 519-533.

[4] Baron, M.H., *Embryonic origins of mammalian hematopoiesis.* Exp Hematol, 2003. 31(12): p. 1160-9.

[5] Haar, J.L. and G.A. Ackerman, *A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse.* Anat Rec, 1971. 170(2): p. 199-223.

[6] Medvinsky, A. and E. Dzierzak, *Definitive Hematopoiesis Is Autonomously Initiated by the AGM Region.* Cell, 1996. 86(6): p. 897-906.

[7] Doulatov, S., et al., *Hematopoiesis: A Human Perspective.* Cell Stem Cell, 2012. 10(2): p. 120-136.

[8] Trumpp, A., M. Essers, and A. Wilson, *Awakening dormant hematopoietic stem cells.* Nature Reviews Immunology, 2010. 10(3): p. 201-209.

[9] Li, Z., et al., *Mouse Embryonic Head as a Site for Hematopoietic Stem Cell Development.* Cell Stem Cell, 2012. 11(5): p. 663-675.

[10] Palis, J., et al., *Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse.* Development, 1999. 126(22): p. 5073-84.

[11] Müller, A.M., et al., *Development of hematopoietic stem cell activity in the mouse embryo.* Immunity, 1994. 1(4): p. 291-301.

[12] Ema, H. and H. Nakauchi, *Expansion of hematopoietic stem cells in the developing liver of a mouse embryo.* Blood, 2000. 95(7): p. 2284-8.

[13] Godin, I., et al., *Stem Cell Emergence and Hemopoietic Activity Are Incompatible in Mouse Intraembryonic Sites.* The Journal of Experimental Medicine, 1999. 190(1): p. 43-52.

[14] Dzierzak, E. and N.A. Speck, *Of lineage and legacy: the development of mammalian hematopoietic stem cells.* Nat Immunol, 2008. 9(2): p. 129-136.

[15] Christensen, J.L. and I.L. Weissman, *Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells.* Proceedings of the National Academy of Sciences, 2001. 98(25): p. 14541-14546.

[16] Kiel, M.J., et al., *SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells.* Cell, 2005. 121(7): p. 1109-1121.

[17] Yang, L., et al., *Identification of Lin–Sca1+kit+CD34+Flt3– short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients.* Blood, 2005. 105(7): p. 2717-2723.

[18] Muller-Sieburg, C., et al., *Stem cell heterogeneity: implications for aging and regenerative medicine.* Blood, 2012.

[19] Spangrude, G.J., S. Heimfeld, and I.L. Weissman, *Purification and characterization of mouse hematopoietic stem cells.* Science, 1988. 241(4861): p. 58-62.

[20] Eliasson, P. and J.I. Jonsson, *The hematopoietic stem cell niche: low in oxygen but a nice place to be.* J Cell Physiol, 2010. 222(1): p. 17-22.

[21] Sitnicka, E., et al., *Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells.* Blood, 1996. 88(1): p. 82-8.

[22] Wilson, A., et al., *Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair.* Cell, 2008. 135(6): p. 1118-1129.

[23] He, S., D. Nakada, and S.J. Morrison, *Mechanisms of stem cell self-renewal.* Annu Rev Cell Dev Biol, 2009. 25: p. 377-406.

[24] Bianco, P., *Bone and the hematopoietic niche: a tale of two stem cells.* Blood, 2011.

[25] Kiel, M.J. and S.J. Morrison, *Uncertainty in the niches that maintain hematopoietic stem cells.* Nature Reviews Immunology, 2008. 8(4): p. 290-301.

[26] Nagasawa, T., Y. Omatsu, and T. Sugiyama, *Control of hematopoietic stem cells by the bone marrow stromal niche: the role of reticular cells.* Trends in Immunology, 2011. 32(7): p. 315-320.

[27] Calvi, L.M., et al., *Osteoblastic cells regulate the hematopoietic stem cell niche.* Nature, 2003. 425(6960): p. 841-846.

[28] Qian, H., et al., *Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells.* Cell Stem Cell, 2007. 1(6): p. 671-84.

[29] Lilly, A.J., W.E. Johnson, and C.M. Bunce, *The Hematopoietic Stem Cell Niche: New Insights into the Mechanisms Regulating Hematopoietic Stem Cell Behaviour.* Stem Cells International, 2011. 2011.

[30] Nilsson, S.K., et al., *Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells.* Blood, 2005. 106(4): p. 1232-9.

[31] Huber, T.L., et al., *Hemangioblast commitment is initiated in the primitive streak of the mouse embryo.* Nature, 2004. 432(7017): p. 625-630.

[32] Sipkins, D.A., et al., *In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment.* Nature, 2005. 435(7044): p. 969-973.

[33] Quesenberry, P.J. and P.S. Becker, *Stem cell homing: Rolling, crawling, and nesting.* Proceedings of the National Academy of Sciences, USA, 1998. 95(26): p. 15155-15157.

[34] Mazo, I.B., et al., *Hematopoietic Progenitor Cell Rolling in Bone Marrow Microvessels: Parallel Contributions by Endothelial Selectins and Vascular Cell Adhesion Molecule 1.* The Journal of Experimental Medicine, 1998. 188(3): p. 465-474.

[35] Frenette, P.S., et al., *Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow.* Proceedings of the National Academy of Sciences, USA, 1998. 95(24): p. 14423-14428.

[36] Hooper, A.T., et al., *Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells.* Cell Stem Cell, 2009. 4(3): p. 263-74.

[37] Audet, J., et al., *Distinct role of gp130 activation in promoting self-renewal divisions by mitogenically stimulated murine hematopoietic stem cells.* Proceedings of the National Academy of Sciences, USA, 2001. 98(4): p. 1757-1762.

[38] Yao, L., et al., *Bone marrow dysfunction in mice lacking the cytokine receptor gp130 in endothelial cells.* Blood, 2005. 106(13): p. 4093-4101.

[39] Sugiyama, T., et al., *Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches.* Immunity, 2006. 25(6): p. 977-88.

[40] Mendez-Ferrer, S., et al., *Mesenchymal and hematopoietic stem cells form a unique bone marrow niche.* Nature, 2010. 466(7308): p. 829-834.

[41] Ding, L., et al., *Endothelial and perivascular cells maintain hematopoietic stem cells.* Nature, 2012. 481(7382): p. 457-462.

[42] Omatsu, Y., et al., *The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche.* Immunity, 2010. 33(3): p. 387-399.

[43] McNiece, I.K. and R.A. Briddell, *Stem cell factor.* Journal of Leukocyte Biology, 1995. 58(1): p. 14-22.

[44] Mebius, R.E. and G. Kraal, *Structure and function of the spleen.* Nat Rev Immunol, 2005. 5(8): p. 606-616.

[45] Brendolan, A., et al., *Development and function of the mammalian spleen.* Bioessays, 2007. 29(2): p. 166-77.

[46] Hecksher-Sorensen, J., et al., *The splanchnic mesodermal plate directs spleen and pancreatic laterality, and is regulated by Bapx1/Nkx3.2.* Development, 2004. 131(19): p. 4665-75.

[47] Burn, S.F., et al., *The dynamics of spleen morphogenesis.* Dev Biol, 2008. 318(2): p. 303-11.

[48] Randall, T.D., D.M. Carragher, and J. Rangel-Moreno, *Development of secondary lymphoid organs.* Annu Rev Immunol, 2008. 26: p. 627-50.

[49] Tan, J.K.H. and T. Watanabe, *Chapter 5-Artificial Engineering of Secondary Lymphoid Organs*, in *Advances in Immunology*, W.A. Frederick, Editor. 2010, Academic Press. p. 131-157.

[50] Dejardin, E., et al., *The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways.* Immunity, 2002. 17(4): p. 525-35.

[51] Honda, K., et al., *Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis.* J Exp Med, 2001. 193(5): p. 621-30.

[52] De Togni, P., et al., *Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin.* Science, 1994. 264(5159): p. 703-7.

[53] Mebius, R.E., P. Rennert, and I.L. Weissman, *Developing lymph nodes collect CD4+CD3- LTbeta+cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells.* Immunity, 1997. 7(4): p. 493-504.

[54] Sun, Z., et al., *Requirement for RORgamma in thymocyte survival and lymphoid organ development.* Science, 2000. 288(5475): p. 2369-73.

[55] Yokota, Y., et al., *Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2.* Nature, 1999. 397(6721): p. 702-706.

[56] Zhang, N., J. Guo, and Y.W. He, *Lymphocyte accumulation in the spleen of retinoic acid receptor-related orphan receptor gamma-deficient mice.* J Immunol, 2003. 171(4): p. 1667-75.

[57] Drenckhahn, D. and J. Wagner, *Stress fibers in the splenic sinus endothelium in situ: molecular structure, relationship to the extracellular matrix, and contractility.* The Journal of Cell Biology, 1986. 102(5): p. 1738-1747.

[58] Bratosin, D., et al., *Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review.* Biochimie, 1998. 80(2): p. 173-195.

[59] Knutson, M. and M. Wessling-Resnick, *Iron metabolism in the reticuloendothelial system.* Crit Rev Biochem Mol Biol, 2003. 38(1): p. 61-88.

[60] Bajénoff, M., N. Glaichenhaus, and R.N. Germain, *Fibroblastic Reticular Cells Guide T Lymphocyte Entry into and Migration within the Splenic T Cell Zone.* The Journal of Immunology, 2008. 181(6): p. 3947-3954.

[61] Anderson, A.O. and S. Shaw, *T cell adhesion to endothelium: the FRC conduit system and other anatomic and molecular features which facilitate the adhesion cascade in lymph node.* Semin Immunol, 1993. 5(4): p. 271-82.

[62] Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nat Rev Immunol, 2007. 7(1): p. 19-30.

[63] Miloud, T., G.J. Hämmerling, and N. Garbi, *Review of Murine Dendritic Cells: Types, Location, and Development*. 2009. p. 21-42.

[64] Geissmann, F., et al., *Development of Monocytes, Macrophages, and Dendritic Cells.* Science, 2010. 327(5966): p. 656-661.

[65] Merad, M., et al., *The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting.* Annu Rev Immunol, 2013. 31: p. 563-604.

[66] Vremec, D., et al., *CD4 and CD8 Expression by Dendritic Cell Subtypes in Mouse Thymus and Spleen.* The Journal of Immunology, 2000. 164(6): p. 2978-2986.

[67] Asselin-Paturel, C., et al., *Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology.* Nat Immunol, 2001. 2(12): p. 1144-1150.

[68] Cheong, C., et al., *Microbial Stimulation Fully Differentiates Monocytes to DC-SIGN/CD209+Dendritic Cells for Immune T Cell Areas.* Cell, 2010. 143(3): p. 416-429.

[69] Wu, L. and Y.-J. Liu, *Development of Dendritic-Cell Lineages.* Immunity, 2007. 26(6): p. 741-750.

[70] Liu, K. and M.C. Nussenzweig, *Origin and development of dendritic cells.* Immunological Reviews, 2010. 234(1): p. 45-54.

[71] Liu, K., et al., *In Vivo Analysis of Dendritic Cell Development and Homeostasis.* Science, 2009. 324(5925): p. 392-397.

[72] Naik, S.H., et al., *Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo.* Nat Immunol, 2007. 8(11): p. 1217-1226.

[73] Wu, L. and Y.J. Liu, *Development of dendritic-cell lineages.* Immunity, 2007. 26(6): p. 741-50.

[74] Rugh, R. and E. Grupp, *Splenic Radioprotective Agent: Particulate or Diffusible?* Radiation Research, 1960. 13(5): p. 657-660.

[75] Kim, C.H., *Homeostatic and pathogenic extramedullary hematopoiesis.* Journal of Blood Medicine, 2010. 1: p. 13-19.

[76] Massberg, S., et al., *Immunosurveillance by Hematopoietic Progenitor Cells Trafficking through Blood, Lymph, and Peripheral Tissues.* Cell, 2007. 131(5): p. 994-1008.

[77] Wolber, F.M., et al., *Roles of spleen and liver in development of the murine hematopoietic system.* Experimental Hematology, 2002. 30(9): p. 1010-1019.

[78] Dor, F.J., et al., *Primitive hematopoietic cell populations reside in the spleen: Studies in the pig, baboon, and human.* Exp Hematol, 2006. 34(11): p. 1573-82.

[79] Tan, J.K. and H.C. O'Neill, *Myelopoiesis in spleen-producing distinct dendritic-like cells.* J Cell Mol Med, 2012. 16(8): p. 1924-33.

[80] Tan, J.K., P. Periasamy, and H.C. O'Neill, *Delineation of precursors in murine spleen that develop in contact with splenic endothelium to give novel dendritic-like cells.* Blood, 2010. 115(18): p. 3678-85.

[81] Mueller, S.N. and R.N. Germain, *Stromal cell contributions to the homeostasis and functionality of the immune system.* Nature Reviews Immunology, 2009. 9(9): p. 618-629.

[82] Gretz, J.E., A.O. Anderson, and S. Shaw, *Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex.* Immunological Reviews, 1997. 156(1): p. 11-24.

[83] Lokmic, Z., et al., *The extracellular matrix of the spleen as a potential organizer of immune cell compartments.* Seminars in Immunology, 2008. 20(1): p. 4-13.

[84] Nolte, M.A., et al., *A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp.* J Exp Med, 2003. 198(3): p. 505-12.

[85] Forster, R., et al., *CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs.* Cell, 1999. 99(1): p. 23-33.

[86] Bonilla, W.V., et al., *The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses.* Science, 2012. 335(6071): p. 984-9.

[87] Katakai, T., et al., *Organizer-Like Reticular Stromal Cell Layer Common to Adult Secondary Lymphoid Organs.* The Journal of Immunology, 2008. 181(9): p. 6189-6200.

[88] Dougall, W.C., et al., *RANK is essential for osteoclast and lymph node development.* Genes Dev, 1999. 13(18): p. 2412-24.

[89] Williams, A.F. and A.N. Barclay, *The Immunoglobulin Superfamily—Domains for Cell Surface Recognition.* Annual Review of Immunology, 1988. 6(1): p. 381-405.

[90] Hynes, R.O., *Integrins: A family of cell surface receptors.* Cell, 1987. 48(4): p. 549-554.

[91] Ellyard, J.I., et al., *Contribution of stromal cells to the migration, function and retention of plasma cells in human spleen: potential roles of CXCL12, IL-6 and CD54.* European Journal of Immunology, 2005. 35(3): p. 699-708.

[92] Periasamy, P., S. Petvises, and H.C. O'Neill, *Development of two distinct dendritic-like APCs in the context of splenic stroma.* Front Immunol, 2013. 4: p. 73.

[93] O'Neill, H.C., et al., *Spleen as a Site for Hematopoiesis of a Distinct Antigen Presenting Cell Type.* Stem Cells International, 2011. 2011.

[94] Periasamy, P., et al., *Splenic stromal niches support hematopoiesis of dendritic-like cells from precursors in bone marrow and spleen.* Experimental Hematology, 2009. 37(9): p. 1060-1071.

[95] Ni, K. and H. O'Neill, *Spleen stromal cells support hemopoiesis and in vitro growth of dendritic cells from bone marrow.* Br J Hematol, 1999. 105(1): p. 58-67.

[96] Wilson, H.L., K. Ni, and H.C. O'Neill, *Identification of progenitor cells in long-term spleen stromal cultures that produce immature dendritic cells.* Proc Natl Acad Sci U S A, 2000. 97(9): p. 4784-9.

[97] Ni, K. and H.C. O'Neill, *Hemopoiesis in long-term stroma-dependent cultures from lymphoid tissue: production of cells with myeloid/dendritic characteristics.* In Vitro Cell Dev Biol Anim, 1998. 34(4): p. 298-307.

[98] Despars, G. and H.C. O'Neill, *Splenic endothelial cell lines support development of dendritic cells from bone marrow.* Stem Cells, 2006. 24(6): p. 1496-504.

[99] Periasamy, P. and H.C. O'Neill, *Stroma-dependent development of two dendritic-like cell types with distinct antigen presenting capability.* Experimental Hematology, 2013. 41(3): p. 281-292.

[100] Tan, J.K., et al., *Identification of a novel antigen cross-presenting cell type in spleen.* J Cell Mol Med, 2011. 15(5): p. 1189-99.

[101] Tan, J.K.H. and H.C. O'Neill, *Hematopoietic stem cells in spleen have distinct differentiative potential for antigen presenting cells.* Journal of Cellular and Molecular Medicine, 2010. 14(8): p. 2144-2150.

[102] Petvises, S. and H.C. O'Neill, *Distinct progenitor origin distinguishes a lineage of dendritic-like cells in spleen.* Front Immunol, 2014. 4: p. 501.

[103] Petvises, S. and H.C. O'Neill, *Characterisation of dendritic cells arising from progenitors endogenous to mu*rine spleen. PLoS One, 2014. 9(2): p. e88311.

[104] Nagai, Y., et al., Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. Immunity, 2006. 24(6): p. 801-12.