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The Adult Hematopoietic Niches – Cellular Composition, Histological Organization and Physiological Regulation

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1. Introduction

For many years the adult bone marrow (BM) was described as a highly vascularised organ consisting histologically of a network of reticular cells, some of them intimately associated with blood vessel walls, so-called adventitial reticular cells, in whose holes mature and developing hematopoietic cells can be found. In long bones, central longitudinal arteries give rise to radial arteries that branch into arterioles near the endosteum where they connect to venous sinusoids that extend back toward the central region where they unite to form a large central sinus. The sinusoidal network occupies 30 ± 5 % of BM volume whereas arterioles comprise a smaller volume, 1.2 ± 0.1 % of total BM [1]. Macrophages, adipocytes, endosteal bone-lining cells and nerve endings also form part of the haematopoietic microenvironment of BM.

Twenty years ago, it was proposed that hematopoietic stem cells (HSCs) and their progeny occupied specific places (niches) in the BM network, a topological arrangement necessary to create a gradient of maturation from the bone endosteum, in which primitive B progenitor cells were identified, to central blood vessels through which mature B lymphocytes migrated into the blood circulation [2]. Thus, the relevance of bone lining cell (endosteal cells)-HSC interface was emphasized for the control of haematopoiesis. However, more recently based on the phenotypic definition of HSCs as CD150+CD48-CD41-Lin-cells, most early hematopoietic progenitors appear to really be in contact with sinusoidal endothelia, defining the so-called vascular niche [3]. Despite this evidence, the cells that organize these different BM microenvironments, the physiological relationships between them and molecules governing their functioning remain elusive. In the present chapter, we will be trying to clarify these issues

providing current evidence on the functional niches operating in the adult bone marrow, their main cell components and the mechanisms governing their homeostasis. Niches involved in the homing and maintenance of distinct lymphoid cell subsets will be also addressed as well as the available information on the organization of hematopoietic niches occurring in primitive vertebrates that lack a functional bone marrow.

2. The existence of an endosteal niche in the adult BM is controversial

Bone development and the appearance of hematopoietic niches occur concomitantly during development. In foetal mice, the vascular invasion of specific chondrogenic sites favours bone formation, organizes the BM and seeds it with HSCs. Prior to the appearance of a bone microenvironment, HSCs occurring in foetal liver cannot home into the BM. In addition, injected HSCs disappear from the blood circulation to home rapidly in the endosteal niche [4]. On the other hand, E14.5 bone tissue grafted under the kidney capsule of syngeneic mice produces bone containing bone marrow that houses HSCs, and grafted CD105+Thy-1-cells isolated from E14.5 bone recruit blood vessels, produce ectopic bone through endochondral ossification and generate a BM capable of recruiting long term (LT) HSCs. In any experimental condition, the presence of bone was mandatory for BM organization [5]. Mixed multicellular spheroids containing CD45-CD105+CD31-Ter119-Sca-1+CD51+osteoprogenitors form a 3D hematopoietic niche that produces CXCL12 and osteopontin, retaining HSCs [6]. Nevertheless, bone marrowless vertebrates contain numerous hematopoietic loci in which HSCs develop in the absence of bone. Remarkably, the stroma of these organs morphologically resembles that of mammalian BM [7].

The existence of an endosteal niche gained considerable support several years ago when it was shown that endosteal cells produced several molecules involved in HSC maintenance, including G-CSF [8], thrombopoietin, angiopoietin [9, 10], CXCL12 [11], Jagged 1, Wnt ligands, among others [12, 13]. They, also, expressed adhesion molecules (VCAM, ICAM-1; N-cadherin, CD44, CD164) that could mediate endosteum-HSC interactions [4]. HSCs express Ca^{++} -sensing receptors that recognize Ca^{++} concentration in endosteum. In mice defective in these receptors HSC migrate into the BM in response to CXCL12 but do not anchor to the endosteum [4]. On the other hand, an increase in osteoblasts or osteolineage cells through enforced expression of parathyroid hormone receptor 1 [14] or by conditional inactivation of BMP receptor I [15] resulted in increased numbers of HSCs. Conversely, conditional depletion of osteolineage cells is concomitant with a loss of HSCs in the BM and extramedullary haematopoiesis [16].

However, recently some of these results have been questioned. Mice deficient in biglycan, a leucine-rich repeat proteoglycan of the connective tissue, have reduced osteoblasts but contain normal numbers of HSCs [17]. Osteoblast depletion in transgenic mice expressing thymidine kinase under the collagen $\alpha 1$ promoter courses with important alterations in haematopoiesis but only slight changes in the Lin-Sca-1+c-Kit+fraction of hematopoietic progenitors [16]. On the other hand, increased numbers of short term HSCs after parathyroid treatment could be mediated through Wnt ligand production by T cells [18] rather than through osteoblast

expansion. Moreover, an increase in osteoblasts is not sufficient to increase HSCs. Thus, the treatment of mice with strontium, a bone anabolic agent, induces expansion of mature osteoblasts but does not affect the number or function of HSCs [19]. Likewise, in mice suffering chronic arthritis that produces osteoblast suppression, HSCs exhibit a normal behaviour [20] and conditional deletion of CXCL12 [21, 22] or SCF [23] from mature osteoblasts has no effect on HSCs, while osteoblasts would promote proliferation and differentiation of lymphoid progenitors [21].

A good example of the controversy about the significance of endosteal niche for haematopoiesis is the role played by the N-cadherin positive cells. As mentioned above, N-cadherin was pointed out to be an important adhesion molecule for endosteal cell-HSC interactions. These interactions were considered to be occurring between osteoblasts and HSCs, but other studies indicated that N-cadherin was largely expressed on immature osteolineage cells called spindle-shaped N-cadherin+osteoblasts [15]. These cells also express high levels of SCF and CXCL12 and support long term repopulating activity of HSCs [24]. In this respect, N-cadherin knock-down or expression of a dominant negative molecule in HSCs inhibited their repopulating capacity [25, 26] but conditional deletion of N-cadherin encoding gene in HSC had no effects on HSC number, mobilization, proliferation or repopulating capacity [25, 27].

In fact, a key issue to understanding these controversial results is the different cell composition attributed to the endosteum in various studies: whereas the earliest studies assigned any effect of endosteal niches to osteoblasts, more recent studies consider that, apart from osteoblasts, osteoclasts, osteoprogenitor cells, blood vessels and reticular cells provide a more accurate idea of the endosteal cell components and their function in the BM microenvironment. It has been proposed that osteoprogenitors rather than osteoblasts provide a functional niche for HSCs [28], whereas osteoclasts contribute to endosteal niches governing osteoblast maturation [29]. In this respect, macrophages interspersed between endosteal osteoblasts, called osteomacrophages, support osteoblast function because their depletion *in vivo* leads to osteoblast disappearance and HSP mobilization [30], and RANK (receptor activator of nuclear factor κ B) ligand, essential for osteoclast maturation, induces mobilization of hematopoietic progenitors to the circulation [31]. In fact, osteoprogenitors, osteoblasts and osteoclasts are in a dynamic balance on the endosteal surface. Furthermore, recent evidence also supports a role for osteocytes in the function of the endosteal niche [32]. An analysis of the kinetics of gene expression in mice treated subcutaneously with injections of G-CSF each 12 hours showed that osteocytes responded more rapidly than osteoblasts to G-CSF. Thus, from the 4th dose onwards a rapid suppression of specific osteocyte genes was observed from the 1st or 2nd dose before HSC mobilization. After 8 doses of G-CSF, osteocytes showed fewer cell processes that connected them with endosteal osteoblasts. Remarkably, the specific elimination of osteocytes that disrupted the bone cell network, resulted in severe impairment of G-CSF-mediated HSC mobilization, as previously observed in other experimental models [33-35]. The authors suggest that these defects in HSC mobilization are due to impairment of the endosteal niche via altered communication between osteocytes and osteoblasts. Thus, changes in osteoblasts would be secondary to osteocyte depletion.

Nevertheless, results that emphasize a close association between HSCs and vascular endothelia, and those emphasizing the relevance of endosteal niche are not discordant because both HSCs and early committed pluripotent progenitors are perivascular but preferentially occupy the highly vascular endosteum [1]. However, it is more difficult to demonstrate that endosteal and vascular niches represent functionally different environments housing quiescent HSC and cycling hematopoietic progenitors, respectively, as suggested by some authors [12, 36]. The endosteal niche would house dormant, primitive HSCs due to its presumptive hypoxic condition [37, 38]. It has been reported that disruption of HIF 1 α (hypoxia-inducible factor) coursed with loss of HSC quiescence and repopulating activity whereas stabilization of HIF-1 α induced opposite effects [39, 40], but CFU-S associated with endosteum has been described as non-quiescent cells and, indeed, endosteum is a highly vascularised and, therefore, presumably well-oxygenated area of the BM. Thus, it has been proposed that hematopoietic progenitors exhibit an intrinsic hypoxic profile independently of their location in the BM [41, 42]. On the other hand, cycling hematopoietic progenitors present in the vascular niches would be ready to respond to the physiological demand of blood cells. However, a recent study which combines three-dimensional BM imaging with computational modelling to carefully analyze relationships between HSCs and BM stromal component, including blood vessels, shows that different vascular niches house quiescent or proliferating stem cells. Whereas arterioles that occur predominantly close to bone [1], contribute to a niche that maintains quiescent HSCs, sinusoids could represent a niche which houses proliferating HSCs [43]. In addition, E-selectin expressed in certain BM sinusoids [44] promotes HSC proliferation and its blockade protects HSCs following chemotherapy or γ -irradiation [45].

3. The existence of a vascular niche

As described above, a restricted analysis of the topological position of HSC identified by the expression of SLAM family molecules CD150, CD244, CD48 and CD41 [46], together with experiments that indicate the relevance of vascular meshwork for the biology of hematopoietic progenitors led to a hypothesis of the occurrence of a so-called vascular niche [36]. Thus, vascular endothelial cells promote haematological recovery in mice following total body irradiation [47] inducing HSCs to enter in cycle after stimulation with different injury factors [48] and conditional deletion of VEGFR2, which inhibits the regeneration of sinusoidal endothelia, impedes the hematopoietic recovery following sublethal irradiation [47]. Also, specific activation of BMPR1A in endothelial cells increases the number of HSCs and hematopoietic recovery after myeloablation [49]. HSC mobilization induced by different agents (cyclophosphamide, G-CSF) that results in extramedullar haematopoiesis is associated with splenic sinusoids [36] and thrombocytopenic mice only recover thrombopoiesis in the presence of BM endothelial cells [50].

4. The cellular components of distinct BM microenvironments

For many years, the characterization of BM stromal cells was merely morphologic identifying a supporting network consisting largely of reticular cells, adventitial reticular cells and blood vessels whose functions were unknown. An important part of our knowledge of the presumptive functions of these BM stromal cells comes from studies in which genes important for HSC maintenance (i.e., CXCL12, SCF, TGF β) are selectively deleted from different niche cell types [42, 51]. Unfortunately, results arising from these studies are controversial partially due to the disparity of these experimental models from physiological conditions and different degrees of specific deletion of the genes studied.

CXCL12, a chemokine implicated in the retention of CXCR4 (the CXCL12 receptor) positive hematopoietic multipotent progenitors is produced by different cell types of BM stroma, including bone-lining cells, but particularly by reticular cells that were named CAR cells (CXCL12 abundant reticular cells) [36]. A strong CXCL12 expression was demonstrated in a small VCAM⁺reticular cell population scattered throughout the BM. It constitutes a relatively homogeneous CD45-CD31-Sca-1-PDGFR β ⁺cell population that in human BM could correspond to CD146-expressing subendothelial cells [31]. Selective depletion of CAR cells results in a drastic reduction of hematopoietic progenitors as well as of both cycling B lymphocytes and erythroid progenitors. On the other hand, CAR cells express adipogenic and osteogenic genes and are capable of differentiating *in vitro* to adipocytes and bone lineage cells.

On the other hand, nestin, a neuroectoderm stem cell marker, has been used to identify another stromal cell type in murine BM [52]. Nestin⁺cells have also been reported in human foetal and adult BM [53]. Previously, other authors identified self-renewing osteoprogenitors from stromal cultures containing all the human BM CFU-F activity, although their phenotype (CD45-CD146⁺ or CD45-CD271⁺CD146^{-/lo}) is a matter of discussion [54, 55]. PDGFR α ⁺CD51⁺stromal cells isolated from human BM form self-renewing clonal mesenpheres and support maintenance and expansion of human hematopoietic progenitors in a dose-dependent manner [56]. Remarkably, these authors indicate that these PDGFR α ⁺CD51⁺stromal cells comprise a subset of CD146⁺cells associated with hematopoietic and MSC activities in foetal human BM. Nestin-positive cells are morphologically similar to pericytes; in fact, they appear to be largely restricted to the perivascular areas in intimate association with HSCs and to heavily express genes known to be involved in HSC maintenance, such as CXCL12, SCF, angiopoietin 1, IL7, V-CAM and osteopontin. Nestin⁺cells are innervated by the sympathetic nervous system, express β 3-adrenergic receptors, mediate G-CSF-induced HSC mobilization and regenerate *in vivo* a BM stroma capable of supporting haematopoiesis. Furthermore, β 3-adrenergic stimulation reduces the expression of the above-mentioned genes involved in HSC biology [52]. Depletion of nestin⁺cells reduces by half the HSC numbers in BM increasing concomitantly in the spleen. Relationships between nestin⁺cells and CAR cells are unclear. They could represent two overlapping BM stromal cells, nestin⁺cells being more primitive because they are less abundant, contain all CFU-F activity of bone marrow, exhibit autorenewal capacity and are able to differentiate into osteoblasts, chondrocytes and adipocytes.

On the other hand, it has been proposed that the BM stromal network, largely formed by CAR cells and nestin-expressing cells, would constitute a functional syncytium through gap junctions capable of affecting the behaviour of hematopoietic progenitors modulating CXCL12 secretion [57]. Nestin+MSCs heavily express connexin (Cx) 43 and Cx45, and G-CSF mediated reduction of CXCL12 expression is intimately related to decreased expression of both Cx43 and Cx45. On the contrary, the pharmacological blockade of gap junctions reduces the CXCL12 expression preventing homing into BM of mononuclear cells. In addition, a tight correlation occurs in confluent cultures of human BM-MSCs between the functional modulation of gap junctions and CXCL12 production. Gap junctions could modulate CXCL12 mediating gene transcription or directly acting on CXCL12 secretion. Loss of function of the Cx45 gene courses with reduced CXCL12 transcription particularly if Cx 43 transcription is also disrupted. On the other hand, Ca^{++} release, due to osteoclast activity in bone, necessary for AMPc-dependent PKA activation involved in CXCL12 secretion, is transmitted by gap junctions [58]. However, other studies have proven that Cx43 deficiency increases CXCL12 secretion, although it compromises HSC homing and hematopoietic recovery following myeloablation [59].

Cre-recombinase expressed under the control of leptin receptor (LepR) regulatory elements in the mouse BM [23] identified another perivascular stromal cell subset that, like the CAR cells and nestin+cells, expresses high levels of CXCL12 and SCF (c-Kit ligand).

A fourth element, in this case related to BM innervation, completes, for the moment, the myriad of stromal cells identified in the mouse BM. GFAP+integrin $\beta 8$ +(a Schwann cell marker), non-myelinated Schwann cells have been identified surrounding sympathetic nerves in BM. They produce important factors for HSC maintenance and appear to be in close contact with high numbers of hematopoietic progenitors [60]. Presumably, BM GFAP+cells are the main cell type processing latent TGF β into an active form of the molecule, key for controlling the HSC niche. In fact, surgical sympathectomy performed by ligation of the sympathetic trunk reduces the HSC frequency and provokes HSC differentiation [33].

On the other hand, at least a subset of the total nestin-expressing cells present in the BM stroma could correspond to adventitial reticular cells identified in the EM studies and in vivo equivalents of the so-called mesenchymal stem cells (MSCs), an in vitro characterized BM stromal cell subset that exhibits interesting immunomodulatory properties and is extensively used in cell therapy [61]. Currently, it is assumed that MSCs represent approximately 0.001% to 0.01% of the BM nucleated cells, 10 fold less than the percentage of HSCs [62]. In 2007, [54] and later other authors [63-65] pointed out that MSC could be identical to, or derived from, pericytes, as previously indicated for nestin+cells. In addition, the CD146+subendothelial cell population of BM stroma, after ex vivo culture, produces bone, adipocytes and a stroma capable of supporting haematopoiesis. Other authors have identified a non-hematopoietic perivascular cell population that expresses both PDGFR α and Sca-1 and, after intravenous injection, produces perivascular cells, but also osteoblasts and adipocytes [66]. In human BM, Stro-1+stromal cells support haematopoiesis, exhibit capacity to differentiate into multiple mesenchymal lineages [67, 68] and after long-term culture express α SMA-actin, as vascular smooth muscle cells. In vivo, α SMA-actin+cells occur in both foetal and adult bone marrow and exhibit long cell processes in intimate contact with HSCs [69], similarly to the alkaline

phosphatase adventitial reticular (AdR) cells described previously [70]. The AdR cells express CD271 and appear in the foetal BM before the presence of HSCs [71]. CD146⁺ cells, another BM-MSc subset [55], are also morphologically and physiologically similar to AdR cells and, after ex vivo expansion, express alkaline phosphatase, α -SM actin and CXCL12 [54].

Although results need further confirmation, [43] two types of nestin⁺ cells have recently been reported in the murine BM. Nestin^{hi} expressing cells appeared along arterioles in both cancellous and long bone BM, closely associated with tyroxine hydroxylase-positive sympathetic nerves and GFAP⁺ Schwann cells. Nestin^{weak} cells were reticular in shape and appeared associated to blood sinusoids. Transcriptome analysis also revealed differences between the two nestin⁺ cell types: periaarteriolar nestin^{hi} cells expressed mainly genes related with HSC biology whereas reticular nestin^{weak} cells were particularly enriched in genes involved in DNA replication and cell cycle.

On the other hand, clonal analysis revealed that BM nestin⁺ cells represent a mixed population composed of neural crest (NC) stem cells plus a few MSCs [72]. Classically nestin⁺ MSC of adult BM were considered to be mesodermal in origin [73], but recent studies have conclusively demonstrated that some adult BM-MSCs derive from the NC [74-78]. The vertebrate NC cells are a multipotent population that gives rise to multiple types of derivatives [79]. In chick embryos, a common mesenchyme-neural progenitor derived from the neural crest has been described [80] and tracing studies using the Sox1 neuroepithelial marker and PO (protein O) NC marker indicate that the first wave of trunk MSC come from the neuroepithelium rather than from the mesoderm, although MSC derived from Sox1⁺PO⁺ cells decrease gradually and appear to be substituted by MSCs from other unknown sources [74]. Murine NC stem cells migrate in the blood to the BM via the AGM region between E12.5 and E15.5 similar to HSCs and form spheres that express nestin as BM-MSCs [75]. It has been pointed out that TGF- β signalling confers mesenchymal potential to NC cells and suppresses their neurogenic potential [81] supporting the hypothesis that GFAP⁺ Schwann cells intimately associated with nestin⁺ cells in the murine BM produce TGF- β that could affect the BM-MSc properties. On the other hand, the neuroectodermal origin of some BM-MSc could confer them a neural potentiality useful for Cell Therapy. In this respect, cranial NC cell-derived MSCs from gingivae show a high capacity to differentiate into neural cells [82] and BM stem cells have been described to differentiate into both neurons and glial cells in vivo and in vitro [83, 84]. However, other studies demonstrate that NC derived BM-MSCs are not more competent than mere MSCs or whole bone MSCs at differentiating into neurons and *in vivo* recovery of the experimentally-damaged dopaminergic system [85].

As mentioned above, it is difficult to establish clear relationships between all these cell types of BM stroma. It has been proposed that they are overlapping cell subsets because LepR is among the top 1% most highly expressed genes in sorted nestin⁺ cells [52] and also in CAR cells [22]. However, conditioned deletion of SCF gene in either osteoblasts, nestin⁺ cells, endothelial cells or perivascular LepR⁺ cells only negatively affects the HSC frequency in the two last experimental conditions suggesting that LepR⁺ cells and nestin⁺ cells are different components of BM stroma [23]. [51] have recently proposed that CAR⁺ cells, which represent the highest BM stromal cell subset (0.26% of total BM nucleated cells), would encompass nestin

+cells (0.08%), GFAP+cells (0.004%) and LepR+cells (0.012%) whereas the nestin+cell subpopulation would partially include LepR+cells. On the contrary, [86] found only a partial overlapping of nestin+MSCs with the other three stromal cell types. In fact, all GFAP+cells express nestin but not PDGFR α , although nestin+cells and PDGFR α cells largely overlap. Thus, BM GFAP+cells seem to differ from PDGFR α MSCs, as further supported by the finding that they do not express smooth muscle actin [60]. Recently, [43] reported that LepR+cells and reticular nestin^{weak} cells largely overlap but they did not find overlapping between LepR+cells and periarteriolar nestin^{hi} cells that these authors consider to be related to pericytes that express Ng2 and α -smooth muscle actin.

Some evidence supports reciprocal interactions between HSCs and the niche. After acute bleeding, HSCs secrete BMP2 and BMP6 that differentiate MSCs to osteoblasts [87]. Also, megakaryocytes located close to the endosteum stimulate osteoblasts by secreting BMP2, 4 and 6 [50, 88].

5. Rhythms and haematopoiesis

HSCs, like other stem cells, are affected by systemic signals, including those associated with nutrition, seasonal and circadian rhythms, exercise, mating, pregnancy, etc. [89]. Recently, [90] have reported that HSCs suffer sexual dimorphism due to estrogens that directly affect HSC biology. More interestingly within the context of this chapter are results that suggest that certain physiological biorhythms known to occur in the BM could be mediated through its stromal components. The close association between nerve endings, GFAP+Schwann cells and some nestin+cells (ultrastructurally identified as adventitial reticular cells [91]), as well as the above mentioned possible neuroectodermic origin of part of this BM cell population suggested by some authors support this hypothesis.

A central clock in the central nervous system [92] orchestrates a uniform temporal programme by synchronizing multiple peripheral clocks that occur in practically all cell types [93]. The haematopoietic system exhibits circadian rhythms that oscillate according to the rest-activity phase that, by turn, depend on whether the species is diurnal or nocturnal. Thus, the numbers of HSCs, committed progenitor cells and most mature leukocytes, except CD8+T lymphocytes [94] peak in the circulation during the resting phase (night for humans and day for rodents) and decrease during the active period [95, 96].

Haematopoietic progenitors and mature immune cells leave the BM to enter the blood circulation at the beginning of the resting phase [97], migrating to tissues predominantly during the active phase [98]. The mobilization from BM is dependent on local sympathetic innervation which down-regulates the CXCL12 expression in the BM in close association with a reduced CXCR4 expression of haematopoietic progenitors [99] and both CD4+and CD8+T cell subsets [94]. On the contrary, the onset of the active phase is related to a peak of glucocorticoids, adrenaline, noradrenaline, TNF and IL1 β [95, 96].

Numerous data support a role for the sympathetic nervous system (SNS) in regulating HSC trafficking. Noradrenalin administration stimulates proliferation and migration of human

HSCs [100], and *Cgt^{-/-}* mice, deficient in the UDP-galactose ceramide galactosyl transferase necessary for sulfatide synthesis, a key component of myelin, have compromised G-CSF-mediated HSC mobilization [33]. Nevertheless, the most remarkable information comes from studies using G-CSF, a cytokine that mobilizes HSCs from the bone marrow to peripheral blood by stimulating adrenergic receptors on target cells resulting in decreased secretion of CXCL12 [33]. The first studies identified osteoblasts as the target stromal cell for the effects observed on HSCs because β_2 adrenergic receptor activation on osteoblasts increases the expression of the vitamin D receptor necessary for G-CSF induced HSC mobilization [34, 101]. Recently, it has been proposed that osteocytes, which indirectly affect osteoblasts on the endosteal surface, could also mediate G-CSF dependent HSC mobilization through a sympathetic tone [32]. This possibility was discarded because adrenergic receptors involved in these processes were largely β_3 receptors not expressed on osteoblasts but on CAR cells and nestin-expressing perivascular cells [33, 52]. Presumably, both adrenergic receptors contribute to regulate haematopoiesis in BM [102].

The circadian rhythms could therefore be explained as a consequence of transmitted information from the central pacemaker to the BM through SNS that rhythmically secretes noradrenalin activating β receptors expressed on different stromal cells. Neural signals could be propagated through the BM stroma via gap junctions whose effects on HSC mobilization have already been commented. Chemical sympathectomy, by using 6-OH dopamine, abolishes the rhythms of circulating blood cell precursors and surgical denervation of mouse tibiae courses with the disappearance of rhythmic CXCL12 oscillations in the denervated limbs [86]. However, recent results suggest that the process could be more complex and the effects of innervation would vary rhythmically and affect different processes in the BM. Systemic activation of β adrenergic receptors does not increase circulating haematopoietic progenitors unless HSC homing is also blocked [97] suggesting that adrenergic stimulation might not only affect mobilization but also homing of haematopoietic progenitors in the BM and/or peripheral tissues [102]. Indeed, when mice defective in E- and P-selectins showing additional α_4 integrin blockade, which inhibited the homing to BM, received isoproterenol, an adrenergic antagonist, during the morning phase they showed considerable HSC mobilization. On the contrary, if isoproterenol administration occurred 1 hour earlier than the onset of light the HSC mobilization was prevented [86].

In any case, it is not easy to establish a direct relationship between sympathetic innervation and mobilizing agents (i.e., G-CSF) that induce haematopoietic progenitor egress from the BM, although some evidence for this is available. Administration of either G-CSF or β_3 adrenergic agonists has similar effects on the haematopoietic niche and HSC mobilization [52]. Tyrosine hydroxylase neurons from the murine superior cervical sympathetic ganglion express G-CSF receptors and this cytokine promotes neuron survival after brain damage [103]. On this basis, [104] have shown that G-CSF increases the sympathetic tone modifying the capacity of neurons to uptake noradrenalin and, consequently, increasing available catecholamine for BM target cells. Furthermore, *in vivo* administration of G-CSF plus noradrenalin re-uptake inhibitors, such as desipramine or reboxetine, induce significantly higher numbers of haematopoietic progenitors in the blood circulation, without changes in BM cell content, than those of animals

receiving only cytokine. These studies support a direct effect of G-CSF on the sympathetic terminals through signalling transmitted by specific receptors on neurons and noradrenalin uptake inhibition.

6. The role of myeloid cells in the hematopoietic niche

We have extensively reported the organization of the main components of BM stroma and their relevance for regulating haematopoiesis. However, many other cells present in the BM that do not form part of the stromal reticular network are also important for this regulation. A clear example is the response of BM to G-CSF administration that is known to have provided such important information on the functioning of the haematopoietic niche. It is obvious that, apart from the BM stromal cells, monocytes/macrophages (Mo/MØ) and granulocytes are the main targets of its action. On the other hand, numerous inflammatory molecules produced mainly by leukocytes affect the behaviour of HSCs in both cell autonomous and non-cell autonomous fashion [105]. Could the response to C-CSF administration between stromal cells and leukocytes be coordinated? or, by contrast, does G-CSF administration induce specific, independent effects on each BM cell type that expresses receptors for this growth factor? Moreover, if this presumptive cooperation exists, how is it established?

Various results support the involvement of monocytes (Mo) and macrophages (MØ) in the functional regulation of the HSC niche [106]. In mice, the loss of MØ results in decreased self-renewal capacity and retention of HSC [107, 108], and it has been reported that G-CSF signalling in a Mo cell line is sufficient to induce HSC mobilization [109]. Is it possible to reconcile these results in which G-CSF mediated HSC mobilization depend on Mo/MØ with those previously described in which HSC egress seems to be mediated by changes in sympathetic tone that affects CXCL12 secretion by CAR cells and/or nestin+cells? Some results try to do this although stromal cell types affected by Mo/MØ activity are controversial. MØ elimination by *in vivo* administration of clodronate-loaded liposomes or use of Fas-induced apoptosis transgenic mice courses with loss of endosteal osteoblasts and reduced HSC mobilization into the blood [108]. In these experiments, endosteal osteomacrophages were particularly affected. In this same study [107] and others, it has been shown that CD169+macrophages directly modulated the activity of nestin+cells by promoting the expression of HSC maintenance molecules, including angiopoietin, CXCL12, SCF and VCAM-1. Factors concerned with these effects are unknown, although some inflammatory molecules have been implicated [107].

Another key question on the regulation of haematopoiesis is how the BM “knows” the necessities of different blood cell subsets at any time and responds to the peripheral demand in each case. Every day there is an enormous production of blood cells and it is necessary to control precisely the number of each blood cell type in the periphery. Some recent results suggest an important role of Mo/MØ and neutrophils (Neu) in this control and show the high plasticity exhibited by the BM stromal components to address these processes. It was reported that BM responds to *Listeria monocytogenes* infection inducing migration of inflammatory Mo

in a process that involves the chemokine receptor CCR2 [110]. CCR2+Mo also migrate from the BM stroma into peripheral blood through vascular sinusoids in response to low concentrations of LPS, a TLR ligand. In these experimental conditions, there is also a significant increase in CCL2 production by perivascular CXCL12+nestin+cells [111]. These same authors showed that conditional deletion of the CCL2 gene under the control of nestin promoter coursed with an important reduction of circulating inflammatory Ly6C^{hi} Mo. The reduction was less severe when CCL2 gene was deleted in endothelial cells. These results show that CCL2 gene up-regulation in nestin+cells, CAR cells and endothelial cells is necessary for inducing migration from the BM into the blood circulation of inflammatory Mo in response to low concentrations of LPS [111]. A similar behaviour was detected after *L. monocytogenes* infection. In mice infected which had deleted the CCL2 gene from nestin+cells, the bacterial clearance was significantly low [111]. It is unclear, however, how the reticular cells of BM stroma govern Mo migration and “perceive” the occurrence of TLR ligands (i.e., LPS) in the circulation, but it is evident that distinct chemokine-chemokine receptor pairs regulate trafficking of haematopoietic progenitors or their mature progeny between BM and the peripheral circulation. Nor is it clear how the migration of a specific cell type affects another blood cell lineage but stimulation of BM-MSK through TLR4 down-regulates the expression of Jagged, a Notch ligand, involved in HSC self-renewal [112].

These results support the existence of mutual influences between BM stromal cells and Mo/MØ but also open interesting concerns about the mechanisms of response of the BM to the peripheral demands of specific blood cell subsets and the possible existence of different stromal cells that define different niches in the BM microenvironment or, alternatively, a unique nestin+cell population capable of adapting its function (i.e., production of different chemokines) in time for the specific demands of the hematopoietic system (see later). The neutrophil is a blood cell type with a short life-span that reflects a fine-tuned turnover between elimination and production, particularly in inflammatory situations. Thus, mice with impaired Neu extravasation, a process necessary for their efficient clearance, results in an imbalance of G-CSF levels and enhanced granulopoiesis suggesting that Neu elimination is part of a homeostatic loop for controlling their level in blood [113]. In this respect, it has been proposed that physiological clearance of Neu in the BM triggers signals that modulate the hematopoietic niche and promote cycles of hematopoietic progenitor mobilization [114]. Neu during aging lose CD62L expression and increase that of CXCR4, that facilitates their migration into the BM [115]. Although the loss of CD62L^{lo} does not contribute to BM homing, within BM CD62L^{lo} Neu are preferentially engulfed by MØ as compared to CD62L^{hi} Neu. On the other hand, the a priori surprising release of haematopoietic progenitors from BM to peripheral blood during Neu clearance could be related to increased levels of G-CSF occurring in that condition [113] that favour HSC mobilization. It may be hypothesized, therefore, that circulating Neu could function as a sensor of the organism status.

7. The other niches of adult bone marrow

Until now we have almost exclusively paid attention to the regulation through the BM niche of early hematopoietic progenitors, their mechanisms of survival, proliferation and differentiation. However, BM stroma houses many developing cells belonging to different blood cell lineages that also need to receive signals from the BM microenvironment for maintenance and maturation. This raises the question of whether the BM constitutes a unique niche capable of numerous functions that vary in their timing and physiological demands or if, instead, the so-called BM niche should be considered as a myriad of different specific “subniches”. In the following pages we will describe some of these “subniches” and their possible relationships to the previously described microenvironments that house and maintain early hematopoietic progenitors.

We have previously emphasized the mutual influences between Mo/MØ and BM stromal cells and their relevance in regulating hematopoietic activity. Erythroblastic islands that are “classical” BM niches, consisting of a central MØ surrounded by developing erythroid cells, and are considered essential for normal erythropoiesis [116] represent other distinct environments in the BM not associated with HSCs but with erythroblast maturation. Recent results suggest a dual role for central MØ contributing to both red blood cell production and clearance in the steady state but playing a supporting role for erythropoiesis during the erythroid recovery that follows haemolytic anaemia, acute blood loss, myeloablation and JAK2 induced polycythemia vera [117, 118]. Thus, during the myeloablative process the depletion of radioresistant CD169⁺MØ impaired the recovery of BM and splenic erythroblasts. A similar condition occurred when antibody blockade of V-CAM was performed suggesting that adhesion molecule-mediated interactions between erythroblasts and MØ are important in this process.

Lymphoid cells constitute another important component of the BM. B lymphocytes mature in the BM whereas other lymphoid cell subsets (i.e., plasmablasts, naïve and memory T-cells) migrate into the BM at any time of their life cycle [13]. The movement of B lymphocytes throughout the central and peripheral lymphoid organs is a good model to evaluate the presumptive occurrence of distinct subniches in the BM. As mentioned above, a pioneer study [2] proposed that primitive B progenitor cells that occurred close to the endosteum progressed during the maturation of distinct cell subsets along the BM reticular network toward the central blood vessels where mature B lymphocytes migrated into the blood circulation. Information on the niches that developing B cells occupy during this journey throughout BM stroma is scarce. The existence of IL7^{hi}- and IL7^{lo}-niches has been reported but their precise location in the BM stroma remains unresolved [119]. On the other hand, pre-BcR signalling necessary for the maturation of pre-pro B cells induces CXCR4 expression and its interaction with CXCL12-expressing stromal cells induces downstream activation of the focal adhesion kinase that could facilitate the movement of pre B cells into the IL7^{lo} niches [120].

In the periphery (i.e., lymph nodes, spleen), naïve B lymphocytes activated by antigens, largely T-dependent ones, become plasmablasts, after passing through the named germinal centres. The conversion of activated B lymphocytes in plasmablasts courses with down-regulation of

both CXCR5 and CCR7 and up-regulation of the S1P1 (sphingosine-1-phosphate) receptor necessary for the exit of plasmablasts from the peripheral lymphoid organs [121]. Plasmablasts that leave the peripheral lymphoid organs migrate predominantly to the BM in a process mediated in part by CXCR4 because sinusoidal endothelial cells of BM express its ligand, CXCL12 [119]. However, other chemokines, including CXCL9, CXCL10 and CXCL11 are also involved in the homing to murine BM [122]. In humans, other chemokines, such as CXCL16 and CCL28 are implicated [123]. Once in the BM, plasmablasts need to home to appropriate niches that guarantee their retention, survival and differentiation to mature plasma cells. CXCL12, some adhesion molecules principally, VCAM1 constitutively expressed by BM stromal cells, and extracellular matrix molecules, such as fibronectin and hyaluronic acid [124] contribute to their retention, but remarkably many arriving plasmablasts fail to establish in the BM. Possible mere competence between arriving plasmablasts, maturing pre-or pro-B cells and/or pre-existing long-lived plasma cells by the space or, more specifically, by the CXCL12-expressing niches could explain these results [125]. Another remarkable finding is that about 50% of the recent immigrant plasmablasts interact with eosinophils (Eos), a smaller number with MØ and some with megakaryocytes [126, 127]. In peripheral lymphoid organs most plasmablasts contact with MØ. The relevance of these cell types for plasmablast colonization of the BM was evidenced in Eos deficient Δ dbl GATA-1 mice in which plasmablasts migrate normally, but only a few are retained and mature to plasma cells. Importantly, the BM stroma in these mutants is normal indicating that VCAM-1+CXCL12+stromal cells are not sufficient to retain and presumptively to allow plasmablast differentiation [128, 129].

At this point it is important to remark that in vitro studies have shown IL5, IL6, TNF α , BAFF (B-cell activating factor), APRIL (a proliferation-inducing ligand) and CXCL12 as well as fibronectin, CD44 and CD28 as plasma cell survival factors [125]. However, in vivo studies have particularly pointed out the importance of APRIL. APRIL-deficient mice or mice deficient in BCMA, the high affinity receptor of APRIL, show a significantly lower number of plasma cells in the BM than WT mice [130, 131]. In addition, plasmablasts transferred into APRIL-deficient mice home normally in the BM but the maintenance of long-lived plasma cells is impaired [129] and blockade of BAFF and APRIL by fusion proteins significantly reduced the plasma cell numbers in the BM [130]. The relevance of APRIL for the survival of plasmablasts and plasma cells is also shown because SLE patients contain numerous plasma cells whose survival is controlled by autocrine expression of APRIL in the self plasma cells [132]. Plasmablasts retained in the BM move from the sinusoids into the stroma where clusters of Eos appear scattered at random throughout the BM parenchyma. These areas containing stromal cells and Eos become evident late after secondary immunization and could constitute the survival niche for mature plasma cells. Remarkably, the number of Eos in BM significantly increases during secondary immune responses when the production of plasma cells peaks. These are activated producing increased amount of cytokines, including APRIL and IL6. It, therefore, appears that a positive feedback governed by the plasma cells activate Eos to induce high levels of plasma cell survival factors.

These results report a new niche in the BM in which stromal cells need APRIL+Eos to home and maintain both plasmablasts and plasma cells. Nothing is known about the stromal cells

associated with Eos, if they are similar to those that support the differentiation of early B lymphocyte progenitors or, by contrast, represent another phenotypic and functional subniche within the BM microenvironment.

8. Adipocytes as negative regulators of haematopoiesis

Adipocytes, other cell component of BM stroma, that were regarded as a passive element in the haematopoietic activity occurring in the BM, in fact appear to be active suppressors of haematopoiesis [133]. Thus, adipocyte-rich BM contains lower numbers of haematopoietic progenitors and fat cells impede their expansion preserving the HSC pool in a process partially mediated through increased expression of neuropilin 1, a semaphorine receptor, involved in angiogenesis and axon guidance [134]. In addition, in lipoathrophic A-ZIP/F1 mice, deficient in adipocytes, or after pharmacological inhibition of adipogenesis, BM exhibits massive osteogenesis and rapid recovery of haematopoiesis [135].

All these data suggest that the BM stromal cells, particularly CAR cells and nestin+cells, represent a highly plastic cell population capable under different physiological conditions, of differentiating either to bone cell lineage or adipocytes, or constitute a niche for homing and maintaining hematopoietic progenitors [136].

9. The haematopoietic niches in the lower vertebrates

When we analyze the characteristics, components and functional relevance of the haematopoietic niches we forget that many vertebrates do not contain a haematopoietic BM but exhibit highly efficient haematopoiesis raising the question of whether they use other mechanisms for controlling blood production, or if blood-forming organs in lower vertebrates have well organized haematopoietic niches equivalent to those described here in mammalian BM. A BM functionally implicated in haematopoiesis appears for the first time in the most evolved urodeles of the *Plethodontidae* family [137]. Earlier in phylogeny, particularly in fish, there are numerous haematopoietic organs which share a structural and functional resemblance to the BM of higher vertebrates [7]. Apparently, in these lower vertebrates any organ that contains a stroma consisting of a reticular network arranged between sinusoidal blood vessels could home and supports differentiation of blood cell progenitors. Obviously, alterations in the histological organization of these organs would result in the loss of their haematopoietic capacities.

The variety of organs involved in haematopoiesis in primitive vertebrates is impressive. In most elasmobranchs, masses of haematopoietic tissue can be found in the oesophageal (Leydig organ) and gonad walls (Epigonal organs) [138]. The kidney is the main haematopoietic organ in many lower vertebrates, including cyclostomes, chondrostei, holostei, dipnoi, polypteriformi, teleostei, some urodelans and embryonic anurans. In this case, cell cords

containing developing blood cells occur arranged among the renal tubules and enlarged blood sinusoids.

The brain and cranium are also suitable sites for haematopoiesis. It occurs in the meninx primitive of some elasmobranchs [139] and in the orbit and subcranium of the holocephali, *Chimaera monstrosa* [140, 141]. An analogous tissue occurs in the meninges of ganoids [142] and in the brain of the urodeles *Ambystoma* [143] and *Megalobatrachus japonica* [144]. The importance of niches for determining the haematopoietic capacity of distinct organs is evidenced during the ontogeny of amphibians and throughout the complex life cycle of lampreys. In amphibians which do not have haematopoietic BM, the kidney retains the blood-forming function. In contrast, in the urodeles of the *Plethodontidae* family which already contain a haematopoietic BM, the kidney is not capable of producing blood cells [137]. In adult anurans with a functional BM, the kidney which had formed blood cells during embryonic and foetal life also loses its hematopoietic capacity [145], although some primitive families (i.e., *Pipidae*) maintain a granulopoietic perihepatic cell layer [146]. In larval lampreys, the typhlosole, a fold of the midgut, and the nephric fold consisting of the larval opistonephros and a little closely-associated adipose tissue are the main haematopoietic organs. All of these have a histological organization similar to that of mammalian BM. During metamorphosis, these organs, particularly the typhlosole, regress completely because the reticular network that constituted the haematopoietic niche is substituted by fibroblasts and masses of collagen fibres. After metamorphosis, when the adult opistonephros is organized, the haematopoietic tissue re-appears there, although the most remarkable adult haematopoietic organ in lampreys is the supraneural body. In larvae, this fat column along the central nervous system only contains adipocytes but just after metamorphosis it begins to home blood cell progenitors in cell cords arranged among big fat cells resembling the organization of BM of higher vertebrates [147].

10. Concluding remarks

It is evident that in a few years the data on the organization, cell and molecular components of adult BM have increased exponentially. However, this mass of information has generated new, important questions that reflect the enormous complexity of the haematopoietic niche and its physiology. The haematopoietic niche is more complex and heterogeneous than the current picture we have owing to the limited cell markers and experimental approaches available. To determine whether this heterogeneity is real, or rather reflects a high plasticity that permits a specific cell type to change its functionality throughout time depending on physiological demands is an important matter that needs an urgent answer. Before this global issue can be resolved we must first address some specific points:

1. An accurate, fine anatomical analysis of the BM is required which permits the precise location of the different components and the relationships between bone, blood vessels (arterioles and sinusoids) and reticular cells to be conclusively established.
2. The relationships between CAR cells, nestin+cells and LepR+cells are unclear. Although they presumably overlap considerably it will be important to define precisely which cell

type (and its possible subsets) is included in each cell population and what is its function in the haematopoietic niche.

3. The physiological significance of the homeostatic changes in the biology of haematopoietic niches also needs clarification and the appearance and maturation of distinct stromal components of BM during ontogeny, aging and disease must be determined.
4. It is also important to define the genes/signals involved in the maintenance and differentiation of various haematopoietic progenitor subsets as well as those that specifically differentiate BM stromal cells to either adipocytes or bone cells.

More importantly, we need to have an integrative model that allows to us to understand the complex interactions that govern haematopoiesis through the BM microenvironment in physiological and pathological conditions. Most self-renewing organs are supported by a myriad of stem cell niche units, distributed throughout the tissue. Moreover, growing evidence points to high heterogeneity in terms of molecular profiles, division patterns and population sizes of stem cells and niches within a given tissue [148]. Organs, therefore, face the considerable challenge of regulating not only each individual niche but also critically, the collective output of all the niches in a given organ. It is, therefore, important to define how multiple, heterogeneous and spatially dispersed units are coordinated in a tissue,

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