HARNESSING THE COMPLEXITY OF NORMAL AND PATHOLOGICAL HEMATOPOIETIC SUPPORTIVE NICHES

EDITED BY: Stéphane J. C. Mancini, Dominique Bonnet and César Nombela Arrieta

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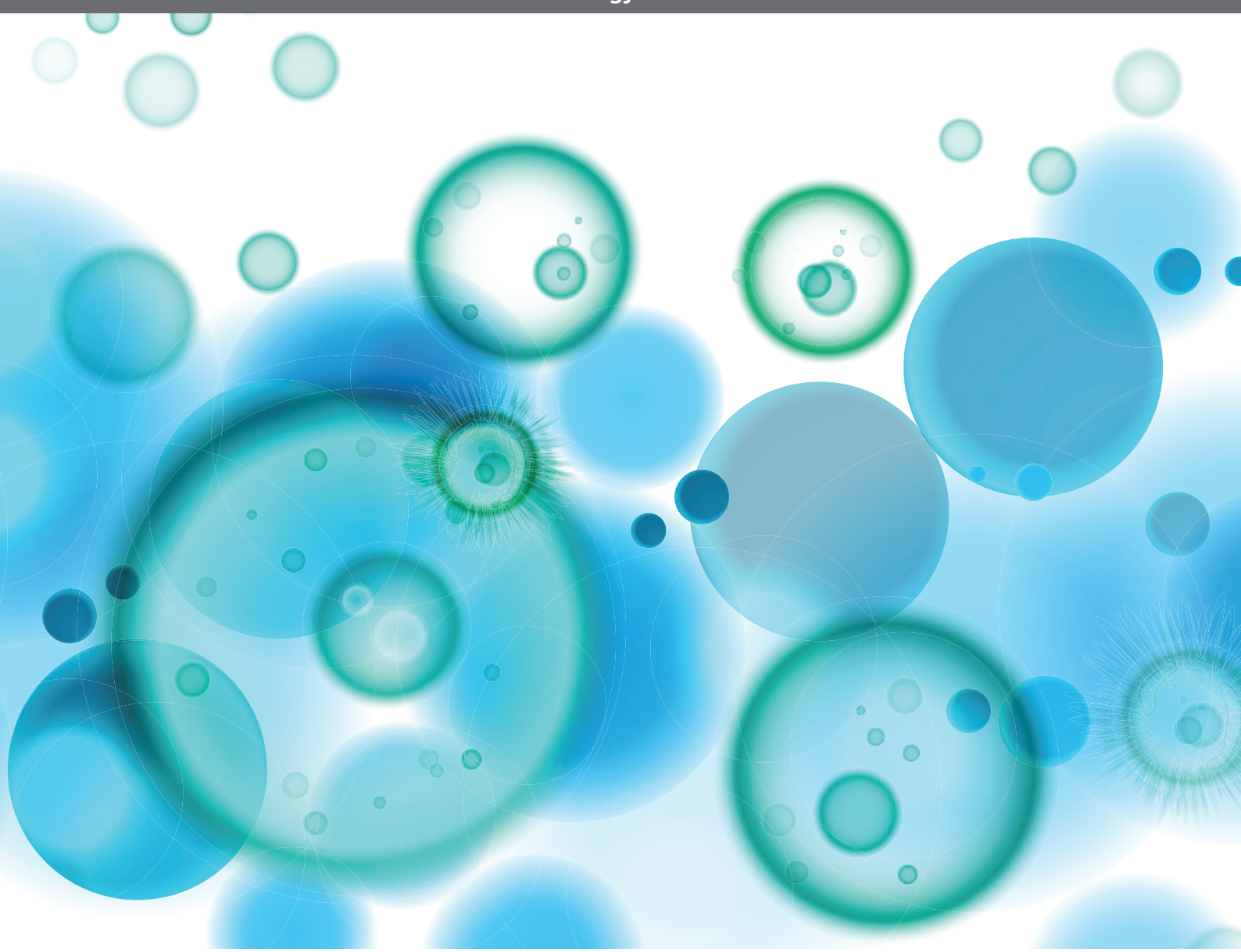

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HARNESSING THE COMPLEXITY OF NORMAL AND PATHOLOGICAL HEMATOPOIETIC SUPPORTIVE NICHES

Topic Editors:
Stéphane J. C. Mancini, INSERM UMR1236 Microenvironnement, Différenciation cellulaire, Immunologie et Cancer, France
Dominique Bonnet, Francis Crick Institute, United Kingdom
César Nombela Arrieta, University of Zurich, Switzerland

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REVIEW
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Harnessing Mesenchymal Stromal Cells for the Engineering of Human Hematopoietic Niches

Alice Pievani[1†], Roberto Savoldelli[2,3†], Juliane Poelchen[4,5†], Elisa Mattioli[2,3†], Giorgio Anselmi[6], Alice Girardot[7], Jochen Utikal[4,5], Pierre Bourdely[7], Marta Serafini[1‡] and Pierre Guermonprez[7‡]*

[1] Department of Pediatrics, M. Tettamanti Research Center, University of Milano-Bicocca, Monza, Italy, [2] The Peter Gorer Department of Immunobiology, Centre for Inflammation Biology and Cancer Immunology, School of Immunology & Microbial Sciences, King's College London, London, United Kingdom, [3] Cancer Research UK King's Health Partner Cancer Centre, King's College London, London, United Kingdom, [4] Skin Cancer Unit, German Cancer Research Center (DKFZ), Heidelberg, Germany, [5] Department of Dermatology, Venereology and Allergology, University Medical Center Mannheim, Ruprecht-Karl University of Heidelberg, Mannheim, Germany, [6] MRC Molecular Hematology Unit, Radcliffe Department of Medicine, Medical Research Council, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom, [7] Centre for Inflammation Research, CNRS ERL8252, INSERM1149, Hopital Bichat, Université de Paris, Paris, France

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Edited by:
César Nombela Arrieta,
University of Zurich, Switzerland

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Medizinische Universität Graz, Austria

***Correspondence:**
Pierre Guermonprez
pierre.guermonprez@cnrs.fr

[†] These authors share first authorship

[‡] These authors share last authorship

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Tissue engineering opens multiple opportunities in regenerative medicine, drug testing, and modeling of the hematopoiesis in health and disease. Recapitulating the organization of physiological microenvironments supporting leukocyte development is essential to model faithfully the development of immune cells. Hematopoietic organs are shaped by spatially organized niches defined by multiple cellular contributions. A shared feature of immune niches is the presence of mesenchymal stromal cells endowed with unique roles in organizing niche development, maintenance, and function. Here, we review challenges and opportunities in harnessing stromal cells for the engineering of artificial immune niches and hematopoietic organoids recapitulating leukocyte ontogeny both *in vitro* and *in vivo*.

Keywords: hematopoiesis, organoids model, bone marrow niche, mesenchymal stroma cell, thymus epithelial cell, 3D culture

INTRODUCTION

Mechanistic studies of the human hematopoiesis, drug testing, immunization and regenerative medicine purposes share a need for immune organoids recapitulating physiological immune niches. Hematopoietic organs supporting leukocyte development are shaped by spatially organized areas defined by multiple cellular contributions. All immune niches contain mesenchymal stromal cells (MSCs) endowed with unique and specific roles in organizing niche development, maintenance and function. MSCs were first described in murine bone marrow (BM) as a population of cells containing fibroblast progenitors and capable to differentiate into cartilage, bones, adipocytes, and to recapitulate a hematopoiesis supporting microenvironment upon transplantation (1, 2). MSC features were subsequently identified in non-hematopoietic cells of the human BM niche (2–6). Here the term "MSCs" will be used from a practical perspective, referring to an often heterogenous populations of mesodermal origin, regardless of their multilineage potential and self-renewal capabilities at the single cell level.

In this review we highlight the challenges and opportunities in harnessing MSCs for the engineering of artificial immune niches recapitulating the physiological bone marrow (BM) or thymic niche.

THE BONE MARROW NICHE FROM THE MSC PERSPECTIVE

MSCs in the Murine Niche

The BM is the primary lymphoid organ deputed to the maintenance, self-renewal and lineage commitment of adult hematopoietic stem cells (HSCs). HSCs originate from the dorsal aorta in the aorta-gonad-mesonephron region of the embryo. Embryonic HSCs seed and expand first in the fetal liver before colonizing specific areas of the trabecular region of the BM called niches where they become proper mature HSCs. Niches are complex 3D environments composed of multiple cell types and factors, such as extracellular matrix (ECM), oxygen tension, soluble molecules, and shear forces, all of which govern the fate of HSCs. Most of our knowledge regarding the components of the BM niche and how they interact to modulate HSC fate has been attained by findings in murine models due to the limited possibilities to access and harness information from human specimens. MSCs are critical contributors of the HSC niche. A key feature of MSCs is the delivery of chemokines and hematopoietic growth factors essential for the maintenance and differentiation of HSCs (2). The chemokine CXCL12 engages the CXCR4 chemokine receptor on HSCs. As a consequence, the CXCR4/CXCL12 axis is essential for HSC retention within the BM niche and maintaining HSC quiescence (7). SCF/KITL engages the KIT tyrosine kinase receptor on HSC which is crucial for their maintenance. Proteolytic cleavage and alternative splicing mechanisms generate membrane bound and secreted forms of SCF/KITL. Selective inactivation of the membrane bound form of KITL in *Sl*/*Sl*^{d} mutant mice impairs long term HSCs despite the presence of soluble KITL (8, 9). In addition, adult deletion of SCF/KITL in inducible knock-out mice depletes HSCs (10). This highlights the importance of cell-to-cell contacts in the delivery of hematopoietic growth factors to HSCs for their maintenance. The production of niche factors has been an important feature guiding the identification of MSC subpopulations relevant for the HSCs niche. For instance, CXCL12^{gfp} mice allowed to identify CXCL12 abundant fibroblastic reticular cells (CAR) (7), while SCF^{gfp} reporter mice showed SCF/KITL expression in both MSCs and endothelial cells (ECs) (10). In addition to ECs and MSCs, multiple other lineages have been reported to also contribute to the maintenance of the niche: for instance, osteolineage cells, adipocytes, neurons and hematopoietic cells. The endosteal niche is found in proximity of the endocortical region of the internal bone shell. It is formed mainly by MSCs together with osteoclasts, osteoblasts, and tissue resident macrophages (11, 12). Multiple lines of evidence locate the HSCs niches mainly in the perivascular areas of BM. The perivascular niche is formed by endothelial and other MSCs. SCF^{gfp} reporter mice have enabled a systematic evaluation of SCF/KITL-expressing cells and revealed a crucial contribution of endothelial and perivascular MSCs compartments (10).

The precise location of HSCs with respect to sinusoidal ECs (sECs) or arterial ECs (aECs) has been controversial (13–18). It is unclear if sinusoids represent a "proliferative" niche whereas arteriole would represent a "quiescent" niche (15) or if sinusoids are the main HSCs localization regardless of their proliferation status (16). The late conclusion has been provided by examining thoroughly HSCs localization defined by KIT^{+} GFP-labeled cells in alpha-catulin^{gfp} reporter mice (16). The development of a fluorescent reporter mouse line fully specific for long term Flt3^{-} HSCs (MFG mice) has enabled to address their localization in un-manipulated environments by intravital imaging (18). This breakthrough revealed that quiescent HSCs seat mostly in vicinity to sECs (and the endosteum), in mildly hypoxic environment (18). Despite the preferential localization of HSCs next to sECs, sECs have recently been reported to express lower levels of SCF/KITL and CXCL12 as compared to aECs, thereby questioning the involvement of aECs in the niche (19–22). Identifying the MSC population of the HSC niche and defining its developmental potential within mesenchymal lineages has proven to be a major challenge. Early work has evidenced BM mesenchymal osteoprogenitors (i) can be engrafted and regenerate bones (2, 23, 24); (ii) are endowed with a multi-lineage potential for osteoblasts, chondrocytes, adipocytes, and fibroblasts (2, 4). Later studies have attempted to prospectively identify self-renewing and multi-potent *bona fide* MSCs (2). A breakthrough in the field has been afforded by studies which reported that murine CD45-Tie2-a$_{v}^{+}$CD105^{+}Thy1.1-fetal BM MSCs are able to recapitulate a functional HSCs niche upon transfer within the renal capsule (25). In addition, Morikawa et al. have shown that PDGFRa^{+}Sca1^{+}CD45^{-}TER119^{-} MSCs from mouse BM give rise to osteoblasts, reticular cells and adipocytes after *in vivo* transplantation (26, 27). Taken together, these studies have defined the so-called "two stem cells" paradigm in which multipotent MSCs are both organizers and components, besides HSCs, of the BM niche (25, 28, 29). The Frenette lab has shown that Nestin^{gfp} reporter mice could be instrumental to identify MSCs with stem cell properties: (i) high CFU-F activity; (ii) a multi-lineage potential to generate fibroblasts, osteoblasts, chondrocytes and adipocytes (in both mice and humanized mice); (iii) a self-renewal potential upon transfer (28). More recently, MSCs activity has been tracked using the LepR^{cre} mice targeting MSCs expressing the leptin receptor (30, 31). Recent scRNAseq studies have re-evaluated the heterogeneity among LepR^{+} cells and highlighted the existence of two subsets, one expressing adipocyte-related genes (Adipo-CAR) while the other expressing osteogenic genes (Osteo-CAR) (19, 21). Micro-dissection analysis suggests that Adipo-CAR would be more associated to sinusoids, as compared to Osteo-CAR, preferring arteriole and other regions (21, 22). Multiple line of evidences, including the inference of developmental trajectories *in silico* (32), suggest that the Adipo-CAR fraction of LepR^{+} cells contains the most primitive MSC progenitors activity giving rise to multiple adipocytes, osteoblasts and chondrocytes (22, 32). Studies from the Morrison lab have established that LepR^{+} cells account for most BM CFU-F activity, represent the main source of bone and adipocytes in adult BM and can give rise to cartilage (30). Various technical approaches have identified multiple cellular entities within the MSCs compartment proposed to play a major role in shaping of the perivascular niche (10, 15, 28, 33):

i) Arteriole-associated pericytes. Pioneer work from the Frenette lab identified arterioles-associated pericytes expressing NG2/CSPG4 coupled with quiescent HSCs in the endosteum region (15). $NG2^+$ pericytes are rare and display bright GFP levels in *Nestin*^{gfp} reporter mice (*Nestin*^{bright}) (15). Conditional and inducible cellular deletion of NG2 pericytes (using NG2-*cre*^{ERTM}xROSA^{iDTR} mice) induces HSCs cycling and reduces long-term repopulating activity (15). *Nestin*^{bright} pericytes express high levels of CXCL12. This feature is relevant for HSCs niche, as conditional inactivation of CXCL12 -but not SCF- in $NG2^+$ pericytes reduces HSCs numbers, perturbs their localization within the BM and induces their peripheral mobilization (34).

ii) Sinusoid-associated pericytes. Sinusoid associated MSCs display high level of GFP in *Cxcl12*^{gfp} mice (7), intermediate level of GFP in *Nestin*^{gfp} reporter mice (15), and are labeled in *LepR*^{cre} mice (10, 30). From the niche point of view, $LepR^+$ positive cells (labeled in *LepR*^{cre} mice) express high levels of SCF/KITL and some levels of CXCL12 (10, 33). Conditional deletion of SCF in $LepR^+$ cells depletes quiescent HSCs (10). Conditional inactivation of CXCL12 in $LepR^+$ cells triggers HSCs mobilization in periphery (33). In addition to SCF and CXCL12, Adipo-CAR produce multiple hematopoietic factors like IL-15, IL-34, Csf1, Bmp4, Ccl19, and Ccl2 (22). Furthermore, $LepR^+$ cells act also as a major source of IL-7, important for the homeostasis of lymphoid-committed progenitors which also express CXCR4, ensuring their correct positioning by responding to CXCL12 from $LepR^+$ MSCs (35).

MSCs in the Human Niche

Compared with the mouse system, much less knowledge exists regarding the architecture of the human BM niche and the function of its different cellular components. Using human bone biopsy specimens, Guezguez et al. provided evidence of HSC propensity to localize to endosteal regions of the trabecular bone area (36). $CD34^+$ cells follow a spatial gradient within the marrow cavities with a maximal concentration in the first 50 μm from the bone trabecula surfaces where the blood vessels are most concentrated (37). Efforts in recent years have also been made to identify the specific human MSC subpopulation that supports HSC activity. In the human BM, a multi-potent $CD45^-CD105^+CD146^+$ sinusoid-associated fraction of MSCs was shown to be able to recapitulate a functional HSCs niche upon transfer under the mouse skin (5). Human pericytes $CD146^+$ express nestin, CXCL12, and LepR similar to mouse perivascular MSCs and directly support the *ex vivo* maintenance of human HPSCs through cell-to-cell contact and activation of Notch signaling (38). A fraction of human $CD146^+$ perivascular MSCs expressing PDGFRα, CD51, and multiple niche factors (e.g., CXCL12, SCF, and angiopoietin1) would correspond to an ortholog of GFP-positive cells in Nestin^{gfp} mice. Upon transplant in immunodeficient mice, this $CD146^+$ MSC subset is mainly localized in close proximity to mouse sinusoids and recruit hematopoietic cells (39). All these evidences seem to suggest that $CD146^+$ perivascular cells could represent the human counterpart of the CAR cells or $nestin^+$ cells described in the mouse. More recently, a population of $CD146^-$ $CD271^+$ MSCs localized in the trabecular region of the human BM has been identified. Like the $CD146^+$ perivascular cells, these $CD271^+$ MSCs showed high clonogenicity, trilineage differentiation capacity *in vitro*, and ability to transfer a HSC microenvironment upon transplantation (40). $CD271^+SSEA4^+$ MSCs have also been shown to express high levels of HSC-supportive genes and to support HSC engraftment potential (41). These data indicate that different subtypes of MSCs exist in the human BM niche and interact with HSCs in specific regions.

MODELING THE BONE MARROW NICHE USING MSCs

A Role for MSCs in Modeling the Niche *in vitro*

HSCs *in vitro* generation is instrumental to understand hematopoiesis as well as to model genetical disorders and cancers. Moreover, large scale manufacturing of HSCs could represent a valuable therapeutic option for many patients. To date, two different approaches have been attempted in this way: (i) the expansion of large number of HSCs from BM or umbilical cord blood (CB) (42, 43); (ii) the *de novo* generation of HSCs from induced pluripotent stem cells/embryonic stem cells or via somatic cell reprogramming (44–48).

Standard culture protocols supporting the proliferation of long term *bona fide* engraftable, self-renewing hematopoietic stem and progenitor cells (HSPCs) with multi-lineage potential remain a challenge. Given the natural role of MSCs in the HSC niche, co-culture with MSCs is a very popular approach to maintain and expand HSCs *in vitro*. However, these conventional systems fail at reproducing the complexity of the BM niche.

The first step in mimicking the physiological HSC niche consist on generating a 3D environment using different biomaterials, such as hydrogels, silicate structures, and human bone-derived scaffolds (49) (**Table 1**).

Those polymers provide structure and support for cell proliferation but also a spatial control of the cell interactions. This is achieved by physical limitation in cell-to-cell contact and controlled availability of soluble factors. Integration of ECM within these scaffolds has been explored. Feng et al. demonstrated how a polyethylene terephthalate (PET) scaffold can be engineered with ECM proteins, such as collagen and fibronectin, to support the expansion and differentiation of CB-$CD34^+$ cells (50). Importantly, those synthetic scaffolds can be colonized with different types of MSCs together with ECs to mimic *in vivo* niches. Ferreira et al. and Raic et al. (54, 55) have developed two independents models based on porous 3D scaffold for the expansion of HSCs *in vitro*. Ferreira et al. tested several natural polymers as scaffolds in combination with MSCs as support and found that 3D fibrin scaffold seeded with MSCs is the most efficient system to expand $CD34^+$ cells. Importantly, expanded HSPCs maintain a more primitive immunophenotype and exhibit strong engraftment and multi-organ repopulation capability (54). Similarly, Raic et al. showed that the positive effect of MSCs on preservation of HSPCs

TABLE 1 | Modeling the BM niche *in vitro* using human MSCs.

Reference	Scaffold-based culture system	Hematopoietic cell source	Findings
Feng et al. (50)	Fibronectin (FN) or collagen-conjugated 3D polyethylene terephthalate (PET) scaffold	CB-derived $CD34^+$ HSPCs	Significant expansion of $CD34^+$ cells with high SCID repopulating function
Nichols et al. (51)	Silicate scaffold coated with PDDA and clay and seeded with stromal cells (human bone marrow stromal HS-5 cell line and human fetal osteoblast 1.19 cell line)	$CD34^+$ HSPCs from different sources	Support expansion of HSPCs and production of functional B cells
Rödling et al. (52)	3D macroporous PEG hydrogel with RGD-peptides seeded with human MSCs and perfused in a bioreactor	CB-derived $CD34^+$ HSPC	Maintenance and differentiation of $CD34^+$ HSPCs in dynamic culture. Importance of perfusion on drug testing (myelotoxic effects of chemotherapeutics)
Braham et al. (53)	Bio printable pasty CPC scaffold with seeded O-MSCs to model the endosteal niche, and Matrigel containing both EPCs and MSCs to model the perivascular niche	Primary $CD138^+$ myeloma cells	Significant increase in the proliferation of myeloma cells. Essential role of the perivascular niche over the endosteal niche in supporting myeloma cells
Ferreira et al. (54)	Comparison of 3D PCL, PLGA, fibrin and collagen scaffold either seeded or not with UC-MSCs	CB-derived $CD34^+$ HSPCs	All scaffolds except PLGA favored the expansion of HSPCs. When the scaffolds are seeded with MSCs the results improve, electing fibrin as the best scaffold
Raic et al. (55)	3D macroporous hydrogel scaffold seeded with UC-, BM-MSCs, or osteoblast-like cells	CB-derived $CD34^+$ HSPCs	HSPCs cultured with BM-MSCs in 3D systems have the highest proliferative status while maintaining stemness
Leisten et al. (56)	3D collagen scaffolds in suspension to generate a double niche, in semi-solid and liquid phase	CB derived HSPCs	Most differentiated cells are found in the liquid phase niche. Differentiation is boosted by UC-MSCs. More immature HSPCs relies in the solid phase of the scaffold
Bourgine et al. (57)	Porous hydroxyapatise scaffold seeded with BM-MSCs within a perfusion bioreactor	CB-derived $CD34^+$ HSPCs	Supported maintenance of HSPCs; possibility to perturb HSPCs behavior by molecular customization or injury stimulation
Sieber et al. (58)	Hydroxyapatite coated zirconium oxide scaffold seeded with BM-MSCs in a microfluidic system	CB-derived $CD34^+$ HSPCs	Successful long-term culture (up to 28 days) of HSPCs with multilineage differentiation potential
Bruce et al. (59)	3D microfluidic model loaded with BM-MSCs and osteoblasts encapsulated in collagen matrix	B-ALL SUP-B15 cell line	Decreased chemotherapeutic drug sensitivity of leukemic cells in 3D tri-culture model from the 2D models
Chou et al. (60)	Perfused PDMS organ chip with "hematopoietic" channel (filled with BM-MSCs in a fibrin gel) and "vascular" channel (lined by HUVECs)	mPB-derived $CD34^+$ HSPCs Shwachman-Diamond Syndrome BM-derived $CD34^+$ HSPCs	Supported differentiation of multiple blood-cell lineages; reproduction of hematotoxicities after chemotherapy/ionizing irradiation; reproduction of marrow recovery after drug-induced myelosuppression; recapitulation of hematopoietic abnormalities of patients with genetic disorders

HSPCs, hematopoietic progenitor stem cells; HUVEC, Human Umbilical Vein Endothelial Cells; PDDA, poly(diallyldimethylammonium chloride); CPC, calcium phosphate cement; O-MSCs, osteogenic multipotent mesenchymal stromal cells; BM, bone marrow; MSC, mesenchymal stromal cells; PDMS, poly(dimethylsiloxane); PEG, polyethylene glycol; RGD, arginylglycylaspartic acid; EPC, endothelial progenitor cells; PCL, poly(epsilon-caprolactone); PLGA, poly (lactide-co-glycolide) acid; UC, umbilical cord; CB, cord blood; PB, peripheral blood; B-ALL, B-cell acute lymphoblastic leukemia.

stemness was more pronounced in a porous 3D hydrogel scaffold in comparison to standard 2D culture systems (55). Moreover, phenotypically immature HSPCs ($CD34^+CD38^-$) with self-renewal and repopulation capacity are shown to be maintained in 3D collagen co-culture with MSCs, in close proximity to the collagen fibers (56). Nichols et al. demonstrated how a silicate and clay 3D structure seeded with heterogeneous stromal cells and human HSCs is able to expand the HSCs and promote B cell development after 28 days, with a significant increase compared to its 2D counterpart (51).

Latest development in the field is the combination of 3D organoids with perfusion-based bioreactor systems, the so-called "4D system," to further increase the amount of resemblance to *in vivo* niches. The BM microenvironment is indeed strictly regulated by the concentration of soluble factors, oxygen levels, and the mechanical stress applied by blood flow. The use of bioreactors and microfluidics devices can than allow modeling the niche situation more closely. Rödling et al. developed a bioreactor system for perfusion of 3D scaffolds seeded with MSCs mimicking the BM *in vivo* and demonstrated the importance of perfusion during drug treatment as results are different with and without perfusion (52). Indeed, while under static conditions the more mature $CD34^-$ subpopulation was more sensitive to 5-fluorouracil treatment, under dynamic conditions both $CD34^-$ and $CD34^+$ cells responded similarly. Bourgine et al. reported the engineering of BM-like tissues in a perfusion bioreactor system partially recapitulating structural, compositional and organizational features of the native human osteoblastic niche environment, resulting in the support of HSPC functions. Their approach consists in the use of bone-like porous hydroxyapatite scaffold functionalized by MSCs and osteoblastic cells and by the ECM they deposited during perfusion culture in bioreactors (57). 3D scaffold-based microfluidic chips have been introduced for the generation of a "BM-on-a-chip." Torisawa et al. in

TABLE 2 | Engineering of MSCs for enhanced human niche activity.

Reference	Cell culture	Ectopic expression on stromal cells	Findings
Patel et al. (66)	BM-derived $CD34^+$ HSPCs co-cultured with OP9-DL1 cell line	IL-7 and FLT3L	T cell progenitor proliferation
Carretta et al. (67)	$CD34^+$ HSPCs co-cultured with human MSCs	IL-3 and TPO over-expression	*In vitro* expansion of $CD34^+$ HSPCs. *In vivo*, humanized models producing IL3/TPO support growth of patient samples
Anselmi et al. (68)	CB-derived $CD34^+$ HSPCs co-cultured with MS5 or OP9 cell lines	FLT3L, SCF and CXCL12	*In vitro* formation of DCs resembling their circulating counterparts. *In vivo* formation of a niche supporting the differentiation of DCs and the maintenance of undifferentiated HSPCs

BM, bone marrow; HSPCs, hematopoietic progenitor stem cells; MSC, mesenchymal stromal cell; CB cord blood; IL-7, Interleukin-7; FLT3L, FMS-like tyrosine kinase 3 ligand; IL-3, Interleukin-3; TPO, thrombopoietin; SCF, stem cells factor; CXCL12, C-X-C motif chemokine ligand 12; DCs, dendritic cells.

a pioneer murine study showed that PDMS (poly-dimethyl-siloxan) device loaded with bone forming factors could be seeded *in vivo* by BM-MSCs, such as $CXCL12^+$ CAR and $Nestin^+$ $LepR^+$ perivascular MSCs. *Ex vivo* culture of the organoid can be achieved in a microfluidics chip (61). Sieber et al. loaded hydroxyapatite-coated zirconium oxide scaffold with human MSCs in a microfluidics chip. This enables HSPCs to maintain multilineage potential up to 28 days *in vitro* (58). Chou et al. seeded a fibrin scaffold with MSCs and $CD34^+$ cells in a microfluidics device embedding vascular channel seeded with human ECs. Upon 4 weeks of culture the vascularized chip recapitulated the ontogeny of multiple blood cell lineages while maintaining undifferentiated HSPCs. Moreover, this system has been used to model the impact of a genetic disorder on myelopoiesis (60).

MSCs in microfluidic devices have also been instrumental in modeling the leukemic niche *in vitro* for the sake of studying chemotherapeutics and immunotherapy (53, 59, 62).

Engineering MSCs to Regulate HSC Self-Renewal or Direct Hematopoietic Differentiation

By virtue of their role in HSC niche, engineering of MSCs offers a window of opportunity to control HSC self-renewal or drive hematopoietic differentiation. Several factors regulate the HSC-supporting activity of MSCs. For instance, primary $Nestin^+$ murine MSCs rapidly lose their hematopoietic supporting potential upon *ex vivo* culture (63). The transcriptional down-regulation of key niche factors (SCF, ANGPT1, CXCL12, VCAM1) is underlying this process (63). An elegant genetic screen has revealed that overexpression of defined transcription factors (Klf7, Ostf1, Xbp1, Irf3, Irf7) can "revitalize" the niche promoting activity of *ex vivo* cultured primary MSCs. MSCs overexpressing those transcription factors become able to maintain transplantable HSCs (63). This important study opens new avenues in the engineering of MSCs. Some niche factors involved in the hematopoietic hierarchy present a limited sequence homology between mouse and human genes. This is limiting the reactivity across species thereby rendering mouse MSCs sub-optimal for applications involving human HSPCs (64, 65). For these reasons, multiple groups have attempted the expression of defined human hematopoietic factors in murine MSCs lines (**Table 2**). For instance, co-expression of IL-7 and FLT3L synergizes with DLL1 expression in OP9 to induce the proliferation of T cell progenitors (66). Building on the MS5 mouse MSC line, Anselmi et al. have developed a screening for combination of human niche factors promoting the efficient generation of dendritic cells (DCs) from human CB-$CD34^+$ cells (68). They have found that a combination of membrane-bound FLT3L and SCF and soluble CXCL12 is efficient in promoting the differentiation of DCs resembling their circulating counterparts (68). Importantly, transwell experiments indicate that this system relies on the establishment of cell-to-cell contacts. Subcutaneous engraftment of engineered MSCs in basement membrane matrix (Matrigel®) in NSG mice defines a niche supporting both the maintenance of a pool of undifferentiated $CD34^+$ cells and the differentiation of DCs (68). Within this niche, it was found that poorly differentiated human $CD34^+$ cells would develop cell-to-cell contact with engineered MSCs. Previous reports have established that membrane-bound forms of hematopoietic growth factors like SCF would be specifically required for niche function *in vivo* (8, 9, 69–71). A practical consequence of this is that engineering of MSCs for the over-expression of membrane-bound SCF (or FLT3L) is an attractive strategy to improve the niche-promoting activity of MSCs *in vitro* or *in vivo* (68, 72) (**Figure 1**). In the same vein, human BM-MSCs have been engineered by Carretta et al. to over-express IL-3 and thrombopoietin (TPO). IL-3/TPO over-expressing MSCs displayed an increased ability to drive the *in vitro* expansion of $CD34^+$ cells and improved capacity to support *in vivo* growth of $CD34^+$ progenitors expressing the MLL-AF9 fusion gene in a humanized scaffold xenograft model (67). *In vivo* delivery of engineering stromal cells could be improved by the implementation of chemically defined scaffold. For instance, Tavakol et al. have shown that collagen coated carboxyl methyl cellulose micro scaffold (CCMs) seeded with OP9 and HSPCs *in vitro* supports the long term maintenance, over 12 weeks, upon engraftment in immunodeficient mice (73).

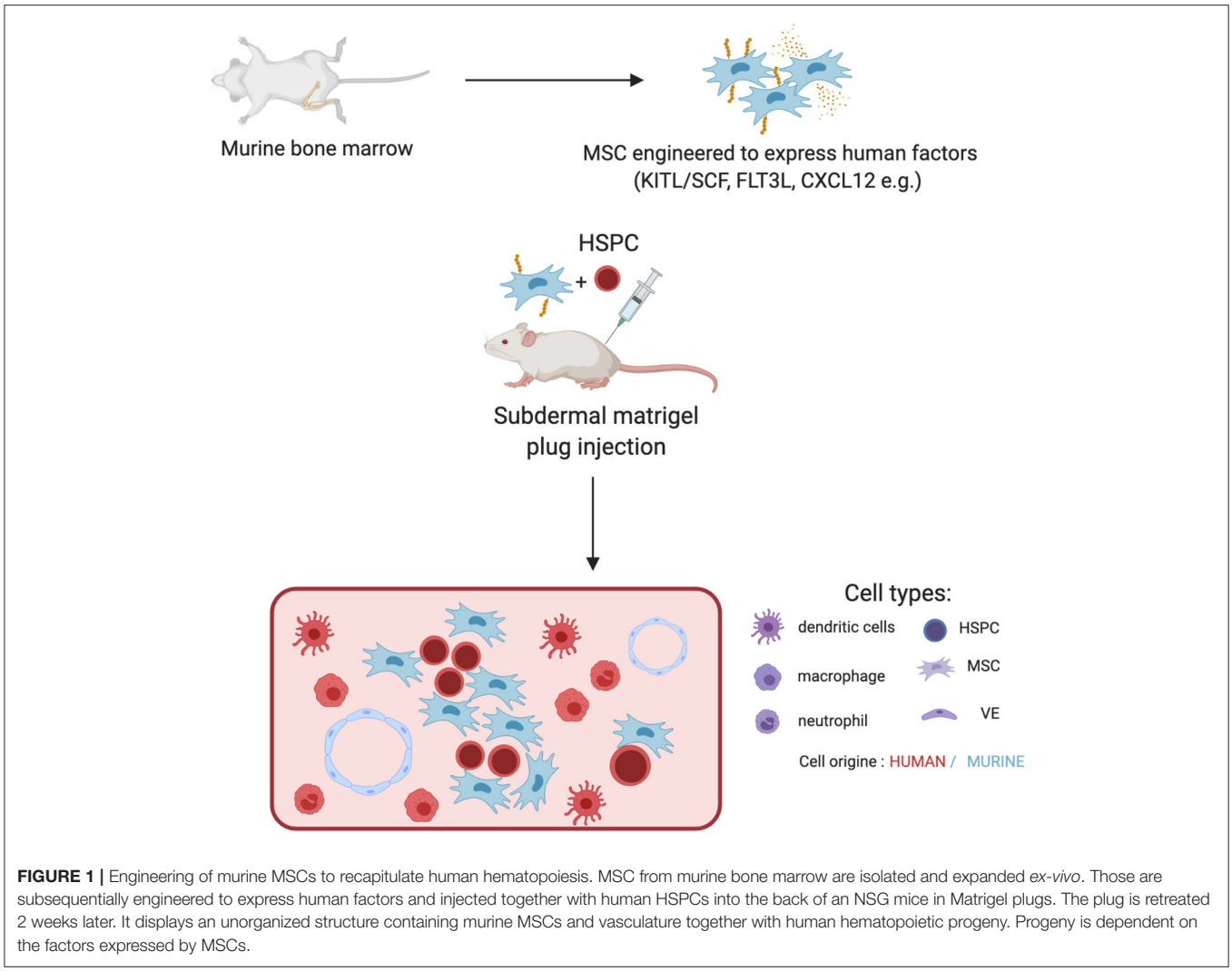

FIGURE 1 | Engineering of murine MSCs to recapitulate human hematopoiesis. MSC from murine bone marrow are isolated and expanded *ex-vivo*. Those are subsequentially engineered to express human factors and injected together with human HSPCs into the back of an NSG mice in Matrigel plugs. The plug is retreated 2 weeks later. It displays an unorganized structure containing murine MSCs and vasculature together with human hematopoietic progeny. Progeny is dependent on the factors expressed by MSCs.

Altogether those approaches highlight the versatility of MSC engineering to control and direct HSPCs fate in health and diseases.

A Role of MSCs in Recapitulating the Human Bone Marrow Niche *in vivo*: The Humanized Ossicle Models

Pioneer studies showing that human MSCs can establish a hematopoietic microenvironment upon transplantation in rodents at non-skeletal sites date back nearly 50 years (74–76). The evolution of bone tissue engineering strategies together with the identification of the human specific osteoprogenitor subpopulations associated with the formation of ectopic bone and bone marrow have been fundamental steps for the generation of humanized BM tissues in mice, the so-called "ossicle" model (5, 25, 28, 29). These human MSC–generated organoids are tissue-specific chimeras, as bone, myelo-supportive stroma, and adipocytes derived from donor MSCs, while blood vessels and hematopoietic tissues are derivatives of host tissues, and they are harmoniously integrated into an overall tissue structure. Several versions of heterotopic transplantation assays have been used, which differ from one another concerning the site of grafting, such as kidney, subcapsular space (25, 77), intramuscular (78), and subcutaneous tissue (79) or type of osteoconductive scaffold/material employed as a carrier (79).

The next level of humanization of human MSC–generated ossicles has been the introduction of human HSCs. In 2010, Vaiselbuh et al. for the first time reported the successful engraftment of human hematopoietic cells in an ectopic humanized niche obtained implanting subcutaneously in immunocompromised mice polyurethane scaffolds coated with human MSCs, giving rise to the first fully humanized bone/BM organoid model (80). The establishment of a functional human BM niche that could support the maintenance of human blood cells opened the possibility to bridge gaps between the interspecies divergence at a molecular and cellular level in the hematopoietic niche and aspires to become an advanced model to investigate human hematopoiesis and leukemogenesis. Although many aspects of the hematopoiesis are conserved

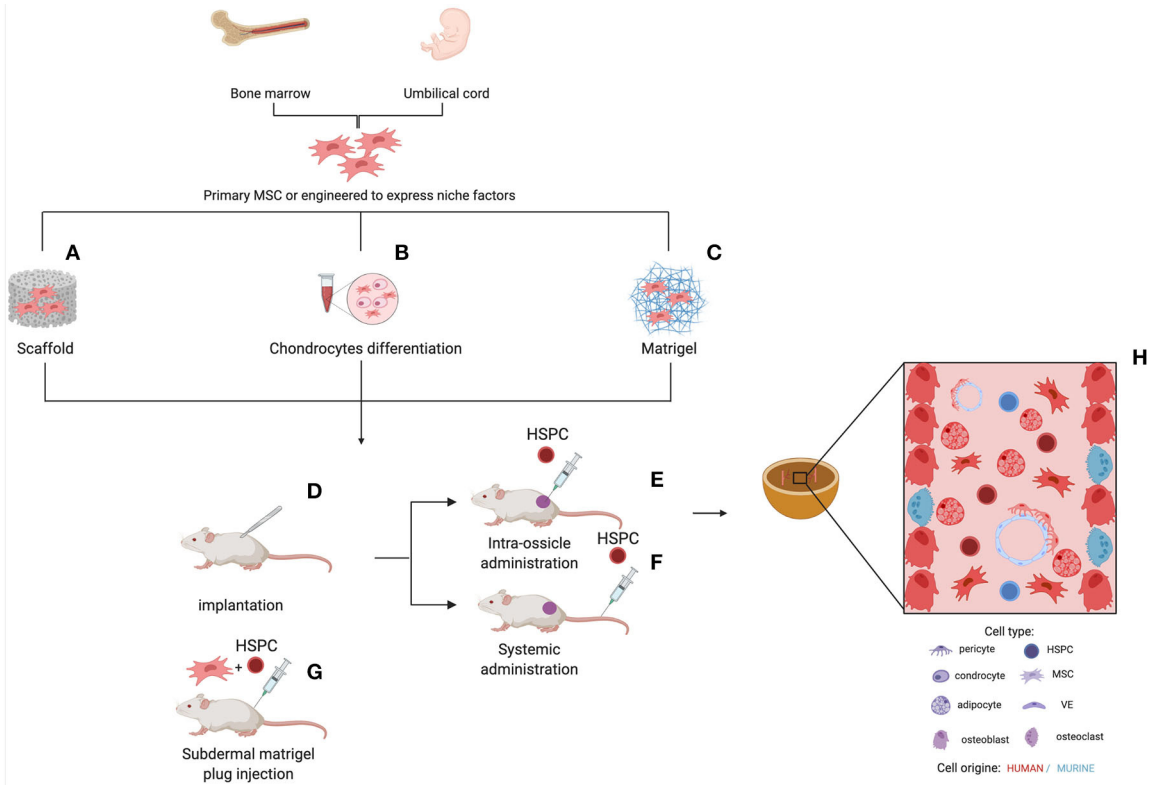

FIGURE 2 | Different strategies for the generation of humanized ossicles. *In vitro* expanded human MSCs (from healthy donors or patients) are seeded onto a scaffold, **(A)** primed to differentiate in cartilage **(B)** or cultured on Matrigel® **(C)** Some protocols include the co-seeding of human ECs and the supplement of osteogenic factors, such as BMPs and PTH. Following the first *in vitro* step, the implantable structures are transplanted subcutaneously into the back of immunodeficient mice for the ossicle formation **(D)**. Aberrant/normal human HSPCs can be added to the system by direct intra-ossicle transplantation **(E)** or intravenous injection **(F)** either before or after the implantation of the ossicle. Irradiation is usually performed to promote engraftment. As an alternative, HSPCs can be seeded onto the Matrigel® plug before the *in vivo* implant **(G)**. The whole process can take several months. The resulting ossicle collected from the mouse is composed of both myelo-supportive marrow stroma and hematopoietic tissues and its progeny of human origin **(H)**. Sinusoidal endothelium, nerve fibers, residual hematopoietic cells, and osteoclasts are derivatives of mouse host.

between mice and humans, several differences need to be taken into consideration before applying results obtained in the mouse to humans, specifically for studies aimed at preclinical testing of new therapies (81). Several humanized ossicle models have been reported to date and each has its specificity, as extensively reviewed recently (**Figure 2**) (82, 83). Current strategies differ for human stromal cell types, carrier material scaffold, human hematopoietic transplantation protocols, and experimental time frames (**Table 3**). Most protocols use stromal cells derived from the BM of healthy donors. Reinisch et al. suggest that only MSCs, as opposed to the umbilical cord-, skin-, or white adipose tissue-derived MSCs, possess the capabilities to form ectopic bone and BM *in vivo* (77). However, other studies reported the formation of ectopic BM niches through endochondral ossification using stromal cells from different sources, such as cord blood (94) and adipose tissue (97), when primed toward chondrogenesis in the presence of transforming growth factor-β *in vitro* before implant. In most protocols, cells are seeded onto ceramic, collagen, calcium phosphate, or hydroxyapatite-based scaffolds or hydrogels before implantation. These scaffolds provide instructive cues to ensure osteogenesis and represent a 3D template that supports the formation of a bone organ. According to their composition and degradation properties, scaffolds can be entirely remodeled during the ossicle formation or remain part of the organoid structure. The persistence within heterotopic ossicles of artificial, mineralized scaffold material that are not resorbable, is not desirable. It prevents the establishment of the completely normal architecture of bone marrow and complicates the analysis of stromal and hematopoietic cell populations contained within the ossicle, particularly their quantitative assessments. The transplantation of cartilage pellets made *ex vivo* by MSCs consent to avoid these limitations due to the use of exogenous scaffold (91, 93). Stimulating factors, such as BMP2 (96), BMP7 (95), or parathyroid hormone (PTH) (87) can be used to promote osteoblast differentiation of MSCs for the successful *in vivo* formation of mature bone and BM tissues. Furthermore, MSCs have also been genetically modified to express BMP2 (98) or BMP7 (99) generating new bone *in vivo*.

Human blood cells (healthy or malignant) can be administered either by peripheral (tail vein or retro-orbital)

TABLE 3 | Humanized ossicle models.

References	Scaffolds	Cell source	Implant generation	Mice strain	Time after implant before human blood cells transplantation	Conditioning	Route of hematopoietic transplantation	Transplanted human blood cells	Engraftment period
Vaiselbuh et al. (80)	Polyurethane discs	BM-MSCs (10 × 10^6)	Seeding on scaffold and culturing in medium + 20% FBS + SDF-1 for 4–5 days	NOD/SCID	Unknown	None	*In situ* injection	Primary AML samples	1, 4, 8, 16, 20 weeks
Lee et al. (84)	Polyacrylamide hydrogel	BM-MSCs (1–5 × 10^5)	Seeding on scaffold and culturingin medium + 10% FCS for 1–3 days	NSG or Nu/Nu	4 weeks	Sublethal irradiation	Intravenous injection	$CD34^+$ HSPC	16 weeks
Chen et al. (85), Jacamo et al., (86), and Reinisch et al. (87)	Matrigel	BM-MSCs (1.5 × 10^6) and ECFCs (1.5 × 10^6); BM-MSCs (2 × 10^6)	BM-MSCs are mixed with ECFCs in Matrigel immediately before implant; daily PTH administration for 28 days in ossicle-bearing mice	NSG	8–10 weeks	Sublethal irradiation	*In situ* injection; intravenous injection	CB derived-$CD34^+$ HSPC or MOLM13 leukemia cells; NALM6 leukemia cells; primary AML, APL, and MF samples	4–11 weeks for $CD34^+$, 2 weeks for MOLM13; 10 days for NALM6, 7–24 weeks for patient samples
Groen et al. (88), Antonelli et al. (89), Sontakke et al. (90), and Carretta et al. (67)	BCP	BM-MSCs; IL-3- and TPO-expressing BM-MSCs	Seeding on scaffold and culturing in osteogenic induction medium for 7 days	RAG or NSG	6–8 weeks	None	Intracardiac or *in situ* injection; intravenous injection	CB derived-$CD34^+$ HSPC or primary multiple myeloma cells; CB-$CD34^+$ BCR-ABL or MLL-AF9 transduced; primary AML or CML samples	8 weeks; from 14 to 38 weeks for AML
Scotti et al. (91), Fritsch et al. (92), and Bourgine et al. (6)	Collagen sponges	BM-MSCs (2 × 10^6)	Seeding on scaffold and culturing for 3 weeks in chondrogenic medium, followed by another 2 weeks of culture in hypertophyc medium	STRG or MISTRG	4 or 6 weeks	Sublethal irradiation	Intravenous injection	CB derived-$CD34^+$ HSPC	8 or 6 weeks
Serafini et al. (93) and Pievani et al. (94)	None	BM-MSCs or CB-BFs (3 × 10^5)	Culturing for 3 weeks in chondrogenic medium supplemented with TGF-B1 as pellet	SCID/beige	3 weeks	Sublethal irradiation	Intravenous injection	CB derived-$CD34^+$ HSPC	6 weeks
Holzapfel et al. (95)	Tubular mPCL	BM-MSCs (3 × 10^5)	Seeding on scaffold and culturing in medium + 10% FCS for 4 weeks, followed by 4 weeks of dynamic cell culture in osteogenic medium	NSG	10 weeks	Sublethal irradiation	Intravenous injection	Pelvic BM derived-$CD34^+$HSPC and $CD34^-$ cells	5 weeks
Abarrategi et al. (96)	Collagen sponges	BM-MSCs (1 × 10^5)	Seeding on scaffold and culturing in medium + 10% FBS supplemented with rhBMP2 for 3–7 days	NSG	48 h or 6–4 weeks pre-implant	None or sublethal irradiation	Pre-seeding in the scaffold or *in situ* injection or intravenous injection	CB derived-$CD34^+$ HSPC or patient AML samples	12 weeks

BCP, biphasic calcium phosphate; mPCL, medical grade polycaprolactone; BMSCs, bone marrow mesenchymal stromal cells; ECFCs, endhotelial colony-forming cells; TPO, thrombopoietin; CB-BFs, cord blood borne-fibroblasts; FBS, fetal bovine serum; SDF-1, stromal derived factor 1; FCS, fetal calf serum; PTH, parathormone; TGF-β1, transforming growth factor β1; NSG, NOD/SCID/IL-2rγ^{null} mice; STRG, Rag2$^{-/-}$IL2rγ$^{-/-}$ mice expressing human TPO and SIRPα; MISTRG, Rag2$^{-/-}$IL2rγ$^{-/-}$ mice expressing human TPO, M-CSF, and SIRPα; CB, cord blood; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ML, myelofibrosis; CML chronic myelogenous leukemia; HSPC, hematopoietic progenitor stem cells; BMP2, bone morphogenetic protein 2.

TABLE 4 | Stromal systems recapitulating T cell maturation.

Reference	Approach	Findings
Röpke (124), Masuda et al. (125), and Corbeaux et al. (126)	Culture of TECs on feeder cells	Notch signaling pathway from thymocytes to TECs is involved in TECs maturation and lymphoid development.
Schmitt and Zuniga-Pflucker (127)	Co-culture of OP9 expressing hDLL1 with fetal liver progenitors with addition of IL-7	Differentiation toward formation of α/β and γ/δ T cells
La Motte-Mohs et al. (128)	$CD34^+CD38^-$ HSPCs cultured on OP9-DL1	Appearance of $CD7^+$ pro-T cells, $CD4^+$ intermediate SP, and $CD4^+CD8^+$ DP
Yeoman et al. (129)	Murine FTOC seeded with human $CD34^+$ HSPCs (human UCB or BM HSPCs)	Formation of human T cells which can rapidly develop into $CD4^+$ or $CD8^+$ SP cells expressing CD3
Poznansky et al. (130), Traggiai et al. (131), Ishikawa et al. (132), and Pearson et al. (133)	Tantalum-coated carbon matrix embedded with murine thymic epithelial cells and human cord blood $CD34^+$ HSPCs	This system supports the differentiation of SP $CD4^+$ or $CD8^+$ mature T cells able to respond to mitogens
Chung et al. (134)	Dissociation and re-aggregation of post-natal human thymus in TEC and thymus mesoderm forming thymic organoids seeded with $CD34^+$ HSPCs	When engrafted within the quadriceps muscle sheath of NSG mice thymic organoids are seeded by T cell precursors (from the cord blood origin). Furthermore, the organoids support differentiation of T cells exhibiting a broad repertoire of TCRβ chains
Parent et al. (135)	Development of human thymic epithelium from iPSCs has opened new avenues for the production of thymic organoids	Human ESCs-derived thymic epithelium supports the development of murine T cells within thymus-deficient mice
Melkus et al. (136), Wege et al. (137), and Kalscheuer et al. (138)	Implantation of fetal thymus under the renal capsule leads to the formation of a competent thymus subsequently seeded by BM HSPCs delivered intravenously	The organoid supports the full maturation of T cells in 15-20 weeks
Seet et al. (139) and Montel-Hagen et al. (116)	3D artificial thymic organoids (ATOs) composed by ectopically expressing DLL4 murine BM MSC line MS5 and HSPCs or iPSCs	This method recapitulates human lymphopoiesis. T cells display a normally broad repertoire and exhibit normal responsiveness (proliferation, cytokines) upon TCR triggering

TEC, thymic epithelial cells; HSPCs, hematopoietic progenitor stem cells; SP, single positive; NSG; TCR, T cell receptor; MSC, mesenchymal stem cells; iPSCs, induced pluripotent stem cells; UCB, umbilical cord blood; ATO, artificial thymic organoid; TCR, T cell receptor. BM, bone marrow; DLL1/4, Delta Like Notch Ligand 1/4; FTOC, fetal thymic organ culture; DP, double positive.

or intra-ossicle infusion for organoids of larger dimensions. By contrast, Abarrategi et al. proposed co-implantation of MSCs and human $CD34^+$ cells within a collagen sponge, thus avoiding the requirement for subsequent transplantation (96). Conditioning before transplantation does not seem to be a requirement for the successful engraftment of ossicles within immunodeficient mice. The human hematopoietic cell transplantation was generally performed within 3–10 weeks after the *in vivo* implant of the ossicles. Four weeks represent the minimum period required for the formation of marrow cavities allowing human engraftment, through osteoclasts resorption of mineralized tissue within the ossicles (93). Engraftment assessment post-transplantation was achieved from 4 to 38 weeks, depending on the type of cells transplanted (malignant cells with fast/slow engraftment) and the functional readout targeted (short/long-term HSCs engraftment). However, numerous challenges remain to be solved and the variability of different protocols does not allow to do a comparison between different systems in terms of accurate reconstitution of the human niche and potential for human blood engraftment sustaining. The topic has been recently well-reviewed by Dupard et al. (82). Despite the optimization efforts, the ossicles remain largely chimeric as both the nervous system and blood vessels are of mouse origin, although human mesenchymal perivascular cells were detected (6). This implies that investigations on the role of specific niche cellular factors or cytokines should consider the influence of their murine counterparts. Human vascular structure can be generated by seeding human ECs (e.g., HUVEC) together with MSCs on carrier materials (85, 96). Stringent quantification of both human MSCs-derived stroma and human blood populations in humanized ossicles is difficult to be performed and required the application of most sophisticated imaging strategies. Most of the reported ossicle approaches are based on the use of primary MSCs isolated from BM of healthy donors. Specifically, these cells are very heterogeneous in terms of proliferation and differentiation capacity and this reflects not only the source- and the donor-dependent variability, but also specific differences in isolation/expansion procedures (100). Furthermore, there is a striking batch-to-batch variability in their ability to form ossicles *in vivo*. All these aspects highlight some limits in the full exploitation of these models although this does not diminish the potential of humanized ossicle approaches for studying human healthy and pathological hematopoiesis.

Application of MSC-Derived Humanized Ossicles to Study Human Normal Hematopoiesis

The establishment of a human BM microenvironment within heterotopic ossicles was associated with enhanced long-term engraftment of human HSCs, as compared to mouse bones (87, 95, 96). The human HSC engraftment was successfully supported also by BM niches generated from cord blood stromal cells (94). Notably, human MSCs, included in the ossicle, release an increased level of cytokines, such as VEGF and IL-6, that accelerates vascularization and enhances the recruitment of human HSCs (84). Moreover, an improved myeloid development was obtained compared with lymphoid-biased human engraftment usually observed in the BM of

intravenously transplanted NSG mice (85, 87, 96). Furthermore, some studies have demonstrated that humanized ossicles maintain the quiescence and the self-renewal potential of human HSCs, which can successfully engraft secondary recipient mice, with a higher efficiency compared to murine BM (87, 92). Within the ossicle, human HSCs $CD45^+CD34^+CD90^+$ have been found in close proximity to human MSCs, suggesting that direct cell-cell contact is fundamental to regulate their fate (6). The ossicle system allows genetic manipulation of human niche components to better understand directly *in vivo* the role of specific factors critical for human hematopoietic reconstitution. Overexpression of CXCL12 by human MSCs in the ossicle results in a specific enrichment in common myeloid progenitors, megakaryocyte/erythrocyte progenitors, multipotent progenitors, and HSC populations expressing the CXCR4 receptor (6).

Application of MSC-Derived Humanized Ossicles to Model Human Malignant Hematopoiesis

Patient-derived xenograft (PDX) mouse models are currently the gold standard for studying the development of human leukemia. However, engraftment and expansion of human acute myeloid leukemia (AML) *in vivo* remain challenging as a substantial number of samples fail to engraft also the most optimized host mice, particularly in the case of malignancies less aggressive (101). The reason may be that some subtypes of AML have low progenitor cell frequency or some samples may be particularly sensitive to the lack of a specific cell type in the mouse BM or a factor that is poorly or not at all cross-reactive between mice and humans. Hence, MSC-derived humanized ossicle models have raised great interest in the leukemia field, as recently extensively discussed in the review of Abarrategi et al. (83). The first study of AML engraftment in humanized microenvironment was reported by Vaiselbuh et al., who demonstrated that primary AML cells injected directly in pre-implanted scaffolds coated with human MSCs or intravenously in mice after implant, successfully engrafted in the ectopic niche (80). Further studies demonstrated that AML samples non-engrafting in mouse BM, such as acute promyelocytic leukemia (APL), were able to efficiently engraft in the humanized microenvironment (87, 96). Importantly, ossicles maintained the clonal heterogeneity in xenografted AML cells and their stem cell self-renewal capacity better than murine BM, as demonstrated by serial transplantation assays (87, 89, 90). Humanized BM ossicles are useful also for the engraftment of small myeloid clones, such as TP53 mutated AML subclones (102). Battula et al. developed a different approach called "human bone implant" that consists of subcutaneous transplants in NSG mice of fragments from freshly collected human BM biopsies using Matrigel® as a carrier (103). The implanted human BM tissue undergoes vascularization and bone restoration in mice, providing a functional human BM microenvironment capable of supporting the human AML engraftment. In addition to AML, a humanized ossicle system has been used to engraft primary multiple myeloma patient samples, which are known to be highly dependent on the human BM microenvironment for their survival and growth (88). Furthermore, humanized ossicles facilitated robust engraftment of myelofibrosis specimens, which has previously shown only limited engraftment with transplantation of large numbers of patient-derived $CD34^+$ cells in conventional xenograft models (87). Genetical manipulation of human niche components can likely help in better understanding the role of factors critical for leukemia engraftment/progression. Deletion of hypoxia-inducible factor (HIF)-1α in human MSCs impaired leukemia engraftment in BM organoids by decreasing CXCL12 expression (85). Another pilot study demonstrated that the blockade of NF-kB activation through IkBα-SR overexpression in MSCs in the humanized ossicle model reduces leukemia burden following chemotherapy, diminishing the stroma-mediated chemoresistance (86). Carretta et al. improved the development of the myeloid compartment from leukemic samples by genetically engineering human MSCs to express IL-3 and TPO (67). Recently, a fully humanized hematopoietic niche system has been exploited to investigate the multidirectional crosstalk among AML, HSCs and the microenvironment and allowed to identify stanniocalcin 1 and its transcriptional regulator HIF-1α as specific mediators whereby AML impairs normal hematopoiesis by remodeling the mesenchymal niche (104). Of note, current 3D models use MSCs isolated from BM of healthy donors, which are molecularly and functionally different from disease-exposed ones. The use of patient-derived niche components may further improve these models and help unravel the role of the niche in the development of hematopoietic diseases. We recently reported an AML stromal niche model obtained using MSCs derived from BM of AML patients (105). AML-MSCs derived ossicles contained a significantly increased fraction occupied by adipocyte and represent an osteoprogenitor-rich niche with the presence of $osterix^+$/$osteocalcin^-$ pre-osteoblasts and $osteocalcin^+$/Dentin matrix acid phosphoprotein (DMP) 1^- immature osteocytes that correlated with the reduced mature bone formation. However, the generation of humanized ossicles from MSCs and hematopoietic cells from the same patient in an autologous setting has yet to be demonstrated but it would provide a personalized *in vivo* model to test new therapies.

RECAPITULATING T CELL ONTOGENY USING MSCs

T cells originate from BM derived lymphoid progenitors differentiating in the thymus. The 3D organization of the thymus is provided by different cell types and creates a complex unique environment for T cell development (106). There are numerous reasons and motivations to recapitulate T cell education in the context of thymic organoids.

i) Primary immunodeficiencies constitute a major cause of deficiencies. BM or umbilical cord transplant represent a clinical approach that is potentially limited by the onset of graft vs. host disease (GvHD) and slow reconstitution of the T cell compartment (107). Also, patients who underwent thymectomy or suffer from the DiGeorge syndrome, a genetic disorder underpinning thymus hypoplasia resulting

from microdeletion on chromosome 22, would benefit from thymus bioengineering.

ii) Thymic involution. After reaching the maximum size during adolescence, the thymus begins to shrink and T cell generation decreases in a process called "thymus involution" (108). Thymic involution is associated with aging and exacerbated by several pathological and environmental influences including viral and bacterial infections, drugs or irradiation, affecting its functionality and leading to a decline in naïve T cell output (109, 110). Defective thymus structure and dysfunction negatively influences the adaptive immune system (110). Therefore, regenerating thymic function through replacing a defective thymus by an artificial thymus organoid is of high clinical interest for overcoming potential immunodeficiency or malignancies and maintaining the adaptive immune system.

iii) Adoptive T cell therapy using infusion of antigen-specific T cells is a promising approach in personalized medicine for the treatment of cancer or chronic viral infections. Engineering TCR-specific T cells starting from $CD34^+$ HSPCs (111–113) or T cell precursors derived from induced pluripotent stem cells (iPSCs) (49, 114–116) represent a promising approach that necessitates to recapitulate T cell ontogeny.

iv) The engraftment of thymic organoids into humanized mice is a promising approach for the induction of T cell tolerance against transplanted tissue (110).

v) Mechanistic studies. *In vitro* models of thymic education offer unique advantage to study and mechanistically dissect thymic selection. For instance, *in vitro* systems are particularly amenable to live imaging approaches (117).

T cells differentiate from BM derived $Lin^-CD34^{+/int}CD38^-CD45RA^+$ progenitors seeding the thymus (118, 119), within the thymus T cell progenitors upregulate CD7, CD1a, and CD4 to generate immature single positive (ISP) cells. ISP cells further develop to $CD4^+CD8^+$ double positive (DP) cells that ultimately differentiate into $CD8^-CD4^+$ or $CD8^+CD4^-$ single positive mature T cells (SP). Thymic epithelial cells mediate positive and negative selection of T cell progenitors cells (TEC). TEC can be classified in cortical (cTEC) and medullary (mTEC) epithelial cells. cTEC deliver chemotactic (e.g., CCL25, CXCL12), differentiation (e.g., DLL4), and survival (e.g., IL-7, SCF) signals to developing T-cells undergoing positive selection (120, 121). mTEC express AIRE, together with dendritic cells, present self-antigens ensuring the deletion of high affinity self-reactive T-cells (122).

Most experimental systems aiming at modeling thymus function relies on the manipulation of TECs and fall outside of the scope of this review focusing on mesodermal components of hematopoietic niches (123). In brief, 2D culture of TECs provided disappointing results in generating lymphocyte progenitors in line with the loss of primary phenotype upon *in vitro* culture (124–126) (**Table 4**). First attempts of 3D cultures were based on murine fetal thymic organ culture (FTOC) (140, 141) (**Figure 3B**). Despite the successes in supporting T cell development, FTOC evolved into reaggregate thymus organ culture (RTOC) (141). RTOC allows to manipulate the cellular composition and thereby to study the role of specific cell types, pathways or key signals including the Notch Delta like ligand (123) (**Figure 3B**).

Mesenchymal stromal cells are part of the physiological thymic microenvironment. Recent single cell studies have highlighted the complexity of the mesenchymal compartment of the thymus. Thymic fibroblasts can be distinguished in type 1 (Fb1) and type 2 (Fb2) fibroblasts (142). Fb1 cells are characterized by the expression of an important key player in innate immunity termed COLEC11 as well as by the expression of the enzyme ALDH1A2 that controls the production of retinoic acid functioning as an epithelial growth regulator. On the other hand, Fb2 cells are characterized by ECM genes as well as semaphorins that regulate vascular development. For that reason Fb2 cells are mainly found close to large blood vessels lined with VSMCs (142). ECM produced by thymic MSCs might play a crucial role in the maintenance of TEC phenotypes. For instance, human fibroblast/MSCs uniquely provides an environment supporting promiscuous gene expression by mTECs associated to AIRE and FoxN1 expression (143).

Here we provide examples of the implementation of MSCs in modeling T-cell education and thymic function.

Harnessing MSCs to Recapitulate T Cell Education

The Zúñiga-Pflücker lab has pioneered the implementation of murine MSCs engineered to express NOTCH ligands to drive T-cell maturation from HSPCs. OP9 is MSC line derived from CSF1-deficient mice with a broad hematogenic activity dependent on SCF/KITL expression (144) but unable to sustain the generation of T cells (127). Schmitt et al. have shown that ectopic expression of the Delta-Like-1 (DLL1) NOTCH ligand is sufficient to confer the ability to support ontogeny of α/β and γ/δ T cells from fetal liver progenitors in the presence of FLT3L and IL-7 growth factors (127) (**Figure 3A**). DLL1 provides a key signal for T cell specification at the expense of B cell development. This approach was later found to also recapitulate the ontogeny of human T cells from umbilical cord blood (UCB) $CD34^+$ HSPCs via the ordered appearance of $CD7^+$ pro-T cells, $CD4^+$ intermediate SP, and $CD4^+CD8^+$ DP (128). Importantly, the OP9 approach (using DLL4) could also be implemented to generate T cells from human embryonic stem cells (145). However, one major drawback of the OP9-DLL system is that T cell differentiation and maturation of TCR^+ SP $CD4^+$ or $CD8^+$ T cells remained inefficient for most sources of human pre or post-natal HSPCs with the exception of UCB (146). Similar to the *in vitro* TECs culture, the culture of Notch ligand expressing OP9 cell lines shows the drawback of lacking a 3D architecture (147). The BLT (BM, liver, thymus) model of humanized mice provides an option to model human thymus function *in vivo*. Implantation of human fetal thymus under the renal capsule leads to the formation of a competent thymus that can be seeded by BM HSPCs delivered intravenously to support the full maturation of T cells in 15–20 weeks (136–138) (**Table 4**).

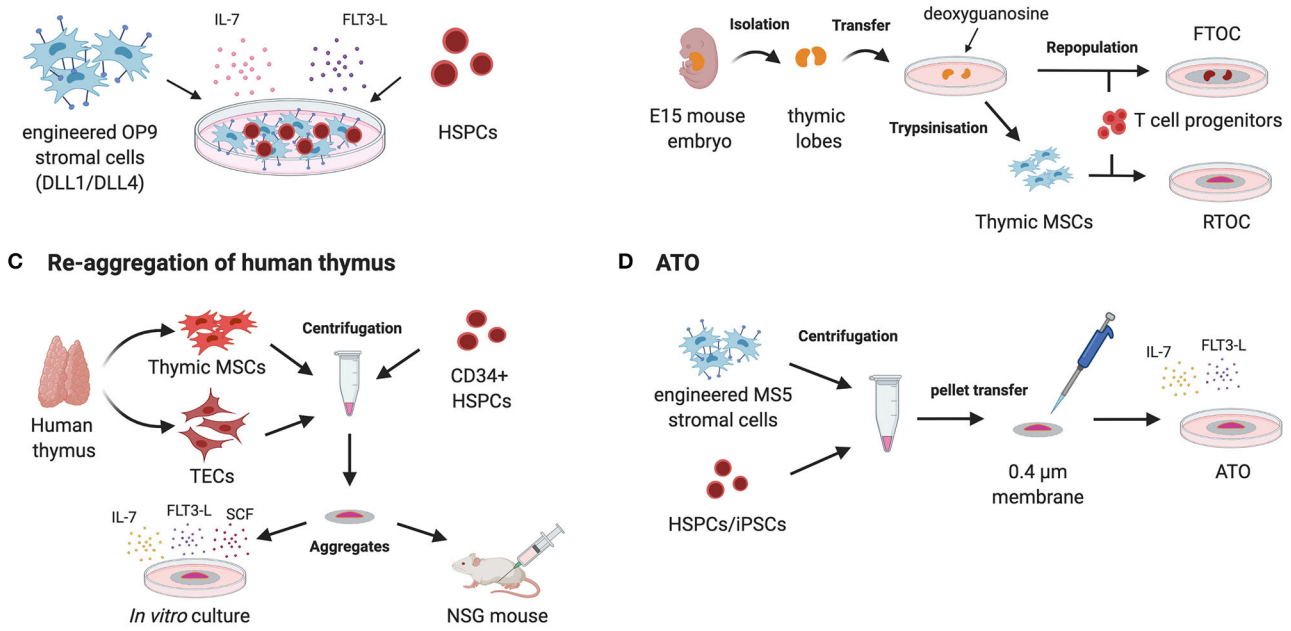

FIGURE 3 | Different strategies to recapitulate T cell ontogeny using MSCs. 2D co-culture of HPCs (hematopoietic progenitor cells) on bioengineered MSC-derived OP9 cells expressing Notch ligands DLL1/DLL4 with recombinant IL-7 and FLT3-L **(A)**. Isolation of murine thymic lobes from day 14 to 15 old mouse embryos followed by their *in vitro* culture with deoxyguanosine for 5–7 days to deplete intra-thymic T cells. The thymic lobes are then repopulated by different T cell progenitors and cultured on the surface of membranes as fetal thymic organ culture (FTOC). For reaggregation thymic organ culture (RTOC) thymic stromal cells (thymic SCs) are extracted from the lobes and reaggregated with T cell progenitors by centrifugation. The cell suspension is cultured on a filter membrane *in vitro* **(B)**. Post-natal human thymus is dissociated in TECs and thymus mesoderm (TM) and further reaggregated with human $CD34^+$ cells leading to the formation of thymic organoids. These organoids support the development of mature human T cells when kept in culture or when engrafted within the quadriceps muscle sheath of NSG mice **(C)**. Artificial thymic organoids (ATOs) are generated by the centrifugation of bioengineered MS5 cells expressing Notch ligands DLL1/DLL4 with HSPCs or iPSCs, respectively. After resuspension in a small amount of culture medium the cell suspension is placed on a membrane at the air-liquid interface to form 3D aggregates. This method recapitulates human lymphopoiesis and offers the ability to generate conventionally naïve T cells from HSPCs or iPSCs *in vitro* **(D)**.

Bypassing the need for fetal thymus mesodermal components have been shown to help building 3D models of human thymus. Using the dissociation and re-aggregation of post-natal humans TECs and thymus mesoderm Chung et al. have recapitulated thymus function (134) (**Figure 3C**). Those thymic organoids supported the development of mature human T cells when seeded with human $CD34^+$ HSPCs (134). This system offers the advantage to be amenable to lentiviral transduction for manipulation of the thymic environment and bypass the use of xenogenic (murine) thymic epithelium (134). When engrafted within the quadriceps muscle sheath of NSG mice previously reconstituted with human CB-$CD34^+$ HSPCs, thymic organoids are seeded by T cell precursors (from the CB origin). Furthermore, the organoids support differentiation of T cells exhibiting a broad repertoire of TCRβ chains (134).

More recently, Seet et al. have shown that MSCs can help modeling thymus function (139). Seet et al. report a method for the formation of artificial thymic organoids (ATOs) in which TECs were replaced by the ectopically expressing DLL4 murine MSC line MS5 (MS5_DLL4) and centrifuged with HSPCs or iPSCs, respectively (116, 139) (**Figure 3D**). ATO simulate the 3D structure of the thymus and can be compared to RTOC (139). After resuspension in a small amount of culture medium the cell suspension was dropwise placed on a membrane at the air-liquid interface to form 3D aggregates. This method recapitulates human lymphopoiesis and offers the ability to generate conventionally naïve T cells from ESCs or iPSCs, respectively (116, 139). Furthermore, this system enables long-term culture and provides improved positive selection due to the 3D organization. T cells developing in ATOs display a normally broad repertoire and exhibit normal responsiveness (proliferation, cytokines) upon TCR triggering. Of crucial relevance for clinical application, this method is amenable to generate TCR-transduced T cells generated after efficient allelic exclusion at the Vβ locus (116, 139). This has been exemplified using TCR specific for NY-ESO or MART1 tumor-associated antigens (116, 139). In sum, the ATO system demonstrates the high versatility of engineered MSCs to recapitulate cellular interactions underlying T cell development.

CONCLUSION AND FUTURE DIRECTIONS

This review highlights the potentially vast range of application for MSCs in the engineering of immune niches supporting leukocyte development. Among the multiple technological challenges raised by the implementation of MSCs, some salient topics emerge defining possible future directions in the field.

Understanding MSCs Developmental Phenotypic and Functional Heterogeneity to Build Better Niches

Recent developments in high dimensional approaches, such as unbiased scRNAseq have brought a fresh look on the heterogeneity of MSCs associated to immune niches (19–22, 148–151). For instance, whole genome expression analysis reveals an exquisite specificity in the distribution of niche factors among the diverse MSC types (22). Important development in spatial transcriptomics and live imaging should unravel the spatial organization of intracellular interactions supporting the function of MSCs within niches (21, 152). Deciphering the functional impact of MSCs heterogeneity and the division of labor between different MSC types should substantially assist tissue engineering purposes.

Engineering MSCs to Improve Their Function

MSCs can be genetically manipulated *ex-vivo* to modulate the expression of key molecules before they are embedded within immune niches. This approach has been developed with success for the establishment of immune niches supporting AML engraftment (67) or supporting the development of dendritic cells (68) in humanized mice. Further programming of MSC transcriptional landscapes (63) broadly impacting on their function might open new avenues for the engineering of immune niches.

Harnessing the Differentiation Pathway of Endogenous Progenitors for MSCs

Understanding the developmental pathways of MSCs populations is of major relevance for tissue engineering. The phenotype and function of differentiated MSC types is potentially difficult to maintain in *ex vivo* cultures (63). Therefore, approaches co-opting the physiological developmental pathway of MSCs are of particular interest. For instance, disentangling the developing hierarchies within early MSC progenitors underpinning the BM niche (30–32, 153) should facilitate the technological implementation of MSCs to build synthetic niches and organoids.

Stimuli-Responsive Dynamic Immune Niches

An essential biological feature of immune niche is their ability to respond dynamically to immune perturbations. BM, for instance, also respond to acute inflammation, often by increasing myelopoiesis, a process termed as "emergency myelopoiesis" (154). In both cases dynamic changes of hematopoietic organs rely on adaptation of the stromal network (19, 22, 150, 151, 155, 156). Assessing this responsiveness feature should be of interest to recapitulate leukocyte development associated to inflammatory settings.

Patient-Specific Immune Niches for Drug Testing

Genetic variation is likely to impact on physiological niches function. One key feature of the BM niche ossicle model is the possibility to transfer in the mouse the human BM microenvironment, either normal or pathological. For instance, the ossicle models represent a valuable tool to unravel the role of cellular and molecular mechanisms underlying the interactions between the hemopoietic and stromal compartment in normal or pathological niche. Ultimately, this analysis could be performed using MSCs and malignant cells from the same patient thereby defining a platform for drug screening. This approach could be applied to targeted therapies interfering with stromal support.

AUTHOR CONTRIBUTIONS

RS, AP, EM, and JP contributed equally to the manuscript writing and figure generation. AG, GA, JU, and PB helped during the manuscript writing and revision. MS and PG supervised the work and took part in the writing process. All authors contributed to the article and approved the submitted version. All figures were created by RS with BioRender.com.

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REVIEW
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Hematopoietic Multipotent Progenitors and Plasma Cells: Neighbors or Roommates in the Mouse Bone Marrow Ecosystem?

Amélie Bonaud[1,2†], *Julia P. Lemos*[1,2†], *Marion Espéli*[1,2*‡] *and Karl Balabanian*[1,2*‡]

[1] Université de Paris, Institut de Recherche Saint-Louis, EMiLy, INSERM U1160, Paris, France, [2] OPALE Carnot Institute, The Organization for Partnerships in Leukemia, Hôpital Saint-Louis, Paris, France

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***Correspondence:**
Marion Espéli
marion.espeli@inserm.fr
Karl Balabanian
karl.balabanian@inserm.fr

†*These authors have contributed equally to this work and share first authorship*

‡*These authors have contributed equally to this work and share senior authorship*

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The bone marrow is a complex ecosystem in which hematopoietic and non-hematopoietic cells reside. In this review, we discuss the bone marrow niches in mice that facilitate the survival, maintenance, and differentiation of cells of hematopoietic origin based on the recent literature. Our review places a special focus on the hematopoietic multipotent progenitors and on plasma cells, corresponding to the last stage of the B-cell lineage, that play a key role in the humoral memory response. We highlight the similarities between the microenvironments necessary for the establishment and the maintenance of these two immune cell subsets, and how the chemokine CXCL12/CXCR4 signaling axis contributes to these processes. Finally, we bring elements to address the following question: are multipotent progenitors and plasma cells neighbors or roommates within the bone marrow?

Keywords: bone marrow, hematopoietic stem and progenitor cell niches, multipotent progenitors, plasma cells, lymphoid lineage, CXCR4, WHIM syndrome

INTRODUCTION

The bone marrow (BM) is a complex organ in which hematopoiesis takes place during adulthood. Both hematopoietic and non-hematopoietic cell types cohabit in the BM and form distinct environments capable of promoting cell differentiation and survival in response to the organism needs. In this complex ecosystem, hematopoietic stem and progenitor cells (HSPCs) co-exist with cells at various intermediate differentiation stages, including fully differentiated cells (e.g., plasma cells [PCs]) (1). In the adult mouse, all functional hematopoietic stem cell (HSC) activity is found within the Lin-Sca1+c-Kit+ (LSK) compartment, which comprises about 0.00125% of total BM cells. This small cellular population is itself subdivided into various subsets based on the expression of cell

Abbreviations: Ab, antibody; BCR, B Cell Receptor; BM, bone marrow; CLP, common lymphoid progenitor; EC, endothelial cells; Emcn, Endomucin; GPCR, G protein-coupled receptor; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cells; LSK, Lin-Sca1+c-kit+; LT-HSC, long-term hematopoietic stem cell; MPP, multipotent progenitor; MSC, mesenchymal stromal/stem cell; PC, plasma cell; PSS, perisinusoidal stromal cell; PVS, perivascular cell; SCF, stem cell factor; ST-HSC, short-term hematopoietic stem cell; vWF, von Willebrand factor; WHIM, warts, hypogammaglobulinemia, infections, and myelokathexis; VCAM1, Vascular Cell Adhesion Molecule 1.

surface markers such as CD34, CD135 (Flk2/Flt3), CD150, and CD48 (2). Adult hematopoiesis is a thoroughly regulated process that is initiated from quiescent pluripotent HSCs, which encompass long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs) (3, 4). Multipotent progenitors (MPPs) are the immediate progeny of HSCs. For several years, MPPs have been considered as a homogeneous population with limited to no self-renewal capacity – in contrast to the more immature CD34-CD150+CD48- HSC compartment –, but with multi-lineage differentiation potential toward the earliest myeloid and lymphoid progenitors in the hematopoietic tree. However, recent studies have shown that the MPP compartment is more heterogeneous than expected and can be divided into four distinct subsets with different lineage fates. MPP1, defined as CD150+CD48-CD135-, shares characteristics with ST-HSCs including multiple-lineage reconstitution ability (5, 6). At steady state, they presumably give rise to functionally distinct lineage-biased MPPs that are more proliferative, devoid of self-renewal potential, and defined as megakaryocyte/erythroid (ME)-biased MPP2 (CD150+CD48+CD135-), granulocyte/macrophage (GM)-biased MPP3 (CD150-CD48+CD135-), and lymphoid-biased MPP4 (CD150-CD48+CD135+) (4–7). Although it is assumed that transition of HSCs from quiescent to more proliferative states associated with differentiation requires a unique set of bioenergetics demands, little is known about the metabolic requirements of MPPs. The MPP compartment is also dynamic and functionally plastic. In particular, the lineage fate of MPPs seems to be not fixed and can be redirected under specific conditions. Accordingly, the Passegué laboratory showed that lymphoid-primed MPP4 with their intrinsic GM poising contributed to myeloid output at steady state and underwent a transient change in their molecular identity that redirected them away from lymphoid differentiation to participate, together with overproduced MPP2 and MPP3, in the burst of myeloid production in blood regenerative conditions (4). Moreover, increased myeloid differentiation is also observed during chronic or infectious diseases, such as chronic myelogenous leukemia and acute viral infection (8, 9), or during homeostatic processes such as aging. Indeed, the Trowbridge group recently reported a progressive loss of lymphoid-primed MPP4 with aging concomitant with expansion of HSCs (10). Apart from CXCL12 and IL-6, two factors released by mesenchymal stromal/stem cells (MSCs) that have been reported to regulate MPP homeostasis and maintenance (11–14), our understanding of how cell-extrinsic niche-related and cell-intrinsic cues drive the lymphoid versus myeloid fate decision of MPPs is still incomplete.

Although not fully understood, it is now well established that BM environmental cues are integrated by hematopoietic cells throughout their differentiation and translate into distinct cell fates. As a typical example, the chemokine CXCL12 produced by non-hematopoietic stromal cells is essential to promote Common Lymphoid Progenitor (CLP) differentiation toward the B-cell lineage (15), through the progressive differentiation of pro-B cells into pre-B cells and then into immature B cells (16, 17). This process is essential for efficient rearrangement of the immunoglobulin loci and production of a functional B-cell receptor (BCR) (18–21). B cells then pursue their maturation in the periphery and, upon activation during an immune response and notably through the formation of germinal centers, some B cells differentiate into PCs, which corresponds to the final differentiation stage of the B-cell lineage responsible for antibody (Ab) production. Most PCs are short lived but some of them can relocate into the BM where they mature into long-term PCs. This occurs in specific niches that support their survival, maintenance and dormancy through cellular and soluble factors and ensure long-term (potentially life-long) protection against reinfection. Whether newly produced PCs and long-lived PCs reside in and/or compete for identical niches is still unknown (22, 23).

In the last two decades, the notion of niches has become an essential part of how we envision the organization and function of the BM ecosystem. Although the definition of "niches" may vary depending on the studies, a unifying view is that they correspond to complex, dynamic microstructures in which several soluble and membrane-anchored factors are produced, allowing the correct positioning of a specific cell type to favor interactions with other cellular actors and access to all the elements necessary for their maintenance or differentiation (24). To date, the definition of what a niche should be is based on the analyses of essential elements constituting the survival and differentiation cues for HSCs. These niches are thought to be composed of perivascular mesenchymal units associated with sinusoids and arterioles (25–28). However, a great heterogeneity may exist in these cell populations and should be integrated into the definition(s) of a niche. Strikingly, the nature of the niche(s) supporting the differentiation and maintenance of other cell types, including MPPs and PCs, has not been studied in detail. In this review, we will discuss these niches, with emphasis on the essential cellular network needed for MPP and PC maintenance within the mouse BM. Based on current literature, we will delineate the roles played by specific stromal cells and various actors in the MPP and PC niches and highlight common factors necessary for the maintenance of these cell populations including the CXCL12/CXCR4 signaling axis. We will also open a discussion on three essential and unresolved questions: (1) Do specific niches or interaction networks exist for each MPP and PC subset?; (2) Do MPP and PC subsets share niches or compete for them?; and (3) Do MPP and PC subsets regulate their own niche or affect each other?

THE VASCULAR VERSUS OSTEOBLASTIC NICHES: HOW FAR FROM REALITY?

Bones can be anatomically divided in four main categories: long, short, flat and irregular bones. The long bones can be structurally divided in the epiphysis, which is filled with spongy bone and red marrow, the diaphysis, a tubular shaft, lined with a dense and compact cortical bone, and the intermediate metaphysis, which contains the epiphyseal plate that allows bone growth (29). Moreover, it can be schematically divided in three major

regions: the cortical bone that forms the hard-outer layer of bones, a central cavity containing the marrow, and the endosteum, which corresponds to the interface between the cortical bone and the marrow. The endosteum is enriched in fully committed bone-forming osteoblasts and bone-resorbing osteoclasts and spreads along the inner bone surface (29). Arteriolar vessels are found near the endosteum, longitudinally aligned along the diaphysis and supply oxygen, nutrients, and growth factors to the marrow. Most vessels within the marrow are specialized venules called sinusoids that form a dense network. The sinusoids finally merge into a central sinus to form the venous circulation (24). In addition to this spatial distribution, different types of BM endothelial cells (ECs) have been phenotypically identified in long bones and in flat and irregular ones as well (30). CD31loEndomucin(Emcn)lo L-type sinusoidal ECs are enriched in the marrow cavity, which is poorly occupied by CD31+Emcn- arteriolar ECs. Arteries co-stain for Sca-1 and CD31 and the distal smaller arterioles are surrounded by Sca-1+ mesenchymal and hematopoietic cells (26). On the contrary, the bone compartment is enriched for arteriolar ECs, few L-type sinusoidal ECs and CD31hiEmcnhi H-type ECs, a small fraction of the ECs at the end of the CD31+Emcn- arteriolar network (30, 31). The last ones were demonstrated to be in close contact with osteoprogenitor cells, providing niche signals that promote bone development and maintenance, besides healing after fracture (30, 32). These well-organized vascular structures, together with the cortical bone, have been classically used to discriminate osteoblastic/endosteal areas versus (peri)-vascular areas within the BM. The recent identification of transcortical vessels crossing the bone cortex (33) suggests that former concepts based on the dichotomy of the osteoblastic and vascular niches are not absolute and led to the reconciling concept of endosteo-vascular niches (25, 26). Indeed, perivascular units likely integrate contributions from ECs and osteoprogenitor cells, including MSCs and perisinusoidal stromal (PSS) cells, as well as from fully differentiated osteoblasts, leading to a more complex definition of the HSC niches (26, 34, 35).

Recently, the use of transgenic mice allowing the tracking of specific cell lineages, associated with single-cell RNA-seq analyses, has permitted to better characterize the diverse components of the BM environment and the heterogeneity of the cell types composing the hematopoietic niches (36–40). Moreover, imaging techniques that allow simultaneous mapping of the HSPCs and interacting stromal cells have been critical for the discovery of BM niches (36–39, 41, 42). Taken together, these key results suggest that HSC niches constitute a critical spatio-temporal regulatory unit composed of multiple mesenchymal, hematopoietic, and neuronal cell populations associated with different vessel subtypes that cross-interact in a highly dynamic setting and where exchange of key signals leads to multi-directional regulation of the different partners. Of note, most of these findings were made using mouse models and extra-caution is required when extrapolating these data to human in which the BM composition might be different (*e.g.*, the human BM contains noticeably more adipocytes). Because the roles of several cell types and many soluble and surface-anchored factors in the BM have been broadly studied and reviewed recently (17, 43–48), we will focus on how the different BM niches can regulate and potentially determine HSC fate and differentiation into lineage-biased MPPs in mice. Moreover, we will discuss whether these early progenitors may share some niche elements with differentiated mature cells, namely PCs.

HSC AND MPP NICHES: DISTINCT OR SHARED?

HSCs are distributed throughout the BM and, under physiological conditions, around 30% of them are in a quiescent stage. Histological and functional assays first indicated that these slow-cycling LT-HSCs preferentially localize in endosteal and sub-endosteal regions, in association with the osteoblasts and the bone surface (49–51). Subsequent studies suggested that mature osteoblasts have an indirect effect on HSC activity and maintenance (52–55), whereas osteoprogenitor cells control HSPC survival and commitment. In particular, depletion of the rare peri-arteriolar osteoprogenitors (also called pericytes) changes the spatial location of HSCs, which move away from the arteries and acquire a non-quiescent state, thereby increasing the proportion of proliferative cells (51, 56). Therefore, most of the fast-cycling proliferative ST-HSCs, committed progenitors, and differentiated cells are distributed in the central region and predominantly localize next to sinusoids (52, 57, 58). This suggests that HSCs require different perivascular niches based on their cell cycle status and that specific endothelial or perivascular reticular/mesenchymal cells orchestrate this process. The enrichment of HSCs in contact with sinusoidal endothelium may ensure a more efficient hematopoietic cell mobilization (52, 59, 60). Several niche factors have been reported to provide HSCs with instructive clues to regulate their location, retention, self-renewing, and fate (57, 61–65). In particular, Stem Cell Factor (SCF) and CXCL12 are required for HSC maintenance and retention in the BM (17, 63, 66, 67). PSS cells identified by surface expression of the Leptin receptor (LepR) promote HSC maintenance and the use of knock-in mouse models showed that these cells are the main source of SCF and CXCL12 (31, 57, 62, 63, 67–69). SCF is present in both membrane-bound and soluble forms, and specific deletion of this factor from PSS cells decreases the numbers of HSC in the BM (28, 56, 63, 69). Similarly, CXCL12 is a chemokine required for HSC retention and localization in the BM, and CXCL12 deletion from PSS cells reduces HSC numbers, while impacting their quiescence status and their distribution (35, 65, 67).

Despite these breakthroughs, it is still unclear whether lineage-biased HSCs and MPPs are broadly distributed through the BM or occupy specific niches. Consistent with this idea, it was shown that platelet and myeloid-biased von Willebrand factor-positive (vWF+) HSCs, which also express high levels of CD150, reside in close association with megakaryocytes in the BM. Megakaryocyte depletion leads to vWF+ HSC expansion, loss of their long-term self-renewal capacity, and lineage-bias after transplantation, suggesting that megakaryocyte-enriched

niches promote HSC quiescence as well as commitment (70–72). On the other hand, vWF- lymphoid-biased HSCs are rather enriched in arteriolar niches, and depletion of peri-arteriolar Neuron-glial antigen 2-positive stromal cells significantly reduces this population with no effect on the myeloid-biased cell numbers (72). Despite our partial understanding of the exact location of lineage-biased HSPCs, it is clear that their positioning within the BM plays a critical role in directing which lineage-specific signals are received by hematopoietic precursors.

In line with this, some studies suggest that MPPs reside further into the endosteal surface, in more perfused areas (59, 73), whereas others indicate that lymphoid specification of MPPs occurs in parallel to their migration away from the endosteal region, which is regulated by G protein-coupled receptors (GPCRs) (74). This suggests that the myeloid versus lymphoid specification of MPPs may occur in different locations within the BM and under different, specific conditions. More recently, CXCR4 expression was shown to be required for proper localization and differentiation of lineage-specific precursors. Indeed, Cordeiro-Gomes et al. showed that conditional deletion of CXCR4 in MPPs reduced their differentiation into CLPs and decreased lymphopoiesis, with a dramatic reduction in B, T, and natural killer cell production and, to a lesser extent, in myeloid progenitors (69). The CXCL12/CXCR4 signaling was not intrinsically required for *in vitro* lymphoid development from CLPs, but for promoting CLP positioning close to IL-7+ cells and proper IL-7R activation *in vivo*. Therefore, MPPs share with HSCs the requirement for CXCL12 signaling and may reside in similar niches, but in closer association with IL-7+ cells. Indeed, depletion of CXCL12 or SCF expression from IL-7+ cells reduced both HSC and MPP numbers in the BM (17, 69). Further insights into the role of the CXCR4/CXCL12 signaling axis on HSPC lineage commitment come from the study of a rare immunodeficiency called the WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome (14, 75–77), that leads to severe chronic pan-leukopenia. Most of the patients present an autosomal-dominant mutation in CXCR4, associated with receptor desensitization resistance and consequently gain of function in response to CXCL12 (78–81). Using an original knock-in mouse model of the WHIM syndrome (82), Freitas et al. reported that the profound circulating lymphopenia was associated with a decrease in lymphoid-primed progenitor numbers in the BM with no difference in the number of myeloid progenitors (14). Thus, efficient CXCR4 desensitization regulates lymphoid differentiation of HSPCs in the BM, and absence of this regulatory mechanism likely contributes to the lymphopenia observed in the mutant mice and likewise in the patients. This study identifies MPPs as the key hematopoietic stage at which CXCR4 signaling termination impacts lymphoid, but not myeloid, lineage commitment. Altogether, these works suggest a pivotal role for GPCRs, including CXCR4 signaling, in regulating the fate of MPPs, for which the BM niches and their precise localization are still largely unknown.

MPPs are not a homogeneous cell population but are composed of distinct subsets that give rise to more differentiated progenitors in a biased manner in mice. Where these subsets are localized in the BM and how they interact with their environment is still unclear. Distinct lineage-biased precursors seem to express different set of adhesion molecules, with myeloid-biased CD34+ precursors expressing more frequently LFA-1 and L-selectin and expressing a lower surface density of CD44, VLA-4, and VLA-5 than lymphoid-biased precursors (83). Moreover, the Kondo laboratory reported three MPP subsets based on CD135 and Vascular Cell Adhesion Molecule 1 (VCAM-1) expression, which differed in their myeloid potential but displayed similar lymphoid differentiation capacities *in vivo* (84). The difference in adhesion molecule expression pattern may thus correlate with different adhesion capacities and stromal cell niche pairs, which in turn could lead to integration of distinct signals driving cell fate (**Figure 1**). Whether MPP subsets share identical niches is unknown but one can speculate that they establish distinct sets of interactions with their niche even if they share one. A recent study by Balzano et al. showed that HSCs and pro-B cells are frequently found in the same niche in contact with LepR+ PSS cells but form specific interaction networks with these cells, through SCF and CXCL12 for HSCs, and through IL-7 and the basement membrane component Nidogen-1 for pro-B cells (31). If this is also the case for the lineage-biased subsets of MPPs, it could explain the different impact of the CXCL12/CXCR4 axis on myeloid versus lymphoid differentiation (14, 69). Further research is required to address whether MPP subsets reside in distinct niches in the BM and how these niches influence their cell program and fate.

PLASMA CELL MATURATION AND MAINTENANCE: MORE THAN ONE NICHE?

PCs correspond to the terminal stage of B-cell lineage differentiation and are the effectors of humoral immunity through the secretion of large amounts of Abs. Following antigenic stimulation of their BCRs and/or activation through the Toll-like receptors, B cells can initiate a differentiation process leading to PC generation (85, 86). *In vivo*, this process occurs in two waves. The first wave is called extrafollicular, is very rapid, and leads to PC differentiation in the next few days after antigen exposure (87–89). By contrast, the second wave is delayed, depends on T-cell help, and relies on the formation of germinal centers, which are an anatomical structure within secondary lymphoid organs (90). After their generation in the secondary lymphoid organs, the vast majority of PCs dies rapidly, with a half-life ranging from a few days to a few weeks at most; however, a small pool of PCs persists in the BM for many years, potentially throughout the life of the individual (91–94). Germinal center-derived PCs are "tailored-made" to be the most efficient cell type against the infectious agent, and considered as constituting the main pool of long-lived PCs that persist in the BM.

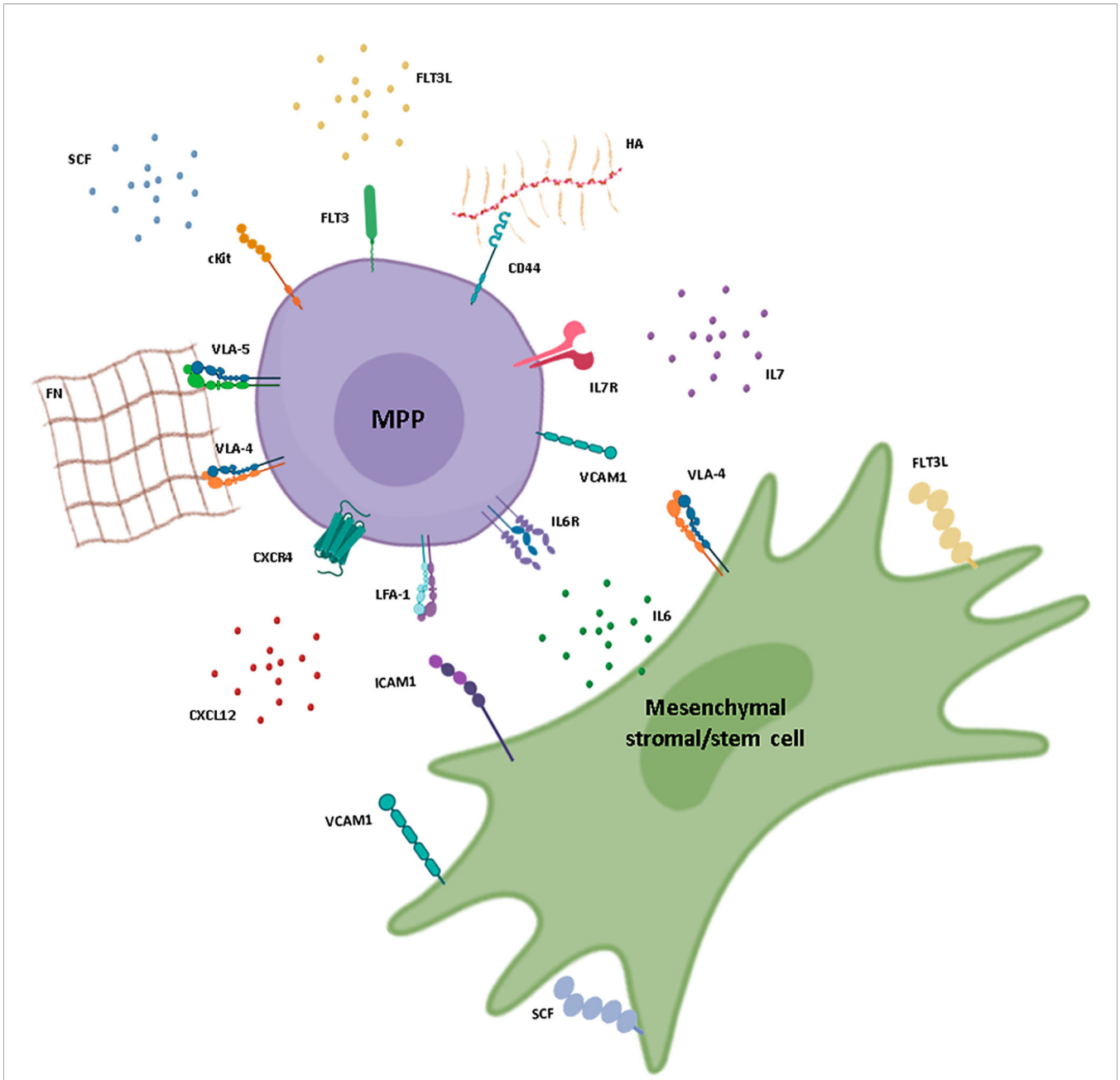

FIGURE 1 | Interactions between hematopoietic multipotent progenitors and mesenchymal stromal/stem cells in the BM. Schematic representation of a multipotent progenitor (MPP) in its BM environment and the possible interactions established with a mesenchymal stromal/stem cell (MSC). Membrane receptors, adhesion molecules, and soluble niche factors previously described for hematopoietic stem and progenitor cell (HSPC) maintenance in the BM are represented. CXCL12, IL-7, IL-6, and SCF are mainly produced by the MSC and bind to CXCR4, IL-7R, IL-6R, and cKit, respectively, at the surface of the MPP. MSCs also produce proteins of the extracellular matrix, such as fibronectin (FN) and hyaluronic acid (HA), which promote cell adhesion through integrin receptors and adhesion molecules expressed by the progenitor cells, such as VLA-4, VLA-5, and CD44. Interactions in the form of cell-cell contacts are also represented and involve VCAM-1 and VLA-4, expressed by both cell types, as well as LFA-1 expressed by MPPs and ICAM-1 expressed by perisinusoidal stromal cells, pericytes, and osteoblasts. Stromal cell factor (SCF) and FLT3L can be found as both soluble and membrane-bound forms. FLT3L is produced mainly by fibroblasts within the BM. IL, interleukin; ILR, interleukin receptor. Figure created using the BioRender icon library.

After their formation in the secondary lymphoid organs, PCs are still immature and are referred to as plasmablasts or short-term PCs. Upon their migration to the BM, they terminate their maturation to become fully differentiated long-term PCs (95). There is still some confusion in the field about the nomenclature that should be used to refer to this intermediate stage of differentiation. The terms plasmablast or short-lived PCs are used interchangeably by some authors, whereas others reserve

the term plasmablasts to *in vitro*-differentiated cells, which are very immature compared to PCs obtained *in vivo* after antigen exposure. These potential discrepancies are due to the lack of proper markers for these different stages of PC maturation, especially in mouse models. Of note, the recently published combination of the CD138, TACI, B220, and CD19 markers offers a new scope to discriminate mouse PCs based on their maturation stage (96). In human, the combination of CD19 and CD138 allows to distinguish three steps of PC maturation with the most mature PCs expressing CD138 and losing the expression of CD19 (97). B-cell differentiation into PCs starts in secondary lymphoid organs and is marked by the shunting of B-cell gene expression program. However, the initial event that allows switching from B cells to PCs is not well understood although a decrease in BCR signaling seems an absolute prerequisite for PC differentiation (98).

Newly generated immature PCs upregulate the expression of the transcriptional factor Klf2, which in turn promotes the expression of the S1P1 receptor essential for PC egress from the secondary lymphoid organs and their migration toward the BM through blood circulation (99–104). Immature PCs express the chemokine receptors CXCR4 and CXCR3 at their surface, essential for their migration into the BM and inflammatory sites, respectively (105, 106). Extravasation of PCs from blood to BM through the sinusoids is still poorly described (107) but may represent a key step allowing PC exit from the cell cycle and final maturation, which is characterized by the loss of the B-cell markers B220 and CD19 (96, 97). Once in the BM, the expression of S1P1 and CXCR3 is downregulated in PCs, while the expression of CD138, CD93, and CXCR4 is increased (103, 105, 106, 108–111). CXCR4 is essential for PC homing and, likely retention within the BM as CXCR4-deficient PCs fail to accumulate in this organ (108). Moreover, fine-tuning of CXCR4 signaling is also critical for PC homeostasis in the BM, because a CXCR4-gain-of-function is associated with aberrant accumulation of immature PCs and decreased detection of germinal center-derived antigen-specific PCs in the BM (112, 113).

Once in the BM, PCs finish their differentiation, while possibly losing their ability to migrate (101) and the last remnants of their B-cell identity, including the expression of the B cell co-receptors B220 and CD19 (96, 97, 114–116). Loss of surface expression of BCR was long considered as a hallmark of PC differentiation. However, several reports suggest that some membrane BCR remain expressed at the surface of IgM+, and perhaps IgA+, PCs and continue to induce signaling in PCs (117, 118). PC terminal maturation is also associated with the expression or re-expression of some markers that might reflect their degree of maturity including CD138 in human, and CD28, CD38, and CD93 in mouse (97, 110, 119, 120). Within the BM, PCs stop cycling and become quiescent. Their long-term maintenance depends on factors produced by specific microenvironments, also called niches, whose functions are still not fully understood.

Many studies have demonstrated the key role of BM stromal cells in the maintenance of PCs, partially through their ability to produce CXCL12 (57, 121). Notably, CXCL12+ stromal cells appear essential for PC maintenance, at least *in vitro*. The recent identification of BM stromal cell diversity is just starting to be integrated into the field of PC research; early work just referred to "MSCs" without any further characterization. These "MSCs" likely represent heterogeneous stromal subsets rather than *bona fide* mesenchymal stem cells (101, 122–124). As mentioned before, all stromal cells may not secrete CXCL12, and PSS cells (sometimes referred to as CXCL12-abundant reticular cells) are one of the main sources of this chemokine in the BM (17, 57, 65, 125). Accordingly, BM PCs were reported to be in close contact with perivascular CXCL12+ stromal cells (57, 126, 127), but further characterization of this presumed dialogue is still lacking.

The close contact established between stromal cells and PCs, notably through integrins and their ligands should be considered as well. Several recent studies have characterized fibronectin as part of the culture-expanded MSC secretome, with an important role in the final maturation and survival of PCs through interaction with the receptor VLA-4 expressed by PCs (102, 122, 124, 128, 129). VLA-4 also interacts with VCAM-1 that is expressed by stromal cells, thus reflecting an important redundancy in adhesion mechanisms (121) (**Figure 2**). Similarly, YWHAZ was found in the human MSC secretome and shown to be essential for PC survival and maturation, potentially through the downregulation of mTORC1 (124). LFA-1 through its interaction with ICAM-1 is also essential, but not sufficient, for PC maintenance because disruption of the LFA-1 signaling axis only causes a transient loss of PCs in the BM (130). *In vitro* experiments have also unraveled a major role for the adhesion molecule CD44, which is highly expressed by BM PCs, for their maintenance. Hyaluronic acid, the ligand of CD44, is a component of the extracellular matrix and the signals induced through CD44 are important to support PC survival (122, 131).

Moreover, PCs require soluble factors for their maintenance. Among them, IL-6 produced notably by eosinophils and stromal cells in BM supports PC survival *in vitro*, although its role *in vivo* is unclear (131–134). PCs also need specific factors for their long-term survival, including TNFα and two cytokines, BAFF and APRIL (135, 136). They express the BR3 (also called BAFF-R), BCMA, and TACI receptors for these cytokines. APRIL is critical, both *in vitro* and *in vivo*, for the maintenance of PCs through the induction of the pro-survival factor Mcl1 (137). In the BM, APRIL is mainly produced by myeloid cells (133, 138–140). Although eosinophils were first reported to be essential for PC maintenance in the BM, it is now accepted that they may play a redundant role in PC maintenance, and that other cell types including megakaryocytes, osteoclasts, monocytes, and even maybe regulatory T cells may contribute to PC survival niches (141, 142). Altogether, these data suggest the existence of a multicellular niche for PCs within the BM with several hematopoietic components and at least one stromal component that may correspond to a CXCL12+ osteoprogenitor. Whether the composition of these survival niches differs between human and mouse is still unknown.

Most of these studies have not discriminated between fully differentiated long-lived PCs and newly produced immature

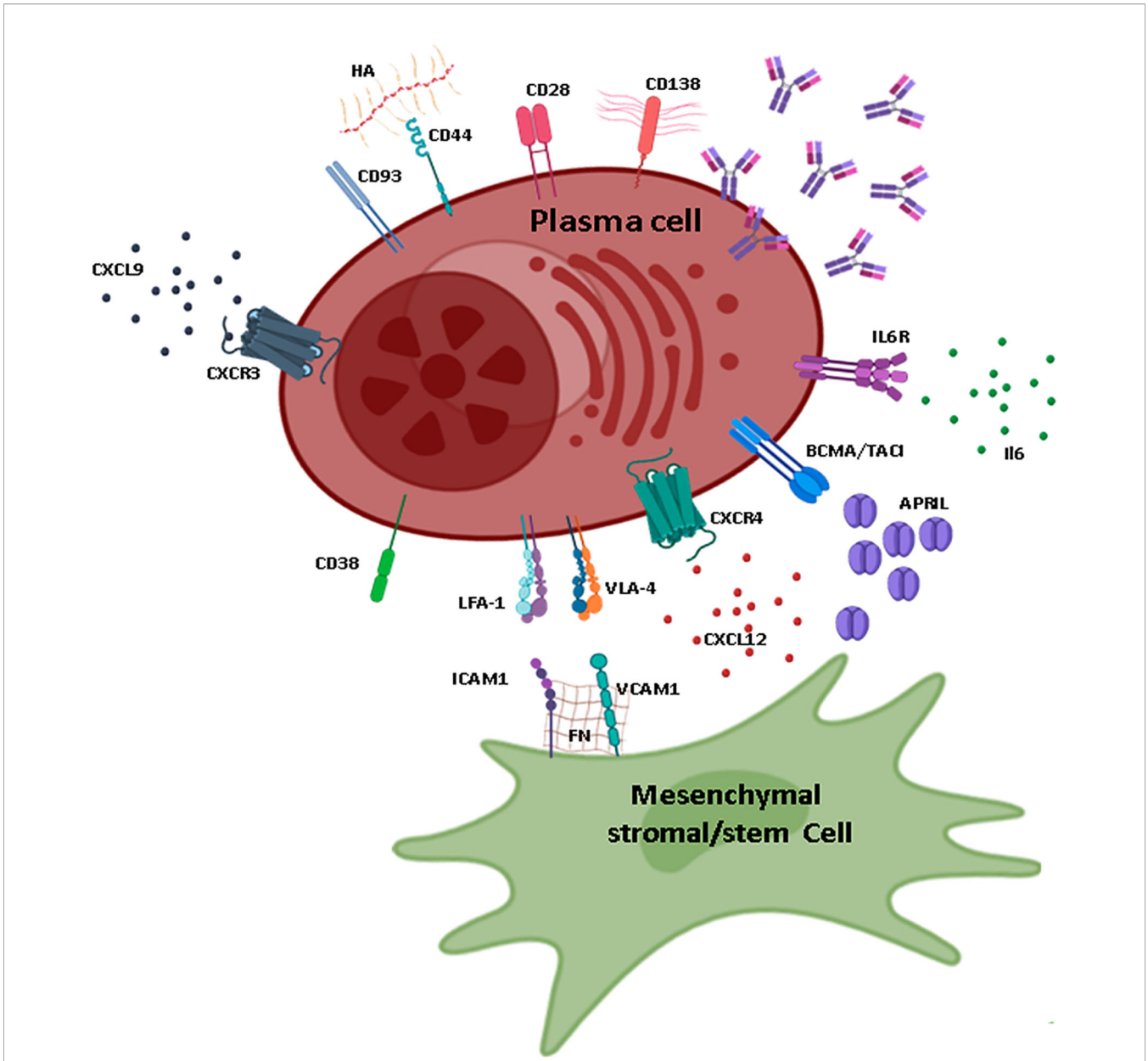

FIGURE 2 | Plasma cell maintenance in the BM. Schematic representation of the main elements necessary for plasma cell (PC) maintenance in the BM, mainly provided by interactions with MSCs. MSCs that sustain PC maintenance are characterized by the production of CXCL12 and are probably CXCL12-abundant reticular cells or PSS subpopulations. MSCs express VCAM-1 and FN that interact with VLA-4 and LFA-1. The main sources of APRIL and IL-6 are cells of myeloid lineage (especially megakaryocytes and eosinophils for APRIL and megakaryocytes, granulocytes, and eosinophils for IL-6). The ligands for CD38 and CD28 are expressed by other cell types and are not represented on this figure. The ligand of CD93 is unknown; however, this adhesion molecule is essential for the maintenance of long-lived PCs. CXCL9, one of the ligands of CXCR3, is produced by osteoblasts. CD138 is one of the main markers for PCs and is characterized by long heparan sulfate chains that trap molecules and allow interactions with the extracellular matrix. HA, hyaluronic acid; FN, fibronectin; IL, interleukin; ILR, interleukin receptor. Figure created using the BioRender icon library.

ones. Although the phenotypic changes occurring in PCs as they mature suggest that immature and fully differentiated PCs have different needs for survival factors, further research is needed to understand the actual mechanism. Moreover, the exact location of PCs depending on their maturation stage has never been assessed. The existence of distinct niches or of a distinct set of interactions within a similar niche for PCs according to their maturation stage will require further investigation. In light of a recent paper suggesting that PCs may actually exit the BM and recirculate (143), it would be interesting to determine whether, like for HSPCs, distinct niches control the quiescence and the reactivation of PCs. Finally, BM PC plasticity, motility, and effector functions through Ab and cytokine secretion also need to be considered in the context of the dialogue established with

their niche(s) (23). Indeed, through their localization and persistence, PCs may play an important role in the maintenance of HSPC niches.

CONCLUDING REMARKS AND UNANSWERED QUESTIONS

Although our understanding of the elements necessary for the maintenance of MPPs and PCs within the BM has tremendously improved during recent years, several open questions remain. For instance, the localization of the different MPP subsets remains unknown. Although PCs are found mainly in close contact with CXCL12+ cells, the nature of these cells and how they drive the precise positioning of PCs in the BM is not well understood. Whether PC localization changes during their final maturation is also unclear. As highlighted in this review, several factors of the BM niches are essential for the maintenance of MPPs and PCs. Both cell types share common characteristics including adhesion molecules such as CD44 and LFA-1, and dependence on cytokine/chemokine such as CXCL12 and IL-6. These similarities, together with the ability of MSCs to produce some of the common factors and to support them *in vitro*, may argue in favor of a unique niche able to maintain both MPPs and PCs (**Figure 3A**). If true, whether MPPs and PCs share or compete for these niches is an open question. Furthermore, it

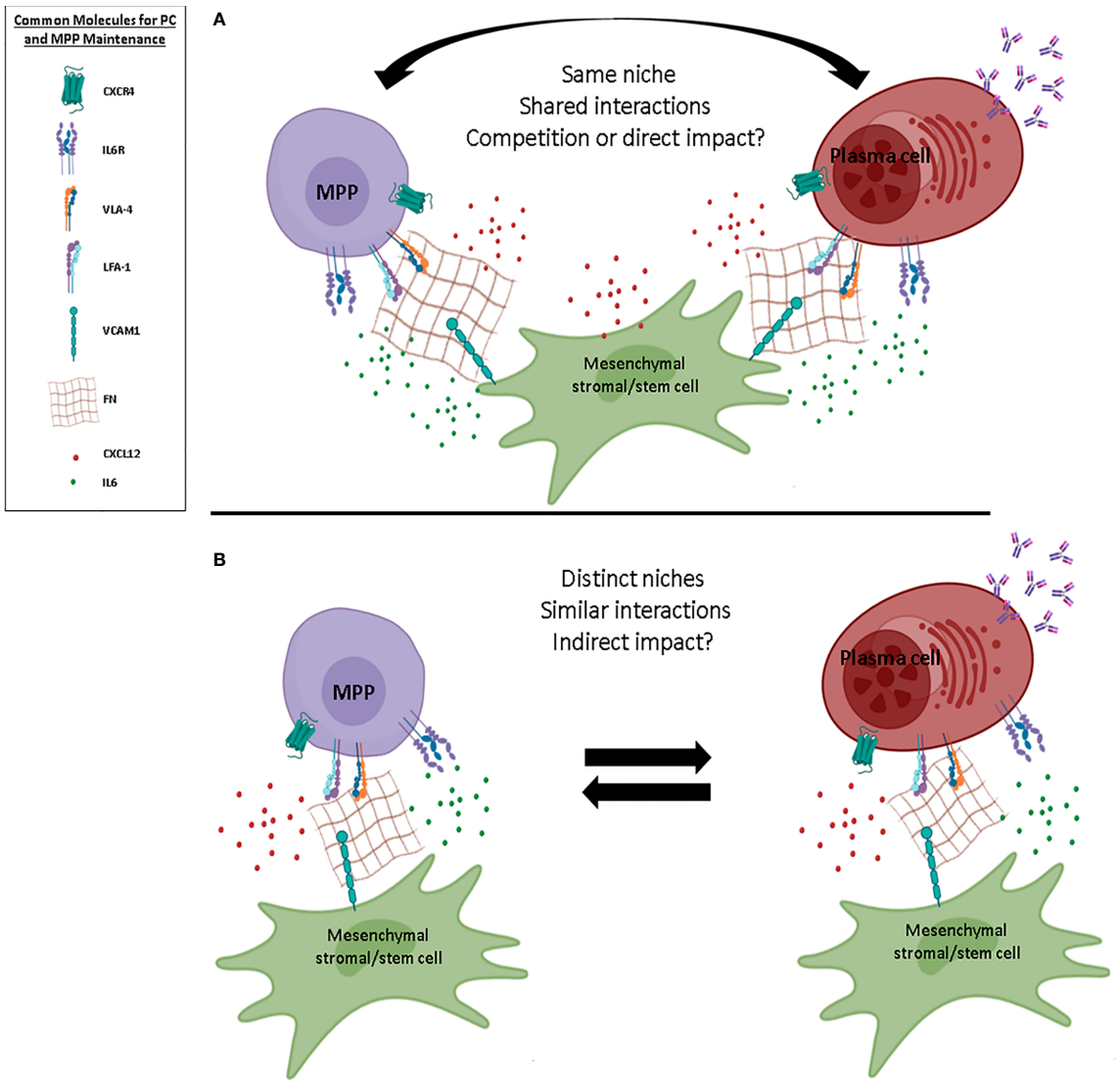

FIGURE 3 | Possible BM niche models for MPPs and PCs. Schematic representation of two different models of multipotent progenitor (MPP) and plasma cell (PC) niches within the BM. **(A)** In the first model, MPPs and PCs share common niches and reciprocally impact their biology, maintenance, and fate. **(B)** In the second model, MPPs and PCs display distinct niches despite common ligand/receptor interactions, and potentially have an indirect impact on each other. Common molecules for PC and MPP maintenance within the BM are shown. FN, fibronectin; IL, interleukin; ILR, interleukin receptor. Figure created using the BioRender icon library.

is worth noting that in conditions where the BM homeostasis is disrupted (*e.g.*, inflammation, aging), PCs can indeed impact HSPCs and MSCs. It is known that both myelopoiesis and the number of PCs increase with aging in the BM. Recently, two studies reported that PC accumulation with age regulates the production of inflammatory factors by BM stromal cells, which in turn promotes myeloid-biased HSCs (144, 145). This impact on myeloid cells is probably due to the ability of PCs to produce IL-10, one of the key drivers of the myeloid differentiation (145, 146). Consequently, both studies suggest a potential impact of PCs on MSCs and on the skewing of MPPs toward myeloid lineage with age (10). Moreover, external factors, such as dietary restriction and exercise, instruct hematopoietic precursors and mature lymphocytes *via* modulation of BM stromal cells (147, 148). Interestingly, in Multiple Myeloma, that is characterized by a massive influx of malignant PCs in the BM, the architecture of the BM is disorganized with numerous lytic bone lesions and the hematopoiesis process is also frequently impaired. Myeloma PCs alter the function of osteoclasts and adipocytes to support their maintenance, through their ability to produce fatty acid and growth factors like IL6 or TNF-α (149–153). In this context, malignant PC-imprinted BM stromal cells support deregulation of the HSPC compartment, suggesting that hematopoietic dysfunction in Multiple Myeloma results from PC-related microenvironmental alterations (154, 155). While the impact of PCs on HSPCs seems clear in pathological settings, it is still unknown whether PCs and MPPs may affect each other in the "young" BM and at steady state.

Based on our current understanding of the BM ecosystem, we cannot affirm that the maintenance of PCs and MPPs is carried out by only one cell subpopulation of stromal cells. If the PSS cells are a good candidate due to their strong expression of CXCL12 and their perivascular localization, this cell population is outnumbered by MPPs and PCs, which, albeit rare, are still about 10 times more numerous than PSS cells. This simple observation questions a model wherein cells may form exclusive pairs in their niche. Furthermore, PCs and MPPs are both heterogeneous populations. At least two populations of PCs (based on their maturation status) and three distinct subpopulations of MPPs have been described in the BM. It is difficult to envision how these different subsets may coexist in common niches. Moreover, long-lived PCs are terminally differentiated and relatively quiescent cells, in contrast to MPPs, which can cycle and differentiate, suggesting that both the MPP and PC pools display a differential turn-over. Such restrictions suggest another model with two distinct niches for MPPs and PCs, featuring common factors and with some possible exchange and/or interactions. However, such niches have not been characterized so far and more insights into the relative functions of the heterogeneous MSC subsets will be necessary to support this model (**Figure 3B**). In conclusion, the two models proposed are equally possible and are not mutually exclusive; indeed, the existence of dynamic interactions between the BM, inter-, and/or intra-niches is probably the closest to reality. This may be linked to the ability of the cells to come and go and, consequently, to modulate their niche to fit their own needs.

AUTHOR CONTRIBUTIONS

AB and JPL made extensive review of the literature listed, drafted different sections of the review, and drawn the figures. ME and KB conceived, designed, and supervised the review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Notch Signaling in the Bone Marrow Lymphopoietic Niche

*Kilian Sottoriva and Kostandin V. Pajcini**

Department of Pharmacology and Regenerative Medicine, University of Illinois at Chicago College of Medicine, Chicago, IL, United States

Lifelong mammalian hematopoiesis requires continuous generation of mature blood cells that originate from Hematopoietic Stem and Progenitor Cells (HSPCs) situated in the post-natal Bone Marrow (BM). The BM microenvironment is inherently complex and extensive studies have been devoted to identifying the niche that maintains HSPC homeostasis and supports hematopoietic potential. The Notch signaling pathway is required for the emergence of the definitive Hematopoietic Stem Cell (HSC) during embryonic development, but its role in BM HSC homeostasis is convoluted. Recent work has begun to explore novel roles for the Notch signaling pathway in downstream progenitor populations. In this review, we will focus an important role for Notch signaling in the establishment of a T cell primed sub-population of Common Lymphoid Progenitors (CLPs). Given that its activation mechanism relies primarily on cell-to-cell contact, Notch signaling is an ideal means to investigate and define a novel BM lymphopoietic niche. We will discuss how new genetic model systems indicate a pre-thymic, BM-specific role for Notch activation in early T cell development and what this means to the paradigm of lymphoid lineage commitment. Lastly, we will examine how leukemic T-cell acute lymphoblastic leukemia (T-ALL) blasts take advantage of Notch and downstream lymphoid signals in the pathological BM niche.

Keywords: lymphopoiesis, hematopoieisis, Notch signaling, T cell development, bone marrow niches

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***Correspondence:**
Kostandin V. Pajcini
kvp@uic.edu

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INTRODUCTION

Notch signaling is a highly conserved pathway activated through cell-to-cell, ligand-receptor interactions. There are five Notch ligands in mammals: Delta like (Dll) 1, 3 and 4 and Jagged (Jag) 1 and 2 which are presented on the surface of multiple cells and tissues (1). When the ligand interacts with one of the 4 mammalian Notch receptors, (Notch1-4) a series of proteolytic cleavages releases the Notch receptor from the plasma membrane (2). Subsequently, the intracellular Notch (ICN) domain translocates to the nucleus, where it binds to Recombining Binding Protein Suppressor of Hairless (RBP-J) and co-activator Mastermind-like (MAML) (2, 3). Ultimately, it is this tri-molecular complex that binds to enhancer and promoter elements to initiate transcriptional activation of target genes. Along with Wtn, Hedgehog, and Bone Morphogenic Peptide/TGF- β, Notch signaling is one of the fundamental pathways essential for mammalian embryogenesis (4). Notch signaling plays a multitude of roles in the differentiation, proliferation, self-renewal, and survival in diverse cell types across many tissues (5). Particularly well studied are the roles of Notch1 in somite segmentation (6, 7), in angiogenesis and vascular development (8, 9),

and the emergence of the definitive hematopoietic stem cell (HSC) in the aorta-gonad-mesonephros (10, 11). In the post-natal murine BM, HSC cell-autonomous and non-cell-autonomous Notch signaling has been implicated in several contexts including aging, regeneration, and mobilization, reviewed in these studies (12–15). Though loss-of-function studies in adult mice do not support a requirement for HSC cell-autonomous Notch activation during homeostasis (16, 17) and in one more recent study in regenerative hematopoiesis (18), Notch signaling has been implicated in the development of several different blood lineages, including megakaryocytes (19), NK cells (20), and erythrocytes (21). Yet, it is the role of Notch signaling in the cell fate determination of the T cell lineage that remains as the archetypic function of the pathway in adult hematopoiesis (22, 23).

The developmental progression from the BM HSC to the production of functional peripheral T cells is physiologically continuous but can be delineated using surface markers and expression of key transcriptional regulators. In both mouse and human, BM lymphoid progenitors give rise to thymic precursors, which progress through well-defined developmental stages in the thymus to become naïve T cells (24). Progression through distinct stages of thymic T cell development requires the careful coordination of several lineage regulatory transcription factors, including: Ikaros, Gfi1, Myb, Runx family proteins, E2A, HEB, TCF1, GATA3, Bcl11b, LEF1, and of course Notch1 (25). In the thymus, the roles of Notch signaling have been well studied. After homing to the thymus, early progenitors activate Notch signaling, which is required for thymocyte development (26–28). Notch is implicated in a variety of functions such as inhibition of progenitor apoptosis, induction of T cell lineage master regulators *Gata3*, *Tcf7*, and *Bcl11b*, as well as activation of genes involved in functional T cell receptor (TCR) production such as *Ptcrα* (29–31). Notch signaling becomes dispensable for T cell differentiation after β-selection occurs, at which point subsequent development is dependent on signals from the pre-TCR complex (29, 32). The main receptor expressed by thymocytes is Notch1, while the major Notch ligand expressed by cortical Thymic Epithelial Cells (TECs) is Delta-like 4 (Dll4) (30, 33). While the role of Notch in T cell development in undeniable, the temporal and spatial aspects of the first requirement for Notch in driving T cell fate have not been fully established. Recently, several findings have begun to address this issue by suggesting that pre-thymic Notch signals influence the ability of primitive BM lymphoid progenitors to produce thymus-seeding cells (18, 34, 35). Here, we will review the work which encompasses our current understanding of the BM populations that give rise to thymic progenitors, and the role of Notch signaling as a niche component in driving this process. Under this new paradigm of pre-thymic Notch activation, we will then examine the pathological Notch-dependent mechanisms of the lymphoid niche in the leukemic BM environment.

BM THYMOCYTE PROGENITORS

In adult mammals, the hematopoietic system is maintained *via* the production of functional blood cells and hematopoietic progenitors by self-renewing HSCs in the BM (36). The BM microenvironment is composed of osteoprogenitors, stromal cells, endothelial cells, and multiple hematopoietic cell types (**Figure 1**) (37, 38). At the apex of BM hematopoiesis is the HSC, which is defined best by its self-renewal and functional capacity to produce all the lineages of blood rather than by a specific set of markers. Even so, for isolation purposes the HSC has been classified by surface markers as $Lin^-cKit^+Sca1^+CD150^+CD48^-$ (39), by the presence of efflux pumps (40) and by the expression of intracellular proteins including Hoxb5 and α-catulin (41, 42). Next in the hematopoietic hierarchy are the HSPCs, which in murine hematopoiesis are generally classified by the combination of $Lin^-cKit^+Sca1^+$ and become increasingly lineage committed. This differentiation potential arises at the expense of the capacity to self-renew (43, 44). As the HSPCs gain lineage specific potential, they begins to express surface proteins which have been used to define specific progenitor populations in the BM, termed Multipotent Progenitor Populations (MPP) (45–47).

In the case of early BM lymphopoiesis, several progenitor populations have been described. Cells within the HSPC pool which express the tyrosine kinase receptor Fms-like tyrosine kinase 3 (Flt3) have been labeled as lymphoid-primed MPPs (LMPP), also termed MPP4 (45, 48–50). LMPP lineage output is functionally distinct from myeloid biased MPP2 ($Flt3^-CD48^+CD150^+$) and MPP3 ($Flt3^-CD48^+CD150^-$) populations as determined *via* murine transplantation experiments (50). LMPPs were shown to have equivalent B and T cell potential, retain some granulocyte and monocyte potential, but lack the ability to produce erythroid and megakaryocyte lineages (49, 51). LMPPs can be further segregated into lymphoid biased cells through expression of a selection of surface proteins. The Interleukin 7 receptor (IL7r), which is required for lymphoid development, is expressed on a subset of LMPPs which efficiently generate T cells and innate lymphoid cells in a murine transplantation setting (52, 53). L-selectin (CD62L) is involved in the trafficking of naïve lymphocytes to peripheral lymphoid organs by binding to a selection of different glycan residues and can be used to specify T lineage progenitors in the BM (54–57). Expression of CD62L separates LMPPs which have transient B cell potential and can yield rapid thymocyte production, but lack the ability to produce cells of myeloid lineages (58). Furthermore, CD62L upregulation has been shown to be an early event in the lymphoid priming of human BM progenitors (59).

Additionally, Vascular Cell Adhesion Molecule 1 (VCAM1) and Flt3 expression can be used to segregate MPPs with combined lymphoid/myeloid ($Flt3^{hi}VCAM1^+$), erythroid ($Flt3^{lo}VCAM1^+$), or B and T cell potential ($Flt3^{hi}VCAM1^-$) (60). VCAM1 is a cell adhesion molecule with roles in vascular adhesion and transendothelial migration of leukocytes (61, 62). Originally identified on the surface of endothelial cells (63, 64), VCAM1 has since been found to be expressed on the surface of multiple cell types including hematopoietic progenitors, macrophages, and BM fibroblasts (65). It is through ligand binding, specifically the α4β1 integrin (CD49d/CD29) and α4β7 integrins expressed on the surface of leukocytes, that VCAM1 mediates adhesion and transmigration of T cells and macrophages (61, 66). The $VCAM1^-$ LMPP population in the BM homogeneously expresses Flt3, and expression of C-C

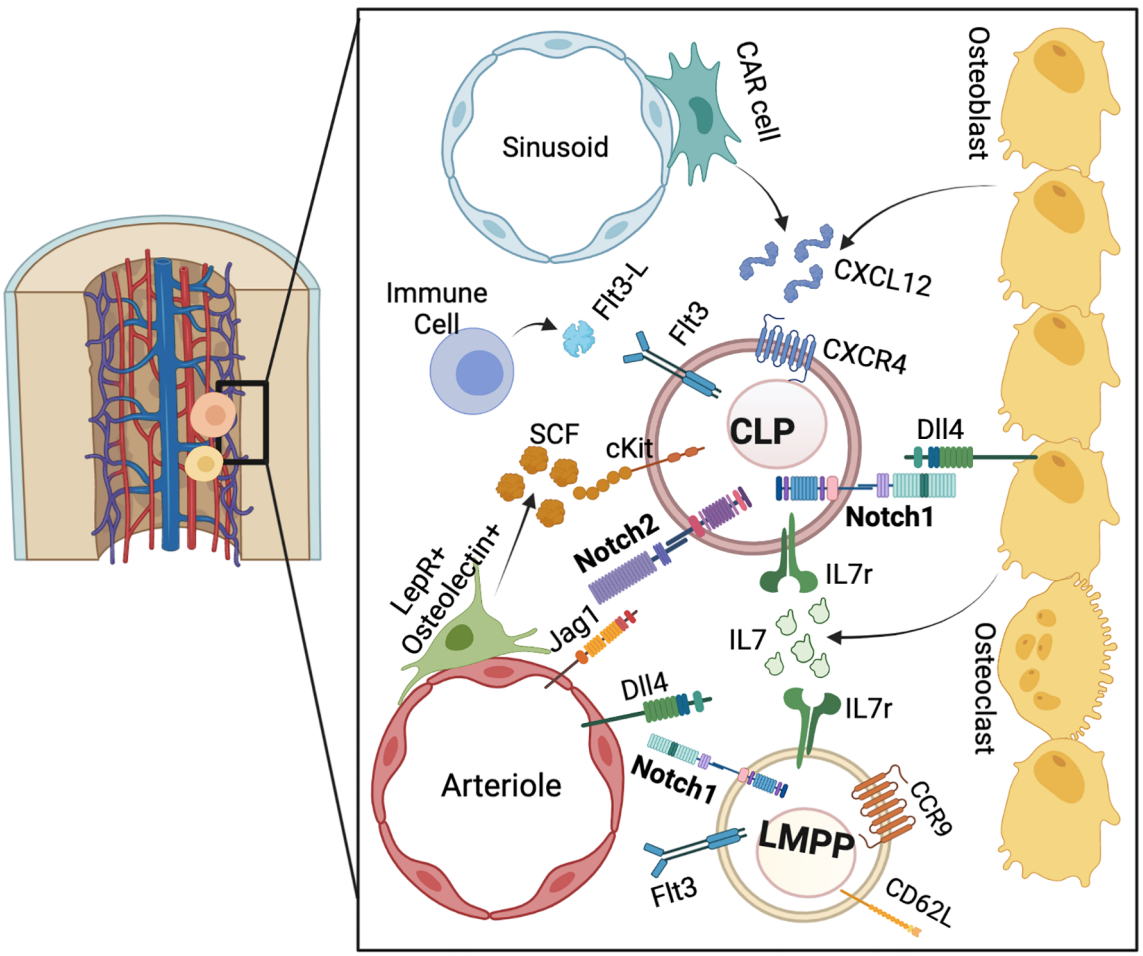

FIGURE 1 | The BM niche for pre-thymic T cell progenitor development. LMPPs and CLPs reside in the endosteal niche. Notch, IL7r, and CXCR4 ligands are derived from the osteoblastic and stromal niche, while SCF is provided from peri-arteriolar cells. Flt3L is provided by mature immune cells. Overall, these signaling pathways converge to stimulate lymphoid progenitors to the T cell lineage.

chemokine Receptor type 9 (CCR9), which also is suggested to play a major role in the recruitment of BM derived cells to the thymus, further delineates a subset of T cell progenitors (67–69). Taken together, MPPs expressing Flt3, IL7r, CD62L, CCR9 and lacking VCAM1 appear to be the main HSPC component contributing to B and T cell development.

Downstream of the LMPP population, the Common Lymphoid Progenitor (CLP), which in murine hematopoiesis is isolated by surface markers $Lin^{-}cKit^{Lo}Sca1^{Lo}Flt3^{+}IL7r^{+}$, represents a canonical branching point between myeloid and lymphoid development and is restricted for lineage production of Natural Killer (NK), B cell and T cell development (70). IL7 signaling is critically involved in BM B cell and thymic T cell lymphopoiesis, and the CLP population is defined by IL7r expression (70–72). Surface expression of Lymphocyte Antigen 6 Family Member D (Ly6D) can be used to divide the CLP population into those with T cell biased potential ($Ly6D^{-}$) and B cell biased potential ($Ly6D^{+}$) (73–75). Downstream of the CLP in bone marrow NK development is the Pre-Natural Killer Progenitor (Pre-NKP) and Refined Natural Killer Progenitor (rNKP) (76). In B cell development, the CLP differentiates into a series of BM sub-populations traditionally referred to as the Hardy Fractions (43), of which Fraction A ($B220^{+}CD43^{+}CD24^{-}BP\text{-}1^{-}$) is immediately downstream of the CLP (77–79). The next well-defined downstream T cell lineage progenitor of the CLP is the early T lineage progenitor (ETP), which is the earliest T cell progenitor in the thymus (80, 81).

While both LMPPs and CLPs possess T cell lineage potential, efforts to determine the exact BM progenitor population which is the Thymic Seeding Progenitor (TSP) have yielded conflicting reports. Although transplantation of CLPs yields thymus engraftment and thymopoiesis, Ikaros-deficient mice have been shown to have thymic ETPs without a detectable CLP population in the BM (82, 83). Additionally, $IL7r^{+}$ LMPPs can generate thymocytes in a CLP-independent manner post-transplant (53). A potential resolution for this issue has been proposed during

pre-natal thymopoiesis, where TSPs are produced in two separate waves, the first of which resembles CLPs and a second resembling LMPPs (84). However, a caveat to these findings is the use of transplantation to determine functional kinetics of TSP generation, which requires removal of these BM resident populations and injection into a recipient's bloodstream. Thus, while TSP generation is determined *in vivo*, the path of the populations in question from the BM to the bloodstream and finally the thymus is in the form of a transplant and does not necessarily mimic natural BM egress and thymic homing. This a principle commonly seen in HSC studies, where it has been recognized that transplantation leads to oligoclonal dominance that does not reflect unperturbed hematopoiesis (85, 86). In order to more accurately determine the BM source for TSP generation, additional methods such as *in vivo* lineage tracing and single cell RNA sequencing should be applied, such as in recent work which unveiled direct production of megakaryocyte progenitors (MkP) from long term HCSs (LT-HSCs) (87). Thus, while the exact BM population for TSP generation has yet to be specifically determined, both the LMPP and CLP populations remain viable sources.

PRE-THYMIC NOTCH SIGNALING IN LYMPHOPOIESIS

Notch signaling is essential for T cell development as shown by the seminal loss-of-function studies by Radtke F et al. (28). However, similar loss of function experiments that deleted the DNA binding member of the trimolecular complex RBPJ (88–90) as well as pan-Notch inhibition with a Mastermind truncation named dominant-negative Mastermind (dnMAML) (17) all indicated that loss of Notch signaling in the BM HSC population had no effect on HSC homeostasis in adult mice. However, as we have described above, several stages of progenitor differentiation occur between the HSC in the BM and the emigrating TPS. The critical temporal question is whether Notch signaling is activated and required for development of the LMPP, CLP or the ETP. Early insight into the role of Notch in BM T lineage lymphopoiesis can be found in studies which showed that the CLP population expresses Notch1 at the mRNA level, and that Notch1 deficient CLP cells erroneously differentiate into B cells in the thymus (91, 92). BM cells transduced with dnMAML failed to produce ETP cells post-transplant, once again hinting at a pre-thymic role for Notch in ETP generation (26). It was further observed that CCR9+ T cell biased MPPs have the potential to activate Notch signaling (68). To determine the expression of individual Notch receptors in HSPC populations, an *in vivo* lineage tracing system has been developed. Cre-recombinase was "knocked into" the individual loci for Notch1-4, which allowed for determination of receptor expression using a fluorescent Cre-reporter mouse strain. This system revealed Notch1 expression in LMPPs, Notch1 and Notch2 expression in CLPs, and an absence of Notch3 or Notch4 in either population (21).

Abrogation of Notch signaling in the BM through inhibition or genetic deletion of Notch receptors or ligands has indicated a role for Notch-dependent T cell progenitor development in the BM. Injection of Notch ligand Dll4 neutralizing antibodies, which have been shown to block ligand specific signaling, leads to a decrease in the BM CLP population (93, 94). Consistently, deletion of either Notch ligand Dll4 or Mindbomb (Mib), which is involved in Notch ligand endocytosis, in Osteocalcin (Ocn) expressing bone cells led to a significant decrease in the CLP population (34, 95). Similar results were obtained when either RBP-J or GDP-fucose protein O-fucosyltransferase 1 (POFUT1) were deleted from BM hematopoietic progenitors (34, 96). The CLP defect observed after conditional deletion of osteoblastic Dll4 underscores the potential existence of an osteoblastic niche for Notch-dependent priming of BM lymphoid progenitors (**Figure 1**). Indeed, it has been shown that C-X-C Motif Chemokine Ligand 12 (Cxcl12) derived from osteoblasts, and not endothelial or hematopoietic cells, is required for CLP and LMPP maintenance in the BM niche (97). The osteoblastic niche has also been implicated in BM B cell progenitor development, through stimulation of HSCs towards the lymphoid lineage *via* $G_s\alpha$ dependent osteoblast IL7 production (98–100). Peri-arteriolar $LEPR^+Osteolectin^+$ cells have also been shown to stimulate CLP development through secretion of SCF (**Figure 1**) (101). This osteoblast-derived SCF secretion decreases in aged mice which have an imbalance in blood lineage output with a propensity toward myeloid populations (101, 102).

While there is mounting evidence supporting the osteoblastic microenvironment as a lymphoid sub-niche in the BM, there are also reports that implicate different niche cells in the priming of lymphoid progenitors. For example, endothelial cells which express high levels of Notch ligands Dll4 and Jag1 (103) have been suggested as an alternative niche for lymphoid progenitor development. Deletion of endothelial expression of Dll4, but not Dll1, leads to a decrease in the frequency of CLP cells, with no effect on the LMPP in the BM (104). However, a direct contribution of endothelial Notch ligand to CLP Notch receptor activation was not shown, and the potential mechanism of CLP depletion was myeloid skewing of upstream HSCs. Additionally, conditional deletion of Cxcl12 in endothelial cells lead to specific depletion of HSCs in the BM, not lymphoid progenitors (97). Furthermore, deletion of SCF derived from peri-arteriolar LEPR+ cells, and not arteriolar or sinusoidal endothelial cells, depleted CLP cells in the BM (101, 105, 106). Taken together these experiments do not support a direct contribution of endothelial derived factors in lymphoid progenitor maintenance during steady-state hematopoiesis in the adult mouse bone marrow.

In most circumstances, the proposed endothelial niche, be it sinusoidal or peri-arterial (101), has been shown to sustain stemness and support self-renewal of HSCs or HSPCs, which by virtue of their hierarchical position in BM hematopoiesis yield more downstream progenitors including CLPs (107, 108). This is evident in several experiments where regenerating or expanding endothelial compartments produce more HSCs and by connection more lineage specific progenitors (109–112). Because Notch signaling is essential for endothelial growth and regeneration, and because the endothelium is a primary niche for HSCs, the effects observed in the CLP compartment could be attributed to an increase in the general abundance in HSC

numbers. Disassociation of the intrinsic role of Notch signaling in arterial cell fate and endothelial function must first be shown to determine if endothelial cells represent the key components of the BM lymphopoietic niche.

Hematopoietic derived signals have also been shown to play a role in CLP homeostasis. Flt3 and Flt3-Ligand (Flt3-L) regulate both myeloid and lymphoid hematopoiesis, and Flt3-L knock-out mice have a severe defect in CLP generation (113, 114). Interestingly, Flt3-L has been shown to be produced in the BM by immune cell populations, including $CD4^+$ memory T cells, rather than stromal, endothelial, or osteoblastic niche cells (115, 116). Although a role for Notch in regulation of Flt3 in homeostatic lymphopoiesis has yet to be established, canonical Notch target Hes1 transcriptionally represses Flt3 expression in Acute Myeloid Leukemia (AML) (117). Additionally, lymphoma/leukemia-Related Factor (LRF), which plays a role in erythroid and late lymphoid lineage decisions, downregulates Dll4 in BM erythroblasts, thus preventing a Notch1 dependent increase in CLP generation at the expense of the HSC pool (118–120).

Confirmation of the existence of the lymphopoietic sub-niche in the BM has been supported by other studies that have shown a cell-intrinsic role for Notch receptor activation in BM lymphoid development. Hematopoietic LRF expression promotes proper B cell development through suppression of Notch signals in CLPs, and hematopoietic deletion of LRF leads to enhanced Notch activity and extra-thymic $CD4^+CD8^+$ T cell generation in the BM (121). Hypomorphic Notch signaling achieved by deletion of the Notch1 Transcriptional Activation Domain (TAD) showed a significant decrease in CLP abundance in the BM (18, 122). Furthermore, an inducible RBPj on/off genetic mouse model has confirmed a role of pre-thymic Notch signaling. Specifically, Notch signaling through RBPj is involved in CD62L+ LMPP generation, with no effect in the T cell primed Ly6D- CLP (35). Collectively, these findings confirm a cell-intrinsic role for Notch signaling in pre-thymic T cell progenitor development in the BM microenvironment.

MECHANISMS OF NOTCH IN CLP DEVELOPMENT

The activation of the Notch receptor is only the first step in the signaling pathway that eventually leads to transcriptional activation of target genes. While a direct role for Notch signaling in pre-thymic progenitor development is evident, the cell intrinsic mechanisms that prime T cell development downstream of Notch in the BM are unknown. A possible mechanism involves the regulation of receptors that are important for cellular migration and tissue retention. Recently, a genetic mouse model for inducible deletion and subsequent inducible expression of RBPj *in vivo* has been developed (35). In this model, floxed *Rbpj* can be conditionally deleted through an inducible Vav-Cre transgene, while a tetracycline responsive element-controlled hemagglutinin (HA)-tagged RBPJ transgene can be induced *via* doxycycline (Dox) injection. RNA sequencing of LMPP cells isolated from $Rbpj^{f/+}$ control, RBPj knock-out, and Dox induced RBPj-HA expressing mice suggested that PSGL1, CCR7, and CCR9 are regulated by Notch signaling in the CD62L+ LMPP population (35). Additionally, deletion of Dll4 in the osteoblastic niche lead to a decrease in CLP cells expressing CCR7 and P-selectin glycoprotein ligand-1 (PSGL1) (34). PSGL1, CCR7 and CCR9 are all involved in the recruitment and migration of BM derived progenitor cells to the thymus (69, 123, 124). However, Notch signaling has also been shown to directly repress CCR9 expression in fetal liver derived T cell progenitors produced *via* co-culture with stromal cells expressing Dll1 (125). The caveat of fetal progenitor acclimation to *ex vivo* co-culture conditions could account for these contrary results. Overall, these observations highlight the possibility that Notch activation in BM lymphoid progenitors prepares cells for thymic migration through induction of genes involved in thymic homing.

Another chemokine pathway involved in BM hematopoiesis is CXCR4/CXCL12, which regulates migration, survival, and quiescence of various progenitor populations (126–130). CXCL12 is expressed by several cell types in the BM, including endothelial cells, osteoblasts, stromal cells, and hematopoietic cells (131). Interestingly, stromal CXCL12 production and HSC release from the BM have been shown to be influenced by circadian neural release of noradrenaline, which activates AdrB3 receptor on Nestin+ osteoprogenitors (132, 133). Although migration of mature leukocyte populations in the BM is regulated in part by circadian rhythms, a direct role for circadian influences on BM lymphoid progenitor biology has yet to be established (134). In humans, there is evidence that $MCAM^+CD146^+$ subendothelial stromal cells express CXCL12 (135). Hematopoietic deletion of CXCR4 results in a reduction of the BM stem cell pool. Specifically affected are HSCs in close contact with CXCL12-abundant reticular cells which surround sinusoidal endothelial cells in the BM (136). CXCR4 has also been shown to regulate the integrity of the vascular barrier in the BM, which further modulates hematopoietic trafficking (137). Work in multiple cell types has revealed dynamic regulation of CXCR4 by the Notch pathway in both mouse and human mesenchymal and endothelial cells (138–142). In the BM, Notch2 has been shown to directly activate CXCR4 expression in HSPCs, while stromal production of CXCL12 has been shown to play a role in CLP maintenance (143, 144). It should be noted, however, that blockade with a Notch2 specific antibody yields only a modest reduction of the CLP population compared to a 30% decrease with antibody blockade of Notch1 (144). Furthermore, mice expressing CXCR4 mutations derived from patients with Warts, Hypogammaglobulinemia, Infections, and Myelokathexis (WHIM) syndrome, which prevent receptor internalization and desensitization, have decreased LMPP and CLP populations (145). These findings suggest a potential role for the CXCR4/CXCL12 axis in the CLP population by placing the CLP near CXCL12-abundant reticular cells, which have further been shown to provide CLPs with the pro-lymphoid cytokine IL7 (72, 126, 146). These observations highlight the potential for the Notch-CXCR4 pathway in lymphoid progenitor development and trafficking within the BM niche.

Lineage commitment of BM hematopoietic progenitors is a complex process involving coordination of cell fate determining

transcriptional networks, which contribute to the heterogeneity of the progenitor pool (44, 86, 147). Given that Notch signaling is essential in the differentiation and maturation of thymic T cells, a direct functional role for Notch signaling in the early BM hematopoietic lineage decisions is a strong possibility (5, 25). Indeed, Dll4 expressed by vascular cells has been shown to suppress the myeloid transcriptional program in HSCs (104). Abrogation of Notch within hematopoietic progenitors leads to an altered myeloid differentiation program, with an increase in GMP production at the expense of MEPs and CMPs (19, 148). In the context of lymphoid progenitors, RNA sequencing has shown that Notch inhibits the myeloid program in both the thymic DN1a/b population and the $CD62L^+PSGL1^+CCR9^+$ subset of LMPPs, which constitutes a putative TSP population (35, 58). Additionally, deletion of Dll4 in Ocn^+ BM osteoblasts yields specific depletion of the T lineage primed $Ly6D^-$ CLP population, hinting at a role for endosteal Notch signaling in influencing B cell vs T cell fates in BM CLPs (34, 73). Such a role has also been established in the thymus, as thymocyte Notch signaling inhibits expression of B-lineage specific factors EBF1 and Pax5 (80). Taken together, there are likely multiple distinct roles for the Notch pathway in the priming and generation of lymphoid progenitors in the BM, including activation of receptors involved in niche trafficking and repression of alternative lineage potential.

NOTCH IN BM LEUKEMIC NICHE

Notch signaling has been implicated in progression of various types of cancer, including Acute Lymphoblastic Leukemia (ALL) (149). Notch signaling plays a well-established role in T-cell acute lymphoblastic leukemia (T-ALL), which is a neoplasm of T-cell blasts accounting for 25% of adult ALL (150). Greater than 60% of patient samples contain mutations in the Notch pathway, with several gain of function mutations in the Notch1 gene, and inactivating mutations in negative regulators of Notch signaling, including FBW7 (151–158). Notch3, which is a Notch1 target gene, has also been shown to play a role in T-ALL (159–161). Mechanisms of Notch signaling in T-ALL oncogenesis include promotion of anabolic cell growth and chemoresistance, activation of the PI3K-AKT-mTOR pathway, and induction of genes involved in G1/S cell cycle progression (158, 162, 163). Notch has also been implicated in B cell leukemias. Hyperactive Notch1 and Notch2 have been shown to sustain B cell Chronic lymphocytic leukemia (B-CLL) (164–167). Conversely, all Notch receptors and the Notch target Hes5 have been shown to act as tumor suppressors in B cell ALL (B-ALL). Even so, Notch3 and Notch4 can prevent apoptosis of human B-ALL cells cultured on human stromal cells *ex vivo* (168–170).

In the context of the BM microenvironment, leukemic cells have been shown to modulate the hematopoietic niche to form a pro-leukemic microenvironment at the expense of homeostatic hematopoiesis (**Figure 2**) (171). Interestingly, and unlike homeostatic lymphoid progenitors, Notch driven T-ALL cells are not maintained by a specific BM niche, but lead to remodeling of the endosteal niche and loss of osteoprogenitors (172). Such remodeling leads to perturbations in BM hematopoiesis, including reduced quiescence of HSCs and more severe leukemia progression (173). Additionally, a multitude of cell extrinsic signaling molecules have been implicated in the pathogenesis of T-ALL (174). The CXCL12/CXCR4 pathway is involved in homing of T-ALL cells to the bone marrow and in Leukemia Initiating Cell (LIC) activity (175). LICs propagate leukemia progression *via* their ability to both self-renew and produce clonal daughter blasts (176). Similarly to homeostatic HSPCs, T-ALL cells are subject to increased CXCR4 expression and activity downstream of Notch activation (144, 175, 177, 178). In human Chronic Lymphoid Leukemia (CLL) and Multiple Myeloma (MM), CXCR4 is also a direct transcriptional target of Notch1 (179, 180). Furthermore, CXCL12 receptor CXCR7 has been shown to be transcriptionally activated by Notch signaling in T-ALL and potentiates CXCR4 signaling and migration (181, 182). This pathway has become clinically relevant since CXCR4 inhibition has shown therapeutic potential in T-ALL (183). Specifically, direct CXCR4 antagonism prevents migration of $CD4^+/CD8^+$ leukemic cells from the thymus to the bone marrow in hypermorphic Notch3 transgenic mice (178). Furthermore, CXCR4 deletion in Notch1-induced T-ALL cells or CXCL12 deletion in endothelial, but not perivascular cells, limits T-ALL progression in mice through induction of cell death (177, 183). Thus, the CXCR4/CXCL12 axis, regulated in part by a hyperactive Notch pathway, is involved in the homing and progression of several leukemia subtypes in the BM niche.

IL7 signaling is critical for lymphopoiesis and, in the context of T-ALL, it plays a role in activation of the JAK/STAT5 and PI3K/Akt/mTOR pathways, with 10% of patient leukemia samples containing activating IL7r mutations (184–186). Stromal derived IL7 has been shown to activate the PI3K/Akt pathway, which is the dominant pathway mediating the proliferative and pro-survival signals downstream of IL7 in T-ALL cells (187–189). Notch1 activates IL7r transcription in human hematopoietic progenitors, as well as in murine leukemia cells (190–192). Additionally, in a human T cell leukemia cell line, IL7r has been shown to be directly co-regulated by Notch1 and RUNX1 (182). Thus, hyperactive Notch signaling contributes to the IL7 dependent proliferation of BM T-ALL cells. Another growth factor involved in BM lymphopoiesis is Insulin-like growth factor 1 (IGF1), which is released from osteoblasts, osteoclasts, and stromal cells in BM and is critical for bone growth (193, 194). Bone marrow levels of IGF1 decrease with age, resulting in increased myeloid bias of HSCs, while temporary IGF1 stimulation of murine hematopoietic progenitors *ex vivo* promotes lymphoid differentiation post-transplant in recipient mice (195). Notch1 has been shown to directly activate expression of Insulin-like growth factor 1 receptor (IGF1R) in T-ALL, which contributes to leukemia survival through the PI3K/Akt pathway (196, 197). IGF1R inhibition yields therapeutic benefits in several solid tumor types and leukemias (198). However, not all T-ALL cell lines are sensitive to IGF1R inhibition, with co-expression of surface IGF1R and tumor-suppressor PTEN indicating IGF1 dependence (196, 199). Interestingly, miR-233 has also been shown to be a Notch target which separately regulates IGF1R expression *via* targeting of the 3′ UTR and reduction of IGF1R protein levels in T-ALL (200). Taken together, Notch signaling in T-ALL allows for optimal

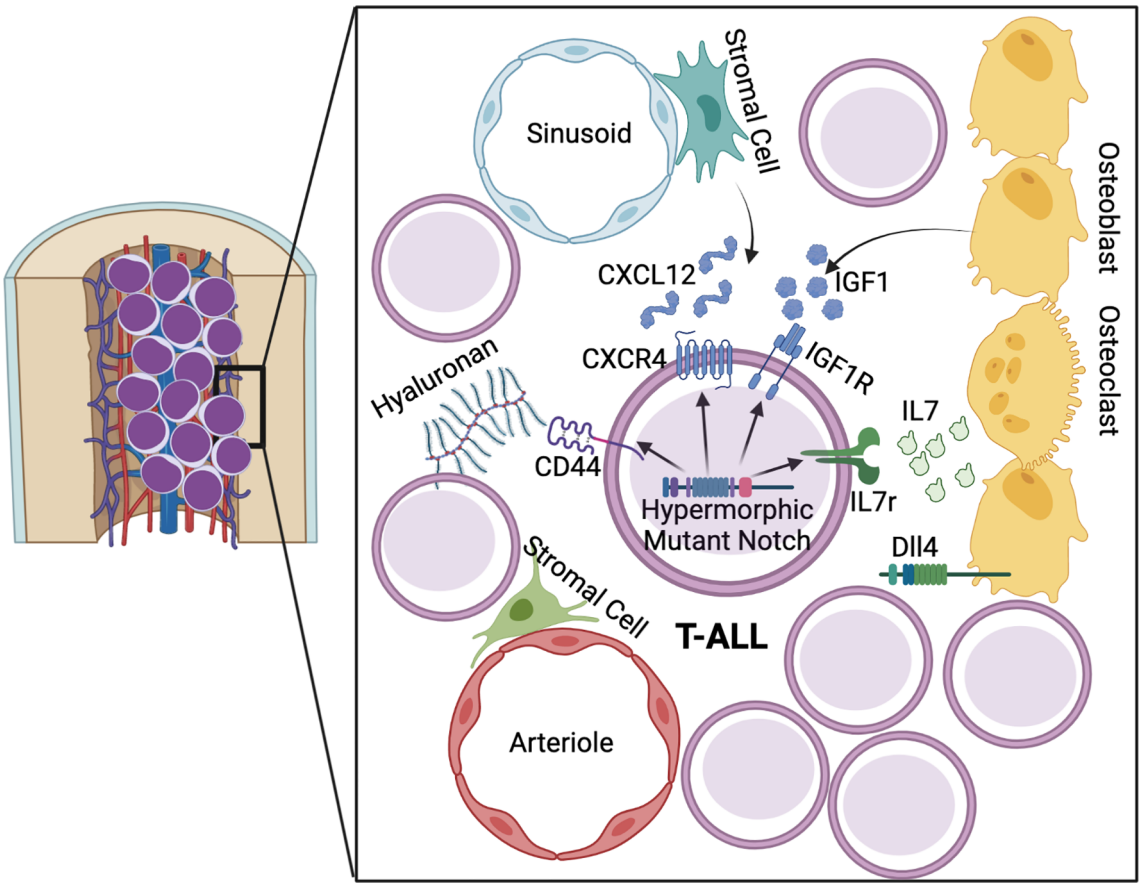

FIGURE 2 | Notch driven mechanisms of T-ALL in the lymphoid niche. Hypermorphic Notch signaling promotes T-ALL progression and amplification of pathways involved in early BM lymphopoiesis. Growth factor signaling from IL7 and IGF1 are augmented *via* Notch driven expression of IL7r and IGF1R. CXCR4 and CD44 promote maintenance of LIC blasts in the BM microenvironment.

signaling of BM derived growth factors, through regulation of their receptors.

Another important, though understudied, component of the BM niche is the extra-cellular matrix (ECM), which has been proposed to regulate both HSPCs and leukemia cells (201–204). The ECM is a vital component of structural and signaling mechanisms in all tissues and consists of collogens, proteoglycans, and glycoproteins (205–207). One protein involved in ECM binding is CD44, which is a cell adhesion molecule that binds to hyaluronan, fibronectin, collagen, E-selectin, and is involved in migration of fetal liver HSCs to the fetal BM (208–212). CD44 is also expressed on adult HSPCs and is involved in progenitor egress from the bone marrow and entry into the thymus (213, 214). Conversely, CD44 has been proposed to play a role in HSPC retention and quiescence, and contributes to apoptosis resistance in LICs (215). A potential mechanism of CD44 mediated chemoresistance in leukemia is through induction of drug efflux (216). In human T-ALL, CD44 has been proposed as a target of Notch1 and suggested to be required for BM engraftment of early leukemic cells (217). Additionally, CD44 can be used as a marker of LICs and is positively regulated by Notch signaling (218). In many tissues, additional ECM components have been shown to influence Notch activity, and there is cross-talk between ECM mediated signaling pathways and Notch (219). Microfibril Associated Glycoprotein-2 (MAGP-2) is found in elastic fibrils and has been shown to regulate Notch activity in COS cells and endothelial cells *via* binding to Notch1 Epidermal Growth Factor (EGF) repeats (220–222). The Cyr61, CTGF, and NOV, (CCN) family of ECM proteins influence osteogenesis and angiogenesis by binding to and enhancing Notch1 signaling (223–226). Additionally, Epidermal Growth Factor-Like Protein 7 (EGFL7), which is secreted by endothelial cells into the vascular microenvironment, regulates angiogenesis in part through antagonization of Notch signaling (227–230). These findings support the need for further exploration into the cross-talk and direct regulation between ECM components and the Notch pathway in T-ALL.

CONCLUDING REMARKS

This review serves to highlight recent work which describes a pre-thymic niche in the BM where Notch signaling influences

lymphoid, and specifically T cell, development. BM lymphoid progenitors receive Notch signals primarily in the osteoblastic niche, which also provides important signals involved in lymphoid development, including CXCL12, IL7, and SCF. Mechanisms of pre-thymic Notch signaling in BM lymphoid development include induction of molecules involved in bone egress and thymus migration. Ultimately a key outcome of this Notch signaling agenda is the early repression of the myeloid transcriptional program. However, while we know that Notch is active and functions in the BM, the downstream target genes of Notch activation in BM lymphopoiesis, particularly with respect to proliferation and survival, have yet to be fully established. We also examined the roles for aberrant Notch signaling in the BM migration, maintenance, and proliferation of T-ALL. Taken together, the works described here underscore the need for careful study of BM Notch signaling in lymphoid hematopoiesis.

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REVIEW
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Drosophila as a Model to Study Cellular Communication Between the Hematopoietic Niche and Blood Progenitors Under Homeostatic Conditions and in Response to an Immune Stress

*Ismaël Morin-Poulard†, Yushun Tian†, Nathalie Vanzo and Michèle Crozatier**

MCD/UMR5077, Centre de Biologie Intégrative (CBI), Toulouse, France

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***Correspondence:**
Michèle Crozatier
michele.crozatier-borde@univ-tlse3.fr

†These authors have contributed equally to this work

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In adult mammals, blood cells are formed from hematopoietic stem progenitor cells, which are controlled by a complex cellular microenvironment called "niche". *Drosophila melanogaster* is a powerful model organism to decipher the mechanisms controlling hematopoiesis, due both to its limited number of blood cell lineages and to the conservation of genes and signaling pathways throughout bilaterian evolution. Insect blood cells or hemocytes are similar to the mammalian myeloid lineage that ensures innate immunity functions. Like in vertebrates, two waves of hematopoiesis occur in *Drosophila*. The first wave takes place during embryogenesis. The second wave occurs at larval stages, where two distinct hematopoietic sites are identified: subcuticular hematopoietic pockets and a specialized hematopoietic organ called the lymph gland. In both sites, hematopoiesis is regulated by distinct niches. In hematopoietic pockets, sensory neurons of the peripheral nervous system provide a microenvironment that promotes embryonic hemocyte expansion and differentiation. In the lymph gland blood cells are produced from hematopoietic progenitors. A small cluster of cells called Posterior Signaling Centre (PSC) and the vascular system, along which the lymph gland develops, act collectively as a niche, under homeostatic conditions, to control the balance between maintenance and differentiation of lymph gland progenitors. In response to an immune stress such as wasp parasitism, lymph gland hematopoiesis is drastically modified and shifts towards emergency hematopoiesis, leading to increased progenitor proliferation and their differentiation into lamellocyte, a specific blood cell type which will neutralize the parasite. The PSC is essential to control this emergency response. In this review, we summarize *Drosophila* cellular and molecular mechanisms involved in the communication between the niche and hematopoietic progenitors, both under homeostatic and stress conditions. Finally, we discuss similarities between mechanisms by which niches regulate hematopoietic stem/progenitor cells in *Drosophila* and mammals.

Keywords: *Drosophila*, lymph gland, niche, hematopoiesis, immune stress

INTRODUCTION

Hematopoiesis is the process that leads to the constant formation of blood cells throughout metazoan life. In vertebrates, hematopoietic stem and progenitor cells (HSPCs) give rise to all blood cell types. In adults, HSPCs are found in the bone marrow, and its microenvironment, termed 'niche', ensures hematopoietic homeostasis by controlling the proliferation and differentiation of HSPCs, both under normal conditions and in response to a stress such as infection or systemic inflammation (1–4). The 'niche' concept was proposed in 1978 by R. Schofield (5) and refers to the cellular context that maintains and regulates HSPC self-renewal and differentiation. The bone marrow hematopoietic niche is now described as a complex multicellular network that supports HSPCs, either *via* direct adhesive interactions or *via* the secretion of many different factors acting in a paracrine manner to control their localization, maintenance, proliferation and differentiation. At least two anatomically distinct HSPC niches exist in the bone marrow. Imaging studies indicate that HSPCs localize around arterioles in the endosteal area, which is in close proximity to the bone surface and is called the endosteal niche (6), and around sinusoids located in the inner bone marrow and called the vascular niche (7–12). Recent advances in single cell technologies allowed the identification of various populations of niche cells with distinct transcriptional profiles, revealing the huge complexity of the cell population within the bone marrow hematopoietic niches (8, 13–16). Furthermore, the HSPC pool itself is heterogeneous, raising the possibility that distinct and specific niche cell types control subsets of HSPCs (6, 17, 18).

For 15 years, *Drosophila melanogaster* has proven to be a suitable model organism to investigate the mechanisms controlling hematopoiesis, based both on limited blood cell lineages and on functional parallels with the vertebrate system. In flies, blood/immune cells are called hemocytes and are related to vertebrate myeloid cells

Drosophila hematopoiesis occurs in two waves during development (19, 20). The first wave takes place during embryogenesis (21). A cluster of cells derived from the head mesoderm gives rise to hematopoietic progenitors, which differentiate into plasmatocytes and crystal cells. Plasmatocytes, which are involved in phagocytosis of cellular debris and pathogens, are equivalent to mammalian macrophages (21–24). Crystal cells contain crystalline inclusions of prophenoloxidases, which are required for the synthesis of melanin (25, 26) and are involved in clotting and wound healing (27–29). These hemocytes of embryonic origin persist in larval and adult stages (**Figures 1A, B** and (30–34). The second wave of hematopoiesis takes place in larval stages at two distinct hematopoietic sites: the hematopoietic pockets and the hematopoietic organ called the lymph gland. Hematopoietic pockets are aggregate of embryo-derived hemocytes segmentally repeated in epidermal-muscular clusters underneath the larval cuticle (**Figure 1C**). In addition, *de novo* blood cell specification occurs in the lymph gland (see below) from hematopoietic progenitors and give rise to plasmatocytes, crystal cells and a third blood cell type called lamellocyte. Lamellocytes are not

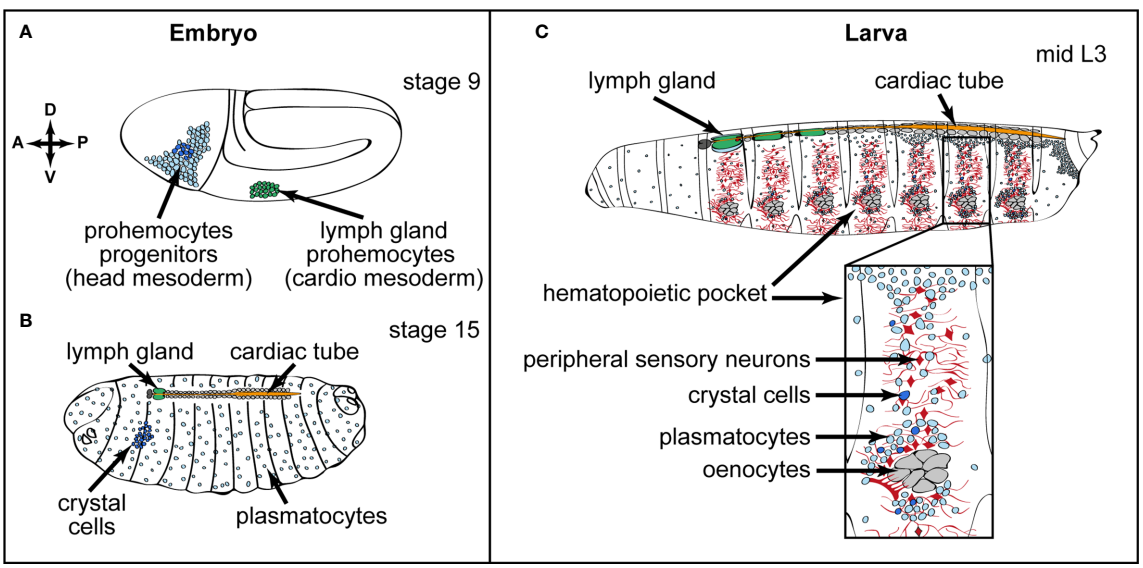

FIGURE 1 | Embryonic and larval hematopoiesis. **(A, B)** Embryonic hemocytes (blood cells) originate from the head mesoderm in the embryo and differentiate into plasmatocytes (macrophages, light blue) and a small number of crystal cells (dark blue). Lymph gland progenitors (green) are specified from the thoracic cardiogenic mesoderm in the embryo **(A)** Anterior (A)/Posterior (P) and Dorsal (D)/Ventral (V) axes are indicated. **(B)** At the end of embryogenesis, crystal cells remain clustered in the anterior part, whereas plasmatocytes are dispersed throughout the embryo. The lymph gland is composed of one pair of lobes and is localized at the anterior part of the dorsal vessel/cardiac tube. **(C)** In third instar larvae, plasmatocytes (light blue) and crystal cells (dark blue) of embryonic origin are found in circulation and colonizing local microenvironments, in particular the hematopoietic pockets, where they expand. Close up of a hematopoietic pocket where neurons are in red, oenocytes in grey, and plasmatocytes and crystal cells in light and dark blue, respectively. Activin-β produced by PNS neurons promotes plasmatocyte proliferation and adhesion. The lymph gland (green) is composed of several pairs of lobes aligned along the cardiac tube.

found in larvae under normal conditions, but they massively differentiate in response to an immune stress such as wasp parasitism. Lamellocytes are required for the encapsulation of foreign bodies too large to be engulfed by phagocytosis (29, 35). The lymph gland is localized dorsally, in close association with the *Drosophila* dorsal vessel, which is the vascular system. At metamorphosis, the lymph gland disrupts and all cells are released into the circulation (29, 36). Both embryo and lymph gland-derived blood cells are present in the adult fly and accumulate in the respiratory epithelia and fat body (33, 34, 37). Their numbers continuously decrease with aging (29, 38), and whether adult flies are able to produce new blood cells is currently under debate. Ghosh et al. identified active hematopoietic hubs, localized in the abdomen, and supporting hematopoiesis in adults (34). However, this conclusion is strongly questioned by a recent analysis which studies hemocytes localized in the head and thorax regions and where no indication of *de novo* blood cell production was observed, even after bacterial infections (37). Since recent single cell RNAseq analyses identify different hemocyte populations (39–42), it is possible that hemocytes characterized in these two distinct locations might have a different potential. This point deserves further investigation.

While no data indicate that embryonic hematopoiesis is niche-dependent, several studies established that larval hematopoiesis is under the control of distinct niches. In this review, we will give an overview of the various *Drosophila* hematopoietic niches identified so far and of the molecular cascades that regulate the communication between niche cells and progenitors, both under homeostatic and immune stress conditions.

NEURONS AS A MICROENVIRONMENT CONTROLLING EMBRYONIC-DERIVED HEMOCYTES IN HEMATOPOIETIC POCKETS

At larval stages, most embryonic-derived hemocytes are differentiated macrophages. They are either circulating in the hemolymph or residing in clusters, which are segmentally repeated along the larval body wall and called hematopoietic pockets (29, 43, 44) (**Figure 1C**). There is a continuous and dynamic exchange between circulating and resident/pocket macrophages (44–47). Large hepatocyte-like cells called oenocytes, and sensory neurons from the peripheral neuronal system (PNS), are in close contact with resident macrophages in the hematopoietic pockets (**Figure 1C**). A subset of sensory neurons that produces Activin-β, a ligand of the TGF-β family, regulates their proliferation and adhesion to hematopoietic pockets (48). It should be emphasized that the neuronal niche in hematopoietic pockets and the niche in the lymph gland (see below) have distinct functions. While the neuronal niche is regulating differentiated macrophages, the niche in the lymph gland is controlling both differentiated hemocytes and hematopoietic progenitors. In vertebrate, tissue-resident macrophages regulate tissue homeostasis and contribute to inflammation (49, 50). Resident macrophage proliferation is strongly dependent on the tissue microenvironment, and whether vertebrate neuronal sensing, as described in *Drosophila*, regulates locally macrophage behavior remains to be addressed.

Finally, several studies report on the plasticity of embryonic-derived hemocytes. Within hematopoietic pockets, plasmatocytes can trans differentiate into crystal cells (51, 52). Furthermore, embryonic-derived hemocytes can also give rise to lamellocytes following parasitism (32, 53–56). A puzzling question was whether signals from the neuronal niche might also regulate blood cell plasticity in hematopoietic pockets. A recent study established that in hematopoietic pockets localized at the caudal end of the larva, the trans differentiation of macrophages into crystal cells is promoted by the neuronal activity of a specific subset of oxygen sensing neurons (52). This study establishes that environmental conditions, such as oxygen levels, control *in vivo* blood cell trans differentiation. Whether neuronal control of blood cell trans differentiation in response to environmental conditions is conserved during evolution, deserves further investigation.

THE PSC ACTS AS A NICHE TO CONTROL LYMPH GLAND HEMATOPOIESIS

In third instar larvae, the mature lymph gland is composed of paired lobes: one primary pair and several secondary pairs. The anterior lobes, which are the largest in size contain progenitors, differentiating hemocytes and mature blood cells, while posterior lobes are composed of a heterogeneous population of progenitors, which do not undergo terminal differentiation (23, 36, 57, 58). Each anterior lobe is divided into several zones (**Figure 2A**). A central zone, called the medullary zone (MZ), contains tightly packed blood cell progenitors (prohemocytes) characterized by the expression of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) receptor *domeless* (*dome*) (23, 59). Recently, the most internally localized subpopulation of MZ progenitors was further characterized by expression of specific markers such as the Thioester-containing protein-4 (Tep4) and Col (60). This subpopulation is defined as "core progenitors". The neighboring progenitors lacking *tep4* and *col* expression are called "distal progenitors" (61). Recent advances in single cell technologies established the transcriptional profiles of lymph gland cells under homeostatic conditions and at various developmental time points (41). The molecular signatures, provided by single cell transcriptomic analysis, define an additional prohemocyte sub-cluster called PH1 (prohemocyte 1). At the periphery of anterior lobes, the cortical zone (CZ) is composed of differentiated blood cells that can be identified through the expression of specific markers for plasmatocytes and crystal cells. Between the MZ and the CZ, cells undergo the transition from progenitors to specified blood cells and correspond to intermediate progenitors. They simultaneously express markers for prohemocytes and for early differentiating cells [**Figure 2A** and (59)]. At the posterior end of the primary lobe is the PSC, identified by its expression of the Notch ligand

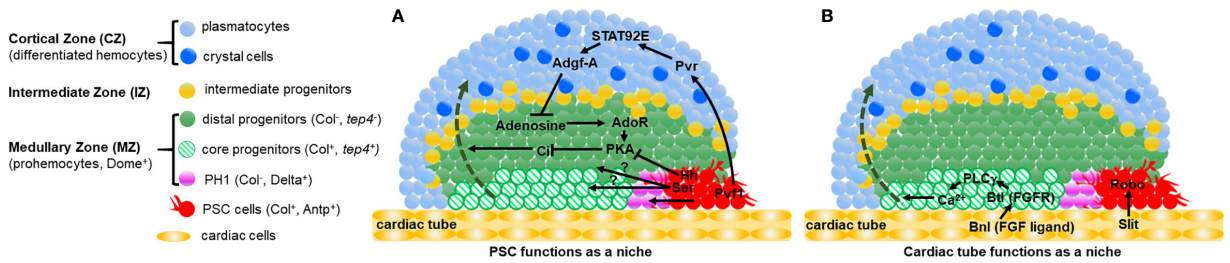

FIGURE 2 | Two niches control lymph gland homeostasis. **(A, B)** Schematic representation of third instar larva lymph gland anterior lobes. The medullary zone (MZ) contains three types of progenitors: distal progenitors and core progenitors are in green and hatched green, respectively, and the PH1 is in pink. Intermediate progenitors are in yellow, plasmatocytes and crystal cells in the cortical zone (CZ) are in light and dark blue, respectively. The PSC and the cardiac tube/vascular system are in red and orange, respectively. **(A)** Differentiated hemocytes result from progenitors' differentiation (green dashed arrow) In a wildtype (WT) lymph gland, under homeostatic conditions, the PSC regulates the maintenance of a subset of MZ progenitors. Hedgehog (Hh) is required for maintaining distal progenitors. PSC signals required for controlling PH1 remain to be identified, as well as the progenitor subset controlled by Ser expressed in the PSC. Pvf1 secreted by the PSC, controls progenitor maintenance *via* differentiated hemocytes. **(B)** The cardiac tube corresponds to a second niche present in the lymph gland. The FGF ligand Branchless (Bnl) activates its receptor Breathless (Btl) in progenitors. Btl-FGF activation regulates intracellular Ca^{2+} levels *via* PLCγ, and controls the maintenance of core progenitors and in turn the whole progenitor pool. The ligand Slit produced by cardiac cells activates its Robo receptors in the PSC. Robo signaling controls PSC cell clustering and proliferation.

Serrate (Ser) (23, 62), the homeobox protein Antennapedia (Antp) (63) and high levels of Collier/Knot (Col/Kn), an orthologue of mammalian Early B cell factor (EBF) (57, 64). In late third instar larvae the mature PSC is composed of 30-40 cells [**Figure 2A** and (57, 63, 64)] and it plays a role similar to a niche to control lymph gland homeostasis.

Most studies on the PSC's role as a niche were performed in third instar larvae. The number of PSC cells is tightly controlled and several intrinsic and extrinsic factors regulate their proliferation. Since several recent reviews report on genes and molecular mechanisms that control PSC cell numbers, we shall not develop this specific issue but provide a table summarizing the information [see **Table 1** and reviews by (19, 20, 77)]. PSC cells produce cytoplasmic processes called filopodia that extend over 2 to 3 cell diameters. An interesting possibility is that filopodia could be engaged in direct cellular contacts between PSC cells and MZ progenitors (63, 64).

The PSC requirement to control the balance between hemocyte differentiation and progenitor maintenance (homeostasis) in third instar larvae was first reported by Mandal et al. and Krzemien et al. In this context, Hedgehog (Hh) secreted by PSC cells is a key regulator of lymph gland homeostasis (63) and Col is required for PSC specification during embryonic development (64). It has been proposed that by controlling hematopoietic progenitor maintenance within the lymph gland, the PSC plays a role similar to a niche. However, several studies questioned the genuine interactions that take place between MZ and PSC cells, since ablation of PSC cells driven by the expression of the proapoptotic gene *reaper* (*rpr*) does not affect MZ progenitor maintenance but rather reduces crystal cell differentiation (78). Another study reported that reduction of PSC cell numbers or alteration of PSC signaling increases hemocyte differentiation without affecting the pool of "core progenitors". Altogether, these two studies establish that core progenitors are maintained independently from the PSC [**Figure 2A** and (60, 78)]. More recent studies shed some light on these discrepancies. Baldeosingh et al. examined the effects on MZ progenitors of specific PSC cell ablation induced by *rpr* expression. For this, they analyzed the expression of Odd-skipped (Odd), a transcription factor expressed in all MZ progenitors. In the absence of PSC cells, a prohemocyte subpopulation with Odd-positive/Col-negative cells differentiated into mature hemocytes, whereas the Odd-positive/Col-positive cells remained undifferentiated (79). The study further reported that Hh from the PSC is required to maintain Odd-positive but not Col-positive prohemocytes, establishing that the MZ cell population is composed of Hh-independent (core progenitors) and Hh-dependent (distal progenitors) progenitors (**Figure 2A**). Altogether these data confirm that the PSC only regulates a subset of MZ progenitors and that this is achieved through Hh signaling.

Blanco et al. investigated the role of Ser in the PSC. *Ser* knockdown in PSC cells leads to increased plasmatocyte and crystal cell numbers, which is in agreement with previously published data (64). Furthermore, they report that Notch knockdown specifically in core progenitors leads to a reduction of their numbers. These data indicate that Ser in PSC cells restricts hemocyte differentiation [**Figure 2A** and (61)]. Ser requires cell-cell contact to activate the Notch pathway, raising the possibility that PSC filopodia could mediate Notch signaling, although the subset of progenitors controlled by Ser remains to be identified.

A recent study further established a role of the PSC in L1 larvae (80). At this stage the lymph gland is composed of PSC cells and hematopoietic progenitors and no differentiation occurs. The PSC counts 2-4 cells that express Col and Antp. Through the expression of different markers, it has been shown that two types of progenitors are present. One subset of progenitors, expressing Notch, is aligned along the cardiac tube, and this cell state is transient, since Notch positive cells are only found during the first 20 hours of larval development.

TABLE 1 | Genes and pathways involved in controlling the number of PSC cells and their cohesion.

Gene	Cell type	Genetic conditions	Function	References
Collier/knot	PSC	LOF (*col* RNAi)	Reduces PSC cell number	(65)
Wnt/Wingless	PSC	LOF (UAS-Dfz2^{DN}) GOF (UAS-wg)	Promotes PSC cell proliferation	(66)
BMP / Decapentaplegic	PSC	LOF (*dpp* RNAi; UAS-tkv ^{DN})	Inhibits PSC cell proliferation	(65)
Dally-like	Not determined	*dlp* mutant	Reduces PSC cell number	(65)
Dmyc	PSC	LOF (d*myc* RNAi) GOF (UAS-*dmyc*)	Increases PSC cell number	(65)
Insulin/TOR	PSC	LOF (InR RNAi) GOF (UAS-PI3K ^{CAAX})	Increases PSC cell number	(67, 68)
Bantam	PSC	LOF (UAS-sponge) GOF (UAS-*bantam*)	Increases PSC cell number	(69)
Bag of Marbles	PSC	LOF (*bam* RNAi)	Inhibits PSC cell proliferation	(70)
Thor/4EBP	PSC	LOF (*elf4A* RNAi)	Increases PSC cell number	(70)
Retinoblastoma-family protein	PSC	LOF (*Rbf* RNAi) GOF (UAS-*Rbf*)	Inhibits PSC cell proliferation	(70)
ARF1-GTP	PSC and hemocytes	LOF (*arf1* RNAi)	Increases PSC cell number	(71)
Jumu	progenitors	LOF (*jumu* RNAi)	Inhibits PSC cell proliferation Promotes PSC cell clustering	(72)
Jumu	PSC	LOF (*jumu* RNAi) GOF (UAS-*jumu*)	Increases PSC cell number	(72)
Slit/Robo	PSC and cardiac cells	LOF (*robo* and *slit* RNAi)	Inhibits PSC cell proliferation Promotes PSC cell clustering	(73)
DE-cadherin	PSC	LOF (*DE-cad* RNAi)	Reduces PSC cell number Promotes PSC cell clustering	(73)
Cdc42	PSC	LOF (UAS-cdc42^{DN}) GOF (*UAS*-cdc42^{CA}	Increases PSC cell number Promotes PSC cell clustering	(73)
Coracle	PSC	LOF (*cora* RNAi)	Reduces PSC cell number	(74)
Neurexin IV	PSC	LOF (*nrxIV* RNAi)	Reduces PSC cell number	(74)
Lar	PSC	LOF (*Lar* RNAi) GOF (UAS-*Lar*)	Reduces PSC cell number	(75)
NUP98-HOXA9	PSC and hemocytes	GOF (UAS-NA9)	Promotes PSC cell proliferation	(76)
E2F	PSC	LOF (*E2F* RNAi)	Increases PSC cell number	(70)

Lineage tracing experiments established that this cell population gives rise to most lymph gland cells at later larval stages, leading the authors to propose that they correspond to genuine Hematopoietic Stem Cells (HSCs). The presence of these HSCs in L1 larvae is also niche-dependent. They rely on Dpp/BMP signaling issued from the PSC.

In summary, these studies reveal a temporal role for the PSC during larval development to regulate lymph gland hematopoiesis and further establish that different signals, long *versus* short distance, are produced by PSC cells throughout larval development to regulate different progenitor subsets. Thus, the lymph gland is a valuable model to investigate the spatial and temporal role of the niche. Additional analyses are required to identify other yet undetected PSC signals, define which progenitor sub-clusters respond to which PSC signals, and finally define how these various niche signals are integrated in progenitor subtypes to control the balance between progenitor maintenance and blood cell differentiation.

THE PSC INDIRECTLY CONTROLS HEMATOPOIETIC PROGENITOR MAINTENANCE *VIA* DIFFERENTIATED HEMOCYTES

In third instar larvae, the PSC indirectly controls hematopoietic progenitor maintenance *via* differentiated hemocytes. The PSC also secretes another ligand called PDGF and VEGF-related factor 1 (Pvf1), which binds and activates the Pvr tyrosine kinase receptor. Pvf1 is produced by PSC cells and transported by vesicles into CZ cells that express Pvr. Pvr activation in the CZ induces a Stat92E-dependent but JAK-independent signaling cascade, leading to the overexpression of adenosine deaminase-related growth factor A (Adgf-A). Stat92E activation is dependent on the ARF1/Asrij complex that encodes a ras small GTPase and an endocytic protein (71). Adgf-A downregulates adenosine levels in neighboring MZ cells, leading to a reduced activity of PKA (cAMP-dependent protein kinase 1). PKA controls the degradation of active Cubitus interruptus (Ci), the transcription factor mediating Hh signal transduction. This backward signal to the MZ is called the "equilibrium signal" (81, 82). Overall, signals from CZ and PSC cells regulate the balance of Ci activity within the MZ, thereby controlling progenitor maintenance (**Figure 2A**).

THE CARDIAC TUBE FUNCTIONS AS A HEMATOPOIETIC NICHE

Within the MZ, "core progenitors" that express *col* and *tep4*, are in close contact with the cardiac tube and are maintained independently from PSC activity [**Figure 2A** and (60, 78, 79)]. This raises the possibility that cardiac cells contribute to the regulation of lymph gland homeostasis. Two recent studies investigated the role of the cardiovascular system under homeostatic conditions and established that cardiac cells act

both i) indirectly *via* the PSC, and ii) directly on MZ progenitors to control lymph gland hematopoiesis. An initial study showed that the *Drosophila* cardiac tube is required to maintain the integrity and function of the PSC through Slit/Robo signaling. The Slit ligand secreted by cardiac cells activates Robo signaling in the PSC. Slit/Robo activation controls both the number of PSC cells and their cohesion, and in turn PSC function (**Table 1**). It controls PSC size by repressing BMP signaling, and maintains PSC cell clustering by regulating the activity of the Cdc42 small GTPase and the accumulation of DE-Cadherin. This study was the first to highlight an inter-organ communication between the cardiac tube and the lymph gland in order to control PSC morphology and consequently its function [**Figure 2B** and (73)]. In a second study, which investigated whether cardiac cells can directly act on MZ progenitors *via* secreted signals, the authors performed a candidate RNAi screen in cardiac cells to identify new potential signaling pathways involved in the crosstalk between the vascular and the hematopoietic systems. This study provided evidence that cardiac cells play a role similar to a niche through the activation of Fibroblast Growth Factor (FGF) signaling. The FGF ligand Branchless (Bnl) secreted by cardiac cells was detected in MZ progenitors as cytoplasmic punctate dots; it is internalized by MZ cells, most likely through FGF-receptor-mediated endocytosis. Bnl binding to its receptor Breathless (Btl) leads to Bnl/Btl-FGF pathway activation in MZ progenitors, where it controls calcium levels *via* the activation of phospholipase C (PLCγ). A previous study showed that reduction of cytosolic Ca^{2+} in lymph gland progenitors leads to the loss of progenitor markers and to increased blood cell differentiation (83). Altogether, these data indicate that through the activation of Fibroblast Growth Factor (FGF) signaling, the vascular system prevents hematopoietic progenitors from massive differentiation, ensuring the proper balance between blood cell populations within the lymph gland. For the first time, this study provides evidence that the vascular system, which directly controls blood cell progenitors independently from the PSC, acts as a niche [**Figure 2B** and (84)]. In conclusion, two distinct niches, the PSC and the cardiac tube, control lymph gland homeostasis.

EMERGENCY HEMATOPOIESIS: KEY ROLE FOR THE PSC

Drosophila blood cells are the effectors of the cellular arm of the innate immune response (24). Wasp parasitism is commonly used to induce an emergency hematopoiesis, which culminates in the massive differentiation of lamellocytes, a cryptic blood cell type (29, 85). Lamellocytes are specialized hemocytes, which mediate the encapsulation and killing of pathogens too large to be phagocytosed. Resistance to wasp parasitism depends on the ability of the *Drosophila* larva to reroute basal hematopoiesis and produce lamellocytes, in a timely manner, to neutralize wasp eggs before they hatch inside the fly larva. Following wasp egg-laying in a *Drosophila* second instar larva, the egg is identified as a foreign body and differentiation of lamellocytes from lymph gland MZ progenitors and circulating/sessile hemocytes is triggered (29, 32, 53–57). In response to wasp parasitism, lymph gland hematopoiesis is drastically modified and shifts to emergency hematopoiesis, leading to increased progenitor proliferation 4-6 hours post-parasitism (59, 86). 20 hours post parasitism, lamellocytes massively differentiate at the expense of MZ progenitors, ultimately leading to the premature dispersal of lymph gland anterior lobes [**Figure 3** and (86)]. The PSC is absolutely required for this emergency response, since lamellocytes fail to differentiate when PSC cells are ablated by targeted expression of *rpr* (60, 64, 78). It has been shown that in *Drosophila* larvae, parasitization increases Reactive Oxygen Species (ROS) levels in PSC cells, leading to the secretion of Spitz (sSpi), one ligand of the Epidermal Growth Factor Receptor (EGFR) signaling pathway (87). Spi issued from the PSC activates the EGFR pathway, both in circulating embryo-derived hemocytes and in MZ progenitors, which triggers their differentiation into lamellocytes. [**Figure 4** and (86, 87)]. Furthermore, it has been established that the Toll/NF-κB pathway is activated in PSC cells in response to wasp parasitism (86, 88). Activation of the pathway is triggered by high ROS levels in PSC cells, which leads to expression of Spätzle (Spz), the Toll/NF-κB pathway ligand, and subsequent activation of the pathway in the PSC. This pathway controls, in a non-cell

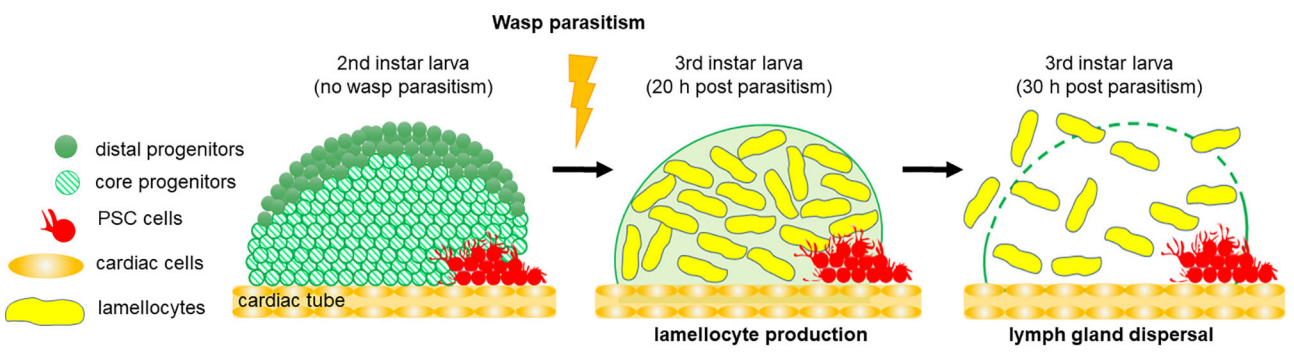

FIGURE 3 | Lymph gland response to wasp parasitism. Schematic representation of 2nd instar larval lymph gland, composed of PSC cells and progenitors. Twenty hours post parasitism, lamellocytes differentiate at the expense of progenitor maintenance. Thirty hours post parasitism, the lymph gland disrupts and cells are released into the hemolymph, where they encapsulate the wasp egg.

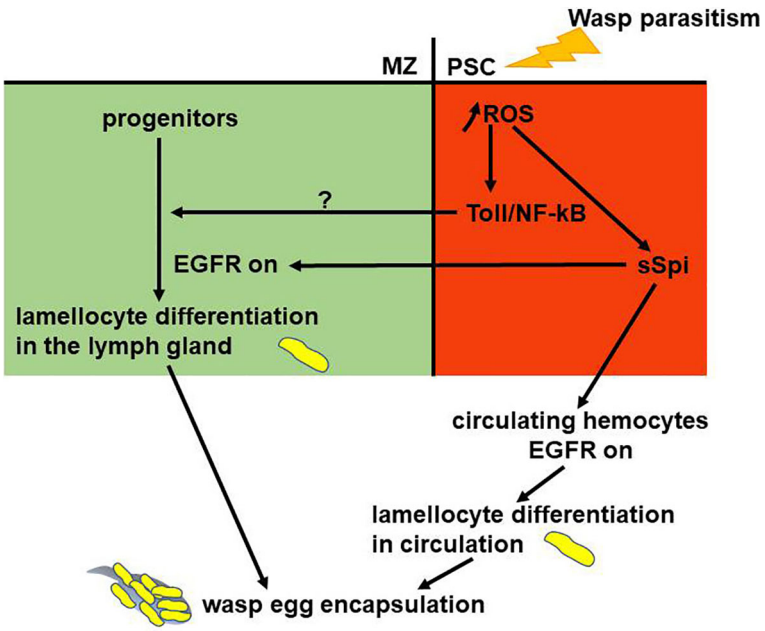

FIGURE 4 | Gene regulatory network controlling larval emergency hematopoiesis. The PSC (red) plays an essential role in mounting the cellular immune response. In response to wasp parasitism, increased Reactive Oxygen Species (ROS) levels in the PSC cause lamellocyte differentiation from lymph gland progenitors (green) and circulating hemocytes. ROS in PSC cells activate Toll/NF-kB and Spitz secretion (sSpi). sSpi, the EGFR ligand, induces lamellocyte fate. Toll/NF-kB activation in the PSC regulates non cell-autonomously lamellocyte differentiation in the lymph gland. EGFR and Toll/NF-kB activation are required to regulate lymph gland stress hematopoiesis.

autonomous manner, lymph gland lamellocyte differentiation in the MZ, which leads to premature disruption of lymph gland anterior lobes, and *in fine* successful wasp egg encapsulation by lamellocytes. It seems that in response to wasp infection, the EGFR and Toll/NF-κB pathways act in parallel to trigger lamellocyte differentiation from MZ cells [**Figure 4** and (86)]. How Toll/NF-κB activation in the PSC acts on MZ progenitors remains to be investigated.

JAK/STAT signaling is one of the evolutionarily conserved signaling pathways involved in immunity (89) and specifically in *Drosophila* for lamellocyte differentiation upon parasitism (90, 91). Under normal conditions, JAK/STAT is activated in lymph gland MZ hematopoietic progenitors; in response to wasp parasitism, the pathway is switched off in progenitors, thus triggering their differentiation into lamellocytes (90). Furthermore, wasp parasitism leads to JAK/STAT activation in larval somatic muscles, which in turn controls the number of circulating lamellocytes and the efficiency of wasp egg encapsulation (92).

In depth analysis of lymph gland hematopoiesis focuses on lymph gland anterior lobes. In response to wasp parasitism, hemocytes from posterior lobes do not differentiate into lamellocytes (23, 36, 58). The JAK/STAT pathway, which is activated in posterior lobes in response to parasitism, is required to prevent lamellocyte differentiation. Furthermore, while the PSC is essential in anterior lobes for the response to wasp parasitism, it plays no role in posterior lobes to prevent lamellocyte differentiation. Altogether, these data indicate a differential response to parasitism between anterior and posterior lobes. Finally, under wasp infection, cell coalescence is observed in posterior lobes, and this response is prevented when the PSC is ablated, suggesting a role for the PSC in this response (58). In conclusion, a complex regulation of JAK/STAT signaling is induced in response to wasp parasitism, and whether JAK/STAT activity in the different cell types could depend on the two niches, namely PSC or/and cardiac tube, certainly deserves further investigation.

Besides wasp parasitism, *Drosophila* can be infected by bacteria, fungi or viruses, which activate the humoral response (24). Interestingly, a recent study established that bacterial infection also alters lymph gland hematopoiesis, since it reported increased plasmatocyte and crystal cell differentiation at the expense of MZ progenitors upon infection. However, in contrast to wasp parasitism, no lamellocytes differentiated. The study further showed that septate junctions form a permeability barrier at the PSC that is disrupted following bacterial infection that trigger prohemocyte differentiation probably by enabling PSC signals to extend into the MZ (74). The authors further established that activation of the Toll/NF-κB and Immune Deficiency (Imd) pathways in PSC cells leads to the loss of the PSC permeability barrier. However, whether bacterial infection disrupts the niche permeability barrier *via* the activation of NF-κB pathways in the PSC is not known yet. Since the Toll/NF-κB pathway is activated in PSC cells and is required for lamellocyte differentiation, it is possible that the permeability barrier modification in PSC cells in response to wasp parasitism

contributes to niche hematopoietic progenitor signaling. These data open novel insights into the cellular communication between the PSC and MZ progenitors.

In mammals, systemic bacterial infection activates the Toll/NF-κB pathway in mouse bone marrow endothelial cells, provoking an "emergency granulopoiesis" (93, 94). This, again, underlines the evolutionary conservation of molecular mechanisms controlling stress-induced hematopoiesis between *Drosophila* and mammals. As a conclusion, our comprehension of the mechanisms regulating emergency hematopoiesis in *Drosophila* should improve our fundamental understanding of how inflammatory signaling regulates hematopoiesis in health and disease conditions.

CONCLUSIONS AND PERSPECTIVES

Drosophila is a powerful *in vivo* model system to study the dialogue between a hematopoietic niche and progenitor cells, since several signaling pathways and transcription factors involved in the *Drosophila* microenvironment play comparable roles in mammals. Under homeostatic conditions, the transcription factor Col/EBF, expressed in PSC cells, is required for PSC specification (57, 64) and controls PSC cell numbers and function through BMP/Dpp pathway activation (65). In mouse osteoblasts, EBF2 is an essential component of the endosteal niche, where it controls osteoblast numbers and regulates HSPC maintenance (95, 96). The Notch pathway is also involved both organism. The Notch ligand Serrate is expressed in the *Drosophila* PSC, where it prevents progenitor differentiation (61, 62). Similarly, in mammalian osteoblasts, Notch1 and 3 and the ligands Jagged1 and Delta1 are all expressed and regulate hematopoiesis, although the precise regulatory mechanisms remain unclear (8, 97, 98). Furthermore, in *Drosophila*, Slit secreted by cardiac cells activates Robo receptors expressed by PSC cells. Silt/Robo activation controls PSC cell numbers and their function (73), while, in mouse bone marrow Slit2/Robo4 controls HSPC localization in the perivascular niche (99–101). In *Drosophila*, the FGF pathway is a key player in the communication between cardiac tube and hematopoietic progenitors. In mammals, this pathway remodels bone and the bone marrow microenvironment to support bone integrity, HSPC maintenance and expansion, and plays a crucial role for proper hematopoiesis during stress recovery (102). Finally, the high similarity between *Drosophila* and mammalian bone marrow hematopoiesis is further emphasized by our recent identification of the cardiac tube as a second niche for lymph gland hematopoiesis, reminiscent of the two niches, endosteal and perivascular, controlling HSPC self-renewal and differentiation in mammals.

Recent data based on single cell analysis revealed an unsuspected heterogeneity among lymph gland hematopoietic progenitors (41). Single-cell RNA sequencing performed on circulating *Drosophila* larval hemocytes highlighted a similarly unexpected heterogeneity among these cells, which were so far believed to consist of merely two cell types, crystal cells and plasmatocytes (40, 42, 103). These results raised many questions about the heterogeneity of the *Drosophila* blood cell pool and their regulation by different niche cell types. Likewise, in mammalian bone marrow, single cell approaches revealed a considerable heterogeneity among both niche and hematopoietic stem progenitor cells (10). Further analyses are now necessary to decipher which niche cells control which progenitor subset, to identify the signals involved in this crosstalk, and finally to determine how information provided by the diverse niche cells is integrated to control hematopoiesis under homeostatic conditions and after infection.

Mechanisms regulating emergency hematopoiesis are poorly understood. Oxidative stress regulates hematopoiesis *via* ROS both in Mammals and in *Drosophila* (87, 104, 105). In mammalian bone marrow, bacterial infection induces an "emergency granulopoiesis" that leads to *de novo* production of neutrophils. In this context, the TLR (Toll-like Receptor)/NF-κB pathway is activated *via* TLR4 in mouse bone marrow endothelial cells, a component of the vascular niche (93, 94). In *Drosophila*, the cellular immune response to parasitism is a typical emergency hematopoiesis. ROS levels increase in the PSC, thus activating both Toll/NF-κB and EGFR signaling pathways, which act in parallel to mount a stress hematopoiesis (86). Whether the EGFR pathway plays a role in mammalian hematopoiesis has not yet been established (106). Altogether, those studies are in favor of evolutionary parallels between *Drosophila* and mouse in the control of stress-induced hematopoiesis. The recent identification of the cardiac tube as a niche controlling lymph gland homeostasis under homeostatic conditions obviously raises the question about its potential role during emergency hematopoiesis.

Malignant hematopoiesis and inflammation in mammals is often associated with an abnormal microenvironment (2, 3, 12, 107, 108). Thus, deciphering the mechanisms at play in the HSPC/niche dialogue is of most importance and *Drosophila* stands as an invaluable model to do so.

AUTHOR CONTRIBUTIONS

IM-P, YT, NV, and MC wrote the manuscript. IM-P and YT made the figures and NV the table. IM-P, YT, NV, and MC designed the review. All authors contributed to the article and approved the submitted version.

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Leukemic Stem Cells: From Leukemic Niche Biology to Treatment Opportunities

Tony Marchand[1,2,3]* *and Sandra Pinho*[4]

[1] Service d'Hématologie Clinique, Centre Hospitalier Universitaire de Rennes, Rennes, France, [2] Faculté de médecine, Université Rennes 1, Rennes, France, [3] Institut National de la Santé et de la Recherche Médicale (INSERM) U1236, Rennes, France, [4] Department of Pharmacology & Regenerative Medicine, University of Illinois at Chicago, Chicago, IL, United States

Acute myeloid leukemia (AML) is one of the most common types of leukemia in adults. While complete remission can be obtained with intensive chemotherapy in young and fit patients, relapse is frequent and prognosis remains poor. Leukemic cells are thought to arise from a pool of leukemic stem cells (LSCs) which sit at the top of the hierarchy. Since their discovery, more than 30 years ago, LSCs have been a topic of intense research and their identification paved the way for cancer stem cell research. LSCs are defined by their ability to self-renew, to engraft into recipient mice and to give rise to leukemia. Compared to healthy hematopoietic stem cells (HSCs), LSCs display specific mutations, epigenetic modifications, and a specific metabolic profile. LSCs are usually considered resistant to chemotherapy and are therefore the drivers of relapse. Similar to their HSC counterpart, LSCs reside in a highly specialized microenvironment referred to as the "niche". Bidirectional interactions between leukemic cells and the microenvironment favor leukemic progression at the expense of healthy hematopoiesis. Within the niche, LSCs are thought to be protected from genotoxic insults. Improvement in our understanding of LSC gene expression profile and phenotype has led to the development of prognosis signatures and the identification of potential therapeutic targets. In this review, we will discuss LSC biology in the context of their specific microenvironment and how a better understanding of LSC niche biology could pave the way for new therapies that target AML.

Keywords: leukemic stem cell (LSC), acute myeloid leukemia, stem cell niche, genetic heterogeneity, therapeutic targets

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***Correspondence:**
Tony Marchand
tony.marchand@chu-rennes.fr

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INTRODUCTION

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults. AML is characterized by the clonal proliferation of abnormal hematopoietic progenitors leading to blood and bone marrow infiltration and consequently hematopoietic failure (1). Over the past decades, intensive research has significantly improved our understanding of AML biology, highlighting the role of clonal evolution and identifying potential therapeutic targets based on recurrent molecular abnormalities (2, 3). However, therapeutic progress has been limited (4). Despite a promising initial

response to intensive chemotherapy, relapse occurs in the majority of patients and prognosis remains poor with a long-term overall survival of 40-50% in patients younger than 60 years old (5–8). In older patients not able to endure intensive chemotherapy, therapeutic options are limited, and long-term overall survival remains low at 15% (9, 10).

Leukemic stem cells (LCSs), also sometimes referred to as leukemic initiating cells, were first described 25 years ago, when Lapidot et al. showed that a small subset of leukemic cells could be transplanted and give rise to leukemia in immunocompromised recipient mice (11). The same group latter identified the $CD34^{pos}CD38^{neg}$ phenotype as a way to enrich the LSC population. Similar to normal hematopoietic stem cells (HSCs), LSCs are able to differentiate and self-renew suggesting a leukemic hierarchy (12–16).

Like their normal counterpart, LSCs reside in the bone marrow in a specialized microenvironment termed "niche". Schofield first described the concept of niche in 1978 and defined it as a limited specific anatomical site where stem cells could be maintained, undergo self-renewal, and where differentiation is inhibited (17). Over the past 20 years, the development of transgenic mice and the improvement of imaging techniques has led to several breakthrough discoveries suggesting that the bone marrow microenvironment plays a central role in normal and pathological hematopoiesis (18). Within the niche, LSCs are thought to be protected from chemotherapy (19–22). Therefore, targeting the LSCs niche represents a promising option to cure AML.

LEUKEMIC STEM CELLS ONTOGENY AND PHENOTYPE

The concept of LSCs is based on the idea that a small subset of cells is able to continually replenish the bulk of leukemic cells. Leukemic stem cells are defined by their capacity to self-renew, incompletely differentiate, and reinitiate leukemia upon serial transplantation in immunocompromised mice (11, 23). Initially thought to originate from the healthy HSC compartment, recent studies have shown that LSCs may instead emerge from committed progenitors (24, 25). Most of human AMLs have at least two molecularly hierarchically ordered distinct LSCs populations (24). Interestingly, the more mature LSC population most closely mirrors normal granulocyte-macrophages progenitors (GMP) whereas the immature LSC population is functionally similar to lymphoid-primed multipotent progenitors (LMPPs). Leukemia originates from the acquisition of driver mutations by HSC or early progenitors (26–28). Identification of clonal hematopoiesis of indeterminate potential (CHIP) has recently generated a significant interest (29). The sequential acquisition of mutations in HSCs and progenitors over a lifetime is suspected to favor hematological malignancies. However, given the high frequency of CHIP in the general population, the exact significance of these mutations and implication in leukemogenesis still needs clarification. To add more complexity, LSCs ontogeny seems to be reversible as opposed to the previously accepted idea that LSCs unidirectionally differentiate into mature AML cells. Indeed, PU.1 gene suppression in differentiated AML-derived cells has been shown to revert AML cells to an immature, clonogenic leukemogenic state (30).

Following the pioneering work done by John Dick's group, showing that LSCs are enriched within the $CD34^{pos}CD38^{neg}$ fraction, several surface markers have been described. Indeed, studies showed that when compared to normal HSCs, LSCs displayed a higher expression of CD25 (31), CD32 (31), CD44 (32), CD96 (33), CD123 (34–36), GPR56 (37), C-type lectin-like molecule-1 (38), IL1RAP (Interleukin 1 Receptor Accessory Protein) (39, 40), N-cadherin, and Tie2 (41). However, a high intra and inter-patients' heterogeneity prevents the use of a single surface marker to easily isolate LSCs.

THE HEALTHY HEMATOPOIETIC NICHE

Hematopoietic stem cells reside in a highly specialized microenvironment or niche within the bone marrow (18). Cellular and molecular interactions between niche constituents and HSCs tightly control their self-renewal, proliferation, and differentiation properties. The development of reporter mice and the improvement of imaging techniques has led to a better understanding of the niche since the concept was first proposed in 1978 (17). Studies have identified several cell populations, sometimes redundant, implicated in homeostatic and pathologic hematopoiesis. Similar to the heterogeneity of the hematopoietic system, niche cells are also highly heterogeneous (42–46).

Early studies have suggested a major role of osteoblasts in hematopoiesis by showing hematopoietic stem and progenitor cells (HSPCs) and osteolineage cells in close proximity at steady state and after bone marrow transplantation, additionally osteoblasts have the capacity to support HSPCs *in vitro* (47–50). Other studies showed a correlation between the number of osteoblasts and $Lin^{neg}Sca1^{pos}c\text{-}Kit^{pos}$ HSPCs (51, 52). However, the specific genetic deletion in osteoblast of two key cytokines required for HSC maintenance, *stem cell factor* (*Scf*) and *CXC-chemokine ligand 12* (*Cxcl12*), did not have a major effect on HSCs (53–55). In addition, 3-D imaging of the bone marrow revealed that HSCs were preferentially localized close to the vascular network but not to the endosteal surface (56, 57). However, osteolineage cells form a niche for early lymphoid progenitors (53, 54, 58), and are implicated in the development and progression of several hematological malignancies like leukemia (54, 58–61).

The identification of the SLAM cell surface markers allowed the imaging of purified HSCs in their native niche (62). This study and others revealed the close proximity of HSCs and blood vessels suggesting the existence of a vascular niche composed by different types of blood vessels and associated perivascular cells (18). Bone marrow mesenchymal stem cells (BM-MSCs) represent a rare and heterogeneous population of stromal cells

characterized by their ability to self-renew and differentiate into osteoblasts, chrondrocytes and adipocytes (63). In the bone marrow, MSCs are located around the blood vessels where they closely interact with HSCs and support hematopoiesis. The development of new transgenic mice models led to the identification of several MSC subsets with significant overlap between the different populations identified (53, 55, 64–68). BM-MSCs are major sources of key niche factors important for the maintenance, proliferation and retention in the mouse bone marrow of HSCs (69). Deletion of *Scf* or *Cxcl12* in stromal cells directly affects HSC number and localization (67, 70, 71). Recent single cell RNA sequencing-based studies have confirmed the high heterogeneity among stromal cells in the bone marrow in particular within the MSC compartment at an unprecedented resolution (42, 44, 45).

The bone marrow is highly vascularized which provides nutrients and oxygen and furthermore allows HSCs and newly generated hematopoietic cells to leave the bone marrow and circulate throughout the body. Bone marrow vascularization is composed of thin-walled arterioles paralleled to the long bone axis and mostly closed to the endosteal region. Arteriolar vessels are connected to the dense network of highly branched sinusoids by type-H vessels at the proximity of the bone (72). Endothelial cells are also key regulators of HSC maintenance and function, and most HSCs localize within 5μm of a bone marrow vessel (56, 62). Indeed, endothelial cells express several factors that regulate HSC function such as SCF, CXCL12, and Notch ligands among others. Depletion of these factors has a dramatic effect on HSC number at steady state and hematopoietic recovery following myeloablative treatment (53–55, 73, 74).

The nervous system plays a crucial role in bone and bone marrow homeostasis (75). Whereas parasympathetic fibers only innervate the compact bone, the bone marrow cavity is innervated by both sympathetic and sensory nerves (76, 77). Although sympathetic nerves do not regulate HSC directly, they are important regulators of HSC mobilization from the bone marrow in response to G-CSF (78). HSCs are also released into the circulation in a circadian manner in response to adrenergic signals from the sympathetic nervous system (SNS) that regulate the synthesis of MSC derived CXCL12, critical for the retention of HSCs inside the bone marrow (65, 78, 79). Interestingly, nociceptive nerves collaborate with the SNS in HSC maintenance and G-CSF-induced mobilization *via* the secretion of calcitonine gene-related peptide (80). Bone marrow neuropathy observed in aging or after the administration of genotoxic drugs induced a profound remodeling of the HSC niche and affected bone marrow regeneration (81–83). Non-myelinating Schwann cells are also involved in HSCs maintenance by converting the latent Transforming Growth Factor β (TGFβ) into the active form inducing HSCs quiescence (84).

In addition to bone marrow stromal cells, healthy HSCs are also directly and indirectly regulated by their own hematopoietic progeny including megakaryocytes, macrophages, regulatory T cells, neutrophils and other myeloid cells, reviewed elsewhere (18).

THE LEUKEMIC NICHE

Although the exact location of LSCs within the bone marrow niche still needs to be clarified, it is now clear that the microenvironment plays a role in leukemogenesis and that leukemic cells can also alter the bone marrow at the expense of physiological hematopoiesis.

A Potential Role of the Microenvironment in Leukemogenesis

Leukemogenesis was long regarded as a cell autonomous process. This dogma was challenged by the early description of donor cell derived leukemia in bone marrow transplanted patients (85). These observations supported the "seed and soil" theory proposed by Paget in 1889 who suggested that tumor metastasis required favorable interactions between tumor cells (the "seed") and their microenvironment (the "soil") (86). The role of non-hematopoietic cells in leukemogenesis was first demonstrated by the development of transgenic mice and the capacity to delete genes in a cell-specific manner. In the context of hematological malignancies, the proof of concept came from the description of a myeloproliferative disorder induced by deregulated expression of Jagged 1 in IκBα deficient hepatocytes. In contrast, mice with a conditional deletion of IκBα specifically in the myeloid lineage did not develop any myeloproliferative neoplasm (MPN) (87), suggesting that premalignant hematopoietic disorders can be initiated by nonhematopoietic cells. Walkley, et al. demonstrated the role of the retinoic acid receptor-γ (RARγ) in niche-driven MPN. Mice deficient in RARγ developed a MPN-like phenotype even when transplanted with wild-type cells (88). The same group investigated the role of the retinoblastoma protein (RB) in hematopoiesis and demonstrated that the deletion of *Rb* induced a MPN-like phenotype only when deleted in both the hematopoietic and non-hematopoietic compartments (89). These studies support the role of the interaction between hematopoietic cells and their microenvironment in the development of hematological malignancies.

Bone marrow MSCs play a central role in the regulation of HSCs during homeostatic hematopoiesis while also involved in the development of myelodysplasia and leukemia. Indeed, specific deletion of the gene encoding Dicer 1, an enzyme involved in micro-RNA processing in osteoprogenitors induces myelodysplasia and sporadic secondary leukemia (59). This phenotype was not observed when *Dicer1* was deleted in the hematopoietic cells demonstrating that the myelodysplasia was environmentally induced. Deletion of *Dicer1* induced the downregulation of *Sbds*, a gene mutated in Schwachman-Bodian-Diamond syndrome, which is a rare human disease characterized by bone marrow failure and a predisposition to leukemia. Specific deletion of *Sbds* in MSCs induced mitochondrial dysfunction, oxidative stress, and activation of the DNA damage response in HSPCs ultimately impairing hematopoiesis and favoring leukemogenesis (90). This effect is a consequence of the secretion of the pro-inflammatory

molecules, S100A8 and S100A9, by MSCs. Conditional expression of a mutated form of *Ptpn11*, the gene encoding for the protein tyrosine phosphatase SHP2, in MSCs and osteoprogenitors also induced a MPN-like phenotype (91). To further support the role of the osteolineage compartment in leukemogenesis, activating mutation of beta-catenin in osteoblasts induced AML by activation of Notch signaling in HSPCs (92). By contrast, the defective activation of Notch in the microenvironment leads to myeloproliferative disease (93). This effect is attributed to a Notch-dependent repression of the micro-RNA miR-155, regulating the inflammatory state of the bone marrow niche (94).

Healthy hematopoiesis is the consequence of close and highly regulated interactions between HSPCs and their microenvironment. Overall, cumulative evidence suggests that niche constituents can also drive hematopoietic malignancy.

Remodeling of the Hematopoietic Niche by Leukemic Cells

As our knowledge of the normal hematopoietic niche improved in the past 20 years, the role of the microenvironment in leukemia development captured the attention of the field. Leukemic cells can remodel the niche creating a favorable microenvironment at the expense of the normal hematopoiesis

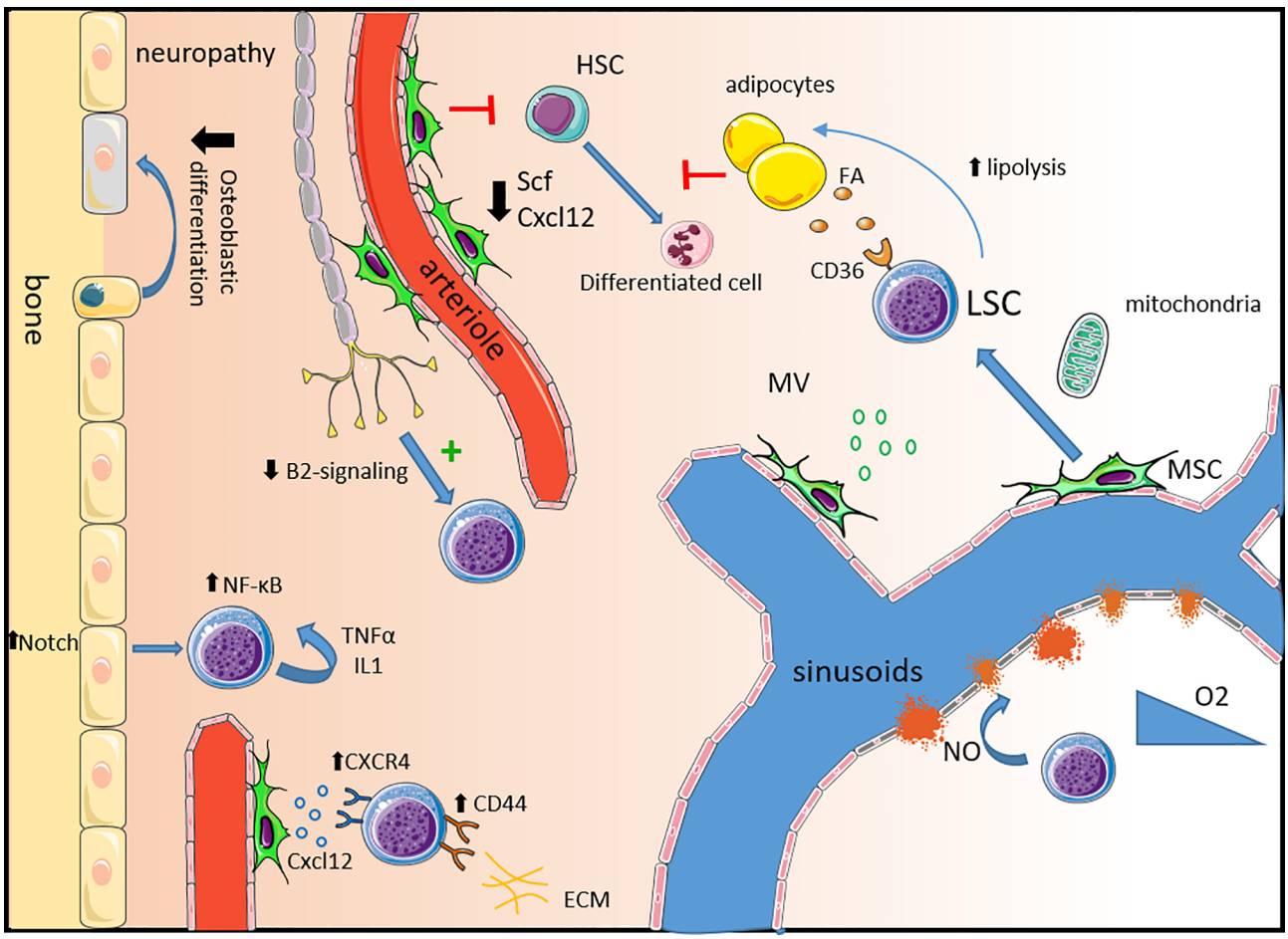

FIGURE 1 | Remodeling of the healthy niche into a permissive leukemic niche. *Neuropathy:* Leukemic progression is associated with sympathetic neuropathy. Loss of β2-adrenergic signaling directly promotes leukemic progression and triggers the expansion of MSCs primed for osteoblastic differentiation but with a defect in terminal maturation leading to a reduction in mineralized trabecular bone. *Mesenchymal stem cells*: In leukemia, MSCs are dysfunctional expressing lower levels of key healthy HSC niche factors such as *Scf* and *Cxcl12* impairing healthy hematopoiesis. LSCs express high levels of the CXCL12 receptor CXCR4 and other adhesion molecules such as CD44 and VLA-4 to usurp the adhesion mechanisms of healthy HSCs. MSC also contribute to LSC survival by the production of microvesicules and *via* mitochondria transfer, providing energy support. *Alteration of the vascular niche:* The expression of VEGF in the leukemic niche induces an increase in vascular density and the production of NO by endothelial cells increases vascular leakiness contributing to hypoxia. In leukemia, endosteal blood vessels are more disrupted than the central bone marrow ones. *Adipocytes:* Leukemic cells support their own metabolism and survival by stimulating lipolysis which fuels fatty acid oxidation in chemotherapy resistant LSCs expressing the fatty acid transporter CD36. *Inflammatory niche*: Activation of Notch signaling in osteolineage cells leads to the activation of the NF-κB pathway in leukemic cells supporting their survival and proliferation. An autocrine secretion of pro-inflammatory molecules like IL-1 and TNF-α also activates the NF-κB pathway. HSC, hematopoietic cells; SCF, stem cell factor; FA, fatty acid; LSC, leukemic stem cell; MSC, mesenchymal stem cell; MV, microvesicule; NO, nitric oxide; ECM, extracellular matrix.

(**Figure 1**) (95). Imaging studies in mice have shown that chemotherapy resistant human LSCs primarily home to and engraft close to the endosteal region where they closely interact with different microenvironmental structures (19).

The bone marrow vascularization is altered in AML with an increased micro-vessel density consequence of the production of pro-angiogenic factors like vascular endothelial growth factor (VEGF) (96–99). AML progression induces the production of nitric oxide (NO) which increases vascular permeability and maintains overall hypoxia (100). Interestingly AML leads to a differential remodeling of vasculature in central and endosteal regions (101). A preferential disruption of the endosteal blood vessels leads to progressive remodeling of the endosteal stroma and the progressive loss of stromal cells. Inhibition of the AML-driven vascular remodeling was shown to improve chemotherapy efficiency in mice (100, 101).

Leukemic cells can reprogram MSCs to create a pro-tumoral niche. MSCs reprogramming can occur following direct cell-to-cell contact, *via* secreted factors, or *via* exosomes (102–104). In addition, human MSCs isolated from AML patients (AML-MSC) displayed *in-vitro* reduced proliferative potential and increased levels of apoptosis (105). Compared to MSCs isolated from healthy donors, AML- MSCs have a lower expression of several niche factors such as SCF, THPO, ANGPT1, VCAM1 and BMI1 (106). In mice, MSCs support AML cells by transferring mitochondria to provide additional energy (107, 108). This transfer is enhanced by some chemotherapies and provides a survival advantage to leukemic blasts and LSCs. This transfer occurs through AML-derived nanotubes. Study in mice showed that superoxide produced by AML cells NADPH oxidase-2 (NOX2) stimulates the nanotubes formation in MSCs. Interestingly, inhibition of NOX2 was able to prevent mitochondrial transfer and improved survival in a xenograft model (107). MSCs also help LSCs to cope with increased reactive oxygen species (ROS) levels, consequence of the mitochondrial transfer by providing increased bioenergetics and detoxifying enzymes (109). Furthermore, MSCs protect AML from chemotherapy through increased Notch and Wnt signaling and inhibition of apoptosis (110–113). Dysregulation of the cytokine profile is suspected to create a pro-tumoral niche in AML (114, 115). LSCs reside in a pro-inflammatory environment known to favor LSCs survival and proliferation. As opposed to normal HSCs and differentiated blasts, LSCs exhibit constitutive NF-κB activity. This activity is partly the consequence of an autocrine tumor necrosis factor-α (TNF-α) secretion, formed by an NF-κB/TNF-α positive feedback loop (116). Activation of Notch signaling also contributes to the activation of the NF-κB pathway (111). Similarly, LSCs aberrantly express the co-receptor for interleukine-1 (IL-1), IL1RAP. Downregulation of IL1RAP inhibits the clonogenic activity of AML cells and leads to increased apoptosis (39). Interestingly, LSCs express IL-1 suggesting another pro-inflammatory autocrine loop. Within the leukemic niche, cytokines can be produced by either immune or leukemic cells. Several cytokines and soluble factors have been shown to affect leukemic cells survival and growth *in-vitro* (117). While pro-inflammatory cytokines such as IL-1β, GM-CSF, IL-3, TNF-α seem to promote AML cells growth, anti-inflammatory molecules such as IL-1Rα, TGF-β and IL-10 have an inhibitory effect (117–119). The function of a specific cytokine is dependent on multiple complex molecular interactions within the microenvironment. Therefore, despite a major improvement in our understanding over the past decade, further studies are needed to clarify the cytokine network in AML.

Adipocytes are classically considered negative regulators of normal hematopoiesis (120). However, this negative action seems to depend on adipocytes anatomical location. Indeed, adipocytes in the active red bone marrow support blood regeneration and myelo-erythroid maturation (121, 122). In the context of AML, leukemic cells repress bone marrow adipocyte maturation impairing myelo-erythoid differentiation (122). Leukemic cells induce the lipolysis of triglyceride to free fatty acids supporting their proliferation and survival (123). Interestingly, outside the bone marrow, gonadal adipose tissue represents a reservoir for LSCs. Within this adipose tissue, leukemic cells create an inflammatory environment triggering lipolysis and the released of fatty acids that fuel LSCs expressing the fatty acid transporter CD36, contributing to chemo-resistance (124).

The sympathetic nervous system is a critical regulatory component of the bone marrow microenvironment that controls the plasticity of bone marrow stromal cells under homeostatic conditions (78, 79, 125). Aging, a condition associated with myeloid biased hematopoiesis and an increased risk of myelodysplastic syndromes and leukemia is associated with sympathetic neuropathy and decreased β3-adrenergic signaling (82, 83). In a MLL-AF9 mouse model, AML infiltration induced sympathetic neuropathy which further promoted AML (60). This neuropathy was associated with an expansion of Nestin-GFP^{pos} MSCs primed for osteolineage differentiation, and HSC exhaustion. Loss of β2-adrenergic signals directly promotes an expansion of LSCs expressing the β2-adrenergic receptor. Studies using primary AML cells from patients showed that leukemic cells altered adipogenesis in favor of osteolineage differentiation (122, 126). However, sympathetic neuropathy impairs terminal osteoblastic lineage differentiation leading to a reduction in mineralized bone density (60). Sympathetic neuropathy was also induced by the pro-inflammatory environment observed in a JAK2^{V617F} MPN mouse model (127). In this context, Nestin-GFP^{pos} MSCs are reduced, which in turn led to the expansion of altered HSPCs and disease progression.

Similar to their healthy counterpart, LSC localization is dependent on the expression of cytokines and adhesion receptors. Leukemic cells adhere to the bone marrow through three main receptors: CXCR4, Very Late Antigen-4 (VLA-4) and CD44 (128). The high expression of these adhesion molecules facilitates the homing and retention of leukemic cells in the niche impairing chemosensitivity (32, 129–131). In addition, interactions between VLA-4 expressed by leukemic cells and

VCAM1 expressed at the surface of BM-MSC mediates chemoresistance *via* activation of the NF-κB pathway in stromal cells (20).

LEUKEMIC STEM CELLS: A THERAPEUTIC OPPORTUNITY

LSCs as a Prognostic Marker

Patients with AML are treated according to a risk stratification aiming to identify the patients with low, intermediate, and high risk of relapse based on the disease characteristics at diagnosis (9). Since LSCs have been implicated in treatment resistance and relapse, quantification of the LSC pool could be an additional prognostic factor beside the traditional genetic and molecular abnormalities. As we discussed before, a clear definition of the LSC phenotype does not exist, and different approaches have been used to estimate the LSC pool in patients. Using flow cytometry, Zeijlemaker W. et al. showed that CD34-positive AML blasts were associated with an increased incidence of relapse compared to CD34-negative AML (132). More recently, the prognostic impact of LSC frequency defined by the $CD34^{pos}CD38^{neg}$ phenotype combined with minimal residual disease (MRD) evaluation was demonstrated in a prospective study (133). High level of $CD34^{pos}CD38^{low}/CD123^{pos}$ blasts at diagnosis is predictive of an adverse outcome (134). Interestingly, a recent study performed in older AML patients showed that this predictive impact is only seen in patients treated by intensive chemotherapy but not by hypomethylating agents (36). Leukemic stem cells frequency seems to be correlated with a lower white blood cell count, an adverse cytogenetic risk, and less frequent NPM1 mutation (36, 135).

Stem cell gene expression signatures have been shown to have a prognostic impact in AML, also highlighting the potential role of leukemia stemness in treatment response (25). Based on this observation, a 17 genes score (LSC17) that compared the gene expression profiles between 138 LSC^{pos} and 89 LSC^{neg} isolated from 78 AML patients was developed (136). A high score is associated with a poor outcome after standard treatment including HSC transplantation (136). The LSC17 was recently challenged by the newly developed AML prognostic score (APS), a 16 gene expression signature score, derived from RNA-sequencing and whole exome sequencing results (137). Interestingly, the authors hypothesized that APS can outperform the LSC17 because of its capacity to capture signal from the microenvironment.

How to Target the Leukemic Stem Cell Niche

Compared with other hematological malignancies, therapeutic progresses have been limited in AML highlighting the need for new strategies. The microenvironment shelters LSCs, protects them from genotoxic drugs and therefore represents a possible cause of treatment failure and relapse. Different strategies have attempted to target the LSC-niche interactions and several studies are currently ongoing (**Figure 2**). LSCs can also be directly targeted based on their phenotypic and functional differences compared to healthy HSCs. These strategies are beyond the scope of this article and have been reviewed elsewhere (31, 138–140).

Adhesion Molecules

Adhesion molecules maintain LSCs in the hypoxic niche protecting them from cycling-dependent chemotherapies. Targeting adhesion molecules aims to mobilize LSCs out of their protective niche in order to expose them to chemotherapy. LSCs express the receptor CXCR4 and migrate in response to CXCL12 (141). Moreover, high levels of CXCR4 expression are associated with relapse and poor overall survival in patients (142). Plerixafor, a potent inhibitor of CXCR4, is currently used in association with G-CSF to induce HSCs mobilization (143). In an acute promyelocytic leukemia murine model, treatment with plerixafor in combination with cytarabine and daunorubicine improved chemosensibility and overall survival (144). Since this early study, plerixafor has been tested in phase I-II studies, in combination with various chemotherapies and hypomethylating agents with promising results (145–147). Other CXCR4-CXCL12 axis inhibitors are under clinical development like CX-01, BL-8040 and ulocuplumab. These drugs showed encouraging results in combination with chemotherapy in phase I-II studies (148–151). However, larger phase III studies are needed to confirm the benefit and the exact place of the CXCR4-CXCL12 axis inhibition in AML treatment strategy.

Bromodomain and extra-terminal domain-containing (BET-containing) proteins (BETPs)-inhibitors, can also target adhesion molecules. Sustained degradation of BETPs induced the downregulation of CXCR4 and CD44 expression, decreased the LSC population, and improved overall survival in a patient-derived xenotransplantation model (152). Importantly, BETPs inhibition significantly reduced the number of LSCs when used alone or in combination with chemotherapy. CD44 represents an exciting target since it is differentially expressed between LSCs and normal HSCs (130, 131). Administration of H90, a monoclonal antibody directed to CD44, in immunocompromised mice transplanted with human AML reduced the leukemic burden. Interestingly, H90 seemed to specifically target the LSCs population since no leukemia was observed in serially transplanted mice (32).

Vascularization Remodeling and Hypoxia

VEGF was early identified as a promising target given its pro-angiogenic and anti-apoptotic effects on leukemic cells (153). However, results of clinical studies using bevacizumab, a humanized recombinant monoclonal antibody directed against VEGF have proven disappointing (154, 155). A recent study in mice suggests that inhibition of NO production by endothelial cells could restore the normal vascularization and improve response to cytarabine (100). Targeting NO production by inhibiting the NO synthase could therefore represent a new therapeutic target. The niche represents a hypoxic environment that maintains LSCs in a quiescent state. Moreover, hypoxia inducible factor-1 (HIF-1α) expression induced by hypoxia

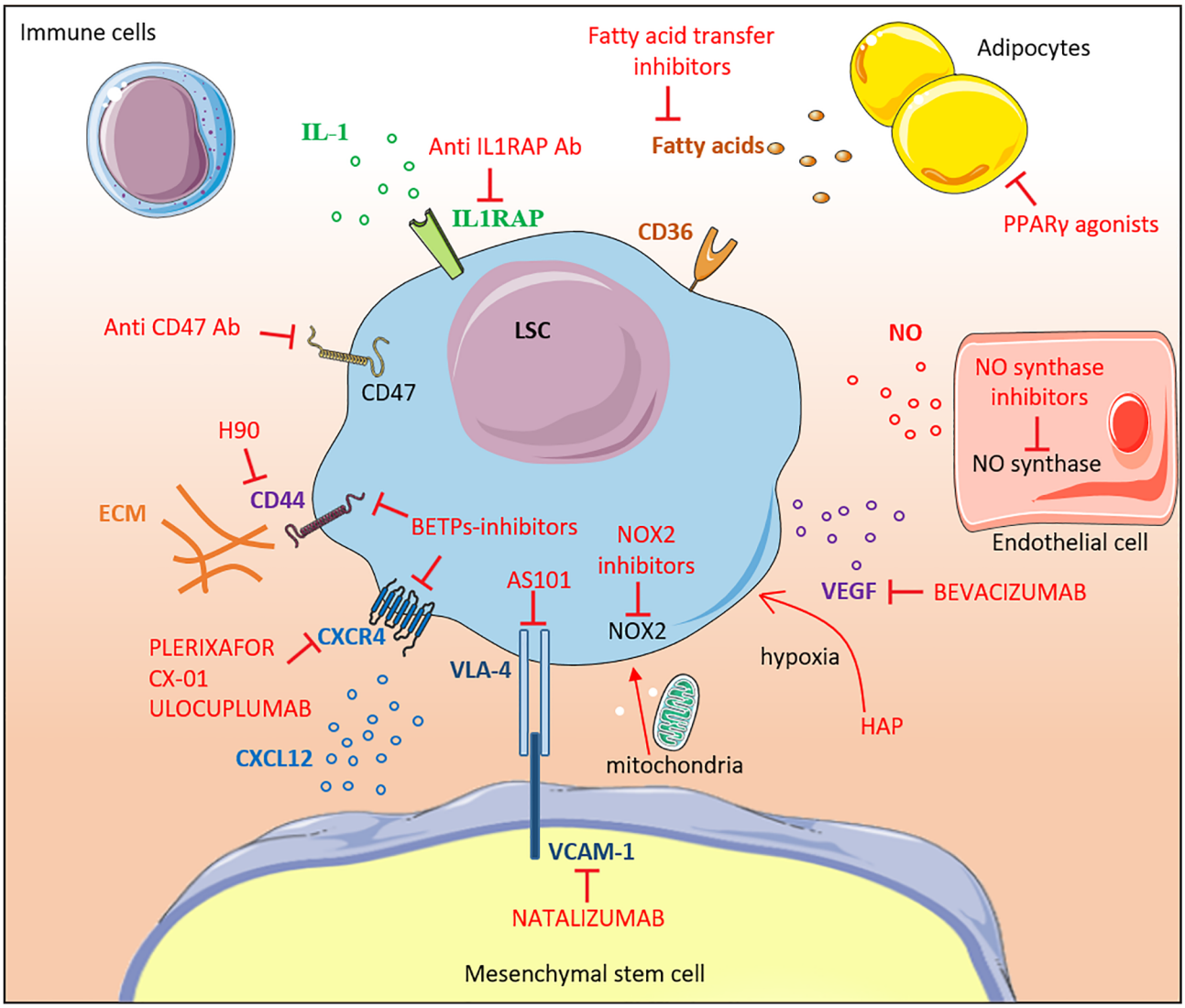

FIGURE 2 | Therapeutic targeting of the leukemic niche. The different molecular interactions between LSCs and the bone marrow niche constituents are shown. Inhibitors are labeled in red. Most of the drugs shown in the figure are under pre-clinical or early clinical development. IL-1, interleukine-1; Ab, antibody; CD, cluster of differentiation, FA, fatty acid; LSC, leukemic stem cell; MSC, mesenchymal stem cell; NOX2, NADPH oxidase 2; NO, nitric oxide; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; HAP, Hypoxia-activated prodrugs; PPARγ, Peroxisome Proliferator-activated Receptor gamma; VCAM-1, Vascular Cell Adhesion Molecule-1; BETPs, Bromodomain Extra-Terminal Protein.

upregulates CXCR4 expression at the membrane surface of LSCs (19). However, the exact impact of HIF-1α inhibition is still debated (156, 157). Another way to target the hypoxic microenvironment is to use hypoxia-activated prodrugs (HAPs) (158) specifically designed to form cytotoxic agents under hypoxic conditions while limiting the toxicity on normal tissues. Evofosfamide (also known as TH-302) is a 2-nitroimidazole-linked prodrug. *In vitro*, evofosfamide treatment promotes a dose- and hypoxia-dependent apoptosis and cell death in AML cells. Interestingly, in a xenograft model, evofosfamide reduces LSC pool with limited toxicity on normal hematopoiesis (159, 160). However, a phase I study conducted in 49 patients with advanced leukemia showed disappointing results with an overall response rate of only 6% only (161). Other HAPs are currently under development.

Cytokines and Soluble Factors

Targeting the pro-inflammatory environment represents another interesting strategy considering the importance of cytokines like IL-1, IL-6 and TNFα for LSC survival and proliferation. IL-1 and IL-6 inhibitors are already commercially available for the treatment of autoimmune disease and cytokine released syndromes (162, 163). It would be interesting to test these inhibitors in combination with chemotherapy even if caution is

needed regarded the risk of infections. Given the higher expression of IL1RAP at the surface of LSCs compared with normal HSCs, targeting IL1RAP is an attractive option. Indeed, in a preclinical study, targeting IL1RAP using a monoclonal antibody induced selective killing of AML $CD34^{pos}CD38^{pos}$, and $CD34^{pos}CD38^{neg}$ cells both *in vitro* and in a xenograft model (164).

Since leukemic cells trigger lipolysis and use fatty acids as a source of energy, targeting the adipose tissue represents another possible strategy. Studies in mice have shown that restoring normal adipocyte maturation using PPARγ agonists inhibits leukemic growth. Similarly, inhibiting fatty acids transfer to leukemic cells improved survival in a xenograft model (123). However, further studies in human are warranted.

CONCLUSION

According to the cancer stem cell theory, LSCs sit at the top of the hierarchy and are the source of the more differentiated leukemic blasts. Even if these cells represent an attractive target, eradicating LSCs is highly complex, notably due to the lack of specific markers. AML is associated with a remodeling of the hematopoietic niche where HSCs and LSCs reside, however, modifications of the microenvironment also contribute to leukemia development at the expense of normal hematopoiesis. Since the first description of LSCs more than 25 years ago, our understanding of this small subset of leukemic cells has greatly improved with the identification of potential therapeutic targets paving the way for the development of new treatment strategies in a still deadly disease.

AUTHOR CONTRIBUTIONS

TM and SP conceptualized and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Patient-Derived Bone Marrow Spheroids Reveal Leukemia-Initiating Cells Supported by Mesenchymal Hypoxic Niches in Pediatric B-ALL

Juan Carlos Balandrán[1,2†], José Dávila-Velderrain[3,4], Antonio Sandoval-Cabrera[5], Gabriela Zamora-Herrera[1], Vanessa Terán-Cerqueda[6], Lilia Adela García-Stivalet[6], José Alejandro Limón-Flores[6], Erick Armenta-Castro[1], Aurora Rodríguez-Martínez[1,7], Bertha Alicia Leon-Chavez[7], Verónica Vallejo-Ruiz[1], Duane C. Hassane[8], Sonia Mayra Pérez-Tapia[9], Vianney Ortiz-Navarrete[2], Monica L. Guzman[8‡] and Rosana Pelayo[1*‡]*

[1] Laboratorio de Oncoinmunología y Citómica, Centro de Investigación Biomédica de Oriente, Instituto Mexicano del Seguro Social Delegación Puebla, Puebla, Mexico, [2] Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Mexico City, Mexico, [3] Computer Science and Artificial Intelligence Lab, Massachusetts Institute of Technology (MIT), Cambridge, MA, United States, [4] The Broad Institute of MIT and Harvard, Cambridge, MA, United States, [5] Hospital para el Niño de Toluca, Instituto Materno Infantil del Estado de México (IMIEM), Toluca, Mexico, [6] Servicio de Hematología, Unidad Médica de Alta Especialidad, Hospital de Especialidades "Manuel Ávila Camacho", Instituto Mexicano del Seguro Social, Puebla, Mexico, [7] Posgrado en Ciencias Químicas, Area de Bioquímica y Biología Molecular, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico, [8] Division of Hematology and Medical Oncology, Department of Medicine, Weill Cornell Medicine, New York, NY, United States, [9] Unidad de Desarrollo e Investigación en Bioprocesos (UDIBI) and Unidad de Investigación, Desarrollo e Innovación Médica y Biotecnológica (UDIMEB), Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico

B-cell acute lymphoblastic leukemia (B-ALL) results from the expansion of malignant lymphoid precursors within the bone marrow (BM), where hematopoietic niches and microenvironmental signals provide leukemia-initiating cells (LICs) the conditions to survive, proliferate, initiate disease, and relapse. Normal and malignant lymphopoiesis are highly dependent on the BM microenvironment, particularly on CXCL12-abundant Reticular (CAR) cells, which provide a niche for maintenance of primitive cells. During B-ALL, leukemic cells hijack BM niches, creating a proinflammatory milieu incompetent to support normal hematopoiesis but favoring leukemic proliferation. Although the lack of a phenotypic stem cell hierarchy is apparent in B-ALL, LICs are a rare and quiescent population potentially responsible for chemoresistance and relapse. Here, we developed novel patient-derived leukemia spheroids (PDLS), an *ex vivo* avatar model, from mesenchymal stromal cells (MSCs) and primary B-ALL cells, to mimic specialized niche structures and cell-to-cell intercommunication promoting normal and malignant hematopoiesis in pediatric B-ALL. 3D MSC spheroids can recapitulate CAR niche-like hypoxic structures that produce high levels of CXCL10 and CXCL11. We found that PDLS were preferentially enriched with leukemia cells displaying functional properties of LICs, such as quiescence, low reactive oxygen species, drug resistance, high engraftment in immunodeficient mice, and long-term leukemogenesis. Moreover, the combination of PDLS and patient-derived xenografts confirmed a microenvironment-driven hierarchy in

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Pablo Menendez, Josep Carreras Leukaemia Research Institute (IJC), Spain
Anastasia Tikhonova, University Health Network, Canada

***Correspondence:**
Rosana Pelayo
rosana.pelayo.c@gmail.com; rosana.pelayo@imss.gob.mx
Monica L. Guzman
mlg2007@med.cornell.edu

†Present address:
Juan Carlos Balandrán, Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Department of Cell Biology, Albert Einstein College of Medicine, New York, NY, United States

‡*These authors have contributed equally to this work and share last authorship*

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their leukemic potential. Importantly, transcriptional profiles of MSC derived from primary patient samples revealed two unique signatures (1), a *CXCL12^{low} inflammatory and leukemia expansion* (ILE)-like niche, that likely supports leukemic burden, and (2) a *CXCL11^{hi} immune-suppressive and leukemia-initiating cell* (SLIC)-like niche, where LICs are likely sustained. Interestingly, the CXCL11^{+} hypoxic zones were recapitulated within the PDLS that are capable of supporting LIC functions. Taken together, we have implemented a novel PDLS system that enriches and supports leukemia cells with stem cell features driven by CXCL11^{+} MSCs within hypoxic microenvironments capable of recapitulating key features, such as tumor reemergence after exposure to chemotherapy and tumor initiation. This system represents a unique opportunity for designing *ex vivo* personalized avatars for B-ALL patients to evaluate their own LIC pathobiology and drug sensitivity in the context of the tumor microenvironment.

Keywords: acute lymphoblastic leukemia, leukemia-initiating cell, bone marrow niche, mesenchymal stromal cells, tumor microenvironment, B-cell development

INTRODUCTION

Childhood cancer, a global health priority, remains a leading cause of death from disease in scholar age, with B-cell acute lymphoblastic leukemia (B-ALL) exhibiting substantial number of years of life lost and increasing rates of unfavorable outcome cases in low- to middle-income countries (1, 2).

B-ALL starts and progresses in the bone marrow (BM), where malignant precursor cells expand in the context of pro-inflammatory microenvironments and a highly complex and dynamic BM topology, endowed with the ability of selecting pre-malignant clones able to evolve into tumor (3–6). A number of genetic abnormalities associated with high-risk B-ALL suggest stem cell-like properties, such as colonization of hematopoietic niches, and highlight the cooperation between leukemia cells and BM microenvironment by intrinsic and extrinsic signals. Furthermore, the evaluation of the hematopoietic organization structure in B-ALL has challenged the traditional hierarchy of the differentiation, revealing that cell fate decisions are indeed supported by heterogeneous hematopoietic stem/progenitor cell (HSPC) niches in a more stochastic structure. A new model of hematopoietic forming units suggests that HSPC can respond to environmental cues driving intra- and inter-communication networks that may create adaptable niches (7, 8).

Accordingly, CXCL12-abundant reticular (CAR) niches, formed by specialized mesenchymal stromal cells (MSCs) (9) overlapping with nestin and leptin receptor (LepR) expression (10, 11) and producing high levels of CXCL12, SCF, and IL-7, are critical for B-cell lymphopoiesis (12). The essential roles of the CXCL12/CXCR4 axis in niche positioning and cell cycle status of leukemia stem cells have been highlighted by the specific deletion of CXCL12 from BM MSCs, suggesting the differential use of CXCL12-niches by CXCR4^{+} malignant cells (13). The professional cytokine-secreting CAR cells that create stage-specific micro-niche configurations crucial for maintenance, cell cycling, and differentiation fate decisions of lymphoid, and myeloid progenitors, have been recently defined by transcriptomics single-cell approaches (14, 15), and confirmed the critical interdependence of normal and malignant HSPC with their niches (16). Although the lack of a phenotypic stem cell hierarchy is apparent in B-ALL, leukemia-initiating cells (LICs) have been recognized as a rare subpopulation endowed with stemness properties and potentially responsible for chemoresistance and relapse (17, 18). Therefore, due to their clinical and therapeutical implications, it is critical to characterize the relationship between LICs and their microenvironment. Computational modeling approaches have recently inferred a unique inflammation-inducible CXCR7^{+} B-precursor cell population, displaying abnormal phenotypes and presumably able to colonize distinct emergent inflammatory niches producing CXCL11 (19). Moreover, three-dimensional (3D) hematopoietic structures have been instrumental to advance our knowledge on cell-to-cell intercommunication, nutrient diffusion, oxygen gradients, hypoxic zone formation, and HSPC expansion (20, 21).

Thus, to better investigate the LICs in their microenvironment, we sought to implement a co-culture method capable of mimicking the BM niche and sustain primary B-ALL cell growth and survival from B-ALL patients. The resulting patient-derived leukemic spheroids (PDLS) showed a remarkable ability to enrich leukemia cells with stem cell properties. RNA-seq data from pediatric B-ALL-derived MSCs provided evidence of two putative MSCs subpopulations with unique and distinguishable immunological expression profiles and potential clinical implications (1): a *pro-inflammatory and leukemia expansion* (ILE)-like niche, and (2) an *immune-suppressive and leukemia-initiating cell* (SLIC)-like niche. Strikingly, PDLS recapitulated the hypoxic CXCL11^{+} zones that support LICs, revealing the previously undescribed relevance of CXCL11^{+} mesenchymal niches for cell maintenance of long-term leukemia initiating and relapse population.

METHODS

Patient Characteristics and Sample Collection

This research has been performed in accordance with the Declaration of Helsinki and was approved by the Ethics,

Research and Biosafety Committee from IMIEM (CIEICE-007-01-13) and by the National Committee of Scientific Research at IMSS (R-2012-3602-29 and R-2015-785-120). All samples were collected after informed consent from parents. The study included 147 B-ALL pediatric patients, 8 months to 16 years old (8.15 ± 4.47), referred to the IMSS Specialties Hospital and the IMIEM Children's Hospital. At clinical diagnosis, 85% of patients were classified as high risk and 41.5% as ProB/PreB-, 30.6% as ProB-, and 27.9% as PreB-ALL, with only 30% exhibiting prognostic translocations. Control BM was obtained from 12 healthy children undergoing minor orthopedic surgery. BM specimens were collected by aspiration before any treatment and according to international and institutional guidelines. (**Supplementary Table S1**).

Isolation of Primitive Hematopoietic Cells

Mononuclear cells (MNCs) were separated by Ficoll-Paque Plus (GE Healthcare Bioscience, NJ, USA) gradient. No sample pooling was performed for any of the experimental strategies. By immunophenotyping with fluorochrome-conjugated antibodies (**Supplementary Table S2**), Pro-B cells were identified as $CD45^{low/-}CD34^{+}CD10^{+}CD19^{+}$ and Pre-B as $CD45^{low/-}CD34^{-}CD10^{+}CD19^{+}$, before sorting in a FACSAria II flow cytometer (BD Biosciences, USA) (**Supplementary Figures S1A, B**).

Cell Lines

REH and RS4;11 B-ALL cell lines were purchased from ATCC (VA, USA) and cultured according to instructions. Nalm6 cell line was kindly provided by Dr. JL Maravillas (INNSZ, Mexico). Cell lines were tested for mycoplasma and authenticated using STR assays.

Primary Mesenchymal Stromal Cells

MSCs were isolated by adhesion, as previously reported (22).

Patient-Derived Leukemic Spheroids

A total of 25,000 MSCs were plated on 96-well round-bottom plates previously coated with 1% agarose to induce spheroid formation for 24 h, before co-culture with leukemic cells (22). For harvesting, PDLS were incubated with 0.05 mM PBS-EDTA for 5 min to detach cells from the surface, followed by 10 min enzymatic treatment (TrypLE Express, Gibco, CA, USA) and mechanical disruption. Cell suspension was recovered from the inside of PDLS (PDLS-in) and separated from outer cells and supernatant (PDLS-out), before staining with fluorochrome-conjugated antibodies and/or direct FACS analysis (**Supplementary Figures S1C, D**).

Cell Tracking Strategies

MSCs or B-ALL cells were stained with fluorescent dyes Cell Trace Violet®, Cell Trace CFSE®, or Cell Trace Far Red® (Invitrogen, Life Technologies, CA, USA), according to the manufacturer.

Fluorescence Microscopy

PDLS were fixed with 4% PFA and 2 h treated with 0.01% Triton X-100 (Bio-Rad, MX). Upon 1 h blocking with 3% BSA, they were incubated overnight at 4°C with primary unlabeled antibodies in PBS 3% SFB, washed, and incubated for 1 h with conjugated secondary antibody before 10 min DAPI staining and Vectashield. BM biopsy staining was performed as described (22).

Cytokine Detection

Supernatants were collected after 24 h of 3D culture and investigated for cytokines by multiplex assays (Milliplex Map, Millipore, Merck MX).

Proliferation Assay

FACS-sorted B-ALL cells were stained with CellTrace CFSE® (Invitrogen, Life Technologies, CA, USA), co-cultured with MSC spheroids, and further assayed for fluorescent dye dilution by flow cytometry.

Pimonidazole Incorporation and Hypoxia Detection

Hypoxia was detected by the Hypoxyprobe-1 Plus Kit (Pimonidazole Hydrochloride, Chemicon International, Temecula, CA, USA). Pimonidazole incorporation was confirmed by flow cytometry and fluorescence microscopy. Image-IT green hypoxia (Invitrogen, Life Technologies, CA, USA) was used to track low oxygen levels.

Side Population Assay

Harvested cells were adjusted to 10^6 cells/ml and incubated with Hoechst 33342 to a final concentration of 5 µg/ml (Sigma-Aldrich, MX), 37°C for 2 h, prior to staining with anti-human CD45.

Patient-Derived Xenografts

In vivo experiments were conducted according to the WCM Institutional Animal Care and Use Committee (IACUC) and the CINVESTAV Committee for Animal Care and Use (CICUAL) guidelines and regulations. NOD/SCID gamma chain (NSG) mice from the Jackson Laboratory (JAX, CA, USA) were i.v. injected with primary B-ALL cells from 48 h cultures. Animals were euthanized after 5 weeks or when exhibiting clinical signs of leukemic disease. Human $CD45^{+}$ cell frequencies in peripheral blood and BM were investigated for engraftment monitoring.

Limiting Dilution Assays

Serial dilutions of leukemic cells were injected into NSG mice. After 4 weeks, the engraftment was determined by flow cytometry and documented as positive when human $CD45^{+}$ cells recorded within mouse BM cells were >1%. ELDA program was used to calculate the LIC content for each culture condition (23).

RNA-Sequencing Library Preparation and Analysis

Whole RNA was extracted from 5×10^5 MSC (RNeasy kit, QIAGEN, MX), and samples with RIN > 8 were used for experiments. Libraries were constructed by using the TruSeq Stranded mRNA Library Prep Kit (Illumina, CA, USA) before

mRNA sequencing on a NextSeq 500 instrument at INMEGEN (Mexico). Paired-end reads were aligned to the human genome reference GRCh38/hg38 (build 38.2) with the R software package Rsubread (24) and read mapping statistics were reported (**Supplementary Table S3**). Mapped reads were summarized to gene level counts featured by counts function of Rsubread, considering the built-in NCBI RefSeq gene annotation for gene reference. Protein coding genes with detected counts in at least one sample library were retained and normalized using TMM normalization. Differential expression analysis was performed with the edgeR package (25). Statistical analyses and plots were performed using the programming language R (R Core Team, 2012). Gene ontology and functional enrichment analyses were performed by Metascape (26). The original contributions presented in the study are publicly available. RNA-seq data can be found in E-MTAB-10838 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10838/).

Data Analysis and Statistics

FlowJo 10.0.8 (TreeStar Inc., Ashland, OR, USA) and Infinicyt 1.8 (Cytognos, Spain) software were used for cytometry data, while Prism 8 (GraphPad, CA, USA) software was used for statistical analysis. Differences within groups were established by non-parametric tests, considering significant probability values <0.05. Mann–Whitney *U* test with α of 5% to define significance was applied. Data were normally distributed and individual data points for independently repeated experiments and mean (SD) were graphed.

RESULTS

Mesenchymal 3D Spheroids Are Capable of Reconstructing Unique B-ALL Niches

As 3D cellular organization is essential to preserve physiological features of BM, we generated 3D structures to characterize the leukemic niche by using MSCs derived from either primary B-ALL at disease onset (ALL-MSC) or from healthy bone marrow donors (HBM-MSC) (**Figure 1A**). We found that MSCs were capable of forming a single multicellular spheroid within the first 24 h of non-adherent culture conditions with a direct cell number–size relationship (**Supplementary Figures S2A, B**). Despite the decrease in the MSC proliferation in 3D settings, the classical MSC markers were conserved (**Supplementary Figures S2C, D**). CXCL12-abundant reticular (CAR) immunophenotype was confirmed in $CXCL12^{hi}SCF^{hi}IL\text{-}7^{hi}$ HBM-MSC spheroids (**Figure 1B**) as well as the expression of Nestin, PDGFRα, and LepR (**Supplementary Figure S2E**). In contrast, a substantially lower abundance of typical $CXCL12^{hi}SCF^{hi}$ CAR cells in ALL-MSC spheroids with weaker expression of CXCL12 and SCF (**Figure 1B**), but increased production of IL-8, Flt3-L, GM-CSF, FGF-2, CXCL10, and CXCL11, was observed in the supernatants evaluated at 24 h (**Figure 1C**). ALL-MSCs in a 3D organization have the ability to *in vitro* recapitulate unique CAR niche-like structures that produce high levels of CXCL10 and CXCL11.

Primary B-ALL Cells Can Be Expanded in Mesenchymal 3D Spheroids

Since we confirmed the ability of the 3D ALL-MSCs spheroids of recapitulate CAR niche-like structures, we sought to evaluate the ability of primary B-ALL cells to migrate to the MSC spheroids by assessing their colonization capacity. First, we stablished the 3D HBM- or ALL-MSCs spheroids, and 24 h later, we seeded 25,000 primary B-ALL cells $CD10^{+}CD19^{+}$ (n = 8), labeled with Cell Trace Far Red. After 24 h of co-culture, spheroids were washed and prepared for the whole-mount fluorescence microscopy analysis or enzymatically disrupted to analyze their cellular content by multiparameter flow cytometry (MPFC). We found a clear advantage for ALL-MSC spheroids to facilitate the colonization of leukemic cells when compared with the HBM-MSC (**Figure 2A**). To evaluate niche saturation, serial spheroid sizes were tested, finding that in all cases, only near 1%–3% of leukemic cells were able to colonize inner niches (**Supplementary Figure S3A**). Since CXCR4 has been implicated in homing of leukemia cells to their niche (27, 28), we tested the effect of plerixafor (AMD3100) in the colonization of AMD3100-treated B-ALL cells to the 3D structures and found that it can only partially prevent B-ALL cell spheroid colonizing, with a 4.6-fold decrease (**Figure 2B**). The effect was similar when the niche positioning of leukemic cells into normal BM spheroids was investigated (**Supplementary Figure S3B**).

Next, we assessed the ability of the spheroids to maintain cells capable of initiating leukemia without enrichment. Thus, we co-cultured 25,000 MNCs from five different B-ALL patients with either stromal-free (SF), MSCs monolayers (2D), or spheroids (3D) for 48 h and then transplanted into NSG mice (**Figure 2C**). We observed that 3D architecture was best at facilitating survival and expansion of primary leukemia cells when compared to other culture conditions (**Figure 2D** and **Supplementary Figure S3C**). Importantly, 3D co-cultures in ALL-MSC expanded more robustly (**Figure 2D**) and exhibited higher leukemic engraftment at week 6 post-transplantation than other culture conditions (**Figure 2D** and **Supplementary Figure S3D**). In addition, cells cultured in the 3D system performed better than freshly thawed MNC and transplanted (**Supplementary Figure S3E**). Taken together, we demonstrated that ALL-MSC spheroids support homing, survival, growth, and efficient engraftment of primary B-ALL cells. This co-culture system is referred to as patient-derived leukemic spheroids (PDLS).

Hypoxic Patient-Derived Leukemic Spheroids Support Leukemia Cells With Stem Cell Features

Despite the fact that LICs in B-ALL have been controversial due to the lack of a specific immunophenotype (18), cells with stem cell features have been shown to be enriched in hypoxic zones within the BM (29). Here, we sought to characterize the cells capable of colonizing the PDLS. Because leukemia initiation in NSG mice is a feature of LICs, this and additional LIC properties were evaluated in different compartments of 3D structures using primary B-ALL samples. At 24 h, we harvested cells from the supernatant (PDLS-out) and, upon enzymatic digestion of the

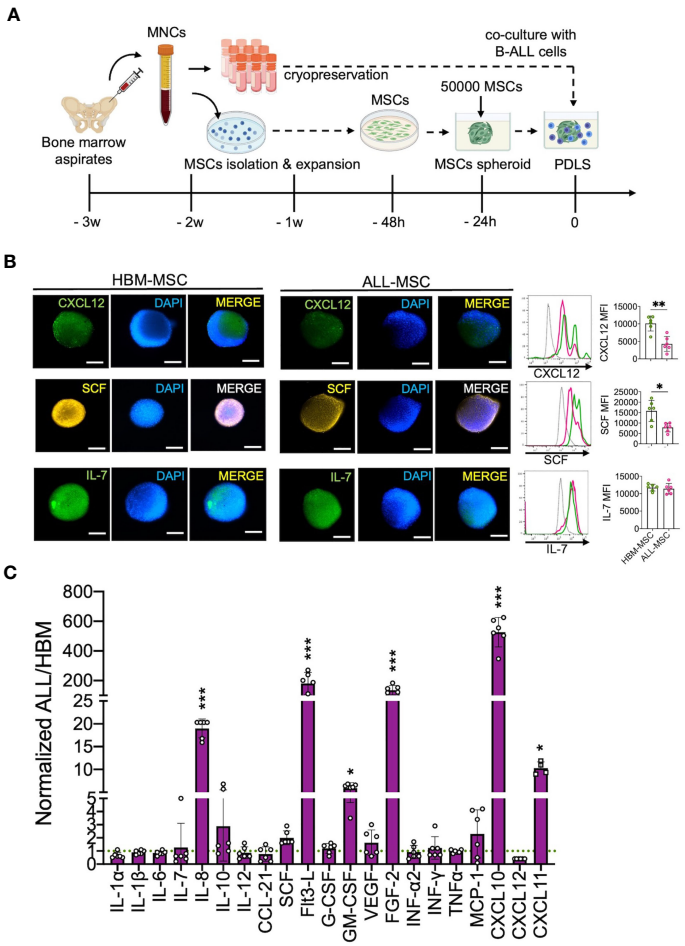

FIGURE 1 | B-ALL BM MSC form 3D spheroids endowed with high CXCL10 and CXCL11 production. **(A)** Mononuclear cells (MNCs) were obtained from healthy BM (HBM) donors or B-ALL patients, and MSCs were isolated by their adherent properties and were cryopreserved to be used in further experiments. **(B)** A total of 50,000 MSCs from HBM or B-ALL were induced to form a stromal multicellular spheroid, and CAR-cell derived factors (CXCL12, SCF, and IL7) were evaluated by immunostaining and FACS (n = 6). **(C)** Growth factors, cytokines, and chemokine production from B-ALL-MSC spheroids (3D) were evaluated after collection of 24-h supernatants and normalized to HBM-MSC spheroids (n = 6). MSC, mesenchymal stromal cell; HBM, healthy bone marrow; B-ALL, B-cell acute lymphoblastic leukemia; CAR-cell, CXCL12-abundant reticular cell; FACS, Fluorescence-activated cell sorting. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars represent SD.

PDLS structure, collected the cells that migrated into the inner spheroid (PDLS-in). More than 90% of spheroid-colonizing B-ALL cells (PDLS-in) showed low proliferation activity when growing inside PDLS, while PDLS-out cells exhibited higher proliferation (**Figure 3A**). Consistently, a quiescent (G0) profile defined the PDLS-in cells (**Figure 3B**), which is a feature of LICs (18). Furthermore, we investigated stem cell features such as "side population" (**Figure 3C** and **Supplementary Figure S4A**) and low ROS production (30) (**Figure 3D**), confirming that PDLS-in cells also displayed such properties when compared with other culture scenarios. Moreover, an increase in HIF-1α expression was recorded (**Figure 3E** and **Supplementary Figure S4B**), consistent with increased hypoxia in the PDLS-in cells, assessed by the image-iT green hypoxia tracker and pimonidazole incorporation. These data confirmed a PDLS-in hypoxic setting for both MSCs and B-ALL (**Figure 3E** and **Supplementary Figure S4C**). Taken together, PDLS provide strong evidence that stem-like B-ALL can be enriched by their function and biological features within hypoxic niches, suggesting that they may be the foundation of leukemia-migrating and -proliferating cells.

PDLS Foster Cells With the Capacity of Leukemia Initiation and Chemoresistance

As PDLS were colonized by leukemia cells with stem cell features, we sought to determine whether cells isolated from PDLS-in are characterized by the increased ability of homing. By serial spheroid seeding assay, we discovered that PDLS-in were capable of re-colonizing spheroids with higher efficiency than PDLS-out cells (**Figure 4A**), highlighting their homing and stem cell potentials. To further characterize the LICs capacity, 3,000 sorted $CD45^+$ RS4;11 cells from 48 h PDLS-in and other culture

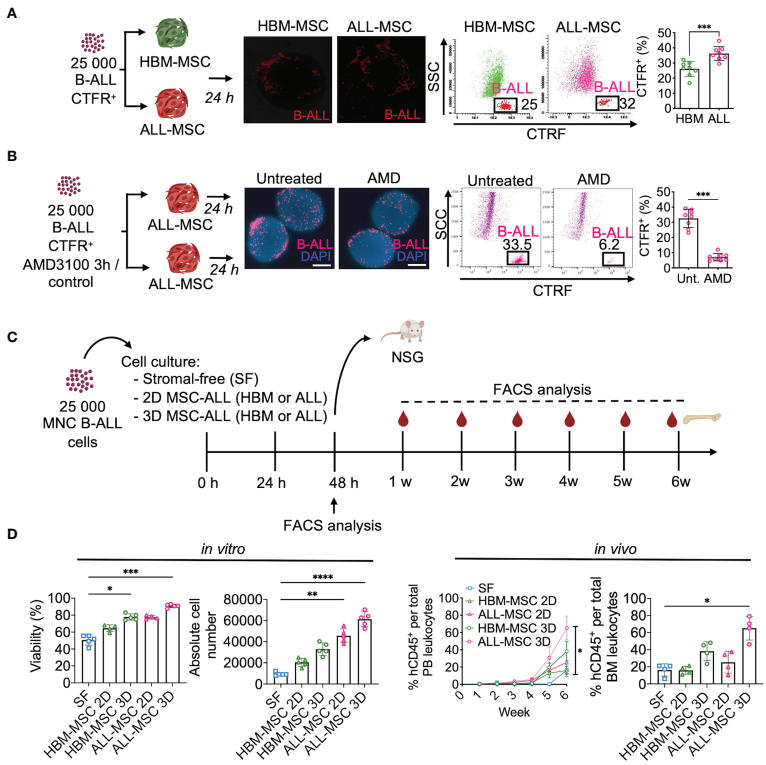

FIGURE 2 | Mesenchymal stromal spheroids support primary leukemogenic B-ALL cells. **(A)** Primary sorted B-ALL CD10$^+$CD19$^+$ blasts were labeled with CTFR and co-cultured with HBM-MSC or ALL-MSC spheroids. After 24 h, spheroids were washed and analyzed by fluorescence microscopy and FACS. CTFR$^+$ colonizer cell frequencies determined upon enzymatic digestion (n = 7). **(B)** CTFR-labeled primary B-ALL cells were treated with a CXCR4 inhibitor (AMD3100) 5 mM for 3 h and then co-cultured within PDLS. Upon 24 h, CTFR$^+$ content was determined by FACS (n = 8). **(C)** Schematic representation of experimental design, 25,000 mononuclear cells from B-ALL patients were cultured in SF conditions, or co-cultured with HBM- or B-ALL-MSC monolayer (2D) or spheroids (3D). **(D)** Cell viability was analyzed by flow cytometry upon 48 h and absolute cell number was determined (n = 5) (left). Leukemic cells from independent experiments were harvested at 48 h of culture and transplanted into NSG mice. Human engraftment of hCD45$^+$ was weekly monitored in PB by FACS and engraftment in BM was determined 6 weeks after transplantation (n = 4) (right). MSC, mesenchymal stromal cell; HBM, healthy bone marrow; B-ALL, B-cell acute lymphoblastic leukemia; CTFR, Cell Trace Far Red; FACS, Fluorescence-activated cell sorting; SF, stromal-free; PB, peripheral blood. *P < 0.05; **P < 0.01; ***P < 0.001, ***P < 0.0001. Error bars represent SD.

scenarios were used to inject NSG mice. Leukemia burden was weekly monitored, and final engraftment was evaluated at 6 weeks (**Figure 4B**). Mice transplanted with purified PDLS-in cells showed the highest numbers of human CD45$^+$ cells peripheral blood (PB) and exhibited the lowest overall survival (OS) of 44 days (**Figure 4C**). BM analysis confirmed the facilitated engraftment with PDLS-in RS4;11 cells. Such results were validated with three different primary human samples from ProB and PreB pediatric-ALL patients (**Figure 4D**). Limiting dilution assay revealed that LICs frequency was 10 times less in stroma-free settings when compared to PDLS-in conditions (**Supplementary Figures S5A, B**). Remarkably, a LICs enrichment was observed in PDLS-in (1/45.2), compared with PDLS-out (1/858) and SF conditions (1/704) (**Supplementary Figure S5C**). MPFC analysis of PDLS-in confirmed that LICs enrichment by PDLS was likely driven by functional attributes associated with leukemia stemness rather than by immunophenotype (**Supplementary Figure S5D**), supporting the notion of a functional LICs hierarchy driven by specialized microenvironmental cues. Thus, PDLS could be potentially used as a proxy to determine the presence of LICs in pediatric B-ALL patient samples.

As LICs have also been described as chemo-resistant (13, 18, 27, 31), we proceeded to investigate the response of the PDLS-in cells to the most commonly used chemotherapy drugs for B-ALL treatment. To this end, the ability of drugs to diffuse inside the spheroid was investigated. When treating PDLS with the anthracycline daunorubicin, the cells were able to uptake daunorubicin within the first hour, evidenced by their red fluorescence (**Figure 5A**). By examining the viability at 24 h of treatment, we found that daunorubicin, prednisolone, and vincristine, even at high concentrations, were not effective in killing the PDLS-in cells (**Figure 5B**). Of note, combined chemotherapy commonly used in B-ALL, including daunorubicin, prednisolone, vincristine, and methotrexate (P-V-D-M), displayed similar results when investigated in high-risk (HR) and standard-risk (SR) patients (**Figure 5C**). Furthermore, when PDLS-in vehicle or P-V-D-M-treated cells were purified and exposed for an additional 24 h in stroma-free conditions, cells remained chemo-

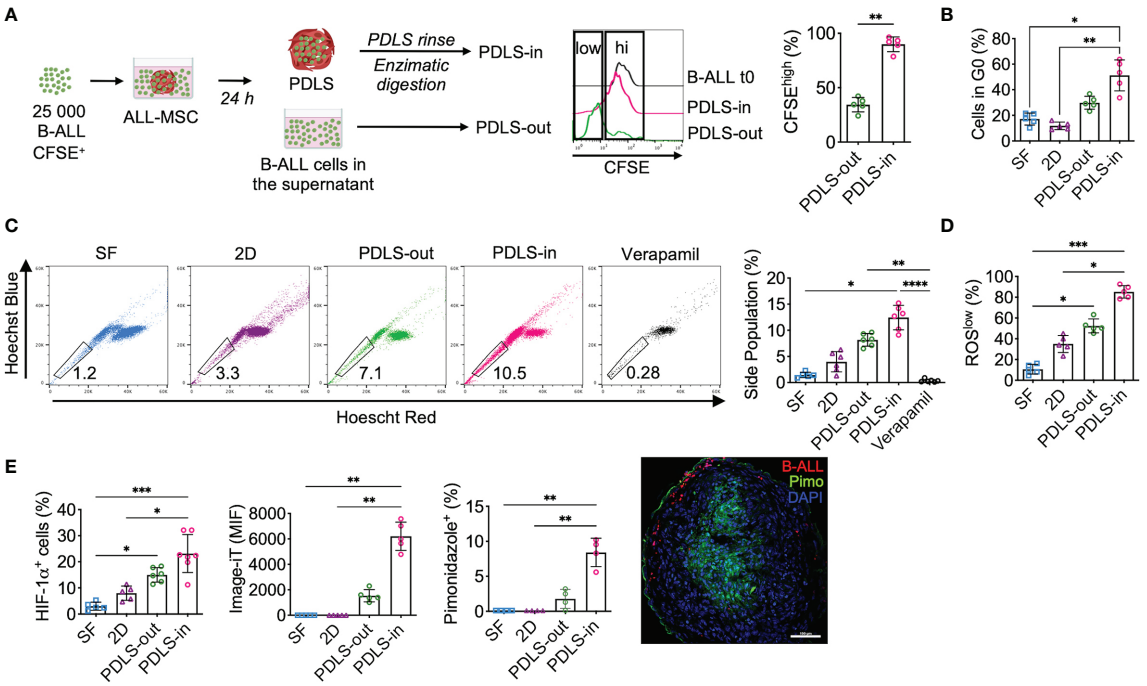

FIGURE 3 | PDLS promote stem cell-like phenotype, quiescence, and hypoxia in a subset of primary B-ALL cells. **(A)** Primary sorted B-ALL $CD10^+CD19^+$ blasts were labeled with CFSE and co-cultured with ALL-MSC to form PDLS. At 24 h, frequency of $CFSE^{hi}$ was determined by FACS in the supernatants after spheroid removal (PDLS-out) and in the PDLS colonizer cells (PDLS-in) after several washes and enzymatic digestion ($n = 5$). **(B)** Primary B-ALL blasts were cultured in stromal-free (SF) conditions and co-cultured with ALL-MSC in monolayer (2D) and PDLS settings for 48 h and cell cycle status was evaluated by Ki-67 staining and DNA content by FACS ($n = 5$). **(C)** Side population cell contents are shown ($n = 5$). **(D)** ROS production was measured by FACS and ROSlow frequency was recorded ($n = 5$). **(E)** Hypoxia was investigated by HIF-1α expression (left), image-iT fluorescent hypoxia probe (middle) and pimonidazole incorporation (right) by FACS. Fluorescence microscopy of pimonidazole incorporation of PDLS is shown ($n = 7$). MSC, mesenchymal stromal cell; B-ALL, B-cell acute lymphoblastic leukemia; PDLS, patient-derived leukemia spheroids; CFSE, carboxifluorescein; FACS, fluorescence-activated cell sorting. *P < 0.05; **P < 0.01; ***P < 0.001, ***P < 0.0001. Error bars represent SD.

resistant (**Figure 5D**). Next, to determine the potential of PDLS-in cells to recapitulate disease after chemotherapy, PDLS were treated with combined chemotherapy for 24 h, washed to remove PDLS-out cells, and cultured again in fresh wells. Strikingly, newly formed PDLS-out cells were harvested upon 120 h and no differences were observed when compared to untreated PDLS (**Figure 5E**), suggesting that PDLS can capture clinical features, such as tumor reemergence after cell survival within internal niches during chemotherapy.

Gene Expression Signatures for BM MSCs Reveals Pro-Inflammatory and Suppressor Niches in B-ALL Patients

In order to investigate the identity of MSCs isolated from primary pediatric-ALL patients, we performed RNA sequencing analysis of three different ALL-MSCs specimens and HBM-MSC. Substantial and heterogeneous dysregulation of gene expression was found when compared with HBM-MSC (**Figure 6A** and **Supplementary Table S3**). Specifically, 103 genes were consistently overexpressed among ALL-MSC (fold change > 2 and FDR < 0.05) (**Figures 6B, C** and **Supplementary Figures S6A, B**) and, of high interest, two major gene ontology (GO) signatures were identified. A pro-inflammatory signature was characterized by a large set of chemokines involved in neutrophil recruitment, IL-17 signaling, metalloproteinase functional activation, and leukocyte migration including *CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CCL20*, and pro-inflammatory molecules like *IL1B, IGF1, MMP1, MMP3*, and *MMP8* (**Figure 6D**). An additional signature, predominantly displayed by ALL-MSC3, showed a TLR signaling, cytokine-mediated signaling, and a negative regulation of leukocyte proliferation signatures. Moreover, high expression of chemokines CXCL10 and CXCL11 and a substantial expression of suppressor molecules like indoleamine 2,3-dioxygenase (*IDO1*) and galectin 9 (*LGALS9*) (**Figure 6D**) were apparent. Importantly, ALL-MSC did not exhibit transcriptional differences in the typical MSCs markers CD73, CD90, and CD105 (**Supplementary Figure S6C**), but a very low transcriptional expression of CAR-niche associated genes *CXCL12* and *SCF* were found in $CXCL10^+CXCL11^+$ ALL-MSC3 (**Supplementary Figure S6D**). When downregulated genes were analyzed, we did not find apparent intersections among samples. However, GO analysis at the individual level showed that some extracellular matrix-associated proteins and

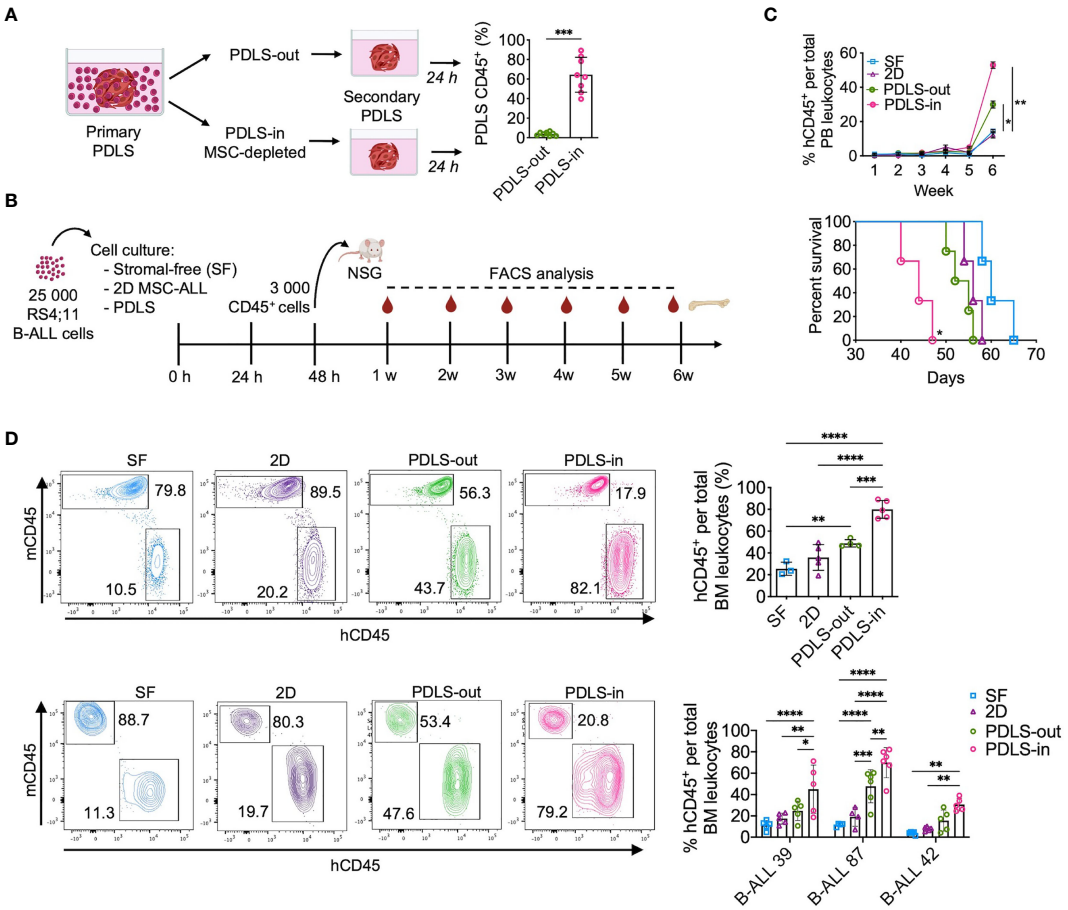

FIGURE 4 | Leukemia-initiating cells (LICs) are enriched in PDLS internal niches. **(A)** Secondary spheroid colonization assay was performed with purified PDLS-in and PDLS-out leukemic cells from primary PDLS (n = 8). **(B)** Schematic representation of experimental design, Leukemic cells were cultured during 48 h on stromal-free (SF), MSC monolayers (2D), and PDLS and 3,000 CD45$^+$ cells were transplanted into NSG mice. **(C)** Leukemia burden was monitored in peripheral blood by FACS (upper panel) and overall survival was plotted (lower panel) when RS4:11 cells were used (n = 5). **(D)** Engraftment was determined in BM after 6 weeks of xenotransplantation of RS4:11 cells (upper panel) or three different primary B-ALL cells (representative plots are shown in lower panel) (n = 5). B-ALL, B-cell acute lymphoblastic leukemia; PDLS, patient-derived leukemia spheroids; MSCs, mesenchymal stromal cells; FACS, Fluorescence-activated cell sorting. *P < 0.05; **P < 0.01; ***P < 0.001, ***P < 0.0001. Error bars represent SD.

cell division-associated networks were dramatically altered in similar extent for all three B-ALL MSCs (**Supplementary Figure S6A**). Taken together, the MSC gene expression profiling suggests two potential niches, according to their functional elements within the B-ALL BM microenvironment. A *pro-inflammatory and leukemia expansion* (ILE) niche, where leukemic clones may proliferate and increase tumor burden in the context of an activating pro-inflammatory milieu, and an *immune-suppressive and leukemia-initiating cell* (SLIC) niche, endowed with immunoregulatory and suppressive properties and high transcription of *CXCL10* and *CXCL11*.

A Hypoxic CXCL11^{hi} Mesenchymal Niche Can Be Recapitulated in the PDLS

In order to assess our transcriptional observations in our PDLS system, we used immunostaining approaches to characterize CXCL11 expression in a leukemia microenvironment. Strikingly, we found that CXCL11^{hi} MSC spheroids were enriched in hypoxic CXCL12^{hi} zones with partial overlapping (**Figure 7A**). Distinct CXCL11^{low} and CXCL11^{hi} cell populations were also evident in ALL-MSC spheroids, while HBM-MSC spheroids did not show CXCL11 expression (**Figure 7B**). Moreover, the occurrence of CXCL11$^{low/hi}$ MSCs in B-ALL BM biopsies was confirmed (**Figure 7C**), where CXCL11 co-stained with CD19. Additionally, B-ALL cells, but not normal CD34$^+$ precursor cells, expressed CXCR3 and CXCR7, the receptors for CXCL10, CXCL11, and CXCL12, suggesting their advantage for selective niche colonization (**Figures 7D, E** and **Supplementary Figure S7A**). CXCL10^{hi}CXCL11^{hi} zones may represent exclusive leukemia-positioning niches where B-ALL cells may also contribute to CXCL11 expression (**Supplementary Figure S7B**) presumably relevant for positioning of CXCR3$^+$CXCR7$^+$ LICs and suitable for immune scape (**Figure 8** and

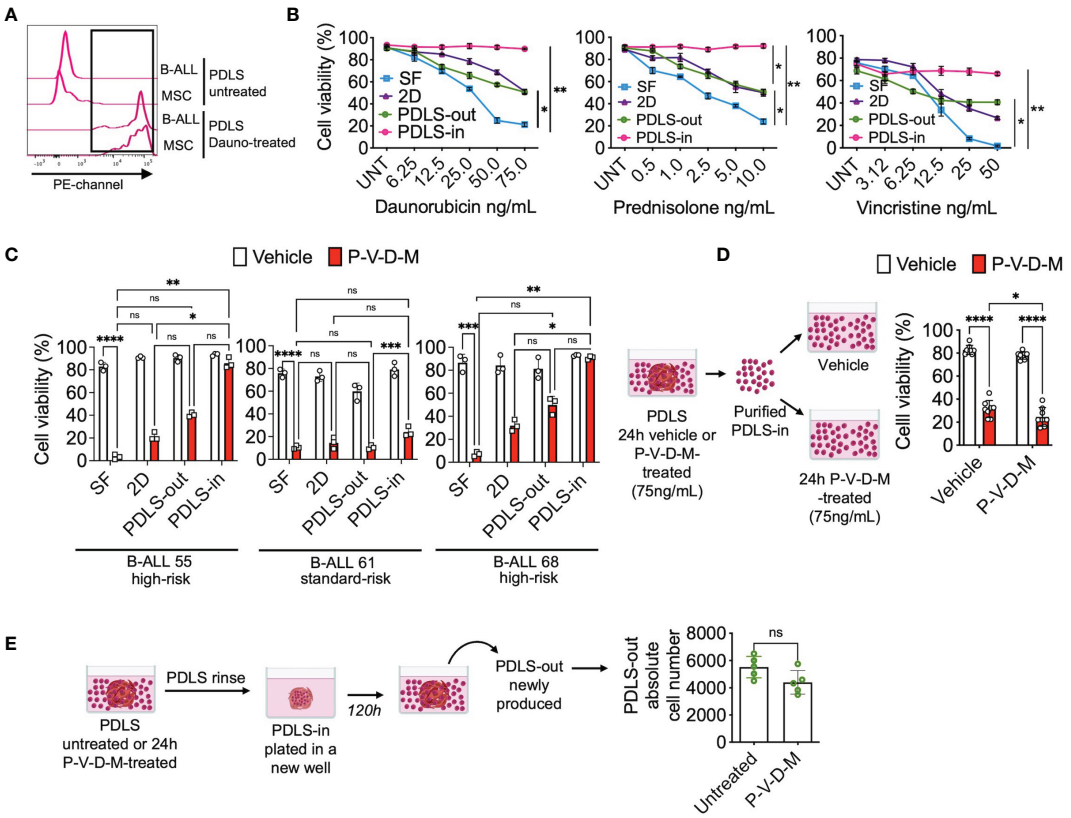

FIGURE 5 | LICs are protected from chemotherapy within PDLS. **(A)** B-ALL PDLS were incubated with daunorubicin (75 ng/ml) for 1 h and enzymatically disrupted; red fluorescence was determined in CD45$^+$ (B-ALL) and MSCs by FACS (n = 5). **(B)** Primary B-ALL blasts were cultured in SF, 2D and PDLS and CD45$^+$ cell viability evaluated upon 24-h treatment with daunorubicin, prednisolone, and vincristine (n = 3), or **(C)** combined chemotherapy: daunorubicin [75 ng/ml], prednisolone [10 ng/ml], vincristine [50 ng/ml], and methotrexate [5 mM] (P-V-D-M). **(D)** Viable leukemic cells recovered from PDLS-in untreated or 24-h P-V-D-M-treated were re-exposed to the drugs for 24 h and their viability was measured by FACS (n = 5). **(E)** PDLS P-V-D-M-treated were replated; upon 120 h, PDLS-out leukemia re-emerging was recorded (n = 7). B-ALL, B-cell acute lymphoblastic leukemia; PDLS, patient-derived leukemia spheroids; MSCs, mesenchymal stromal cells; FACS, Fluorescence-activated cell sorting; NS, non-significant. *P < 0.05; **P < 0.01; ***P < 0.001, ***P < 0.0001. Error bars represent SD.

Supplementary Figure S7C). Taken together, we demonstrate that PDLS are capable of capturing a SLIC niche endowed with a specialized gene expression signature and the selection of malignant cells with stem cell functions.

DISCUSSION

93.5% of poor prognosis cases of pediatric leukemias are registered in low- to middle-income countries, where 90% of the world's children live, with relapses occurring at very early stages of treatment and increasing numbers of high-risk fates (1, 2, 32). Such epidemiology highlights the importance of a comprehensive understanding of the origins and coevolution of the disease in the context of micro/macroenvironmental cues. The phenotypical and functional identity of LICs and leukemia-relapsing cells (LRCs) and the niches where they evolve are critical for the construction of a more integrated view of the nature of leukemia subtypes and their potential control (33, 34).

Here, we have addressed key aspects of the microenvironment-related leukemia etiopathogenesis through implementation of PDLS. This *in vitro* avatar model is a powerful tool to recapitulate malignant niche biology in human–human settings that better mimic natural ecosystems (**Figure 8**). We demonstrated that LIC activity is promoted within PDLS niches and facilitated by hypoxic microenvironments. The CXCL12/CXCR4 axis has been considered the most important player in the chemotaxis and retention of hematopoietic cells into their BM niches (9, 35, 36), and in normal settings, MSC spheroids have shown to increase the CXCL12 expression (20, 22) as a result of hypoxia (37) and to promote high cellular connectivity mediated by connexins (38). However, upon leukemia onset, CXCL12 expression is downregulated in MSCs (13, 22, 39, 40). Several studies have suggested that normal HSPCs live in anatomic regions with lower O_2 levels (29) and it is becoming clear the critical role of the BM hypoxic niches in the low oxidative stress status of quiescent HSPCs that avoid their continuous differentiation and exhaustion. Indeed, pseudohypoxia increases the HSPC engraftment, suggesting that HSPC exhaustion can occur in non-hypoxic conditions (41). Further metabolic studies

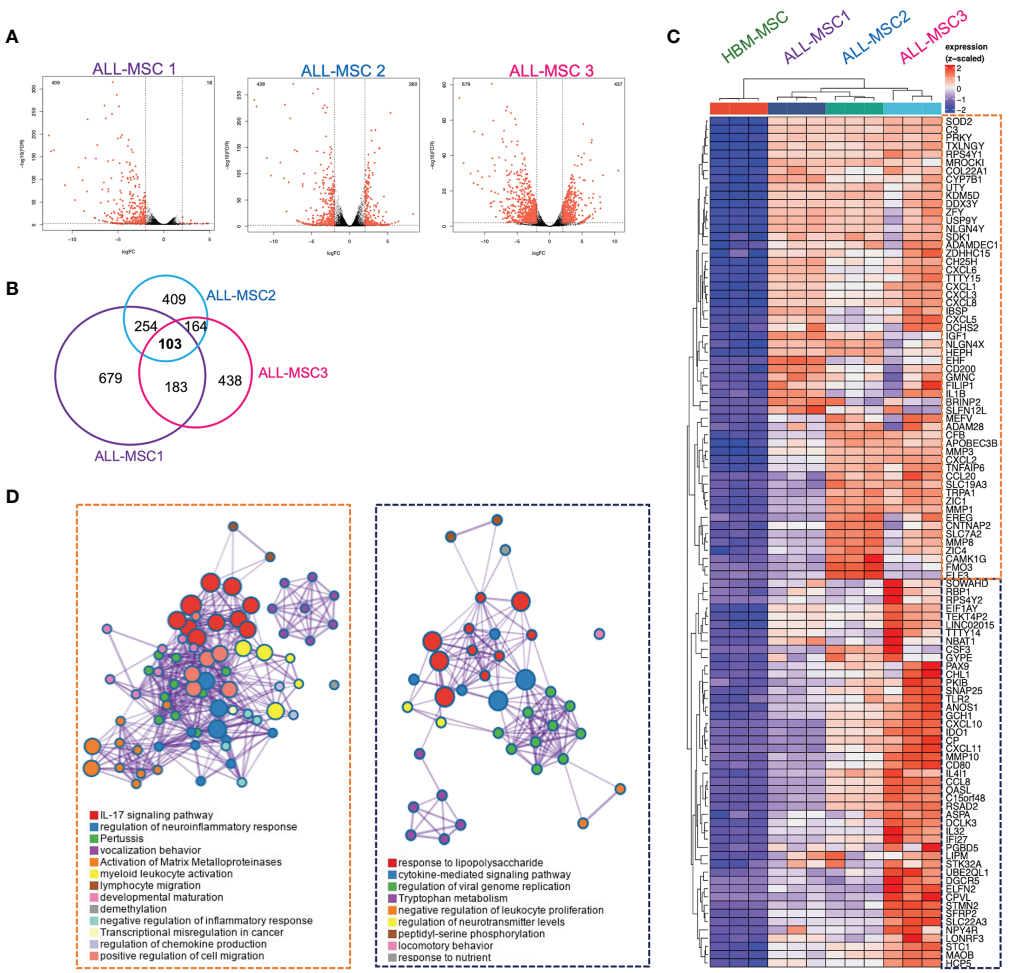

FIGURE 6 | Differentially expressed genes in MSCs derived from B-ALL pediatric patients: RNA-seq approach. **(A)** Volcano plots of statistically significant differentially expressed genes from three B-ALL patients compared to normal counterpart from a healthy donor is shown (n = 3). **(B)** Venn diagram on intersection analysis of overexpressed genes found in **(A)**. **(C)** Heatmap of genes consistently overexpressed after analysis of intersections (fold change > 2 and FDR < 0.05). **(D)** Gene ontology and functional enrichment analysis is shown for two clusters. Dataset E-MTAB-10838.

at single-cell resolution in PDLS may reveal how pseudohypoxia and the hostile hypoxic-inflammatory niches cooperate to preserve LIC functions at the expense of normal hematopoiesis.

One of the crucial challenges when working on primary B-ALL cells is the lack of suitable *in vitro* conditions to maintain or expand them *ex vivo*, to accelerate therapy screening and even target microenvironmental cues (16). Advances in the understanding the microenvironment regulation in B-ALL have been occurring by using mouse models and human co-culture systems to replicate BM niches that support LICs (13, 16). So far, our data strongly suggest that LICs can be enriched in PDLS, by their niche requirements rather than immunophenotypic features, according to the stochastic model proposed for B-ALL (17, 18). LIC gene expression profiles are alike to those from measurable residual disease (MRD) and LRCs, where low metabolic activity and increased cell adhesion are common features (17, 42). Interestingly, when LICs are released from their protective niches, chemoresistance can be reversed as the stem cell characteristics are modified (17). Unfortunately, in B-ALL, the ability of certain niches to induce and support malignant stemness remains unknown. An active competition for the niche may displace normal HSPC, where pro-inflammatory signals provided by leukemic cells or their microenvironment are crucial (14, 22, 27, 28, 39, 43–45). We recently reported the relevance of cortactin-mediated cell migration of B-ALL relapse cells for extramedullary infiltration and intra-niche positioning with high tropism for hypoxic PDLS zones (46). So far, our PDLS model has been only investigated with MSCs and B-ALL cells, but additional niche-associated cells, including those from CNS or gonads, can be further studied at individual or collective levels for their contribution on LICs maintenance.

Two MSC niches with unique and distinguishable expression profiles and potential clinical implications are apparent, and suggest the sequential replacement of normal niches with the

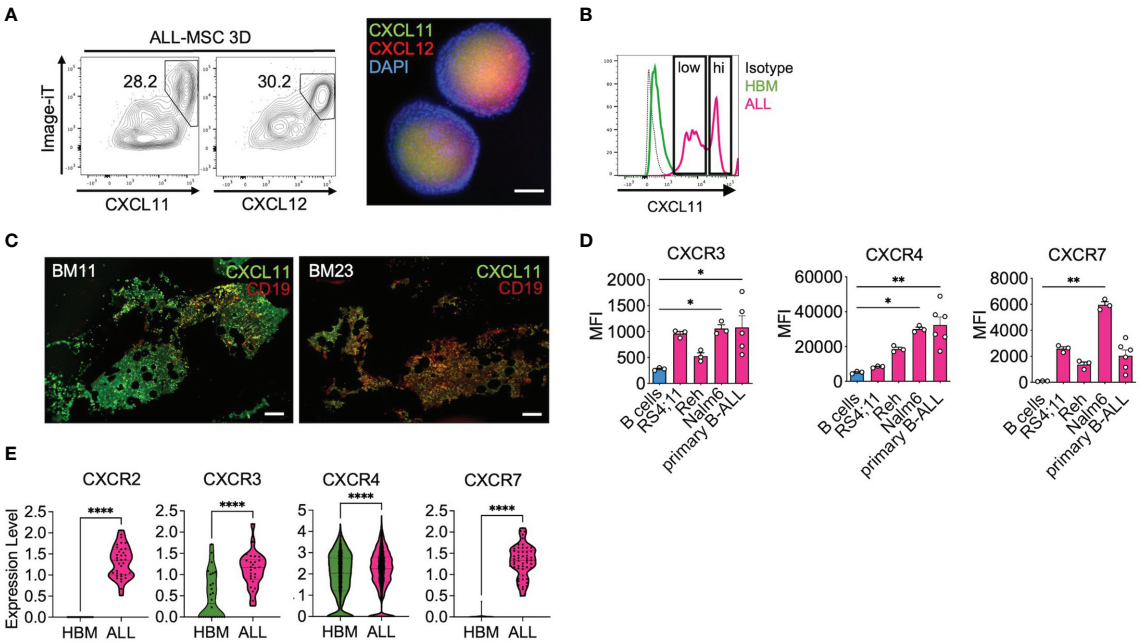

FIGURE 7 | CXCL11 characterize the MSC niche in B-ALL. **(A)** CXCL11 and CXCL12 was hypoxia-tracked by using the image-iT probe and analyzed by FACS and representative CXCL11 and CXCL12 immunofluorescence staining in ALL-MSC spheroids (n = 3). **(B)** CXCL11 was determined by FACS in HBM-MSC and ALL-MSC spheroids (nHBM-MSC = 3, nALL-MSC = 9). **(C)** CXCL11 and CD19 immunostaining in BM biopsies. **(D)** CXCR3, CXCR4, and CXCR7 expression analyzed by FACS in B-ALL cell lines (n = 3) and primary B-ALL cells (n = 6). **(E)** Expression of CXCR2, CXCR3, CXCR4, and CXCR7 in B-ALL and Healthy BM CD19+CD79+ populations obtained from database GSE132509 analyses. MSC, mesenchymal stromal cell; HBM, healthy bone marrow; B-ALL, B-cell acute lymphoblastic leukemia; CAR, CXCL12-derived abundant reticular; FACS, Fluorescence-activated cell sorting. *P < 0.05; **P < 0.01; ****P < 0.0001. Error bars represent SD.

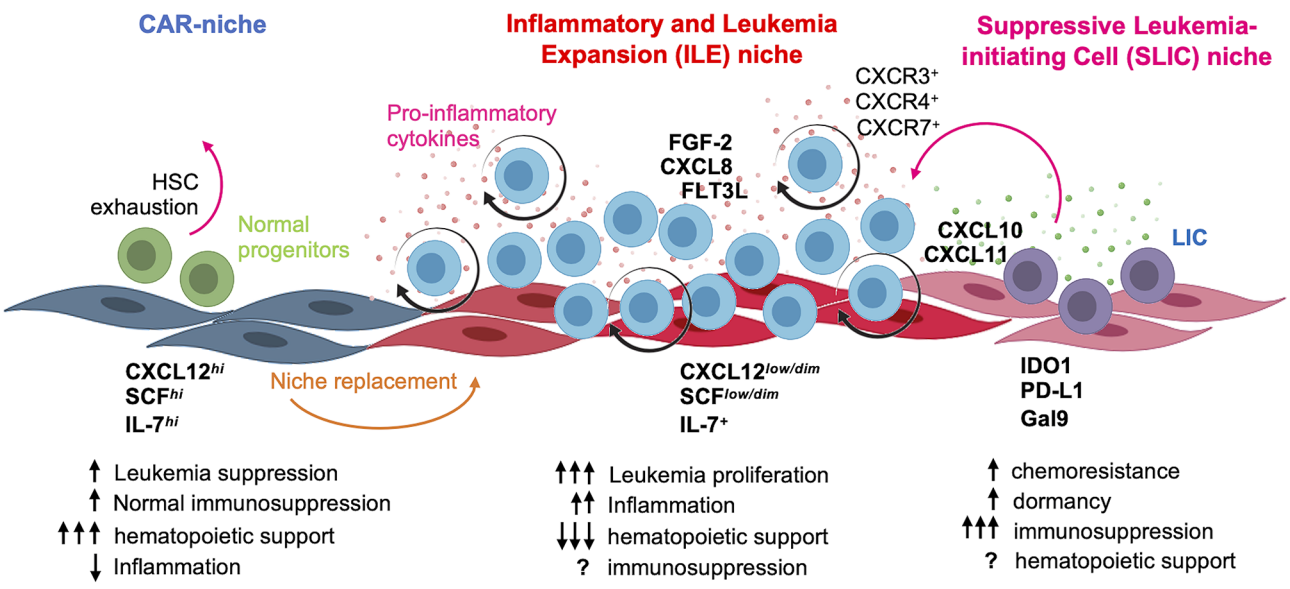

FIGURE 8 | Concluding model. At leukemia debut, B-ALL blasts hijack and remodel CAR niche by inducing CXCL12 and SCF downregulation, concomitant to proinflammatory cytokine production and followed by the emergence of a LIC-supporting niche endowed with suppressor properties. The functional identity of normal CAR niche and the suggested *inflammatory and leukemia expansion* (ILE) and *suppressive leukemia-initiating cell* (SLIC) hematopoietic niches are highlighted. Figure was created using BioRender templates.

inflammatory and leukemia expansion (ILE) niche, followed by the emergence of a $CXCL10^{hi}CXCL11^{hi}$ *suppressive and leukemia-initiating cell* (SLIC) niche, endowed with suppressive capabilities that might be involved in maintenance of long-term initiating or relapse clones. The classical CD73 pan-MSCs marker was found to be increased in some ALL-MSC (data not shown), which may relate to the suppressor role of adenosine (ADO) metabolism in chemoresistance and Treg and suppressor cell development, suggesting a niche-promoted "education".

Although there is increasing evidence of the $Nestin^{+}CXCL12^{+}$ as one essential BM niche (11), dysregulation of CXCL12 and SCF related to pro-inflammatory microenvironment is a feature of ALL (22, 28, 39, 40); the CXCL10/CXCL11/CXCR3 axis has been implicated in chemotherapy resistance and CNS infiltration in B-ALL (47). CXCL10 and CXCL11 share CXCR3 receptor, while CXCL11 is recognized by CXCR7 with more affinity than CXCL12. Theoretical models have suggested an unexpected role of CXCR7 in leukemogenesis (48) and our finding of a $CXCL11^{hi}$ hypoxic niche highlights this. The newly identified $CXCL11^{hi}$ hypoxic niche may play an important role attracting $CXCR3^{hi}CXCR7^{hi}$ leukemic cells even within a $CXCL12^{low}$ scenario. These observations suggest that the remaining $CXCL11^{hi}$ sanctuaries and poor recovery of $CXCL12^{hi}$ niches after treatment are likely to be supportive of relapse or/and poor HSPC engraftment during BM transplantation. In fact, the immunosuppressive landscape associated with such $CXCL11^{hi}$ hypoxic niche supports the notion of a potential transient stage that may function as an attractive therapeutic target as it only occurs in leukemia settings (**Figure 8**). In a very elegant work, Witkowski et al. recently discovered an increased frequency of non-classical monocytes $CX3CR1^{+}$ at diagnosis and relapse (49). Moreover, their elimination improves B-ALL treatment response and survival. Interestingly, we discovered that CX3CL1 is highly produced in the SLIC niche (data not shown). Thus, there is a possibility that non-classical monocytes $CX3CR1^{+}$ are also located in the SLIC niche to cooperate with immunosuppressive/chemoprotective signatures.

Finally, it is well-known that MSCs can protect leukemic cells in the presence of chemotherapeutic agents (13, 16, 31) by several protective mechanisms (50) and now we have shown that LICs enriched by PDLS can be moderately sensitized when they are released form their niche.

Together, our data established, for the first time, an *in vitro* functional 3D hematopoietic-mesenchymal avatar to study human hematopoietic malignancies, which restore important BM mesenchymal niche features with positive impact on primary LICs in pediatric B-ALL. There are great expectations to use this model in precision medicine to predict chemo-resistant leukemic phenotypes, to explore novel therapeutic targets for elimination of LICs in their own niche without affecting normal HSPC or to test abnormal niche elimination strategies that favor niche fitness recovery. PDLS may contribute the comprehensive understanding of mechanisms behind human BM microenvironment alterations, avoiding the use of laboratory animals. Moreover, we have provided strong experimental evidence that supports the idea that LICs are critically dependent on mesenchymal niche interactions and evidenced the existence of a regulatory $CXCL11^{hi}$ MSC niche with a potential role in leukemia initiation. Our new findings contribute directly to understand the pathobiology of childhood leukemias and may be the foundation of niche scoring for Next-Gen patient stratification and design of novel tools for their intervention and prevention.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found here: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10838.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité Nacional de Investigación Científica. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by CICUAL CINVESTAV.

AUTHOR CONTRIBUTIONS

JCB, MG, and RP conceived and designed the work, interpreted results, and wrote the manuscript. JCB performed most experiments. JD-V performed RNA-seq analysis and drafted the work. AS-C, VT-C, LG-S, and JL-F provided patient samples, clinical discussion, and approved the final version. GZ-H, EA-C, AR-M, BL-C, and VV-R performed experiments. DH, SP-T, and VO-N provided reagents, critical discussion, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Deciphering Tumor Niches: Lessons From Solid and Hematological Malignancies

Stéphane J.C. Mancini[1,2,3†], Karl Balabanian[1,4,5†], Isabelle Corre[1,3,6†], Julie Gavard[1,3,6,7†], Gwendal Lazennec[1,8†], Marie-Caroline Le Bousse-Kerdilès[1,9†], Fawzia Louache[1,9†], Véronique Maguer-Satta[1,10†], Nathalie M. Mazure[1,11†], Fatima Mechta-Grigoriou[1,12†], Jean-François Peyron[1,11†], Valérie Trichet[1,3,13†] and Olivier Herault[1,3,5,14,15]*

[1] Centre National de la Recherche scientifique (CNRS) GDR3697, Micronit "Microenvironment of Tumor Niches", Tours, France, [2] INSERM UMR1236, Rennes 1 University, Etablissement Français du Sang Bretagne, Rennes, France, [3] Cancéropole Grand-Ouest, NET network "Niches and Epigenetics of Tumors", Nantes, France, [4] Saint-Louis Research Institute, University of Paris, EMiLy, INSERM U1160, Paris, France, [5] The Organization for Partnerships in Leukemia (OPALE) Carnot Institute, The Organization for Partnerships in Leukemia, Paris, France, [6] Center for Research in Cancerology and Immunology Nantes-Angers (CRCINA), Signaling in Oncogenesis Angiogenesis and Permeability (SOAP), INSERM UMR1232, Centre National de la Recherche scientifique (CNRS) ERL600, Université de Nantes, Nantes, France, [7] Integrated Center for Oncology, St. Herblain, France, [8] Centre National de la Recherche scientifique (CNRS) UMR9005, SYS2DIAG-ALCEDIAG, Montpellier, France, [9] INSERM UMRS-MD1197, Paris-Saclay University, Paul-Brousse Hospital, Villejuif, France, [10] Cancer Research Center of Lyon (CRCL), CNRS UMR5286, INSERM U1052, Lyon 1 university, Lean Bérard Center, Lyon, France, [11] INSERM U1065, C3M, University of Côte d'Azur (UCA), Nice, France, [12] Stress and Cancer Laboratory, Institut Curie, INSERM U830, Paris Sciences et Lettres (PSL) Research University, Team Babelized Ligue Nationale Contre le Cancer (LNCC), Paris, France, [13] INSERM UMR1238 Phy-Os, Université de Nantes, Nantes, France, [14] Centre National de la Recherche scientifique (CNRS) ERL7001 LNOx, EA7501, Tours University, Tours, France, [15] Department of Biological Hematology, Tours University Hospital, Tours, France

Knowledge about the hematopoietic niche has evolved considerably in recent years, in particular through *in vitro* analyzes, mouse models and the use of xenografts. Its complexity in the human bone marrow, in particular in a context of hematological malignancy, is more difficult to decipher by these strategies and could benefit from the knowledge acquired on the niches of solid tumors. Indeed, some common features can be suspected, since the bone marrow is a frequent site of solid tumor metastases. Recent research on solid tumors has provided very interesting information on the interactions between tumoral cells and their microenvironment, composed notably of mesenchymal, endothelial and immune cells. This review thus focuses on recent discoveries on tumor niches that could help in understanding hematopoietic niches, with special attention to 4 particular points: i) the heterogeneity of carcinoma/cancer-associated fibroblasts (CAFs) and mesenchymal stem/stromal cells (MSCs), ii) niche cytokines and chemokines, iii) the energy/oxidative metabolism and communication, especially mitochondrial transfer, and iv) the vascular niche through angiogenesis and endothelial plasticity. This review highlights actors and/or pathways of the microenvironment broadly involved in cancer processes. This opens avenues for innovative therapeutic opportunities targeting not only cancer stem cells but also their regulatory tumor niche(s), in order to improve current antitumor therapies.

Keywords: microenvironment, cancer-associated fibroblasts (CAFs), mesenchymal stem/stromal cells (MSCs), cytokines and chemokines, energy/oxidative metabolism, mitochondrial transfer, angiogenesis, endothelial plasticity

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****Correspondence:***
Olivier Herault
olivier.herault@univ-tours.fr

†These authors have contributed equally to this work and share first authorship

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INTRODUCTION

The bone marrow (BM) is the site where hematopoietic stem cells (HSCs) sustain hematopoiesis after birth and all lifelong in mammals. From early progenitors to committed subsets of myeloid and lymphoid lineages, proliferation and differentiation mechanisms have been extensively studied. They were shown early on, through the use of *in vitro* cultures, to be controlled by cells from the BM microenvironment (1, 2) comprising mesenchymal stem/stromal cells (MSCs), endothelial cells (ECs) and macrophages (3). Since the beginning of the 2000's, the identity and organization of BM niches supporting hematopoiesis have been extensively studied through the use of reporter mouse models (reviewed in (4, 5)). Furthermore, the heterogeneity of BM MSCs and ECs has recently been approached by single-cell RNA sequencing, which has confirmed the presence of multiple subpopulations within these two cell types (6–10). Beside this heterogeneity of the BM microenvironment, it is now moreover clear that the "one progenitor/one niche" rule does not prevail. Indeed, a single niche can support not only hematopoietic subsets at distinct developmental stages, but also mature immune cells homing back to the BM (5, 10).

Because of the contained location of the BM, knowledge on human hematopoietic niches is more limited even if strong similarities could be observed with mice (10–12). For the same reason, the nature of human leukemic niches and the molecular mechanisms regulated by/within them are still unclear and results strongly rely on *in vitro* cultures or on observations obtained in syngeneic or xenograft mouse models [reviewed in (13, 14)]. Furthermore, the immune microenvironment in tumoral BM is also poorly resolved and despite the tremendous progress that came with the use of immunotherapies, resistance and relapse in acute leukemia still concern many patients. Lessons could be learned from solid tumors for which the easier study of microenvironment (i.e., after surgery) has led to important advances. As in hematopoietic malignancies, a complex crosstalk exists between the tumor and the non-malignant cells in its microenvironment. Interestingly, BM is a haven not only for normal and pathological hematopoietic cells from the periphery, but also for metastatic cells from solid tumors, indicating that at least some properties or components of the tumor microenvironment must be shared between hematopoietic and solid tumors.

Tumor development depends on a multidirectional crosstalk between tumor cells, mesenchymal/endothelial cells and immune cells. The immune landscape and modulation of immune responses exerted by tumor cells, directly or through systemic disruption, have been extensively studied (15–17). In this review, we propose to confront and combine the knowledge gained on stromal/endothelial niches of leukemic and solid tumors by focusing on the BM as a common niche.

HETEROGENEITY OF CAFs/MSCs

The term "mesenchymal" is widely spread in the literature to designate stromal cells from the microenvironment of many tissues. MSCs are stromal cells able to adhere *in vitro* to plastic and spread on culture plates as spindle-cells or fibroblast-like cells. Specific MSC shapes are associated with differentiation, for instance rounded MSCs during adipogenic differentiation (18). MSCs are characterized by a specific pattern of surface markers. Namely, they express CD105, CD73, CD90 and CD146 in the absence of CD45, CD34, CD14, CD11b, HLA-DR and lymphocyte-lineage markers. MSCs secrete components of the extracellular matrix (*e.g.* collagens, heparan sulphate, elastin, aggrecan) and metalloproteinases as well as a large variety of mitogenic growth factors, cytokines, chemokines and angiogenic factors (19). These cells have also retained the ability to differentiate into osteoblasts, chondroblasts and adipocytes (20). However, inconsistent definitions of MSCs and varying isolation and culture conditions have resulted in highly diverse outcomes and confusing data (21, 22). The mesenchyme does not constitute a lineage but is an embryonic tissue able to give rise to connective tissue, blood vessels and blood cells that can have different embryonic origins. Therefore, there are no common MSCs in adult tissues, reflecting the fact that the nature and properties of the globally termed "MSCs" likely represent different cellular entities (21, 22). Since their initial definition in the early 1990's (23), the properties of MSCs have been largely explored and debated, even with an attempt at establishing a molecular signature (24). However, a consensus on the definition and use of the term "MSC" is unlikely to be reached. Indeed, with the major improvement of "omic" technological tools in the past years, in particular at the single-cell level, it has become quite obvious that MSCs encompass different subpopulations and states of stromal cells and even fibroblasts. The recent molecular mapping of murine BM niche populations, under homeostatic conditions, by single-cell RNA sequencing, clearly demonstrated a great cellular heterogeneity of BM stromal cells (6–8, 10). This heterogeneity was also identified in human MSCs (from umbilical cord), among which two groups were separated based on differentially expressed genes, including CD73. The first group is characterized by an enriched expression of genes involved in immune response/regulatory activities, muscle cell proliferation and differentiation, stemness and oxidative stress. The second presents a higher expression of genes involved in extracellular matrix production, osteoblast and chondrocyte differentiation, and bone and cartilage growth (25).

In a malignant context, and more particularly in acute myeloid leukemia (AML), the most recent studies report functional abnormalities of human MSCs, which have a significant impact on the aggressiveness of the disease. Among these anomalies, growth deficiency, altered osteogenic differentiation ability and reduced capacity to support hematopoietic cells (26–31) have been described, as well as modifications of the secretome (28, 32), which induce *in vivo* shaping of the stromal niche by leukemic cells (33). Moreover, single cell analyses of murine BM stromal cells recently revealed that leukemia remodels the BM stroma to the disadvantage of normal hematopoietic cells. This notably involves a blockade of the osteoblastic development, as well as of the pathway of bone

morphogenetic proteins (BMPs), including *Bmp4*. It also induces a decreased expression of *Cxcl12* and *Kitl* by leptin receptor expressing osteoprogenitors that regulate HSCs (7) (**Figure 1**).

All solid tumors contain non-tumor stromal supporting cells which are also called tumor-associated stromal cells. In carcinomas, they are well-known as cancer-associated fibroblasts (CAFs). Heterogeneity of tumor-associated stromal cells between tumors and, more recently, within a single tumor, has been disclosed, essentially through flow-cytometry, sorting and single-cell RNA sequencing. Their role in tumor progression is still explored with the use of mouse models. In line with such analyzes, four CAF subsets have been identified in breast and ovarian cancers by combining the study of distinct CAF markers, including the fibroblast activation protein (FAP), smooth-muscle α-actin (SMA) and integrin β1 (CD29) (34–37). Two subsets have also been detected in healthy tissues, reminiscent of normal fibroblasts, while two myofibroblastic subsets (FAP^{High} SMA^{High} $CD29^{Med\text{-}High}$ and FAP^{Neg} SMA^{High} $CD29^{High}$) appear to be restricted to tumors (**Figure 1**). These CAF subsets are respectively characterized by their secretion and organization

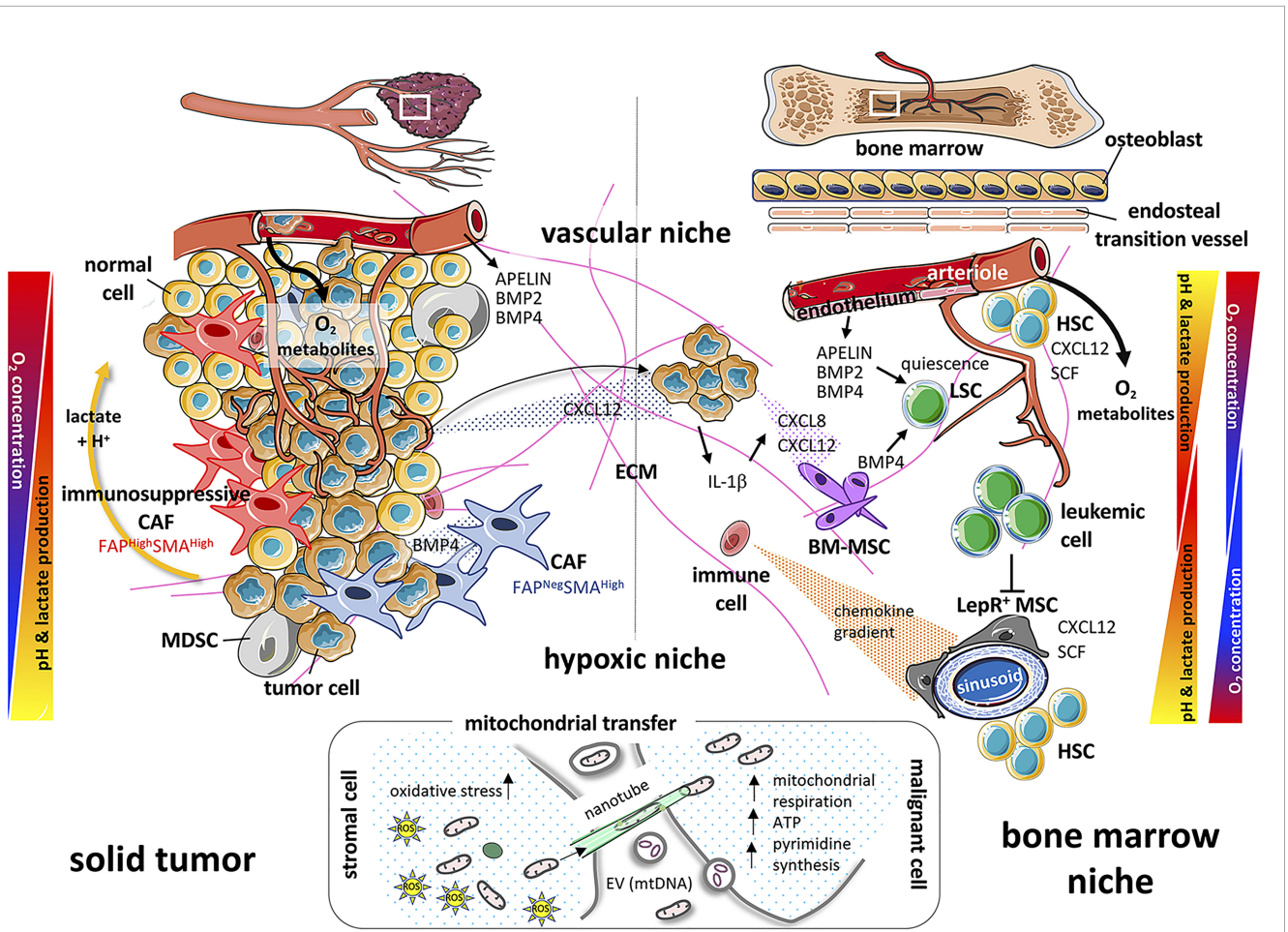

FIGURE 1 | Paralleling the microenvironment and actors of the solid tumor and bone marrow niche. On the left part of the diagram, the solid tumor is governed by cellular components such as healthy cells and tumor cells juxtaposed with immunosuppressive or neutral cancer-associated fibroblasts (CAFs) and myeloid-derived suppressor cells (MDSCs). The different gradients of oxygen, pH and growth factors (BMP2, BMP4), then participate in the tumor cell fate (proliferation, metastasis…). On the right panel, healthy hematopoietic stem cells (HSCs), bone marrow-mesenchymal stem/stromal cells (BM-MSCs), immune cells and tumor cells (attracted from a solid tumor by chemokine gradients such as CXCL12) or leukemia cells, quiescent or not, will be confronted with gradients of oxygen, pH, growth factors (BMP2, BMP4, SCF, APELIN) and cytokines (CXCL8, CXCL12, IL-1β) in a similar way as cells within the solid tumor. All these interrelations and interconnections, controls and feedbacks, will allow the tumor cell to proliferate and spread. Oxygen gradients, on the left, result from diffusion of oxygen from the blood vessels (vascular niche) as tumors grow outward from the local vascular architecture. Vascular niche may also be a source of factors favoring tumor growth (*i.e* Apelin) in solid tumor and in the bone marrow niche. On the right, there is a double gradient between arterioles and sinusoids. The cells will adapt their metabolism along these gradients and create, as a counterpart, a pH gradient due to the release of lactate and H^+ protons. Bottom part: As crucial powerhouses for cell metabolism and tissue survival, mitochondria will transfer horizontally from stromal cells to cancer and/or immune cells, *via* nanotubes, extracellular vesicles (EVs) or freely, to allow recipient cells to adapt and modify their metabolism (mitochondrial respiration, ATP, pyrimidine synthesis) to meet different stresses (oxidative stress) and energy demands.

of extracellular matrix (ECM) components, in particular types 1, 3 and 6 collagen, and by a perivascular contractile gene signature (34, 35, 37). Importantly, the association of the FAP^{High} SMA^{High} CAF subset with poor outcome has been validated by different laboratories in mouse models, as well as in some types of human carcinoma (38–42), highlighting its relevance in distinct species and cancer types.

Consistent with their accumulation in aggressive carcinomas, FAP^{High} $SMA^{Med\text{-}High}$ CAFs favor metastatic spread in breast and ovarian cancers by directly interacting with cancer cells, and the highly contractile FAP^{Low} SMA^{High} CAFs promote cancer cell invasion in 3-dimensions by remodeling the surrounding ECM (34–37, 43–45). Osteosarcoma-associated stromal cells have been characterized by MSC markers and SMA expression, like their healthy counterparts, but with a higher osteoblastic potential and an increase in lung metastases in mouse models (46). Mimicking the acidity of tumor microenvironment has been shown to lead osteosarcoma-associated MSCs to acquire an inflammatory phenotype, with an increased secretion of IL-6 and CXCL8. Such conditions also promoted the stemness of osteosarcoma cells (47). By contrast, healthy MSCs did not modify the quiescent state of osteosarcoma cells (48). Osteosarcoma-associated MSCs were moreover shown to promote not only the invasiveness of osteosarcoma cells, but also angiogenesis through the activation, proliferation and/or differentiation of ECs (49).

Myofibroblastic CAFs (SMA^+) secrete type-I collagen that can modulate immune cells. In a mouse model of pancreatic adenocarcinoma, it was recently demonstrated that the reduction of total type-I collagen secreted by CAFs accelerated the emergence of carcinoma. This was shown to be due to an upregulation of the chemokine CXCL5 (see next section) in cancer cells, leading to the recruitment of myeloid-derived suppressor cells (MDSCs) and impairment of $CD8^+$ cytotoxic T-cells (50). Conversely, the secretion of matrix metalloproteinases (MMPs) can favor tumor cell mobility across collagen fibers and inhibit immune cell activity. Melanoma-associated fibroblasts have thus been described as negative immuno-modulators, through the secretion of MMPs decreasing tumor cell lysis by natural killer (NK) cells (51). The ECM composition can be modified by the protease activity of FAP, which is expressed by tumor and stromal cells in many human carcinomas and sarcomas (43, 52, 53). Consistent with these observations, FAP^{High} SMA^+ CAFs have been identified in aggressive carcinomas to exhibit immunosuppressive activities (43, 44). Indeed, FAP^{High} CAFs are associated with immunosuppression and resistance to immunotherapies in mouse models (37, 54, 55). Interestingly, the FAP^{High} SMA^+ CAF subset promotes immunosuppression by increasing the infiltration of regulatory T lymphocytes (Treg) in human cancers (34, 35). Within the FAP^{High} CAF subpopulation, two distinct subsets exhibiting either an ECM-producing myofibroblastic phenotype (myCAF) or an inflammatory profile (iCAF) were recently identified in different types of cancers (56–60). Importantly, recent single cell sequencing of FAP^{High} CAFs from breast cancer cells allowed identifying eight different FAP^{High} cellular clusters (59). Three of them were further shown to be specifically associated with resistance to immunotherapy in metastatic melanoma and in non-small cell lung cancer patients (59). Taken together, these findings highlight the existence of a network of numerous CAF and MSC subpopulations in solid tumors and underline their relevance in various cancer types and across species.

NICHE CYTOKINES AND CHEMOKINES

The BM is a major location where hematological malignancies affecting myeloid or lymphoid lineages develop and is also an important site of metastasis for solid tumors, especially breast, prostate, and lung cancers (61, 62). The engraftment of metastatic cells from solid tumors into the BM can generate secondary tumors with either osteoblastic properties, notably in early stages of metastasis of prostate cancer, or osteolytic properties in the case of breast cancer (63–65). Osteoblastic lesions correspond to an increased bone mass at the lesion site whereas osteolytic lesions lead to a destruction of the bone structure. In the case of prostate cancer, there is a preferential accumulation of cancer cells in the lateral rather than medial endocortical bone region. This former area is enriched in osteoblasts, which could explain this phenomenon (66). The segregation of bone metastasis location between prostate and breast cancers has been extensively studied. Although this differential tropism is still not completely understood, there is a consensus about the involvement of chemokines in this phenomenon. The activity of chemokines depends on their receptors, a family of G protein-coupled seven-transmembrane-spanning molecules. Chemokines are versatile secreted factors critically required to drive the migration of immune and non-immune cells, notably within lymphoid organs including the BM. Depending on the targeted cell type, they can foster an effective anti-tumor immune response or conversely contribute to a pro-tumorigenic microenvironment. Early work from Zlotnik's lab has shown that high production of the chemokine CXCL12 (SDF-1) by the BM is sufficient to attract breast cancer cells expressing CXCR4, one of the cognate receptors of CXCL12 (67) (**Figure 1**). CXCR4 and CXCL12 are also critical for the homeostasis of the BM ecosystem, with a key role in controlling the production and mobilization of hematopoietic stem/progenitor cells (HSPCs) (68, 69). Indeed, in the BM, HSPC niches are thought to be composed of perivascular stromal units associated with sinusoids and arterioles as reviewed recently (70). In particular, many studies have shown that a population of MSCs termed CXCL12-abundant reticular (CAR) cells overlaps with leptin receptor (LepR)-expressing cells. These CAR cells constitute a major component of HSPC niches by their capacities to produce such niche factors as CXCL12, SCF and IL-7. Similar stromal cells with salient features of CAR cells have been identified in human adult BM (10, 71). In line with these findings, the CXCL12/CXCR4 axis is key in immunosuppression and metastatic spread in solid tumors, through reciprocal crosstalk between FAP^{High} CAFs and regulatory T cells (Tregs), as well as FAP^{High} CAFs and cancer cells, respectively (24, 25, 27).

In addition to CXCR4 and CXCL12, numerous studies have shown that chemokines act at different levels in the progression of the primary tumor, modulating both tumor cell proliferation, apoptosis, invasion, angiogenesis, recruitment of immune cells and resistance to chemotherapy (72–76). It appears clearly that some kind of "chemokine storm" and sustained inflammation take place in the primary tumor. This comforts the notion of a complex interplay between cancer cells, cells from the tumor microenvironment (including CAFs, MSCs and ECs) and a variety of immune cells, such as macrophages, B- and T-lymphocytes, NK cells, neutrophils and dendritic cells (73, 75, 77). The final outcome of the tumor with either sustained resistance of the host or immune escape of the tumor will depend on these interactions.

Within the tumor microenvironment, MSCs are interesting for multiple reasons. First, as stated above, these cells are highly present in the BM but can also be found at lower levels elsewhere such as in adipose tissue, lung or umbilical cord blood. They are moreover detected in multiple types of primary solid tumors (e.g., breast, ovarian, pancreatic cancers) (78–81). Several studies have shown that BM or adipose MSCs [called adipose-derived stromal cells (ADSCs)] have a particular tropism for primary tumors (81, 82). MSCs can either favor or inhibit primary tumor growth and metastasis (78, 81, 83–85)s. Recent evidence has also shown that the nature of tumor cells, in particular their low or high aggressiveness, dictates the type of interactions with MSCs and notably the production of multiple chemokines and prostaglandin E2 upon release of IL-1β by tumor cells. In turn, chemokines produced by MSCs can stimulate the invasiveness and potentially metastatic ability of tumor cells (81, 86, 87). Finally, with BM metastasis of solid tumors, interactions become possible with the other niches of BM MSCs. This interaction might favor a release of new MSCs from the BM to colonize primary tumors but may also affect the properties of MSCs themselves, notably by turning them into CAFs (81).

As stated above, growing tumors establish a chronic state of inflammation that acts locally but also systemically. The BM responds to these stress signals by remodeling the stromal landscape and expanding myeloid cells endowed with anti-inflammatory/immunosuppressive functions, further sustaining tumor progression. Several studies have reported that distant solid or diffuse tumors interfere with hematopoiesis and immune regulation within the BM. Primary breast tumors have thus been shown to generate systemic signals that mobilize BM-derived cells promoting tumor growth and dissemination. Tumor-derived factors also interfere with BM myelopoiesis, increasing the generation of granulocytic-MDSC (88, 89). In a spontaneous model of mammary carcinogenesis, Colombo's lab revealed modifications of the representation of CXCL12-expressing BM-derived MSCs and CXCR4-expressing myeloid cells (90). Such changes in the hematopoietic compartment occurred as early as at preinvasive disease stages and were concomitant with a deregulation of circulating miRNAs. In addition, extracellular vesicles (EVs) produced by follicular lymphoma B-cells have been shown to promote the polarization of BM-derived MSCs to secrete such factors as CXCL12 that could constitute in turn a BM follicular lymphoma permissive stromal niche (91). In AML xenografts, blast-derived EVs convey endoplasmic reticulum stress *in vivo* to the animal's BM stroma. This drives a subsequent osteo-differentiation of MSCs through the incorporation and cell-cell transfer of BMP2 by AML-derived EVs, promoting BM niche remodeling (92). Conversely, many studies provide compelling evidence that the BM can sense distant tissue transformation at premalignant/preinvasive stages and influence cancer progression. Bone-making osteoblasts have the capacity to impact distant cancer progression outside the skeleton in such tumors as melanoma, lung, and breast carcinomas. CXCL12 might constitute one of the systemic bone-derived factors that would directly promote breast cancer cell proliferation and metastasis (93). Other studies indicate that cells of the osteoblast-lineage control cancer progression in the same tumor types at least in part by mobilizing tumor-promoting myeloid cells (94, 95). Finally, BM remodeling could be beneficial or detrimental, depending on the nature of targeted hematopoietic cells, i.e., healthy vs. malignant. For instance, Belkaid's lab recently showed that dietary restriction promoted memory T-cell accumulation in the BM. This was coordinated by glucocorticoids and associated with BM remodeling that involved an increase in such niche factors as CXCL12, erythropoiesis and adipogenesis. Consequently, this was associated with enhanced protection against infections and tumors (96). This work suggests a strategy to optimize immunological memory during nutritional challenges involving a spatiotemporal reorganization of the BM. Unfortunately, the safe haven of the BM can also be remodeled by malignant cells to disturb normal hematopoiesis. For instance, AML can shape the BM landscape to support malignant growth at the expense of normal hematopoiesis. Indeed, AML onset impaired osteogenesis as well as the production of such hematopoietic factors as CXCL12 (7). Likewise, altered cytokine expression such as a decrease of CXCL12 production in the BM of a mouse model of chronic myelogenous leukemia (CML) conferred a growth advantage to leukemia stem cells (LSCs) over normal stem cells (97). Finally, *CXCL12* deletion from MSCs reduced normal HSC numbers but promoted LSC expansion and their elimination by tyrosine kinase inhibitor treatment (98). These findings are consistent with cancer cells impairing normal hematopoiesis and provide a foundation for developing stromal-based therapies.

The relevance of stromal-based therapies is also supported in hematological malignancies by data from Hasselbalch *et al.* suggesting that chronic inflammation can be a driver of clonal evolution in patients with myeloproliferative neoplasms (MPNs) (99). In primary myelofibrosis (PMF), disease severity and treatment complexity have mainly been attributed to the association of clonal myeloproliferation and profound changes in the BM stroma, associated with an excessive production of cytokines, chemokines, growth factors and ECM components. It was initially reported that stromal changes were reactional and secondary to growth factor production by clonal hematopoietic cells. However, the presence of molecular alterations of PMF MSCs has been shown to provide an "intrinsic" osteogenic

signature and an increased differentiation into osteoblasts partly dependent on endogenous TGFβ1 production and activation (100, 101). It has been suggested that the BM stroma of PMF patients is progressively inflammatory-driven by clonal hematopoietic cells towards an "autonomous" state where it becomes independent of hematopoietic cell stimulation. This in turn causes an alteration of the hematopoietic niche and participates in the amplification of the hematopoietic clone. The resulting inflammatory vicious circle becomes unbreakable in the absence of combined stroma targeted therapies (100, 102). Therefore, Stephen Paget's theory (103) of the "seed (cancer/leukemic cell) and soil (microenvironment)" is fully sustained. However, in PMF, the bad soil (altered MSCs) endorse the bad seed (clonal HSCs), revisiting Paget's theory in the "bad seed in bad soil" concept (104, 105). This strengthens the importance of stromal cells and their reciprocal interactions with clonal hematopoietic cells in the development and treatment of neoplasia (106).

CELL METABOLISM AND COMMUNICATION

While it has long been known that oxygen plays a key role in the proper functioning of mammalian cells, the mechanisms by which these cells adapt to the amount of oxygen available have only became to be understood since the 1990's, thanks to the work of the three 2019 Nobel Prize winners in Physiology or Medicine Drs Gregg Semenza, William Kealin and Sir Peter J. Ratcliffe (107–111). The notion of hypoxia in hematopoietic niches is even more recent. While the oxygen gradient created by vascularization is understandable in solid tumors, the idea of such a gradient took longer to emerge in the world of hematopoiesis. It is now accepted, but not necessarily integrated, that the oxygen (O_2) concentration in the hematopoietic niche varies between 1 to 4% of oxygen, strikingly different from the peripheral blood concentration of 10 to 13% (112–114). As in solid tumors, the overexpression of hypoxia-inducible factors (HIFs) has been reported in leukemia to be a marker of poor prognosis. The metabolic adaptation of tumor cells is one of the hallmarks driving aggressiveness in cancer that is clearly emphasized by low oxygen concentrations. Solid tumor cells are often glucose-addicted as sugar provides metabolic intermediates that support proliferation and migration. Thus, lactate metabolism and acidosis, other characteristics of the hypoxic tumor area, must be highly hypoxically controlled to avoid cell death (115). Based on the work of Nobel Prize winner Otto Warburg in 1931 (116), it has been speculated for decades that mitochondria were failing during the tumor process. This theory finally materialized as the Warburg effect whereby anaerobic fermentation is preferred by some tumor cells. However, several studies have now proven that mitochondria function normally in cancer cells and that blocking oxidative phosphorylation (OXPHOS) is an adaptive event (117, 118).

Although glycolytic metabolic reprogramming is common in cancer cells, several types of cells have been reported to prefer OXPHOS for energy production (119–124). AML cells thus highly depend on OXPHOS to satisfy their heightened demands for energy. Mitochondrial and OXPHOS activities greatly influence the sensitivity and *in vivo* efficacy of chemotherapeutic agents (125). Increasing evidence reveals that stromal cells affect the characteristics of cancer cells in the tumor microenvironment (126–129). The niche plays an important role in cancer cell metabolism by secreting metabolites that are used for the tricarboxylic acid (TCA)/Krebs cycle (130). Moreover, CAFs enhance the Warburg effect by interacting with cancer cells and producing lactate used by cancer cells as a fuel for mitochondrial OXPHOS (**Figure 1**). This concept is widely known as the reverse Warburg effect (131–133). Thus, the increase in reactive oxygen species (ROS) promotes the activation of HIF-1α, inducing autophagy, lysosomal degradation and loss of stromal Cav-1, consequently contributing to glycolysis in CAFs. Besides, it has been recently reported that interactions between MSCs and leukemic cells increase oxidative stress in MSCs (134) with a concomitant activation of glutathione (GSH)-based antioxidant defenses, notably through overexpression of *GPX3*, a key determinant of leukemic cell self-renewal (135, 136). These interactions also enhance leukemic blast bioenergetics by increasing OXPHOS and the TCA cycle (136). All these elements suggest that metabolic interactions within their niche are important for the maintenance of mitochondrial OXPHOS in cancer cells.

Mitochondria are not only involved in energy production through the generation of ATP by OXPHOS. They also support important anabolic reactions and are crucial regulators of apoptosis *via* the expression of molecules of the BCL-2 family at their surface (137). Horizontal transfer between two cells of mitochondria and/or mitochondrial DNA (mtDNA) *via* nanotubes, EVs or freely, is likely to have fundamental consequences for the host (**Figure 1**). A first study showed that active mitochondria and/or mtDNA from human bone marrow MSCs could rescue respiration-deficient (ρ0) lung carcinoma cells (138) and apoptotic PC12 cells (139). This effect was described in several non-cancer situations where stressed cells, frequently experiencing hypoxic or ischemic conditions, could recover after the acquisition of mitochondria from their cellular environment (reviewed in (140)). For instance, BM-derived MSCs have been shown to protect lung epithelial cells from lipopolysaccharide-induced injuries through the donation of mitochondria (141). For cancer cells, two seminal publications have shown that ρ0 cancer cells have an impaired tumorigenic potential that can be restored, together with respiration, by the transfer of mtDNA (142) or active mitochondria (143) from surrounding cells, both *in vitro* and *in vivo*. Interestingly, it has also been demonstrated that CAF-derived EVs can transfer mtDNA to OXPHOS-deficient breast cancer cells, leading to the restoration of mitochondrial metabolic activities (144). MSCs moreover could transfer active mitochondria to AML leukemic blasts, especially upon sensitization of leukemic cells by chemotherapy, probably, among other still unclear

mechanisms, *via* AML cell-derived ROS (145, 146). It was also demonstrated that MSCs recognize damaged mitochondria released by leukemic cells under chemotherapy as danger signals and react by stimulating mitochondrial biogenesis followed by transfer of active mitochondria to AML cells (147). Another interesting study showed that BM MSCs from acute lymphoblastic leukemia (ALL) patients harbor a CAF phenotype. Upon chemotherapy and ROS induction, they transfer mitochondria to ALL blasts to support their survival and resistance to chemotherapy (148). The uptake of mitochondria by leukemic cells can increase their mitochondrial mass by up to 14% (145) and is associated with better fitness and a higher resistance to chemotherapy. Since mitochondria-recipient cells become able to resist apoptotic signals, it is possible that this transfer could increase the pool of anti-apoptotic molecules of the BCL-2 family in leukemic blasts. Another obvious effect of mitochondrial transfer is an increase in ATP content (145, 147, 149) and in other important metabolites. A recent study demonstrated that transferred mitochondria were important to sustain pyrimidine synthesis and cell proliferation *via* the dihydroorotate dehydrogenase (DHODH) enzyme present in the mitochondrial membrane (150). Exogenous mitochondria could also support resistance to ferroptosis cell death as DHODH appears to mediate an important protective pathway against ROS-induced lipid peroxidation that triggers ferroptosis (151). Finally, mitochondrial transfer could modulate immune responses as it has been reported that horizontal transfer from MSCs could trigger Treg differentiation to limit tissue damage and inflammation during graft-*versus*-host disease (152). Whether this phenomenon also occurs in the BM hematopoietic niche and affects other lymphoid cell subsets such as cytotoxic T-lymphocytes or NK cells during leukemia development remains to be studied.

VASCULAR NICHE, ANGIOGENESIS AND ENDOTHELIAL PLASTICITY

The vascular endothelium refuels the tumor mass with oxygen and metabolites and settles a favorable microenvironment for tumor growth. This is strikingly illustrated in tumors from the central nervous system, where homeostasis of the cerebral vasculature is crucial. As for embryonic and adult stem cells, cancer stem cells reside within a niche articulated around vascular units (153), defined as the vascular niche. This environment allows privileged control of metabolic conditions, secreted protein dosage as well as fine-tuned regulation of cell adhesion and communication with the surrounding ECM and neighboring ECs (154). Cancer stem cells are indeed located in the close vicinity of tumor blood vessels where ECs are suspected to dictate stem cell identity (155, 156). The concept of (peri) vascular niche is also highly significant in the BM and has evolved through the better characterization of HSPCs. In mice, the HSPC compartment is functionally and molecularly heterogeneous, due in part to an extrinsic control by the BM microenvironment, including ECs. Indeed, recent advances in cell imaging and HSPC reporter-mice have revealed the association of HSPCs with at least two types of blood vessels. The latter are central endothelium featuring sinusoids (157) and an endosteal arterial/arteriolar endothelium which is close to bone diaphysis and epiphysis and defines transition vessels (158) (**Figure 1**). Sinusoidal and endosteal ECs differ phenotypically, the latter expressing high levels of endomucin and CD31, while sinusoidal ECs display low levels of both these markers. The location of endosteal ECs in bone metaphyses, close to osteoprogenitor cells, allows for an efficient coupling between osteogenesis and angiogenesis. Furthermore, sinusoidal and endosteal ECs are surrounded by unique specific perivascular MSCs. Although most CXCL12 and SCF in the BM is produced by CAR/LepR+ cells (159, 160), ECs are also a source of both niche factors and hence are involved in the hematopoiesis process. Arterial and transition vessel ECs by displaying such a higher expression of CXCL12 and SCF maintain HSPC quiescence, while sinusoidal vessels, fenestrated and more permeable, promote BM cell trafficking (158, 161, 162).

Several studies have revealed the role of the BM vasculature in the development of leukemia and chemoresistance. In AML, vascular niches provide signals that regulate proliferation and stem cell-like properties (163, 164). In a reciprocal way, AML cells release inflammatory cytokines that activate the vascular endothelium, inducing the expression of such adhesion molecules as VCAM1, promoting AML proliferation and chemoresistance (165, 166). External cues emanating from ECs can regulate the fate of cancer stem cells both in solid tumors and leukemia. In cerebral tumors, exploration of the endothelial secretome identified the vasopeptide apelin (APLN) as a central regulator for endothelial-mediated maintenance of patient-derived glioma stem-like cells *in vitro* and *in vivo* (167). Further studies confirmed the instrumental role of APLN to sustain tumor cell expansion and progression (168). Likewise, a subpopulation of APLN-expressing ECs in the BM orchestrates HSPC maintenance, and further repopulation in the therapy-induced damaged bone microenvironment (169). In luminal breast carcinoma, the BMP2 was found to be overproduced by ECs from the tumor stroma. This factor is an important actor of the stem cell niche, participating also in the initiation of stem cell transformation (170).

In hematological malignancies, sinusoidal ECs from the BM vascular niche of patients with chronic myeloid leukemia have been shown to be the main source of BMP2 and BMP4, involved in the maintenance and expansion of leukemic stem cells (171). BMP4 overproduction in the AML microenvironment furthermore contributes to blast cells "reprogramming" towards a stem-cell like phenotype (172). In addition, BMP4 produced by the leukemic microenvironment is involved in leukemic stem cell quiescence mediated by Jak2/Stat3 signaling and contributes to relapse and tumor escape (173) (**Figure 1**). Similar data in solid tumors, from many laboratories, have identified the BMP-signaling pathway as a major driver of BM dormancy (174).

Seed and soil interactions have to be considered as reciprocal, signals provided by cancer cells impacting ECs and *vice versa*.

The influence of cancer cells towards EC is to promote angiogenesis and increase vascular permeability to respectively provide the oxygen required for growth and allow for cell dissemination. In solid tumors, pro-angiogenic factors (VEGF, Sema3A), either soluble or delivered through tumor-derived EVs, contribute to an increase of both angiogenic potential and permeability (175, 176). Malignant hematopoietic cells are high consumers of oxygen and evolve in a hypoxic environment that favors angiogenesis. An increase in BM vascular density and angiogenic markers (VEGF-A, FGF2, VEGF-R) has been highlighted in several hematological malignancies (177, 178). The sites of active angiogenesis in tumor BM niches are still not fully characterized, but ECs in the transient zone close to the endosteal niche could mediate the local growth of blood vessels in normal bone (158). In MPNs and leukemia, neo-vessels are also characterized by an abnormal tortuous architecture (178, 179). In MPNs, increased microvascular density and expression of VEGF have been reported to correlate with the allelic charge of *JAK2*-V617F mutation (180, 181). In a subset of thrombotic MPN patients, this mutation has been detected in hepatic and splenic ECs as well as in endothelial progenitors, suggesting their clonality (182). More recently, introduction of the *JAK2*-V617F mutation in ECs has been shown to modify these cells towards a pro-adherent and pro-thrombotic profile (183, 184). These results suggest that, similarly to MSCs, ECs may have acquired intrinsic modifications that participate in the activated/inflammatory state within the BM niche and in leukemic progression. An aberrant increase in permeability is an additional striking feature of tumor blood vessels (185) which strongly alters drug delivery in solid tumors (186). An increased permeability of BM vessels, induced by leukemic cells, could also be associated with an impaired perfusion hampering normal hematopoiesis and supporting malignancy as shown in an AML patient-derived xenograft model (166).

Beside their role in angiogenesis, ECs may also engage in the dynamic process of endothelial-to-mesenchymal transition (EndMT), which drives reprogramming of ECs towards a mesenchymal phenotype (187). Initially described in normal cardiac development, this plasticity has been highlighted in several solid tumors in response to tumor environmental soluble and/or mechanical cues, as well as upon therapeutic assaults (188). EndMT may provide a source of CAFs (189) and contribute to metastasis dissemination by destabilizing the endothelial barrier (190). Furthermore, EndMT has been described as a tumor arm to resist chemo- and radio-therapies (191, 192). Recent data support such a transition process in regenerative human BM, as a subset of ECs in trabecular sinusoid vessels has been shown to display an EndMT transcriptional signature (193). Importantly, this endothelial derived-mesenchymal population harbors properties of pluripotent stromal cells, with multi-lineage differentiation capacity (adipocyte, osteoblast, chondrocyte) and supportive capacity of hematopoiesis. Whether EndMT plays a role in hematological cancer is not confirmed yet, but this process surely could participate in the reconstitution of the hematopoietic BM niche after therapy (193). In the BM and spleen of PMF patients, the presence of microvascular ECs showing functional and morphologic changes associated with the MSC phenotype is in agreement with the potential contribution of EndMT to the BM fibrosis process that characterizes this disease (194).

CONCLUSION

This review highlights how knowledge is progressing, in both solid tumors and hematological malignancies, in identifying the role of the multiple subsets of cells widely referred to as "cells of the microenvironment". The latter clearly constitute a network of interacting subsets, which are increasingly well identified, but still incompletely understood. From cytokine/chemokine release patterns to interactions with angiogenesis and oxygen regulation, much remains to be deciphered. However, this review clearly highlights that solid tumors and hematological malignancies use similar strategies to survive in a microenvironment dedicated to their suppression, in particular by modifying the microenvironment to adapt it to tumor growth, while altering its physiological role.

Information generated by single-cell analyses can be used as a blueprint for the identification of CAFs or MSCs subtypes in various organs in different pathological conditions. Comparison of CAF subtypes' molecular profiles with those of MSCs will be useful to identify potential mechanistic similarities in tumor inflammation and niche alterations across malignancies. Much remains to be done however before transposing the results obtained in mouse models to the primary cells of human tumors and hematologic malignancies.

It is obviously still needed to discover specific means to interfere with the intricate interplay between niche actors that affect cancer/leukemic growth and prevent leukemia relapse. The power of multi-omic analyses of the tumor microenvironment, associated with a pan-tumor integrative approach of cancer niche abnormalities could be decisive in proposing new therapeutic strategies targeting niches in order to eradicate cancer cells.

AUTHOR CONTRIBUTIONS

All authors made extensive reviews of the literature listed and drafted different sections of the review. NMM drew the figure, which was finalized with the help of SM and OH. OH conceived, designed, supervised and finalized the review. All authors contributed to the article and approved the submitted version.

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Anatomy of Hematopoiesis and Local Microenvironments in the Bone Marrow. Where to?

Qingqing Wu[1], *Jizhou Zhang*[1] *and Daniel Lucas*[1,2]*

[1] Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Medical Center, Cincinnati, OH, United States,
[2] Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, United States

The shape and spatial organization -the anatomy- of a tissue profoundly influences its function. Knowledge of the anatomical relationships between parent and daughter cells is necessary to understand differentiation and how the crosstalk between the different cells in the tissue leads to physiological maintenance and pathological perturbations. Blood cell production takes place in the bone marrow through the progressive differentiation of stem cells and progenitors. These are maintained and regulated by a heterogeneous microenvironment composed of stromal and hematopoietic cells. While hematopoiesis has been studied in extraordinary detail through functional and multiomics approaches, much less is known about the spatial organization of blood production and how local cues from the microenvironment influence this anatomy. Here, we discuss some of the studies that revealed a complex anatomy of hematopoiesis where discrete local microenvironments spatially organize and regulate specific subsets of hematopoietic stem cells and/or progenitors. We focus on the open questions in the field and discuss how new tools and technological advances are poised to transform our understanding of the anatomy of hematopoiesis.

Keywords: anatomy of the bone marrow, spatial organization of hematopoiesis, microenvironment, niches, dynamics of hematopoiesis

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Noriaki Ono, University of Texas Health Science Center at Houston, United States

***Correspondence:**
Daniel Lucas
daniel.lucas@cchmc.org

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INTRODUCTION

The bone marrow tissue provides a unique microenvironment -composed of both hematopoietic and non-hematopoietic cells and extracellular matrix- that cooperate to accomplish several functions: promote stem cell and multipotent progenitor self-renewal, regulate the differentiation of each lineage, and provide structural support and spatial organization to the tissue. The microenvironment is defined by three large structures: the bone tissue that encloses the marrow; a vascular network, composed of arterioles that penetrate through the bone and give rise to a large sinusoidal network that drains through a central vein; and a network of reticular stromal cells that wraps around the different vessels. These structures cooperate with and regulate each other to maintain the tissue (1).

Many other cell types regulate -directly or indirectly- hematopoiesis and are thus considered part of the microenvironment. Non-hematopoietic cells include osteoblastic precursors, osteoblasts, osteocytes, adipocytes, Schwann cells, sympathetic and sensory nerves, and fibroblasts. Hematopoietic components

include macrophages, megakaryocytes, myeloid cells, and dendritic cells. In-depth discussions on how these cells were recognized as components of the microenvironment and their precise role on hematopoiesis are available elsewhere (1–3). The components of the microenvironment are not evenly distributed through the bone marrow. As a result the microenvironment is spatially heterogeneous. Different regions of the bone marrow contain specialized microenvironments that organize hematopoiesis and regulate unique progenitors, cell types, and blood lineages. The next section discusses the evidence demonstrating that local microenvironments dictate the anatomy of hematopoiesis.

THE ANATOMY OF HEMATOPOIESIS IS SPATIALLY ORGANIZED BY LOCAL MICROENVIRONMENTS

Spatial Organization of Hematopoietic Stem Cells and Their Niches

The discovery of the SLAM markers allowed imaging of HSC (defined as $Lin^-CD48^-CD41^-CD150^+$ cells) for the first time. This first study showed that most HSC were in perivascular location -in contrast to the established paradigm that stated that HSC were enriched in endosteal regions (4). It also paved the way for many other studies that used imaging to identify proximity between candidate niche cells and HSC and then a functional role for the niche cell was confirmed by genetic loss of function experiments (5–10). The composition, spatial organization, and function of HSC niches has been reviewed in detail elsewhere (1–3). Due to the sheer abundance of sinusoids and perivascular cells in the bone marrow virtually all (99%) hematopoietic cells – including HSC– localize within 30μm sinusoids or perivascular stromal cells (8, 11, 12). Both cell types are key regulators of HSC function (1, 3). Additionally, small subsets of HSC also localize near arterioles and/or the endosteum. Myeloid-biased HSC (detected using von Willebrand factor reporter mice) selectively localized near megakaryocytes -a key niche component that promotes HSC quiescence (9, 13, 14)- in the sinusoids. In contrast, lymphoid-biased HSC selectively localized near arterioles (15). Depletion of megakaryocytes led to expansion of myeloid biased HSC through loss of quiescence while lymphoid-biased HSC were unaffected. Similarly, depletion of $Ng2^+$ periarteriolar stromal cells led to loss of the lymphoid-biased HSC (15). Other studies showed that the fraction of HSC with lowest levels of reactive oxygen species was enriched near arterioles (16); that increases in arteriole numbers also cause increases in HSC frequency (17); and that $Ng2^+$ periarteriolar cells support HSC function (6). Together these results support the concept that sinusoids and megakaryocytes provide a niche for myeloid-biased LT-HSC whereas arterioles provide a niche for lymphoid-biased HSC. There is also evidence supporting the existence of an endosteal HSC niche that promotes regeneration. Imaging of fluorescently labeled HSC shortly after transplantation showed that the donor HSC are selectively enriched near the endosteal surface (18–20). Studies from the Li lab propose that $CD49b^-$ cells represent a small subset of HSC that selectively amplifies in the endosteum -supported by N-$cadherin^+$ stromal cells- in response to chemotherapy (21). In agreement, live imaging analyses showed that a rare HSC subset (MFG HSC) localized and amplified near the endosteum after chemotherapy treatment (22).

It is important to note that although most studies agree with the overall distribution described above there are ongoing controversies regarding whether some HSC selectively localize – and are maintained- by arteriolar and endosteal niches (8, 23); whether HSC localization to different niche components is selective or random [and thus controlled by the relative abundance of each niche component (12)]; and about the motility of HSC in live imaging analyses (22, 24). These are likely because each group has used different cell surface markers, transgenic reporters, and statistical approaches to identify HSC and niche cells and to test for spatial relationships between these cells.

Spatial Organization of Hematopoietic Progenitors Downstream of HSC: Role of the Microenvironment

HSC give rise to several types of multipotent (MPP) and oligopotent progenitors (25–27). The localization of these cells in the microenvironment and whether they map near HSC and their niches is controversial. Early studies relied on short-term tracking of fluorescently-labeled MPP and HSC adoptively transplanted into non-myeloablated recipients. These revealed that the transplanted HSC and MPP did not overlap and that MPP localized further away from the endosteum than HSC (19). Much more recently, the Camargo lab generated $Mds1^{GFP+}$ and $Mds1^{GFP+}$Flt3-cre mice to differentially image subsets of multipotent progenitors and HSC. In the $Mds1^{GFP+}$ mice GFP labels almost all HSC and subsets of MPP. In the $Mds1^{GFP+}$Flt3-cre mice constitutive cre-mediated deletion of the floxed gfp allele restricts GFP expression to a small subset of HSC. They found that GFP^+ cells in $Mds1^{GFP+}$ mice were closer to transition zone vessels and farther away from the endosteum when compared to GFP+ cells in the $Mds1^{GFP+}$Flt3-cre mice. This suggests that MPP and HSC reside in different microenvironments (22). The Pereira lab defined multipotent progenitors as $Lin^-CD41^-CD48^-cKIT^-CD150^-FLT3^+$ [which corresponds to the MPP4 subset (25)] and found a similar spatial distribution and interaction with perivascular stromal cells as HSC suggesting that they occupy the same niches (28). The differences between these studies are likely due to the different mouse reporters and methods used to image the multipotent progenitors.

It is likely that multipotent progenitors and lineage-committed progenitors do not overlap. *In vivo* imaging of adoptively transferred multipotent ($Lineage^-Sca1^+c\text{-}kit^+$) or lineage-committed ($Lineage^-Sca1^-c\text{-}kit^+$) progenitors into non-myeloablated recipients showed that both cells did not cluster and remained largely immobile while contacting the surrounding microenvironment (29). This suggested the existence of discrete niches for multipotent and lineage-committed progenitors. The existence of a distinct niche for erythropoiesis comes from classical electron microscopy studies that showed that rare

macrophages, adjacent to sinusoids, provide a niche for islands of erythroblast maturation (30), and these have been the focus of many studies in the field (31). More recently, Comazzetto et al., demonstrated imaging of unipotent erythroid progenitors and showed that they selectively localize next to perivascular stromal cells that maintain them *via* SCF production (32). These indicate that erythropoiesis takes place in the sinusoids.

Herault et al., imaged Lineage$^-$Sca1$^-$CD150$^-$c-kit$^+$FcγR$^+$ committed myeloid progenitors (33). These are a mixed population, containing granulocyte monocyte progenitors, and unipotent monocyte or neutrophil progenitors (34, 35). These myeloid progenitors were found as single cells evenly distributed through the bone marrow. In response to inflammation they formed large clusters that required signals provided by megakaryocytes to emerge (33). We recently developed strategies to image granulocyte progenitors, monocyte progenitors, monocyte dendritic cell progenitors (MDP) and most steps of terminal myeloid cell production (36). We found that myeloid progenitors do not colocalize with each other or HSC. Instead, they spatially segregate and attach to different sinusoids –away from arterioles and the endosteum– where they cluster with differentiated cells: granulocyte progenitors give rise and cluster with preneutrophils, monocyte progenitors cluster with Ly6C^{hi} monocytes, and MDP cluster with dendritic cells and Ly6C^{lo} non-classical monocytes. CSF1 is a key cytokine required for monocyte and dendritic cell production (37). When searching for microenvironmental signals that regulate this distribution we noticed that dendritic cells -which cluster with MDP- selectively localized to a rare subset of CSF1+ sinusoids (8% of all vessels). Conditional *Csf1* deletion in the vasculature led to loss of MDP, dendritic cells, and non-classical monocytes. The surviving MDP no longer attached to sinusoids nor formed clusters with dendritic cell or monocytes. These demonstrated that myelopoiesis is spatially organized by signals produced by discrete sinusoids and that CSF1$^+$ sinusoids provide a unique microenvironment for dendritic cell production (36).

Several studies indicate that B cell differentiation is spatially organized and regulated by the microenvironment [for a recent review see (38)]. Common lymphoid progenitors distribute between the endosteum and arterioles and are maintained by CXCL12 produced by osteoblastic cells (targeted using *Col2.3-cre* or *Osx-cre* mice) and stem cell factor produced by osteolectin$^+$ periarteriolar stromal cells (39–41). Cordeiro-Gomes found that Ly6D$^+$ common lymphoid progenitors were also in contact with -and maintained by- a subset of IL7-producing perivascular stromal cells but it is not clear whether these stromal cells are evenly distributed through the bone marrow or enriched in specific locations (28). Interestingly, subsets of stromal cells predicted to support lymphopoiesis selectively localize near the growth plate and trabecular regions (42). The Nagasawa lab showed that most Pre-pro-B cells are in contact with CXCL12-producing stromal reticular stromal cells but did not localize near IL7-producing reticular cells. In contrast most Pro-B cells did not contact CXCL12 producing cells but localized near IL7 producing cells (43). Mandal et al., showed that Pre-B cells and Immature B cells selectively localize near IL-7$^-$CXCL12$^+$ reticular cells and that CXCR4 (the ligand for CXCL12) was necessary for Pre-B cell differentiation (44). Yu et al., demonstrated that deletion of IFG1 in Osterix+ progenitors using Osx-cre mice did not affect common lymphoid progenitors but led to arrest of B-cell development at the Pro-B stage (45). Fistonich et al., found that approximately 50% of ProB cells were in contact with IL7$^+$CXCL12$^+$ reticular cells that simultaneously contacted with a PreB cell. This suggested an overlapping niche for these two populations (46). Interestingly, live imaging showed that while ProB cells are largely static and remain attached to the CXCL12$^+$ reticular cells whereas PreB cells migrate between different reticular cells (46). Together these studies strongly suggest that -as B cell progenitors differentiate- they migrate between subsets of stromal cells producing different amounts of IL7, CXCL12, or IGF1. In contrast, the Mancini lab found that most reticular cells coexpress IL7, CXCL12 and LepR, that ProB cells localize near LepR$^+$, and that ~15% of HSC colocalize with Pro-B cells –much higher than predicted from random distributions. They also identified Nidogen-1 as niche derived factor regulating lymphopoiesis (47). Since HSC map near IL7 producing cells (28) these suggest that HSC and B cell lymphopoiesis share overlapping niches.

OPEN QUESTIONS AND FUTURE DEVELOPMENTS

The studies above demonstrate that the bone marrow is a complex organ with a unique spatial architecture where specific lineages are supported by discrete regions of the bone marrow (**Figure 1**). The studies also lead to new questions and reveal major gaps in our understanding of how spatial relationships regulate hematopoiesis.

What Is the Anatomy of Stepwise Hematopoiesis in the Steady-State?

Hematopoiesis occurs *via* stepwise differentiation of progenitors. However, it has not been possible to map the location of many progenitor populations – including different subsets of multipotent progenitors, common myeloid progenitors, and megakaryocyte erythroid progenitors. Additionally, for most progenitors, it has not been possible to simultaneously image multiple types of progenitors. Therefore, it is not known whether different types of progenitors share the same niche (and are likely regulated by the same cells and structures) or different niches (which will suggest differential regulation). The main reasons limiting studies to answer these questions are technological. For example, progenitor populations can be routinely defined using complex multicolor flow cytometry panels (25–27). However, most confocal microscopes can only resolve a much more limited number of fluorescent channels. Additionally, scRNAseq studies demonstrated that many of the different flow gates used to prospectively isolate the different progenitors contain heterogeneous populations [e.g., heterogeneity of myeloid progenitors (34)]. Precise mapping of the different steps of blood maturation will require developing approaches to define each type of progenitor by using fewer fluorescence channels as

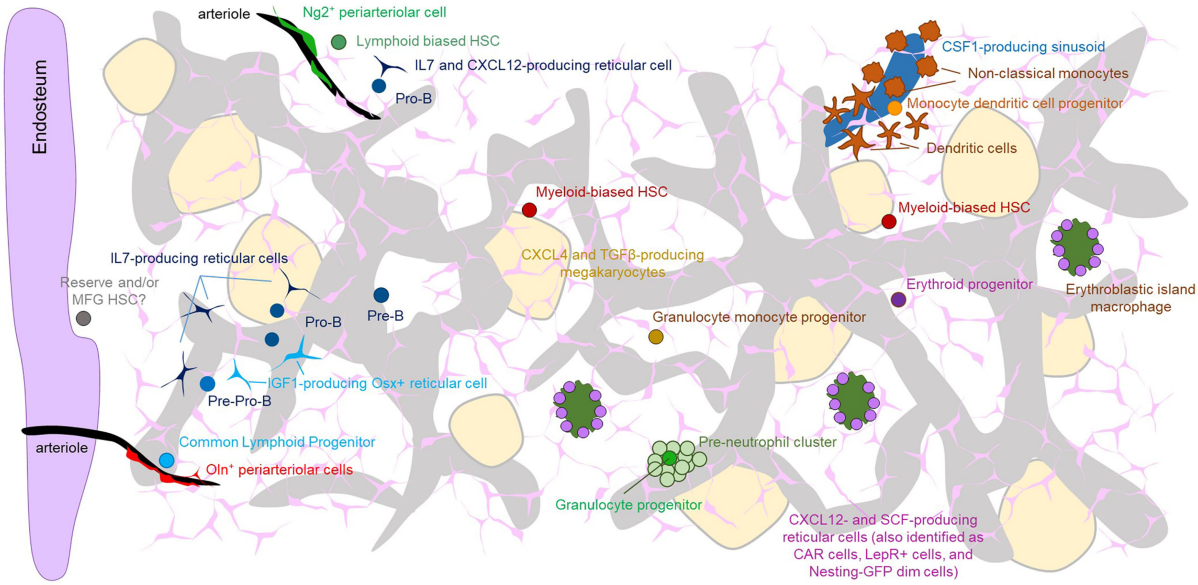

FIGURE 1 | The Figure shows the overall architecture of the microenvironment in the bone marrow as well and the localization of the indicated progenitors with specific microenvironment. Note that because of the abundance of sinusoids and CXCL12- and SCF-producing perivascular cells virtually all cells are proximal to both of these structures. Also note that most types of stem cells and progenitors have been imaged with a limited number of partner cells. Therefore, it is likely that some of the structures depicted overlap (e.g. both erythroid progenitors and HSC have been shown to localize to SCF-producing perivascular cells). The precise location of most multi and oligopotent progenitors remains unknown.

done recently for stepwise mapping of myelopoiesis (36). Alternatively, it might be possible to adapt iterative imaging methods. In these, the samples are stained and imaged with a set of fluorescent probes followed by removal of the fluorescence and staining and imaging with new fluorescent probes. Two of these methods, CODEX and IBEX, are able to resolve dozens of parameters using confocal microscopy (48, 49).

Precise mapping of differentiation will also require clonal fate-mapping to determine developmental relationships between progenitors and adjacent cells. Different studies have used confetti mice [in which cre recombination leads to expression of one out of four fluorescent proteins (50) to examine clonal relationships between cells of interest in the marrow (22, 51)]. However, the confetti model only allows simultaneous detection of a very limited number of fluorescent tags in discrete progenitor populations. A possible way of overcoming this limitation is single-cell spatial transcriptomics, which is developing at a breakneck pace. It might soon be possible to obtain transcriptomic data, track thousands of barcodes for clonal analyses, and obtain spatial information for single cells in the bone marrow (52).

What Are the Cells and Extracellular Matrix Structures Forming These Specialized Microenvironments and How Do They Function?

Answering these might require microdissection of the region of interest followed by transcriptomics analyses to interrogate the identity of the local cells. This technology is already available as shown by a study from the Van Galen lab demonstrating heterogeneity of growth factor production in different regions of the bone marrow (53).

After identification of the components of each local microenvironment the next step will be defining how they function in regulating the proximal progenitors. This has been accomplished by conditional Cre-mediated deletion of one cytokine or growth factor in the candidate cells. However, scRNAseq revealed extraordinary complexity of stromal cell types (53–55) whereas common Cre drivers available to the field target broad, heterogeneous, populations of stromal cells (55, 56). Development of new Cre^{ERT2} mouse models, specific for cells in local microenvironments -as done recently with *Oln-cre*^{ERT} mice to target the periarteriolar stromal cells that maintain common lymphoid progenitors (41)- will greatly facilitate answering these questions.

If Local Microenvironments Regulate Unique Stem/Progenitors What Regulates Progenitor Localization to These Structures?

One possibility is that the specialized microenvironment produces one or more chemotactic cues that selectively attract the desired progenitor. Alternatively, this process might be stochastic with progenitors migrating through the bone marrow transiently interacting with stromal components. This type of transient interactions was shown recently for HSC (24). Eventually, one of these interactions will be of sufficient strength and specificity to retain the progenitor in a specific

microenvironment. In this case the relative abundance of each local microenvironment will profoundly influence the likelihood of successful interactions. A third possibility is that the stem/progenitors themselves remodel local cells into a supportive microenvironment. This type of remodeling has been shown to occur in the zebrafish HSC niche (57). Distinguishing between these possibilities will require live imaging of specific subsets of progenitors. The major technical constrain will likely be the development of fluorescent reporter strains to allow visualization of unique progenitor subsets. Live bone marrow imaging has been done in the mouse calvarium [where the bone is thin enough to allow imaging with sufficient resolution (19, 22)] or by carving a "window" in leg bones to image the marrow within (24, 29, 58). Importantly, recent studies have shown differences in the frequencies of erythroid and lymphoid progenitors across different bones (42, 59). These would have to be considered when deciding which bones to study *via* live imaging.

What Is the Anatomy of Hematopoiesis and Local Microenvironment in Response to Insults?

Hematopoiesis is highly plastic and capable of sensing different insults and respond by quickly adjusting blood cell production to demand. Examples of this plasticity include hemorrhage which triggers emergency red blood cell production and infection which -depending on the infectious agent- can trigger emergency neutrophil, monocyte, and/or dendritic cell production. The bone marrow microenvironment plays critical roles in both sensing and orchestrating the progenitor response to infection [reviewed in (60)]. Importantly, inflammation and infection also profoundly remodel the sinusoidal network and perivascular stromal cells that maintain hematopoiesis and perturb stem cell localization within the marrow (60). Key open questions are a) whether hematopoietic stress responses use the same anatomical structures as normal hematopoiesis or instead depend on stress-specific anatomical cues and b) to what extend remodeling of sinusoids perturbs the anatomical structures that maintain the different progenitors. The most dramatic example of acute insult to the bone marrow is myeloablation. This eliminates not only hematopoietic cells but also the sinusoids and associated perivascular cells whereas endosteal regions and arterioles are more protected (6, 21, 61, 62). In this case the key open questions are: a) how are the local microenvironments restored? and b) what are the anatomical structures that support regenerative hematopoiesis? Identification of these will likely lead to novel therapies to promote restoration of blood cell production after myeloablation.

Chronic insults also lead to progressive remodeling of the microenvironment diminishing its capacity to support normal progenitors and -in some cases- hijacking it to promote pathogenesis. Examples of this pathogenic remodeling occur during physiological aging, leukemia, and other proliferative diseases (63, 64). Most studies have focused on determining how this remodeling perturbs HSC function. Little is known about how the different pathologies affect the structures that support more mature cells and whether protecting these structures can maintain normal hematopoiesis during disease.

CONCLUSION

Hematopoiesis in the bone marrow is spatially and regionally organized by specialized local microenvironments that support different types of stem cells and progenitors. The challenges in imaging the bone marrow tissue have limited progress (65). However, the future is bright. Adapting technological advances validated in other tissues -including live imaging, multiparameter microscopy, new reporter strains, and spatial transcriptomics- will allow systematic examination of blood production *in situ* to define how local cues from the microenvironment control normal and pathological hematopoiesis.

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DL conceived the manuscript who was written by all the authors. All authors contributed to the article and approved the submitted version.

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REVIEW
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Aging of the Hematopoietic Stem Cell Niche: New Tools to Answer an Old Question

Francesca Matteini[1,2], *Medhanie A. Mulaw*[3*] and *M. Carolina Florian*[1,2,4*]

[1] Stem Cell Aging Group, Regenerative Medicine Program, The Bellvitge Institute for Biomedical Research (IDIBELL), Barcelona, Spain, [2] Program for Advancing the Clinical Translation of Regenerative Medicine of Catalonia, P-CMR[C], Barcelona, Spain, [3] Institute for Molecular Medicine and Internal Medicine I, Ulm University and University Hospital Ulm, Ulm, Germany, [4] Center for Networked Biomedical Research on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain

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***Correspondence:**
M. Carolina Florian
mflorian@idibell.cat
Medhanie A. Mulaw
medhanie.mulaw@uni-ulm.de

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The hematopoietic stem cell (HSC) niche is a specialized microenvironment, where a complex and dynamic network of interactions across multiple cell types regulates HSC function. During the last years, it became progressively clearer that changes in the HSC niche are responsible for specific alterations of HSC behavior. The aging of the bone marrow (BM) microenvironment has been shown to critically contribute to the decline in HSC function over time. Interestingly, while upon aging some niche structures within the BM are degenerated and negatively affect HSC functionality, other niche cells and specific signals are preserved and essential to retaining HSC function and regenerative capacity. These new findings on the role of the aging BM niche critically depend on the implementation of new technical tools, developed thanks to transdisciplinary approaches, which bring together different scientific fields. For example, the development of specific mouse models in addition to coculture systems, new 3D-imaging tools, ossicles, and *ex-vivo* BM mimicking systems is highlighting the importance of new technologies to unravel the complexity of the BM niche on aging. Of note, an exponential impact in the understanding of this biological system has been recently brought by single-cell sequencing techniques, spatial transcriptomics, and implementation of artificial intelligence and deep learning approaches to data analysis and integration. This review focuses on how the aging of the BM niche affects HSCs and on the new tools to investigate the specific alterations occurring in the BM upon aging. All these new advances in the understanding of the BM niche and its regulatory function on HSCs have the potential to lead to novel therapeutical approaches to preserve HSC function upon aging and disease.

Keywords: aging, HSC niche, deep learning, bone marrow imaging, vessel remodeling, sinusoidal niche, arteriolar niche

INTRODUCTION

Hematopoietic stem cells (HSCs) were among the first stem cell types that found important clinical applications, and they are used in the laboratory and in clinic for more than five decades. Despite the huge interest and the clinical translation, it is still nowadays not possible to culture these cells and expand them in the lab, and one of the reasons for this pitfall is the importance of the *in vivo* microenvironment, which is critical to preserving the regenerative capacity of HSCs. The physiology of the HSC niche in adult mammals is complex and strictly linked to specific cell types, soluble and circulating factors, extracellular matrix components, and a quite complex three-dimensional architecture within the bone marrow. Importantly, the niche not only is a passive substrate but also exerts active functions in preserving the regenerative capacity of adult stem cells and in instructing their differentiation into progenitors. Recently, the investigation of the HSC niche upon aging revealed many unanticipated changes in the bone marrow (BM) microenvironment, which might play important roles in determining the reduction of the regenerative capacity of aged HSCs and be strongly implied also in disease progression, ranging from leukemia to myelodysplastic syndromes and to immunosenescence. This review focuses on recent work that contributed to identify major cellular players in the HSC niche and highlights the newly reported remodeling of the niche on aging. Finally, we focus on specific techniques and new computational-based approaches that are starting to be explored also in the context of the aging of the HSC niche.

THE BM NICHE SUPPORTING HSCs

The HSC niche is organized in a complex architecture, which comprises many different cell types, extracellular matrix (ECM) components, and soluble factors all involved in regulating HSC behavior. Despite the enormous advances in the understanding of the structure, function, and contribution of the BM niche in regulating HSCs, there are still many unknown aspects that require further elucidation. The emerging view tends to identify the HSC niche not as a unique and homogenous compartment but as a collection of dynamic subsets of micro-niches where different components contribute to regulate specific HSC functions. In line with this view, novel studies based on RNA sequencing and spatial transcriptomic approaches are highlighting the importance of the complexity in cell-type composition within the bone marrow (BM) niche (1, 2). Below, we will review the major niche cell types described to display an important support function for HSCs.

Endothelial Cells

BM endothelial cells (ECs), in collaboration with perivascular cells, form specialized microenvironments shown to be involved in the regulation of HSCs and hematopoietic progenitor cells (HPCs).

ECs have been identified as one of the biggest sources of pro-hematopoietic factors such as Angiogenin, Notch ligands Jagged1 (Jag1), Jagged2 (Jag2), Delta-like ligand 1 (Dll1), and Delta-like ligand 4 (Dll4), selectin E and, in particular arteries, are enriched in the expression of CXC chemokine ligand 12 (CXCL12) and stem cell factor (SCF) (1).

Sca1+ arterial endothelial cells (aECs) constitute more than the 23% of total BMECs and are found in arteries, arterioles, and transitional vessels (or type H vessels). These aECs present a peculiar elliptically elongated nuclear shape and express high levels of vascular endothelial-cadherin (VE-Cad) and Zonulin-1 (ZO1). Type H vessels, composed by ECs highly expressing CD31 and Endomucin ($CD31^{hi}$ and $Emcn^{hi}$ ECs), are a specific subset of capillaries exclusively localized in the endosteal region of the BM and promote angiogenic growth and osteogenesis by providing signals to osteoprogenitor cells (3). Like type H vessels which are exclusively endosteal-localized, the most abundant fraction of arteries in the BM is in close to the endosteum. Both type H vessels and arterioles display low permeability, preserving HSCs from the exposure to high levels of reactive oxygen species (ROS) (4). Taking advantage of whole-mount histological approaches combined with mathematical modeling, arteries and rare neural glial antigen 2-positive (NG2+) perivascular cells in proximity of the endosteum have been identified as the main niche cell types promoting HSC quiescence and involved in HSC retention within the BM (5, 6).

Most BMECs are $Sca1^{-}$ sinusoidal endothelial cells (sECs) and constitute type L vessels, which are characterized by low expression of CD31 and Emcn (3) and display a high permeability. sECs are associated with HSC mobilization, promoting their activation, and providing an exclusive area for mature leukocyte trafficking. In proximity of sinusoids, ROS levels are increased compared to arteriolar areas, and this feature enhances the migration capacity of HPCs (4). Intriguingly, sinusoids have been reported also as a specific localization site for non-dividing HSCs. Indeed, deep imaging of the BM showed that around 85% of Ki67-α-catulin-GFP+ c-kit+ HSCs are located within 10 μm from sinusoids, while Ki67 + α-catulin-GFP+ c-kit+ HSCs are mainly localized in proximity of the endosteum (7), suggesting the involvement of the sinusoidal niche in promoting HSC quiescence. This apparent discrepancy observed by Acar et al. compared to previous work identifying the peri-arteriolar/endosteal niche as the major site preserving HSC quiescence (5) can be explained considering that the staining used to identify HSCs by Kunisaki and colleagues (CD150+ CD48- CD41- lineage cells) differs from the one used by Acar and colleagues (that takes advantage of the $\alpha\text{-catulin}^{GFP}$ mouse model to identify HSCs as α-catulin-GFP+ c-kit+ cells). Moreover, despite the same post-imaging process used for evaluating the distance of HSCs from arteries and sinusoids, the bones used to perform the analysis, the protocol used to perform the staining, and the imaging techniques are not the same, while both authors rely on Ki67 to identify the non-dividing fraction of HSCs. Overall, the data might suggest that both arteries and sinusoids can be a preferential site for quiescent HSCs, hinting at the possible existence of different strategies played by arterial and sinusoidal ECs to promote HSC quiescence.

EC-HSC Interaction: Focus on Notch Signaling

The direct interaction between ECs and HSCs is important to maintain and expand the HSC pool by triggering Notch activation in stem cells (8). Notch signaling is a fundamental player in the specification of HSCs during development (9, 10) and also in the regulation of adult HSCs where it is known that Notch signaling activation maintains HSC self-renewal potential (11), while its inhibition impairs HSC maintenance (12).

ECs express many Notch ligands (1, 8). For example, Jag1 and Jag2 are expressed in ECs upon angiogenic stimuli (8). The endothelium-specific knockout of Jag1, while not affecting the vascular system, exhausts the HSC pool and impairs HSC repopulation ability after transplantation and BM reconstitution after myeloablation. These data strongly indicate a key role of endothelial Jag1 in regulating HSC quiescence and self-renewal (8, 13). Endothelial Jag2 does not influence HSC homeostasis but plays a key role in regulating HSC function after myeloablation. Specifically, the deletion of Jag2 in endothelial cells causes a fast HSC exhaustion after both 5-fluorouracil (5FU) treatment and γ-irradiation (14).

The endothelial-specific inducible knockout of Dll1 does not affect any hematopoietic populations, while the endothelium-specific inducible deletion of Dll4 causes the expansion of myeloid progenitors and the reduction in the frequency of common lymphoid progenitors (CLPs), indicating that endothelial Dll4 expression regulates lymphoid lineage differentiation (1).

Of note, Notch signaling regulates HSC function also by promoting EC regeneration. The endothelium-specific deletion of the Notch1 transcriptional activation domain (TAD) in mice causes a severe reduction of HSCs and progenitor cells in BM after myelosuppression, due to increased apoptosis of ECs. The increased apoptotic rate is linked to the EC insensibility to HSC- and HSPC-dependent Angiopoietin1 (Ang1) stimulation. In control conditions, Ang1 triggers Tie2 activation, which reinforces Notch signaling in ECs and enhances Notch ligand expression, thus improving the HSC-dependent bone marrow repopulation after injury (15).

Mesenchymal Stromal Cells and Perivascular Cells

Mesenchymal stromal cells (MSC) are rare non-hematopoietic BM cells characterized by the ability to form multipotent self-renewing mesenspheres and to self-renew in serial transplantations. These cells are identified by the expression of the intermediate filament nestin (Nes), and this aspect has been used to generate mouse models to study these cells and their contribution to the regulation of HSCs. Different groups developed similar mouse models using the Nestin gene to express the Cre recombinase and trace MSCs (NesCre mice). However, these mouse models do not overlap precisely and they were shown to target different cell types [see ref. (16, 17) for an extensive review]. Further, of Frenette's group derived from NesCre mice a specific mouse model expressing the green fluorescent protein (GFP) under the control of the regulatory elements of the nestin promoter (Nes-GFP+ mice) (18). Based on GFP expression levels, Nes-GFP+ cells can be classified into rare Nes-GFP^{bright} cells, exclusively localized at arteries, and into more abundant Nes-GFP^{dim} cells, prevalently associated with sinusoids (5). Nes-GFP cells are innervated by noradrenergic nerve terminals and respond to this stimulation by retaining HSCs into the BM and promoting HSC and progenitor cell homing by secreting CXCL12, c-kit ligand (c-kitL), interleukin-7 (IL7), angiopoietin-1 (ANG-1), and osteopontin (OPN) (18).

Pericytes are perivascular cells displaying mesenchymal stem cell features, which have also been described as niche-supporting cells. Classically, pericytes have been divided into NG2+ cells, shown to overlap with Nes-GFP^{bright} MSC (19) and into leptin receptor-positive (LepR+) cells (20), largely coinciding with the CXCL12-abundant reticular (CAR) cell population and expressing CXCL12 and SCF (21). NG2+ pericytes are a rare cell population, mainly localized at arteries and arterioles and promoting HSC quiescence (5). LepR+ cells are mainly associated with sinusoids (6, 21, 22) and have been shown to control the stem cell pool size through CXCL12 (6) and HSC mobilization through SCF secretion (23). A recent work based on scRNA-seq data analysis deciphered and highlighted the existence of an additional level of complexity in perivascular cell organization and in their supportive function. Clustering analysis defined NG2+ cells as NG2+ and Nes+ MSC hierarchically located at the apex of differentiation into CAR cells, osteoblast, and fibroblast. Of note, CAR cells appear to include both Adipo-CAR cell population, highly expressing leptin receptor (LepR), and Osteo-CAR cells, highly expressing osterix (Sp7) and displaying low LepR levels. Interestingly, these two CAR cell subtypes contribute to HSC regulation by different cytokine secretory patterns, and due to their distinct localization, HSC function is distinctly influenced based on the specific localization within the BM niche (2).

Osteolineage Cells

Osteoblasts and spindle-shaped N-Cadherin+ (N-cad+) osteoblastic cells are located into the trabecular bone region of the endosteum and were the first cells identified to functionally support HPCs (12, 24). Osteoblast-secreted ANG-1 (25) and OPN (26) maintain the HSC pool by promoting HSC quiescence, while parathyroid hormone (PTH) promotes HSC expansion through a Jag1-dependent activation of Notch signaling in HSCs (12). Osteoblast conditional ablation by ganciclovir-dependent activation of the herpes virus thymidine kinase (TK) gene under the control of a 2.3-kb fragment of the rat collagen 1 type I promoter (Col2.3TKmice) leads to a block in hematopoietic lineage progression with a reduction in lymphoid, erythroid, and myeloid progenitors, subsequently followed also by HSC depletion in the BM (27). Osteoblast-specific deletion of CXCL12 in mice showed that this cell component of the endosteal niche is the main effector in influencing lymphoid differentiation (6). Further, the *in vivo* lineage tracing of N-cad+ bone marrow stromal progenitor cells demonstrated that this supportive progenitor population contributes to osteoblast, adipocytes, and chondrocytes, which maintain the most

quiescent HSC fraction by providing SCF and by protecting them from chemotherapeutic stress (28).

Interestingly, the endosteal peri-arteriolar niche is recently emerging as a specific lymphoid differentiation-promoting site. Indeed, in a very recent work Shen and colleagues demonstrated that peri-arteriolar LepR+ Osteolectin+ osteoblast progenitor cells promote CLP expansion and differentiation by secreting SCF, as the specific deletion of SCF from these cells strongly reduces CLP frequency in BM (29).

Adipocytes

The bone marrow adipose tissue (BMAT) represents 10% of the total body adipose tissue, and, interestingly, BMAT strongly differs from white adipose tissue (WAT) and brown adipose tissue (BAT). The BMAT transcriptomic profile clusters apart from WAT and BAT. Additionally, BMAT displays a higher glucose uptake and a decreased insulin responsiveness (30). BMAT is one of the most affected compartments upon aging, expanding up to occupy 50% of the BM cavity (31). Traditionally, adipocytes are considered negative regulators of the BM microenvironment and HSC function, in contraposition with osteoblasts which exert a positive function on HSC (32). Recent evidence suggests a novel and positive role for adipocytes in promoting HSC maintenance. Mattiucci and colleagues demonstrated that BM adipocytes are closely related to BM-MSCs rather than to other adipocyte populations (such as the subcutaneous adipose tissue population) and that these cells support HSC survival by expressing cell-specific cytokines, like interleukin 3 (IL3), and other MSC-overlapped cytokines (33). In line with this finding, Zhuo and colleagues showed that SCF adipocyte-specific ablation reduces mouse survival by causing HSC deficiency after myeloablation, indicating a positive role of adipocytes particularly in promoting hematopoietic reconstitution after myeloablation (34).

β-Adrenergic Sympathetic Stimulation

The sympathetic nervous system (SNS) in the bone marrow has been shown to innervate both arteries and peri-arterial Nes-GFP+ stromal cells (5). The SNS innervation by β2 and β3 adrenergic receptors (ADR) plays a key role in the circadian mobilization of HSCs. CXCL12 is the major chemokine regulating HSC mobilization and displays an inverse pattern with HSC circadian mobilization. Interestingly, isoprenaline (a non-selective β-adrenergic agonist) treatment of bone marrow stromal cell line reduces CXCL12 expression levels, acting through the regulation of the levels of the transcription factor Sp1, and BM denervation critically alters CXCL12 circadian fluctuation in mice (35). CXCL12 levels are regulated by β3- but not β2-ADR, as both the selective β3-agonist (BRL37344) and the selective β3 antagonist (SR59230A) respectively increase and reduce the CXCL12 expression levels in the MS-5 stromal cell line. This indicates that β3-adrenergic stimulation controls the egress of HSCs from the BM (35). On the contrary, β2-ADR stimulation is involved in the reset of the local circadian clock by upregulating the *Per1* gene in the MS-5 stromal cell line, as the β2-selective agonist (clenbuterol) treatment induces *Per1* expression in the same cell line, indicating that β2-adrenergic stimulation is involved in regulating HSC homing into the BM (36).

Sympathetic innervation has been shown also to regulate HSPC and leukocyte circadian egress and homing in mice. Murine HSPCs and leukocytes preferentially home to the BM at night while during day they are released into the systemic circulation. During the night, the parasympathetic nervous system (PNS) through cholinergic stimulation reduces the egress of HSPCs and lymphocytes from BM by buffering β3-adrenergic stimulation and increasing β2-adrenergic signal, which promotes homing of hematopoietic cells by increasing the expression of vascular adhesion molecules. Conversely, during the day the depression of the β2-noradrenergic activity promotes the β3-AR-CXCL12–dependent exit of hematopoietic cells from the BM (37).

Megakaryocytes and Macrophages

HSC progeny is an important player in regulating HSC function, and evidence shows the involvement of megakaryocytes, macrophages, neutrophils, and regulatory T cells, among others [see ref. (38) for a detailed review].

So far, megakaryocytes (MKs) are the ones mainly implied in HSC regulation and young HSCs are often found in close proximity to MKs (39, 40), which is interestingly not observed upon aging (40, 41).

MKs' control on HSC function is dual: it has been shown that MKs control HSC quiescence by the release of specific factors like CXCL4, as both global MKs and CXCL4 depletion cause an expansion of the HSC pool and an increase in their proliferation (39). Additionally, MKs promote HSC quiescence through TGF-β secretion, which activates the SH2 domain–containing protein tyrosine phosphatase SHP-1 (42). TGF-β also regulates HSC quiescence by promoting SMAD2/3 phosphorylation in HSCs (43). It would be fascinating to verify if these two TGF-β-mediated signalings cross talk in regulating HSC quiescence. Interestingly, MKs also play a role in promoting HSC activation and proliferation after myeloablation, as MK deletion in Pf4-cre; iDTR mice by diphtheria toxin (DTR) treatment before 5FU administration causes a severe impairment of HSC expansion and BM repopulation. Upon stress stimuli, MKs start to express fibroblast grow factor 1 (FGF1), which overcomes TGF-β signaling and promotes HSC activation and proliferation (43).

Of note, the MK and HSC interplay is not unidirectional. A quiescent subpopulation of HSCs, characterized by the expression of platelet integrin CD41 (CD41), has been identified as primed toward myeloid differentiation and strongly increases upon aging (44, 45). Deletion of CD41 in HSCs leads to hematopoietic defects with loss of HSC quiescence and insensitivity of TGF-β signaling (45), supporting the existence of a positive feedback loop between CD41+ HSCs and MKs in regulating HSC quiescence and myeloid differentiation.

Macrophages have been shown to play a key role in regulating HSC quiescence and retention in the BM. DRAC+ macrophages have been shown to regulate HSC quiescence through the activation of the TGFβ1-Smad3 pathway downstream of CD82/KAI1. CD82/KAI1 is predominantly expressed in LT-HSCs, and when knocked out, HSC proliferation increases.

Depletion of DRAC (CD82/KAI1-binding partner) expressing macrophages leads to a reduction of CD82/KAI1 levels in HSCs, increased proliferation, and differentiation (46). CD169+ macrophages constitute another example of cells regulating HSC retention in the BM. In CD169-iDTR mice, it has been shown that the depletion of CD169+ macrophages after DTR treatment causes an increase of HSCs and progenitor cells in peripheral blood (47). Ablation of CD169+ macrophages in CD169-iDTR mice upon DTR administration significantly reduced not only the HSC number in the BM but also the HSC, LSK, and GMP frequencies in the spleen. In line with their function in the BM, macrophages are also responsible for the retention of HSCs into the spleen, selectively through VCAM1-mediated signaling. Knockdown of VCAM1 expression in macrophages, using siRNA targeting VCAM-1 within macrophage-avid lipidic nanoparticles, causes reduced retention of splenic HSCs, LSKs, and GMPs, without affecting HSC retention into the BM (48).

AGING OF THE BM NICHE: PHENOTYPIC AND FUNCTIONAL REMODELING

Aging is a very complex physiological process that causes substantial changes in the whole organism together with tissue-specific changes in gene expression and cell composition (49). In particular in the BM, upon aging the HSC pool is expanded, and HSCs display a skewed differentiation to myeloid progenitors (50) at the expense of the lymphoid ones (51) and an impaired regenerative potential (51). The analysis of the mitotic history of HSCs and progenitors cells upon aging highlighted that HSCs and MPPs maintain their quiescent nature in a steady state, while GMLPs increase their proliferation rate (52) in line with an increase in their self-renewal potential at the expense of differentiation. Upon aging, HSCs display also loss of cell polarity (53), an intrinsic increase in Wnt5a non-canonical signaling (54), deregulated autophagy (55, 56), deregulation of the mitochondrial unfolded protein response (57), downregulation of mitochondrial acetylation mediated by SIRT3 (58), epigenomic alterations (59–62), and increased symmetry of epigenetic division (63), indicating that aging directly affects HSC function independently from the BM niche: a phenomenon described as "intrinsic" HSC aging and extensively reviewed elsewhere (64, 65). Interestingly, transplantation of young LT-HSC into aged recipient mice induces the expansion of the stem cell pool (66) and a differentiation skewing toward the myeloid lineage (67), while transplantation of aged HSCs into young recipients has been shown to rejuvenate their transcriptomic profile, despite the poor contribution to progenitor cells and the maintenance of a myeloid differentiation bias (68). Interestingly, the transplantation of rejuvenated HSCs into an aged niche restrains their rejuvenated function (69), suggesting again that BM microenvironmental aging contributes to promote an aging-associated phenotype in HSCs. This niche-dependent aging phenotype is called "extrinsic" HSC aging [reviewed in ref. (38, 70)]. Recently, this concept has been further dissected by analyzing the contribution of the middle-aged BM microenvironment, which identified the decrease in IGF1 BM levels as an essential aging-promoting factor for both HSCs and niche cells. Restoring the IGF1 signal has been shown to rescue Cdc42 and tubulin polarity, to reduce γH2AX focus and myeloid differentiation skewing in middle-aged LT-HSCs (71). In contrast, previous reports identified in the fasting-induced decrease of IGF1-dependent stimulation of PKA activity as a key factor to promote HSC self-renewal, balanced differentiation, stress resistance, and regenerative capacities after chemotherapy in aged mice (72). This apparent disagreement can be explained considering the downstream pathways activated by IGF1. The fasting-induced IGF1-mediated pathway has been described to pass through PKA activation (72), while IGF1 effects observed upon aging promote mTOR pathway activation (71). Interestingly, the mTOR pathway is dependent on nutrients and growth factors (73), suggesting more broadly that probably there is still more to understand about the regulation of HSC function by IGF1 during aging. Upon aging, many different niche compartments undergo degeneration and remodeling, affecting, on different levels of HSC behavior and function.

Vascular Remodeling

The BM vascular niche is profoundly changed upon aging. Despite the preservation of the endothelial area occupancy and the overall vascular volume, the frequency of endothelial cells (ECs) is reduced during physiological aging (40). These changes not only are associated with a vascular remodeling including reduction of arteries and type H vessel density but also directly affect HSC behavior (74).

In a young niche, the small arterioles located into the endosteal compartment in specific association with NG2+ pericytes represent the main quiescent niche for HSCs (5). HSCs in proximity (within 20 μm of distance) to arteries and NG2+ pericytes display high retention of EdU and negative Ki67 staining. In agreement, upon induced activation or mobilization, respectively by polyinosinic-polycytidylic acid (Poly(I:C)) or G-CSF, the quiescent HSC fraction changes its localization relative to Nes+ perivascular cells and their proliferation rate increases. Interestingly, the conditional deletion of NG2+ pericytes impairs HSC long-term repopulation ability by inducing HSC cycling. This indicates that the proximity to NG2+ pericytes preserves HSCs from genotoxic insults (5). During aging, arteries and arterioles degenerate (75), decreasing their length and orientation, which becomes disorganized and not supportive anymore for the preservation of HSC quiescence (40, 76) (**Figure 1A**). Type H capillaries are also affected by aging, and their number strongly reduces over time, contrarily to sEC number which is not altered in aged mice (3). Recently, it was shown that in young mice arteries and arterioles are characterized by the expression of netrin-1 (ligand of neogenin-1), which is decreased upon aging. Neogenin-1 is exclusively expressed by quiescent HSCs and promotes the maintenance of self-renewal and quiescence. However, the aging-dependent decline of netrin-1 expression in

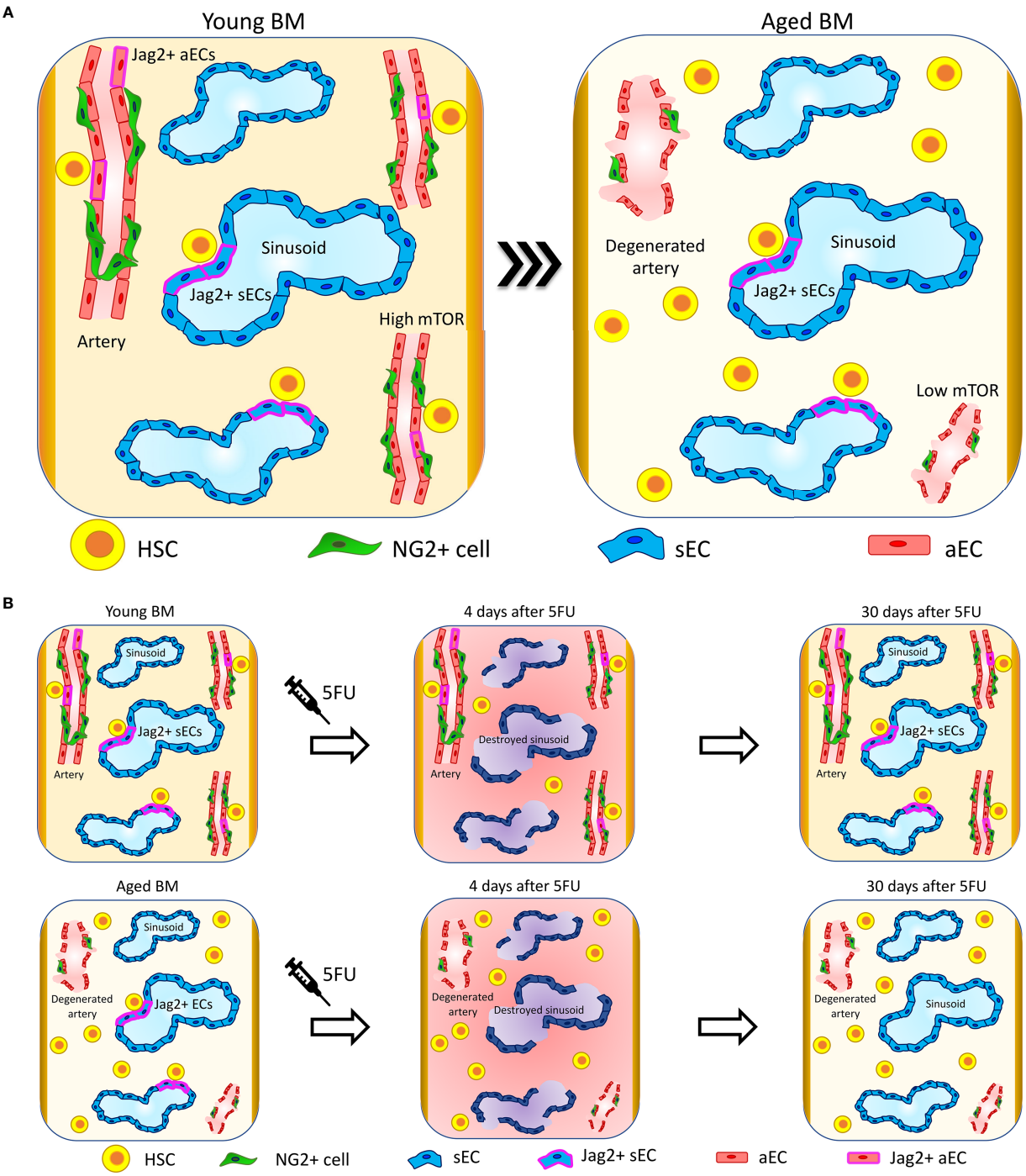

FIGURE 1 | Vascular niche upon aging. **(A)** Upon aging, arteries and arterioles degenerate, changing their orientation and reducing their length (41, 75). Interestingly, specific HSC-supporting signalings are lost at the arteries. In particular, Jag2 expression is lost at arterial ECs (aECs) (40) and mTOR is downregulated (77). The sinusoidal niche preserves its structure and functionality, and sinusoidal ECs (sECs) maintain the signaling involved in the support of HSC functions. In line with these changes, HSC localization in proximity to arteries and arterioles is reduced and HSCs migrate far away. Conversely, HSCs retain their localization in proximity to sinusoids (40). **(B)** Four days after 5FU treatment, sinusoids are destroyed and there is a global increase in inflammation in both young and aged mice. Thirty days after 5FU administration, young mice recover sinusoidal vascular integrity and Jag2 expression while aged mice show only a partial restoration of sinusoidal vascular integrity and almost no recovery of Jag2. These correlate with aged HSCs localizing significantly further from sinusoids compared to control not 5FU-treated and presenting with increased clustering (40).

arteries and arterioles impairs this signaling axis, leading to HSC expansion and to the reduction of their regenerative potential (76). HSCs divide rarely, and in SCL-tTAxH2B-GFP mice, the less dividing HSCs retain the pulsed histone H2B-green fluorescent protein (H2B-GFP) label *in vivo* after doxycycline (Dox) treatment and for this are defined as label-retaining HSCs (LR-HSCs) (78, 79). The analysis of LR-HSC localization in aged mice demonstrates further that quiescent HSCs in aged mice are mainly localized at sinusoids (40), in line with the expansion of the non-endosteal neurovascular niche at the expense of the endosteal niche (41).

In young mice, the sinusoidal network occupies around 30% of the total BM volume as assessed by whole-mount histological analysis in long bones (5). Contrary to what happens to arteries and arterioles, sinusoids are largely not affected by aging and maintain the same volume occupancy, length, diameter, and orientation of the vessels, as observed in young samples (40) (**Figure 1A**).

Bone marrow ECs display a high expression of the Notch ligand Jag2 in comparison to the same cells localized in other tissues (14), and Jag2 expression is retained upon aging (40). While in young animals the endothelial-specific Jag2 knockout alters neither the proliferation rate of LT-HSCs nor their lineage composition in the BM or peripheral blood (PB) in steady state (14), the *in vivo* blockade of the endothelial Jag2 signal in aged mice causes an increase in HSC proliferation and clustering, and aged mice display a physiologic reduction of Jag2 expression at aECs (40) (**Figure 1**). It has been shown that Jag2 expression is upregulated in the recovering phase after BM myelosuppression by both 5-fluorouracil (5-FU) and lethal γ-irradiation and promotes HSPC expansion upon BM reconstitution (14). The administration of the chemotherapeutic agent 5FU, which induces in addition to myeloablation a specific sinusoidal damage, highlights critical differences in niche regeneration when comparing young versus aged mice. Indeed, in young animals there is a complete niche reconstitution and Jag2 at sinusoids is re-expressed after 30 days from treatment. In aged mice, the sinusoidal niche damage is persistent and HSC localization is affected, which results in impaired hematopoietic reconstitution and decreased overall survival after 5FU (**Figure 1B**) (40).

As for other Notch ligands, Jag1 expression in ECs regulates HSC homeostasis and regeneration capacity (13), while EC-expressed Dll4 inhibits the activation of the myeloid transcriptional program in HSCs (1). However, the endothelial expression of these markers is not affected upon aging (40).

Notch signaling has been also demonstrated to play a key role in regulating EC proliferation and artery and type H vessel formation. The endothelium-specific overactivation of Notch signaling in aged mice increases arterial and type H vessel density, regulating HSC number. However, the endothelium-specific Notch overactivation by deletion of the *fbxw7* gene mediating Notch proteasomal degradation does not overcome the intrinsic aging of HSCs. Competitive transplantation of HSCs isolated from aged EC-specific Notch-overactivating mice do not show increased regenerative capacity nor rescuing of DNA damage accumulation (γH2AX foci) in HSCs, both classic hallmarks of intrinsic HSC aging (74).

Additional signaling pathways have been identified to regulate the functional interplay between ECs and HSCs as for example the mTOR pathway. Upon aging, ECs downregulate mTOR signaling, which induces a reduction in their support to hematopoiesis. Specific deletion of mTOR in ECs ($mTOR^{(ECKO)}$ mice) leads to loss of α-tubulin polarity, accumulation of γH2AX foci, and change in the transcriptome of HSCs, and transplantation of young HSCs in $mTOR^{(ECKO)}$ mice is sufficient to induce an aged phenotype in stem cells (77).

The reduced expression of heme oxygenase 1 (HO-1) in ECs and CAR cells upon aging has also been reported to impair HSCs. Upon HO-1 reduction, ECs and MSCs reduce their release of hematopoietic factors, promoting the acquisition of an aged phenotype in HSCs. Transplantation of young HO-1 wild-type HSCs into HO-1-deficient mice leads to a premature aging phenotype in transplanted cells, with the exhaustion of their regenerative potential and inability to reconstitute the BM upon secondary transplantation (80).

Collectively, this evidence indicates the importance of the vascular niche in supporting HSC quiescence, function, and stress response during aging, highlighting the importance of some specific endothelial-dependent pathways in preserving HSC regenerative potential.

Endosteal Niche Degeneration

The endosteum in young mice strongly contributes to the maintenance of HSC quiescence (12, 25, 26), and upon aging the degeneration of the bone is dramatically affecting the endosteum and the arteriolar and peri-arteriolar compartment. Nes-GFP^{bright} cell frequency is reduced at the endosteum and increased in the proximity of the central vein. Moreover, these cells reduce their colony-forming ability, while the more abundant Nes-GFP^{dim} cells do not change their localization at sinusoids (75) (**Figure 2**). Interestingly, the analysis of HSC localization showed that the frequency of HSCs in proximity to Nes-GFP^{bright} cells is reduced while there are no significant alterations in HSC proximity to Nes-GFP^{dim} cells (40). In the stromal compartment, MSC number and their colony-forming activity are reduced during aging. Moreover, these cells downregulate CXCR4 expression, which leads to an increased ROS production and DNA damage accumulation (**Figure 2**). Competitive transplantation experiments showed that deletion of CXCR4 in MSCs is sufficient to drive the acquisition of an aged phenotype in young HSPCs, which show a reduced BM repopulation capacity and myeloid differentiation skewing. Interestingly, *ex-vivo* pretreatment of CXCR4-deficient MSCs with the ROS scavenger N-acetyl-L-cysteine (NAC) restores normal ROS levels in MSCs and ameliorates the phenotype of HSPCs (82). In line with these changes, intravital multiphoton microscopy analysis of the BM of young and aged mice showed the decrease of the bone matrix coupled with a reduction in the frequency of mature osteoblasts and the expansion of the mesenchymal cell population (81). Upon aging, MSCs increase IL6 and TGF-β expression and the TGF-β pathway has been

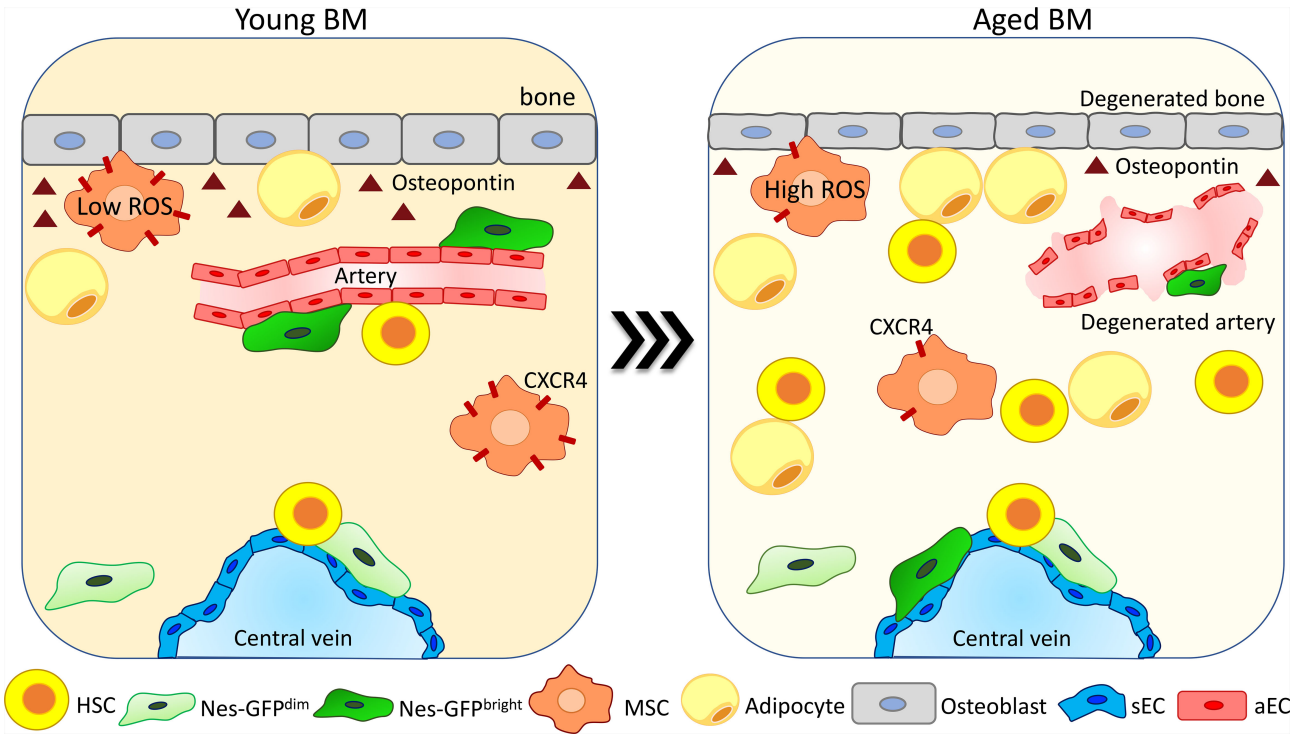

FIGURE 2 | Degeneration and reorganization of the endosteal niche upon aging. The endosteal niche is one of the most affected compartments upon aging. The bone area is compromised due to a reduction in the number of osteoblasts and in osteopontin (OPN) release (26, 66, 81). Arteries and arterioles degenerate, becoming no more able to support HSC function (40, 41, 75). NES^{bright} cells change their localization from arteries moving in proximity to sinusoids, while NES^{dim} cells do not change their localization (40, 75). The MSC population is increased upon aging (81). MSCs reduce CXCR4 expression and increase ROS production, reducing their colony-forming ability and becoming less supportive for HSC maintenance (82). Adipocytes increase in number due to an enhanced pro-adipogenic differentiation of MSCs (83), promoting HSC myeloid differentiation skewing (84).

demonstrated to be a key regulator of HSC aging, as its inhibition in aged HSCs reverts their enhanced platelet lineage bias and restores the lymphoid output upon transplantation (85).

During aging in human bones, the number of adipocytes is increased and correlates with a change in their milieu of secreted cytokines (86) (**Figure 2**). Studies conducted in mice showed that the expansion of adipocytes upon aging is due to a pro-adipogenic differentiation shift of osteo-adipogenic mesenchymal precursor cells, causing a reduction of hematopoietic progenitors and HSC number and repopulation capacity (83). Interestingly, a recent paper showed that the fraction of CD34+ HSPC as well as the number of differentiated myeloid cells in proximity to adipocytes is increased in the BM of aged individuals, suggesting a possible role of adipocytes in the increase of myeloid cells during aging by promoting myeloid differentiation skewing (84).

In line with changes in the adipogenic population, osteoblast (defined as CD45, Ter119, CD31, Sca1, and CD51+ cells) frequency in the BM is reduced upon aging, as well as their production of OPN (**Figure 2**). This has been shown to confer an aged phenotype to HSCs (26), and thrombin-activated OPN treatment rescues aging-related HSC phenotypes like loss of cell polarity and myeloid differentiation skewing (66). An additional study performed using OPN knockout mouse models displays also that OPN regulates the repopulation ability of aged HSCs upon transplantation (87).

Recently, new data showed that the frequency of LepR+ Osteolectin + osteogenic progenitors decreases upon aging, contributing to the reduction of the amount of CLP within the BM in the elderly. Strikingly, the reduction of LepR+ Osteolectin + osteogenic progenitors observed on aging is dependent on a change in the mechanosensing of the endosteal environment because, in concomitance with physiological or induced bone demineralization, the LepR+ Osteolectin + osteogenic progenitor population is reduced, as well as CLPs, without any significant change occurring in the frequencies of HSCs, MPPs, GMPs, MEPs, or CMPs (29). Therefore, it is likely that the aged HSC-intrinsic myeloid skewing is paralleled by a niche-dependent age-associated degeneration of the lymphoid niche.

Sympathetic Adrenergic Signal Alterations

Sympathetic adrenergic signals play a key role in regulating homing and egress of HSCs and hematopoietic cells from the BM (88), and it has been shown that the SNS innervation is strongly changed upon aging (**Figure 3**). However, there is not a clear consensus on the nature of the changes occurring to adrenergic fibers *per se* during aging, and analyses on the

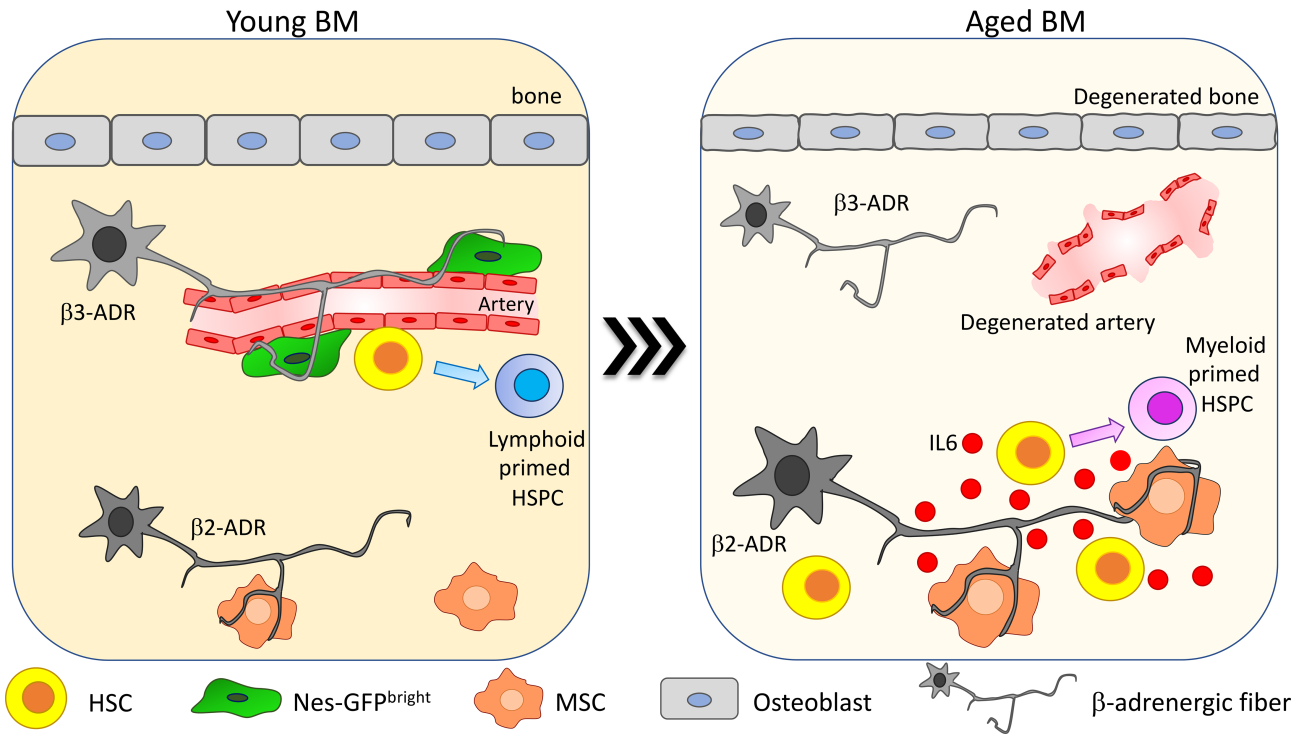

FIGURE 3 | β-Adrenergic signaling alteration upon aging. β-Adrenergic innervation is strongly altered upon aging (41, 75). In young mice β3-adrenergic stimulation is predominant and promotes lymphoid differentiation while β2-adrenergic stimulation is involved in regulating myeloid differentiation. Upon aging, β2-adrenergic stimulation is increased and promotes MSC secretion of IL6, thus increasing HSC myeloid differentiation skewing.

changes in sympathetic adrenergic innervation showed contrasting results.

In Maryanovich et al., the analysis of adrenergic fibers by staining for tyrosine hydroxylase revealed a strong and general reduction in nerve density in old BM, coupled with a reduction of perivascular Nes-GFP^{bright} cell innervation (75). Consistently, also synaptic contacts between adrenergic nerve fibers and BM-innervated cells are reduced upon aging. Taking advantage of hind-limb denervation to recapitulate the decrease of SNS stimulation occurring upon aging, it has been shown that after denervation HSCs increase their proliferation and lose their polarity for Cdc42 and tubulin and myeloid-biased CD41+ HSCs are expanded (75). Moreover, upon transplantation, HSCs collected from denervated bones display reduced engraftment compared to the non-denervated counterpart. Interestingly, β3-ADR deletion in young mice causes a premature aging phenotype and treatment with the β3-ADR agonist BRL37344 is able not only to rejuvenate HSCs by improving their engraftment potential upon transplantation and normalizing their differentiation skewing but also to rescue the acquisition of the aged phenotype in HSCs after hind-limb denervation. These findings suggest that β3-adrenergic stimulation is one of the main players in maintaining HSC regenerative potential (75).

Conversely, a more recent paper described an increase in SNS innervation upon aging. Taking advantage of the whole-mount analysis of thick-bone sections, Ho and collaborators demonstrated an increase up to 2.5 times in SNS innervation with aging in both flat and long bones, in association with a reduction of the endosteal niche. This change in adrenergic stimulation promotes myeloid differentiation skewing through the enhanced secretion of IL6 from BM stromal cells. IL6-increased secretion is triggered by the increased β2-ADR-mediated stimulation. In line with the described role for β3-adrenergic stimulation in maintaining HSC function, HSC frequency and myeloid progenitor differentiation are increased, and lymphoid differentiation is reduced in β3-ADR knockout mice (**Figure 3**). Transplantation of HSCs isolated from the progeria mouse model bearing mutation in the gene codifying for the nuclear envelope protein LaminA/C (*Lmna*$^{G609G/G609G}$) into healthy recipients did not recapitulate the aged phenotype observed into the progeria mouse model, while chronic treatment with the β3-ADR agonist ameliorated the aged phenotype observed in *Lmna*$^{G609G/G609G}$ mice, reducing the HSC frequency into the BM. Altogether, these data support a key role for the niche and in particular for β3-adrenergic stimulation in regulating the premature aging phenotype observed in the context of LaminA/C mutation (41).

Intriguingly, it has been shown that knockout of LaminA/C alters the epigenetic and chromatin architecture of HSCs similarly to what was observed in aged HSCs, which also present with very low levels of LaminA/C compared to young stem cells (59, 60). It would be therefore interesting to understand if the SNS in the BM microenvironment can impact on the epigenetic and chromatin architecture of HSCs.

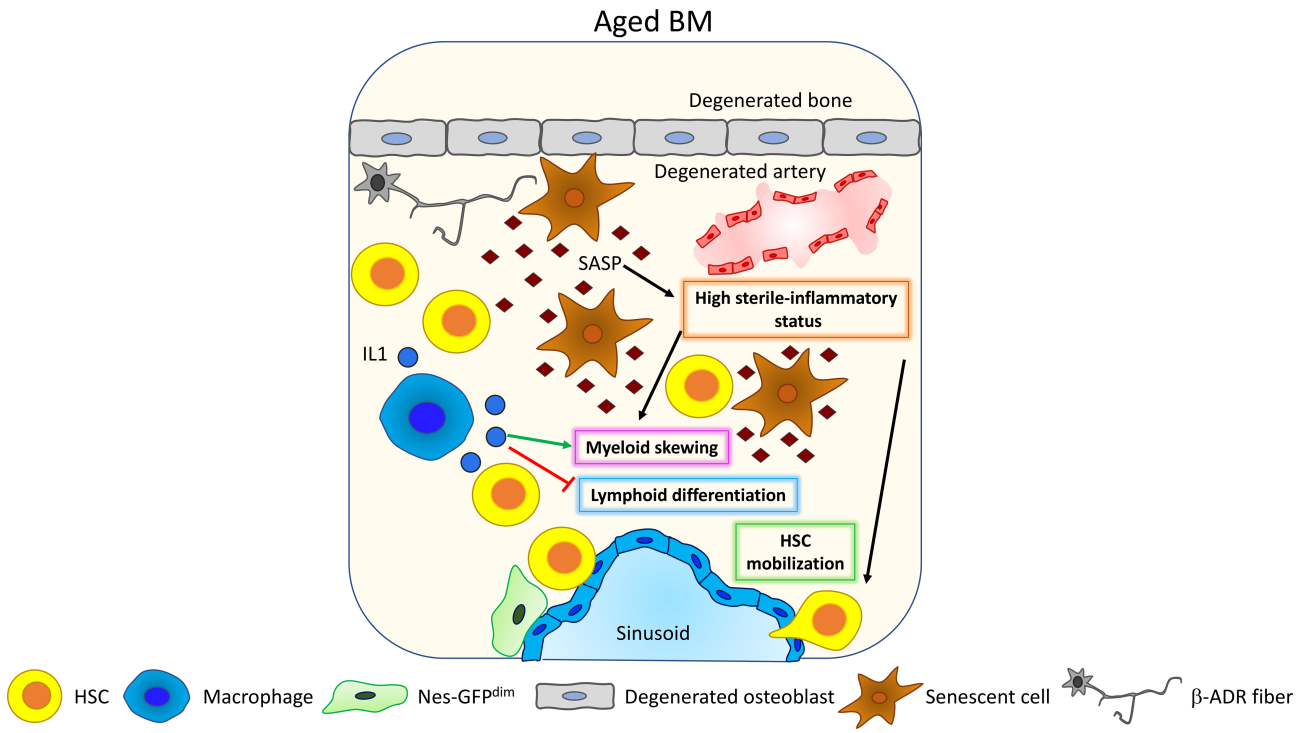

FIGURE 4 | Inflammaging. Upon aging, senescent cells accumulate and acquire the so-called senescent-associated secretory phenotype (SASP) (89). SASP contains different pro-inflammatory factors that increase the general level of sterile inflammation within the BM, promoting HSC myeloid differentiation skewing (81) and HSC mobilization (91, 92). Interestingly, HSC myeloid differentiation skewing is also affected by macrophage's activity. Upon aging, macrophages increase their release of IL1 thus promoting HSC myeloid differentiation skewing (93) and inhibiting lymphoid differentiation (94).

Despite the absence of a consensus on the extent of the alteration of the SNS upon aging, it is clear that this niche cell type strongly affects HSCs, contributing to the aging-associated myeloid skewing.

Inflammaging

One of the major changes occurring upon aging in the BM is the insurgence of a low-grade inflammatory state developed in absence of any triggering infection defined as "sterile inflammation". This systemic, chronic, and low-grade inflammation in the BM occurring during aging is termed "inflammaging" (89), and it has been postulated as one of the major stimuli promoting HSC aging and lymphoid to myeloid differentiation skewing (90). Inflammaging is mainly driven by senescent cells that accumulate upon aging (**Figure 4**). ChIP-seq analysis coupled with machine learning approaches hinted at alterations of the transcriptional and epigenetic landscape as the primary driver of the upregulation of the inflammatory response occurring upon aging (95). Senescent cells are characterized by a senescence-associated secretory phenotype (SASP), which refers to the secretion of pro-inflammatory molecules including chemokines and cytokines, bioactive lipids, and exosomes. As soon as the roles of senescent cells and SASP were identified, new classes of drugs were developed to selectively kill senescent cells (senolytics or senolytic drugs) or to inhibit the inflammatory function of the SASP components (senomorphic drugs) (96, 97). Targeting senescent cells, inflammation and SASP with senolytic drugs may represent a powerful rejuvenation tool, since a reduction of circulating levels of inflammatory cytokines has been associated with increased lifespan in several models and has also been shown to improve aged HSC function (98–101). In a recent work, Helbling and collaborators confirmed in mice the increased transcription of inflammatory cytokines, such as IL1β and IL6, and in inflammatory chemokine, such as Ccl5, Ccl6 CXCL9, CXCL10, and CXCL11, in aged BM stromal cells and endothelial cells. Interestingly, the transcriptional signature of these cells is overlapping with the signature of young BM stromal cells and endothelial cells upon lipopolysaccharide (LPS) stimulation (102). Consistently, human MSCs isolated from aged donors display a reduced colony-forming ability and a senescent-like phenotype characterized by increased β-galactosidase and SASP factors. Importantly, umbilical cord-blood (CB)-derived CD34+ HSPCs exposed to aged MSC-conditioned medium increase the expression of the inflammatory cytokines MCP1 and IL8 and reduce cell clonogenicity. This outcome is rescued if CB-derived CD34+ HSPCs are cultured in conditioned medium derived from MSCs of aged donors treated with steroids, suggesting that the main trigger in HSPC alteration is represented by the increased secretion of SASP factors from MSCs upon aging (103). Other cell types have been demonstrated to be involved in the regulation of changes in HSC and HSPC function by triggering an inflammatory response

upon aging. In mouse and human aged BM, a megakaryocytic skewing with an increase in CD41+ HSCs and megakaryocytic progenitor frequency concomitant with the insurgence of an inflammatory state has been observed. Experiments in aged mice correlate this inflammatory state to an increase in activated macrophages (Mφs) with a reduced phagocytic function and increased release of IL1 (**Figure 4**). Interestingly, impaired phagocytosis in Mφs young mice, due to *alx* gene deletion, recapitulates the age-dependent myeloid skewing of HSC differentiation (81). Additionally, IL1 is sufficient to drive myeloid skewing in HSC: aged mice lacking IL1 receptor display a specific decrease of myeloid biased MPP3, while IL1 chronic administration *in vivo* expands Mac-1+Gr-1+ granulocytes, simultaneously reducing B220+ B cells (93). Consistently, treatment of aged mice with the IL1 antagonist Anakinra improves HSC repopulation ability after 5FU treatment (104). Complementary to this evidence, studies in mice (105) and in rabbits (106) demonstrate that the adipocyte-promoted myeloid expansion and IL1β production inhibit B lymphopoiesis.

Also, tumor necrosis factor alpha (TNFα) has been shown to be upregulated in HSCs upon aging, promoting myeloid differentiation skewing, HSC survival, and changes in the immunomodulatory properties through the activation of a nuclear factor-κB (NF-κB)-dependent gene program (94). Inflammaging has been shown to promote HSC mobilization (91), and Cymer and colleagues suggest the increased release of extracellular adenosine triphosphate (eATP) in the BM as a trigger for inflammation-dependent HSC mobilization (92).

Clonal hematopoiesis of indeterminate potential (CHIP) is defined as the presence in the peripheral blood of a somatic mutation with a variant allele frequency equal to or greater than 2%. CHIP is characterized by the expansion of HSC clones bearing somatic mutations, its incidence increases upon aging, and it is considered as a predisposing step to the development of hematological cancer and cardiovascular diseases (107). Proliferation and acquisition of a malignant phenotype have been linked with the presence of an inflammatory environment (107, 108). It seems likely that the pro-inflammatory changes occurring in the BM microenvironment upon aging can promote clonal hematopoiesis and its transformation to a malignancy (109). However, further experiments are needed to determine a direct cause–consequence relationship between inflammaging and CHIP.

Taken together, this experimental evidence highlights that inflammaging plays multiple and transversal roles in promoting different features of the aged-associated functional impairment of HSCs and increased cancer predisposition in the elderly.

TOOLS TO INVESTIGATE THE AGING BM NICHE

Mouse Models

Mouse models represent one of the most important tools to study and mechanistically investigate hematopoiesis and the function of BM niche cells. For this reason, the development of new murine models has increased exponentially in the last years.

Xenotransplantation of human hematopoietic cells in mice highlighted the existence of defined but important differences in hematopoiesis and BM niche structure and supporting functions between mice and humans. However, this limitation has been partially overcome by a progressive "humanization" of several murine models, through the expression of specific human cytokines, and by the development of promising alternative strategies to mimic a human BM niche *ex-vivo* (110). In fact, the use of mouse models to study human hematopoiesis has increased exponentially in the last decades [see ref. (111) for a full list of all mouse models used for recapitulating human hematopoiesis in mice]. More in general, mouse models have been instrumental for understanding the supportive function of the niche in regulating HSCs [for an extensive review see ref (16)]. Mouse models also play a key role for deciphering the molecular mechanisms that govern many complex physiological processes, such as aging.

For example, Poulos and colleagues, using an *in vitro* coculture system, reported that young BMEC can improve aged HSPC function (112). However, only the use of a mouse model to induce the knockout of mTOR specifically in BMEC allowed the discovery of the molecular mechanism governing this supportive role of BMEC. In fact, young mice upon BMEC specific deletion of mTOR display a premature aging phenotype with increased frequency of HSCs and myeloid cells and reduced lymphoid cells. Moreover, the same mouse model allowed the analysis of the vasculature in BMEC mTOR KO animals and to point out that the observed premature aging phenotype in HSPC was due to changes in the instructive signals arising from the endothelial niche, excluding a possible role for vascular degeneration, as the vasculature of knockout mice did not manifest gross alterations (77).

An additional example of the important role of mouse models to dissect the molecular mechanisms driving the niche-dependent HSC aging is represented by OPN knockout mice.

OPN is a matrix glycoprotein secreted in the BM extracellular matrix by osteoblasts and osteocytes (113, 114). OPN levels are reduced upon aging (66), and taking advantage of the complete knockout of OPN coding gene "secreted phosphoprotein 1" (spp1), different null OPN viable mouse models have been developed (115, 116). Thanks to these mouse models, it has been possible to identify that OPN positively regulates lymphopoiesis and erythropoiesis in aged mice and directly promotes HSC regenerative capacity. Transplantation of HSCs isolated from OPN null mice into lethally irradiated mice fails to repopulate the donor BM, leading to a premature death of the transplanted mice (87). *Ex vivo* thrombin-cleaved OPN treatment of HSCs obtained from OPN null mice reverts their premature aging phenotype (66).

The work of Ho and colleagues represent an additional example of the essential role of the use of mouse models for understanding the age-dependent alteration of BM niche cells. Adrenergic stimulation is altered upon aging (75). However, different receptors participate to modulate the adrenergic response. Taking advantage of two different mouse models bearing the specific deletion of β2-ADR and β3-ADR, Ho and

colleagues demonstrated a different role of adrenergic stimulation in driving HSC myeloid differentiation skewing. They observed that in aged mice, β2-ADR stimulation specifically drives myeloid differentiation skewing by a niche-dependent signal, while β3-ADR stimulation is involved in lymphoid differentiation. Aged Adrb2$^{-/-}$ mice display a reduced frequency of myeloid progenitors, and transplantation of wild-type BM cells into Adrb2$^{-/-}$ recipients recapitulates the megakaryocyte and platelet loss. On the contrary, β3-ADR knockout mice display a reduced frequency of lymphoid-biased HSCs in association with an increase in LT-HSC frequency and myeloid progenitors. Intriguingly, the double knockout for β2-ADR and b3-ADR does not display myeloid skewing, suggesting that the increase in adrenergic signal and the overcoming of β2-ADR signaling over the β3-ADR one are the main drivers in promoting myeloid differentiation skewing upon aging (41).

Another interesting example of the critical role of mouse models to mechanistically dissect the role of the BM niche on aging was recently provided by Frisch and colleagues (81). They demonstrated that aged mice display an impaired phagocytic activity in macrophages, in correlation with a decrease in the expression of the efferocytic receptor Alx. Taking advantage of the deletion of the tyrosine receptor Alx (117), the authors modeled a mouse with impaired phagocytosis. Young Alx knockout mice display macrophages with an impaired phagocytic activity, and this defect was sufficient to drive premature megakaryocytic skewing of HSCs (81). These data reveal the importance of mouse models as tools to decipher specific molecular pathways responsible for the interplay between stem cells and niche cells upon aging.

Mouse models can also represent a powerful tool to explore the contribution of aging in cancer development and progression. In a very recent paper, Hao and collaborators took advantage of a chronic myeloid leukemia (CML) mouse model to test how aging is affecting tumor progression in aged mice, highlighting the role of the niche in the oncogenic process (118).

Engineering the Human BM Niche

The unique structure and architecture of BM represent a limitation for its study in humans. The inability or the difficulties in directly analyzing the whole BM in human samples stimulated the development of novel technologies to mimic and study the human BM (hBM) niche outside of its natural localization (110) also to address its role in hematological malignances. As a matter of fact, recent evidence highlighted a crucial role for the niche in disease development and leukemia expansion (119). The classical model for studying hematological malignancies is represented by xenotransplantation assays, where human hematopoietic cells are transplanted into mouse recipients. However, this type of approach excludes the possibility to investigate the signaling coming from the human niche. In these experimental setting, engraftment analysis plays a key role to define the disease and its aggressiveness. Unfortunately, mouse models sometimes fail to properly recapitulate the disease due to the murine (not human) microenvironment where the cells are transplanted. Improvements in this sense have been done by implementing immunocompromised mouse models expressing human cytokines [refer to ref (110, 120). for an extensive review]; nevertheless, this aspect requires further investigations and additional strategies are raising.

The subcutaneous implantation in mice of scaffolds supporting human niche cells represents one of the most promising strategies to mimic and study the hBM and to model hematological malignancies.

Ossicles have been described for the first time by Urist and colleagues (121) and by Freidenstein and colleagues (122) as human-derived bone formations containing in their inside structured BM (120).

Friedenstein and collaborators extensively demonstrated that freshly isolated BM cells by both trypsin digestion and bone flushing are able to generate ossicles when absorbed into porous sponges and transplanted under the renal capsule of mice (122). Subsequently, Robey and Bianco have extended the use of ossicles to model the pathogenesis of McCune-Albright fibrous dysplasia, demonstrating the relevance of this tool for clinically related investigations (123–125). Humanized ossicles can be generated by seeding hMSC into EMC-based 3D scaffolds and subcutaneously implanted in NSG-recipient mice. Abarrategi and colleagues used a porous Gelfoam® scaffold composed by partially dehydrated gelatin. Human HSPCs or leukemic cells can be directly seeded into the scaffold 48 h after MSC seeding or directly injected into the mouse tail vein 4–6 weeks after the implantation of the scaffold with comparable engraftment. Once implanted, the host provides vascularization to the scaffold and is colonized with hematopoietic cells (126). Interestingly, this approach has been used to generate a humanized niche model to analyze the influence of leukemic cell remodeling of the mesenchymal niche and its effect on normal HSPC proliferation. For example, Waclawiczek and colleagues demonstrated that patient-derived AML cells impair normal hematopoiesis by influencing the release of HSPC-supporting factors by MSCs, leading to the suppression HSPC proliferation and differentiation (127).

A complementary approach to develop ossicles was described also by Reinisch and colleagues. Using this method, hMSCs are directly subcutaneously seeded into the flanks of immunocompromised NGS mice. hMSC differentiation and ossification are induced by PTH injections within 10 weeks after seeding. Normal or malignant hematopoietic cells are directly seeded into the ossicle after myeloablation (irradiation or busulfan-based chemotherapy) (128).

Formation of LT-HSCs into ectopic niches derived from fetal bone and implanted *in vivo* under the kidney capsule requires ossification (129); however, additional approaches have been developed to mimic *in vitro* the human niche. For example, the "bone marrow–on–a–chip" represents one of such approaches (130, 131) and it consists of a poly(dimethylsiloxane) (PDMS) device coated with bone inducing materials, which is subcutaneously implanted in mice to obtain an engineered BM (eBM). The eBM can be subsequently cultured *in vitro* maintaining a functional hematopoietic system (130). However, in line with the ossicle technology, this strategy still requires the *in vivo* implantation step.

To overcome this issue in the BM-on-a-chip approach, Sieber and colleagues used a hydroxyapatite-coated Sponceram 3D ceramic scaffold to seed hMSCs and form an eBM. The similarity

of the scaffold with the bone allows the formation of an eBM completely *in vitro* which functions in association to a microfluidic device to provide nutrients. Moreover, the chip system allows hHSPC seeding and differentiation, forming an eBM stable up to 28 days (131). Another very interesting approach that excludes the *in vivo* step is the *ex vivo* perfusion bioreactor model. This system consists of a hydroxyapatite ceramic scaffold inserted into a perfusion system. hMSCs are seeded into the ceramic scaffold and induced to differentiate by administering an osteogenic medium, leading to the formation of an engineered niche (eN) where CD34+ HSPCs and recombinant growth factors (SCF, TPO, FLT3-L) are subsequently added. The eN induces the expansion of phenotypic HSPCs and promotes the maintenance of stem cells, mimicking the human osteoblastic BM niche (132).

Another approach to mimic the human BM niche *in vitro* is represented by decellularized matrix scaffold [see ref. (133) for detailed information]. These scaffolds are produced by the deposition of ECM by the immortalized MSC cell line SCP-1. After the decellularization, CD34+ human HSPCs (obtained from peripheral blood after mobilization) are seeded on the scaffold. With this approach, Krater and collaborators demonstrated that these scaffolds support HSPC functionality and that they can also modulate it through integrin-mediated signaling (134).

All these strategies have proved to be extremely useful in the study of the human BM niche, and it would be intriguing to explore their potential application also for investigating aging of the human BM niche. These approaches could be useful for dissecting the contribution of specific cell types, secreted factors, and signaling pathways in impairing human HSC function over time. An engineered aged niche would offer a novel approach for defining and recreate *in vitro* the time-line cascade of events involved in aging of the BM niche, allowing not only the functional characterization of the processes involved in driving aging of the hematopoietic system but also the possibility of exploring new therapeutic approaches targeting the stem cell niche directly.

Imaging Approaches

The BM is a densely packed tissue with a gelatinous consistence that limits its investigation and the preservation of its three-dimensional architecture by classical histological approaches. New imaging approaches have been recently developed to overcome these limitations [see ref. (135) for an extensive review].

A 3D analysis of BM architecture taking advantage of extensive BM sectioning and analysis after perfusion has been extensively used by the group of Nilsson to demonstrate that HSC localization is not random after transplantation. While progenitor and differentiated cells mainly localize in the inner marrow, HSCs prevalently localize at the endosteal area (136), requiring SCF (137) and hyaluronate (138) for their lodgment. The key aspect of this analysis is the sectioning of the BM every 3.5 μm to evaluate each stem cell only once. This approach requires extensive sectioning, and the BM 3D architecture might be easily compromised. To overcome this aspect, different strategies have been developed, like 3D-quantitative microscopy (3D-QM) and BM whole-mount histology.

3D-QM has been used by the group of Nombela-Arrieta to image the BM niche and its components (139, 140), allowing the modeling of the entire bone surface. This approach has highlighted the real abundance and complex organization of the sinusoidal network and mesenchymal reticular subsets and its maintenance upon aging (141). The same approach has been used to demonstrate that HSPCs are mainly localized at the endosteal niche in close proximity to sinusoidal and non-sinusoidal microvessels and that these cells display a hypoxic profile (142). Recently, this technique has been also applied to study the leukemic stem cell (LSC) niche. In a chronic myeloid leukemia model, the importance of CXCL12 in promoting LSC localization and clustering in close proximity of MSCs has been highlighted. Moreover, it has been shown that CXCL12 deletion in MSCs increases LSC clearance upon TKI treatment (143).

BM whole-mount histology represents one of the best strategies to preserve and analyze the BM 3D architecture. This approach allows the study of the BM niche directly in fixed bone samples by immunostaining and confocal or multiphoton microscopy imaging. This technique has been now more and more used to study different components of the BM niche and their changes upon aging (40, 41, 75). The possibility to image the BM niche from the exposed surface to the inner marrow spanning from the endosteal region to the perivascular one allowed the identification of the HSC preferential localization at arteries and endosteum in young mice (75). Moreover, this technique made possible to image and dissect the localization of rare label-retaining aged HSC at sinusoids in aged mice (40). Similarly, this technique has been used to identify the changes in the adrenergic stimulation occurring upon aging and how these are affecting HSC behavior (41, 75). However, this technique is limited by the nature of the sample and by the imaging power of the confocal or multiphoton microscopes. Samples used for whole-mount histology must be fixed in order to preserve the 3D BM structure. UV and visible light lasers used in confocal microscopy usually allow a penetrance of about 100 μm into the BM, which is further reduced in the case of combining multiple fluorophores.

To improve the possibility of resolving the composition of the BM niche by combining multiple fluorochromes together, Schroeder's lab developed a multicolor quantitative confocal imaging approach. This technique applies to thick-bone sections from PFA-fixed long and flat bones, cleared and decalcified before the imaging process. Using a sequential staining based on primary, secondary, and tertiary antibody combinations, it is possible to image up to eight colors by confocal microscopy without linear unmixing (144). This approach has been used to map non-hematopoietic cells in the BM (145) and to demonstrate that young cycling HSCs are preferentially located in proximity to CXCL12 stromal cells and far from sinusoids and megakaryocytes (146). Interestingly, this approach has been also extended to study the functional distribution and differentiation of hMSCs seeded in ossicles (147). Moreover, this multicolor quantitative confocal imaging takes advantage of the specific image analysis software "XiT," able to analyze large data sets and to provide internal controls for determining preferential cell localization (144).

Similarly, Lucas' lab combined the whole-mount histological approach with staining of different antibodies and the Ubc-Cre^{ERT2}:Confetti mouse reporter to map the spatial segregation of myeloid progenitors during differentiation. They took advantage of the specific cytosolic localization of the Confetti reporter *vs*. the localization at the cell membrane of the antibodies to use the same color channel for staining different markers. The discrimination of the fluorescent signal localization (intracellular *vs*. cell surface) allows the clear identification of the specific cell type and its localization within the BM compartment (148).

Another powerful strategy to overcome confocal imaging limitations is represented by the use of multiphoton microscopy and by the two-photon excitation fluorescence (TPEF) microscopy. By using the near infrared light (700–1000 nm) to excite the fluorophores, it is possible to combine more colors simultaneously, reducing photobleaching in comparison to the lasers used in confocal microscopy. In addition, TPEF takes advantage of the second harmonic generation to image collagen 1 fibers in the bone (135) and the use of the infrared light for imaging increases the resolution and the penetration of the light into the samples up to a depth of 150 μm in calvaria (149). The key advantage of TPEM is its applicability both on fixed samples and for *in vivo* imaging. Currently, intravital imaging coupled with TPEM represents the best strategy to analyze the BM niche in living animals taking advantage of fluorescent reporter mouse models. The group of Von Andrian extensively used this approach to study HPC homing to BM after transplantation (150), HSC, and HSC's progeny trafficking and homing (151–155). One of the most investigated bone for this analysis is the calvarium, because this thin skull bone does not require major manipulation prior to imaging, but it can also be applied to other bones like for example the tibiae (53, 156, 157). Of note, this imaging approach has been extensively applied to analyze young animals; however, it has been rarely used on aged mice (53, 156). This is probably due to the increased challenges of applying this technique in aged mice, which are more fragile animals, limiting the applicability of this technique. Recently, intravital imaging has been used to analyze the physiological localization of HSCs in the calvaria in relation to hypoxic areas within the BM, observing that HSCs are not found in deep hypoxic areas (158). In this example, the authors took advantage of the Mds1$^{GFP/+}$ Flt3^{Cre} reporter mouse models to trace HSCs. The use of reporter mouse models is a key aspect of this technique, as non-viable staining cannot be performed. Other reporter mice that can be used are for example α-catulin$^{GFP/+}$ and labeling-retaining models (146). Of note, the use of these HSC reporter lines can be combined with other reporters to image at the same time different subsets of niche cells, such as those currently used in histology as well [refer to ref (16, 17). for a detailed list]. Intravital microscopy has been also extensively used by Lo Celso's lab to analyze the interplay between leukemic cells and the BM niche. As an example, Duarte and colleagues demonstrated that acute myeloid leukemia (AML) cells induce a massive remodeling of the endosteal BM niche by releasing pro-inflammatory and anti-angiogenic cytokines and that the degenerated endosteal niche displays a reduced capacity to support non-leukemic HSCs. Interestingly, HSC loss and the reduction of normal hematopoiesis are spatiotemporally correlated with the AML-dependent endosteal remodeling (149).

COMPUTATIONAL TOOLS TO INVESTIGATE THE BM NICHE

Next-generation sequencing (NGS) has created a paradigm shift in medical and biological research. The advent of single-cell sequencing has further enhanced the importance of NGS and has enabled investigators to ask questions that would normally not be feasible to address *via* bulk sequencing. Single-cell sequencing methodologies enable the analysis of transcriptome, mutatome, protein–DNA interaction, and broadly the epigenome. Combined with increased statistical power and advanced analytic tools geared toward single-cell analysis, one can then look at, but not limited to, tissue heterogeneity, clonality, analysis of gene- and allele-specific expression, and single-cell level mutational analysis. For example, our groups have also applied single-cell RNA-seq and single-cell Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) to look at transcriptional and chromatin accessibility of HSC daughter pairs (63) and allelic-specific expression associated with inactivation of chromosome X upon aging (60).

More recently, combinatorial approaches have been developed that would allow simultaneous interrogation of macromolecules (multi-omics) and spatial context of cells. This is especially highly interesting in tissues and systems with relatively higher complexity and heterogeneity. Stoeckius et al. (159) developed such a single-cell sequencing technique, which they named as Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq). Using this method, one can look at both the protein markers and transcriptome profile of the same cell. By combining single-cell and spatially resolved transcriptomics where the positional information of cells is also deduced, Baccin et al. (2) mapped the molecular, cellular, and spatial compositions of distinct bone marrow niches. A more recent approach with potentially significant impact in broadening our understanding of the relationship between open chromatin and transcriptome at the single-cell level is SHARE-seq (the simultaneous high-throughput ATAC and RNA expression with sequencing) (160). Along these lines, the single-cell method has also been modified to specifically fit the needs of analysis of the niche or microenvironment in the body. NICHE-seq, developed by Medaglia et al. (161), combined fluorescent reporters, two-photon microscopy, and single-cell RNA sequencing (scRNA-seq) to infer the cellular and molecular compositions of niches. They stated that, using this technique, one can sort and analyze cells from a given region in a transgenic mouse.

In combination with the aforementioned single-cell technical advances, a seemingly obvious but still not fully exploited analytical approach that we strongly believe could significantly expand our understanding of cell biology and allow in-depth analysis of single-cell sequencing is deep learning. We have, for

instance, successfully utilized deep learning to understand the positional proximity of HSCs with niche cells in the bone marrow and how, solely based on this information, one can predict whether a given HSC is obtained from a young or aged mouse (40). Our work shows the untapped potential of deep learning, even with limited number of cells, in immunofluorescence and imaging-based studies. Fortunately, most of the current single-cell technologies generate data in the range of thousands, providing a conducive platform for deep learning models that could be optimally trained and validated. In agreement with our statement and not surprisingly, Raimundo et al. (162) reported that single-cell omics has seen a surge in use of machine learning for dimensional analysis, batch normalization, classification, trajectory analysis, and inference, emanating from the flexibility and scalability of the method. Li et al. (163), for instance, used an unsupervised deep embedding algorithm to gradually removes batch effects. Yan et al. (164) discussed the potential of machine learning in single-cell sequencing, where one can use structure of cells and subpopulations with differentiation potential for stem cell therapy.

The multiome (multiple macromolecules being simultaneously interrogated in a given cell) enhances the application of machine learning by providing an additional and potentially orthogonal set of information for a given cell, thereby helping in further refinement of the deep learning model. As also stated by Li et al. (163), machine learning has been used in gene regulatory network inference or multimodal data integration based on single-cell sequencing.

CONCLUSIONS: WHERE WE STAND AND PERSPECTIVE FOR THERAPEUTIC APPROACHES

Implementation of deep learning, by integrating various datasets, is a highly promising novel approach to a long-standing question regarding niche composition, dynamics, and cell-to-cell communication. As a proof of principle, we show in **Figure 5** a model workflow on how to integrate data acquisition and analysis

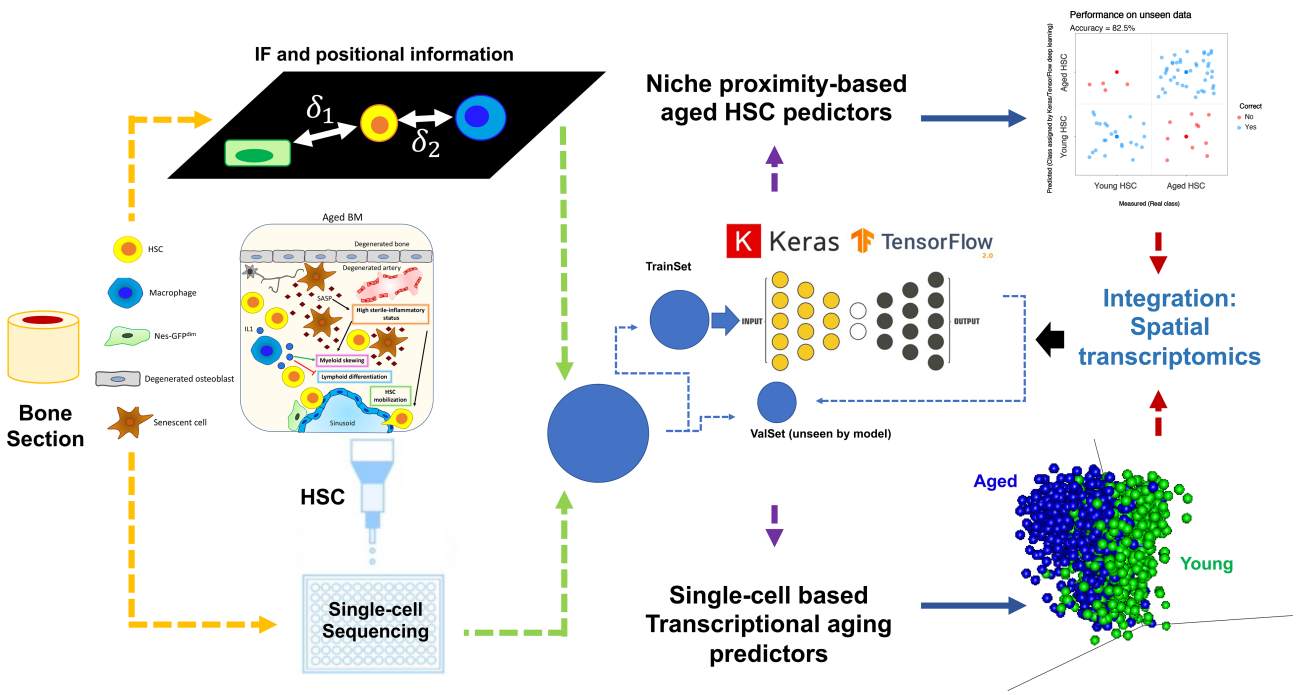

FIGURE 5 | Implementation of deep learning, by integrating various datasets, to elucidate the impact of aging-mediated niche dynamics on HSC positioning and function. Here are shown two types of datasets: 1) upper part: immunofluorescence positional information showing the relative distance of HSC to niche cells and 2) lower part: single-cell sequencing of sorted HSCs. Due to the adaptability and flexibility of deep learning methodologies, both datasets are analyzed using, for instance, an R implementation of Keras/TensorFlow (165). Models are trained using a subset of the data (TrainSet) and different kernel initializations and varying numbers of hidden layers, depending on the dataset at hand. After validation of the model using a subset of data not included in the training (ValSet), prediction accuracies are calculated. For the positional information, we assess if we can predict whether a given cell is young or aged HSC purely based on their relative proximity/distance to a given niche cell and each other. As shown in our recent study (40), using such an approach, we could show ~83% prediction accuracy in defining the age status of HSCs using positional information. Using single-cell dataset and deep learning, we have also recently developed an aging signature with very high prediction accuracy (>95%; unpublished data), where the top predictors could show clear segregation of young and aged cells using principal component analysis (PCA; lower right part of the figure). We propose here that by integrating spatial transcriptomics of specific regions of a bone section (including HSCs and niche cells) and deep learning, one can perform an integrated analysis that will not only show transcriptional signatures of the cells assayed but also shed light on how the HSC-niche cell proximity and transcriptional dynamics change upon aging.

to elucidate the impact of aging-mediated niche dynamics on hematopoietic stem cell (HSC) positioning and functionality. Two types of datasets are shown: 1) immunofluorescence-based positional information correlating the relative distance of HSC to niche cells and 2) single-cell sequencing of sorted HSCs. As aforementioned, due to the adaptability and flexibility of deep learning methodologies, both datasets are analyzed using, for instance, an R implementation of Keras/TensorFlow (165). Models are trained using a subset of the data (TrainSet) and different kernel initializations and varying numbers of hidden layers, depending on the dataset at hand. Prediction accuracies are calculated after validation of the model using a subset of data not included in the training (ValSet). For the positional information, we assess if we can predict whether a given HSC is young or aged purely based on their relative proximity/distance to a given niche cell and each other. As shown in our recent study (40), using this approach, we could show ~83% prediction accuracy in defining the age status of HSCs using positional information. Of note, it would be interesting to refine this further, for instance, by applying spatial transcriptomics of specific regions of a bone section (including HSCs and niche cells) instead of classical single-cell sequencing.

As aging is a multifactorial biological dynamic, various types of datasets should be considered to improve our understanding of both intrinsic and extrinsic processes affecting the aging process. This also has a significant advantage in developing novel and powerful deep learning models with improved performance. By deducing the relative significance of the factors under consideration, prioritization of intervention schemes including rejuvenation and maintenance of cells of interest in an *in vivo* setting can be planned.

More broadly, investigation of the BM niche is now gaining growing attention for its new potential therapeutic and translational angle, and also other approaches, ranging from single-cell profiling, to spatial transcriptomics, to humanized niche models will all contribute to consolidate and deepen our understanding of how the BM niche supports HSC function over time. At the moment, the most general consensus view indicates the intrinsic aspects driving aging of HSC as largely fixed within the cells and with few options to be influenced by the microenvironment or by systemic rejuvenation interventions (166). However, it is interesting to underline that rejuvenation of aged HSCs proves to be beneficial to different tissues and it could be also impacting on the BM niche itself. We are just starting to explore the boundaries between intrinsic and extrinsic HSC aging and their mutual interplay. Based on the current view, it is quite likely that intervention strategies able to affect contemporarily both aspects might have a much more profound impact on hematopoiesis. Further, it would be very intriguing to explore if this combined approach targeting hematopoietic stem cell intrinsic and extrinsic aging could extend to other somatic stem cells and tissues and contribute to eventually extending lifespan and slowing aging of the whole organism.

AUTHOR CONTRIBUTIONS

FM, MAM, and MCF wrote the manuscript together. All authors contributed to the article and approved the submitted version.

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Adhesion Molecules Involved in Stem Cell Niche Retention During Normal Haematopoiesis and in Acute Myeloid Leukaemia

*Julien M. P. Grenier, Céline Testut, Cyril Fauriat, Stéphane J. C. Mancini† and Michel Aurrand-Lions**

Aix-Marseille Université, Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM), Institut Paoli Calmettes, Centre de Recherche en Cancérologie de Marseille (CRCM), Equipe Labellisée Ligue Nationale Contre le Cancer 2020, Marseille, France

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Jean-Pierre Levesque, University of Queensland, Australia
Louise Purton, University of Melbourne, Australia

***Correspondence:**
Michel Aurrand-Lions
michel.aurrand-lions@inserm.fr

†Present Address:
Stéphane J. C. Mancini, INSERM U1236, MIcroenvironment, Cell differentiation, iMmunology And Cancer (MICMAC), Rennes, France

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In the bone marrow (BM) of adult mammals, haematopoietic stem cells (HSCs) are retained in micro-anatomical structures by adhesion molecules that regulate HSC quiescence, proliferation and commitment. During decades, researchers have used engraftment to study the function of adhesion molecules in HSC's homeostasis regulation. Since the 90's, progress in genetically engineered mouse models has allowed a better understanding of adhesion molecules involved in HSCs regulation by BM niches and raised questions about the role of adhesion mechanisms in conferring drug resistance to cancer cells nested in the BM. This has been especially studied in acute myeloid leukaemia (AML) which was the first disease in which the concept of cancer stem cell (CSC) or leukemic stem cells (LSCs) was demonstrated. In AML, it has been proposed that LSCs propagate the disease and are able to replenish the leukemic bulk after complete remission suggesting that LSC may be endowed with drug resistance properties. However, whether such properties are due to extrinsic or intrinsic molecular mechanisms, fully or partially supported by molecular crosstalk between LSCs and surrounding BM micro-environment is still matter of debate. In this review, we focus on adhesion molecules that have been involved in HSCs or LSCs anchoring to BM niches and discuss if inhibition of such mechanism may represent new therapeutic avenues to eradicate LSCs.

Keywords: adhesion, haematopoietic stem cell, leukemic stem cell, haematopoiesis, bone marrow, acute myeloid leukaemia

INTRODUCTION

Haematopoiesis takes place in the bone marrow of adult mammals and is the process leading to the formation of blood components throughout life. Haematopoietic stem cells (HSCs) are at the apex of the haematopoietic hierarchy and are able to self-renew and to differentiate into all blood cell types. The balance between differentiation and self-renewal is controlled by intrinsic properties of HSC and extrinsic cues delivered by the bone marrow microenvironment in micro-anatomical sites called "niches".

The concept of niche has been formulated by R. Schofield in 1978 who proposed that stem cell association with other cells prevents maturation while its progeny proliferate and differentiate, unless they can occupy a similar 'niche' (1). Although this working hypothesis turned to be true, its formal proof has long time been hampered by the lack of methods allowing precise localization of un-manipulated HSC within their niche (2, 3). In addition, because HSC activity has been essentially studied in transplantation assays, it has been difficult to decipher whether experimental assays were measuring intrinsic HSC stemness of engrafted cells or their ability to find a supportive niche in which they can self-renew (4, 5). The development of constitutive knock-out mouse models in the early 90's, and conditional or inducible models later on, has represented a breakthrough to study the contribution of niche components to mammalian haematopoiesis (6, 7). Accordingly, a bibliographic search using combination of the words "haematopoiesis, adhesion and niche" reveals that only seven publications combine such words between 1989 and 2000, while more than hundred papers have been published thereafter. This likely indicates that adhesion was initially considered as an intrinsic property of HSC, while it has been integrated to the niche concept later on. This review is focused on adhesion molecules implicated in HSC or acute myeloid LSC interaction with the BM microenvironment (**Figure 1**).

ADHESION MOLECULES INVOLVED IN HSC RETENTION IN THE BONE MARROW

With the exception of CD44, haematopoietic adhesion molecules belong to the immunoglobulin superfamily (Ig Sf), the cadherin family, the selectin family or the integrin family. Adhesion molecules promote cell/cell or cell/extracellular-matrix (ECM) interactions and deliver survival signals to haematopoietic cells. Reciprocally, stromal and endothelial cells express adhesion molecules interacting with haematopoietic cells or ECM contributing to the maintenance of bone marrow architecture.

Integrins

Integrins are non-covalent heterodimers of α and β chains. In mammals, 18 α and 8 β subunits form 24 different integrin heterodimers involved in embryonic development and maintenance of tissue homeostasis. α/β chain pairing and integrin interaction with ECM, cell surface molecules or soluble factors have been extensively reviewed in the past and will not be described in further details here (8–11).

One key property of integrins is that they can be expressed in inactive, activated or clustered state on the surface. The switch between inactive and active state results in increased ligand affinity as a consequence of inside-out or outside-in signalling. Integrin clustering further induces cytoskeleton rearrangement and enhanced cell signalling (**Figure 2**).

Among $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$and $\alpha_9\beta_1$ integrins that have been involved in interaction of HSC with bone marrow microenvironment (12–18), $\alpha_4\beta_1$ is the most studied. The integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are activated by inside-out signalling that involves cytokines and divalent cations present in the bone marrow microenvironment, suggesting that they are essential for HSC retention in the bone marrow (19, 20). Accordingly, HSPC mobilization using G-CSF is correlated to decreased α_4 integrin expression (21) and deletion or inhibition of $\alpha_4\beta_1$ integrin result in accumulation of HSC in the blood circulation (22–25). Similar results were obtained using antibody against VCAM-1, suggesting a central role of $\alpha_4\beta_1$/VCAM-1 axis in HSC retention in the bone marrow (26). This is consistent with the finding

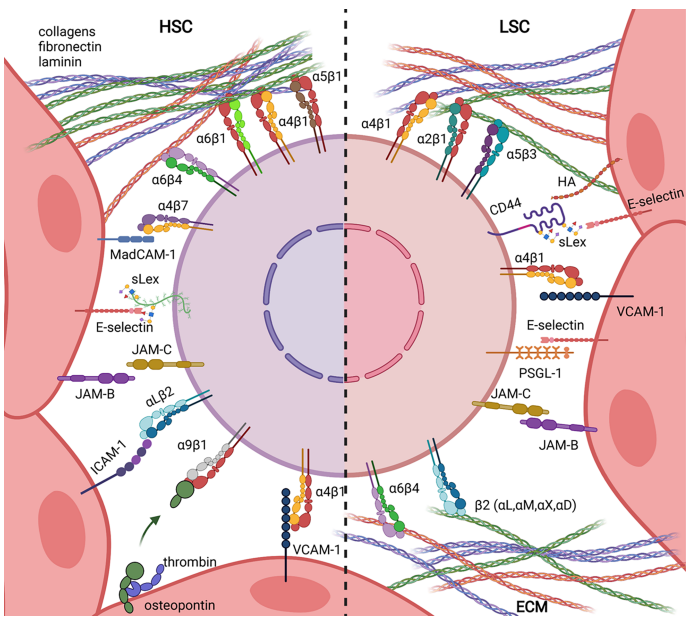

FIGURE 1 | Ligand/Receptor adhesion pairs involved in Haematopoietic Stem Cell (HSC, left) and Leukemic Stem Cell (LSC, right) retention in bone marrow niches.

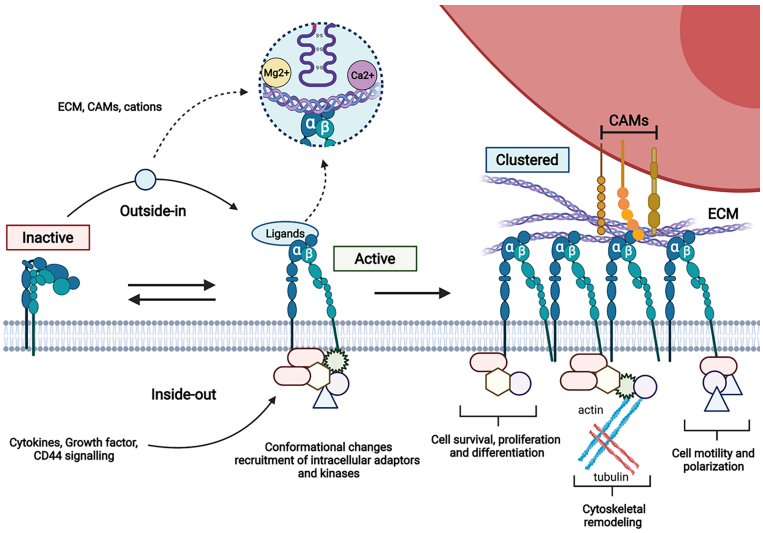

FIGURE 2 | Schematic representation of integrin activation. The variety of intracellular protein complexes involved in integrin signalling (kinases, adaptors…) is depicted by forms recruited to the cytoplasmic tails of integrins.

that β_1 null HSC fail to engraft in irradiated recipient and that β_1 null HSC from chimeric embryos are unable to seed foetal liver (27, 28).

Along this line, β_7-deficient mice do not have defects in HSCs function (29), while interaction between $\alpha_4\beta_7$ and MadCAM-1 (mucosal addressin cell adhesion molecule-1) accounts for half of the α_4-integrin mediated homing activity to the bone marrow (30, 31). Therefore, it seems that β_1 integrin heterodimers play a prominent role in bone marrow HSC retention as further supported by the fact that the dual $\alpha_9\beta_1$/ $\alpha_4\beta_1$ inhibitor BOP ((N-(benzenesulfonyl)-L-prolyl-L-O-(1-pyrrolidinylcarbonyl) tyrosine) induces a rapid mobilization of HSCs including those that are located in the endosteal region which bind thrombin-cleaved osteopontin with high affinity (32). This is also supported by the finding that patients treated with natalizumab, an anti-α_4 integrin antibody, present increased levels of circulating $CD34^+$ progenitor cells associated with an higher migratory profile as compared to GM-CSF mobilization (33, 34).

Finally, it has recently been reported in zebrafish that $VCAM\text{-}1^+$ patrolling macrophages can interact with HSCs in an $\alpha_4\beta_1$ dependent manner and contribute to their retention in the niche (35). This study confirms earlier findings in mouse models showing that macrophages contribute to HSC retention within niches through integrin-mediated interactions (36–38).

Selectins

The selectin family encompasses three members: E- (Endothelial), P- (Platelets) and L- (Leukocyte) selectins expressed by endothelial cells (E- and P- selectins), platelets (P-Selectin) and leukocytes (L-Selectin). They have been initially involved in the rolling of haematopoietic cells along vessels in flowing blood (39–41).

The minimal requirements for Ca^{2+}-dependent ligand binding to selectins are the tetra-saccharides Sialyl Lewis X (Sle^x) and Sialyl Lewis A (Sle^A) (42, 43). As reviewed elsewhere (44), Sle^x and Sle^A synthesis requires several enzymes including α (1–3)-fucosyltransferase activities as illustrated by defective selectin-dependent leukocyte trafficking in FucT-VII deficient mice (45). This is reminiscent of the phenotype of P-Selectin deficient mice that harbour elevated number of circulating neutrophils, loss of leukocyte rolling in mesenteric venules and delayed leukocyte recruitment in peritonitis model (46). In contrast, E-selectin deficient mice have no defect in neutrophils trafficking suggesting a compensatory mechanism mediated by P-selectin (47).

The study of double knockout mice for E- and P-selectin has revealed defect in haematopoiesis with increased extramedullary erythropoiesis and reduced haematopoietic progenitor cell homing in irradiated deficient mice upon transplantation (41, 48). However, such functions were mostly attributed to HSPC homing and it is only in 2012 that E-selectin was shown to mediate HSC proliferation at the expense of self-renewal (49). In contrast to E- and P- Selectin, early haematopoietic defects in L-Selectin-deficient mice have not been reported so far (50).

Cadherins

Cadherins are transmembrane glycoproteins characterized by tandemly repeated sequence motifs in their extracellular segments that allow homophilic interactions in a Ca^{2+} dependent manner (51). N-cadherin is not only expressed by neural cells but also by HSCs and spindle shaped osteoblastic cells lining the bones, called "Spindle-shaped $N\text{-}cadherin^+CD45^-$ Osteoblastic" (SNO) in the original publication. Because conditional inactivation of BMP receptor type IA (BMPRIA) led to expansion of both SNO and HSC, with asymmetric N-Cadherin distribution between SNO and HSC adjacent cells, it has been proposed that N-cadherin-mediated adhesion contributes to HSCs maintenance in endosteal niche (52). This concept was further supported by the fact that the knock-out of

N-cadherin in LSK cells impairs long term engraftment in the bone marrow but not in the spleen (53). However, the latter demonstration used LSK cells, a compartment in which less than 20% of the cells are HSCs. Therefore, the function of N-cadherin mediated adhesion in HSC maintenance has been challenged in several studies. First, it was demonstrated that N-cadherin is not expressed on purified HSCs and that osteoblasts are dispensable for HSC maintenance (54). Second, the conditional deletion of N-cadherin in HSC using Mx1-Cre did not affect haematopoiesis, nor did its specific deletion in osteoblasts (55–57). Therefore, the controversial function of N-cadherin in HSC maintenance has been revisited in the light of the methodology used to study its function (engraftment *versus* knock-out) and with respect to heterogeneous expression of N-cadherin by HSC subsets (58, 59). This led to the most recent concept that N-cadherin mediated adhesion of HSC to BM stromal progenitor cells (BMSPC) may only be revealed during emergency haematopoiesis such as the one needed by "reserve" HSC to survive chemotherapy (60).

Ig Sf Adhesion Molecules

Several Ig Sf adhesion molecules such as ALCAM (CD166), ESAM, JAM-A or JAM-C are expressed by HSPCs and BM stromal or endothelial cells (61–64). Some others such as ICAM-1 or VCAM-1 are expressed in the BM microenvironment and interact with integrins expressed by HSPCs or contribute to more complex adhesive networks involving IgSf/Integrin as well as IgSf/IgSf interactions such as the JAM family members (65–68). Therefore, early haematopoietic defects reported for IgSf deficient animals have to be interpreted with caution unless specific conditional knock-out mouse models are combined with orthogonal methods such as long-term engraftment. Defects in early haematopoiesis following knockout have been reported for ALCAM, ESAM, VCAM-1, JAM-C, JAM-B and ICAM-1 (**Table 1**).

ADHESION MOLECULES INVOLVED IN LSC RETENTION IN THE BONE MARROW

Similar to HSCs, LSCs are retained into specialized microanatomical sites by adhesive interactions. Indeed, AML development originates from LSC which share with HSCs the ability to self-renew (79, 80). After disease initiation, acute myeloid leukemic burst is accompanied by a remodelling of bone marrow niches that alters the physiological adhesive network of HSC (81–83). Whether adhesive remodelling occurs already at disease initiation in immunocompetent context remains to be addressed, but several adhesive Ligand/Receptor pairs have been involved in AML development in mouse models. Among them, only a limited number of Ligand/Receptor pairs that cross barrier species have been validated as putative therapeutic targets in preclinical setting using patient derived xenograft (PDX) models. This has encouraged some clinical trials targeting LSC adhesion to the niche in order to sensitize these cells to chemotherapy as recently reviewed by A. Villatoro et al. (84). In the next section, we will discuss the adhesion molecules known to contribute to LSC stemness maintenance that belong to the emerging class of adjuvant therapies for LSC eradication in AML.

CD44

CD44 is a class I transmembrane glycoprotein that does not belong to an adhesion molecular family and that interacts with ECM ligands such a as osteopontin, fibronectin or hyaluronan (HA). When CD44 is sialo-fucosylated and bears Sle^X glycan, it is called HCELL and interacts with E- and L-selectin (85, 86). In addition, several isoforms of CD44 are generated by alternative splicing and associated with different cellular processes (87). CD44 isoforms are widely expressed on AML cells and expression of the CD44-6v isoform has been associated with poor prognosis (88, 89). Functionally, CD44 has been involved in AML cell adhesion to bone marrow stromal cells (90, 91) and ligation of CD44 with HA or activating antibodies such as H90 has been shown to reverse differentiation blockage in AML cells (92). The same H90 activating antibody inhibited homing of AML-LSC to microenvironmental niches reducing the leukemic burden in a PDX setting. This was attributed to opposing effects of the H90 antibody which increases adhesion of normal $CD34^+CD38^-$ cells to HA but inhibits adhesion of $CD34^+CD38^-$ AML blasts to HA (93).

Integrins

Overexpression of the integrins $\alpha_M\beta_2$ (CD11b/Mac1), α_2, α_6 and $\alpha_4\beta_1$ by AML cells has been associated with poor prognosis (94–96). Indeed, it has early been shown that both β_1 and β_2 integrin chains are necessary for AML blast adhesion to BM stromal cells (97).

Among the β_1 integrins, $\alpha_4\beta_1$ seems to play the most prominent role through its interaction with fibronectin (FN) and VCAM-1. Interaction of integrin $\alpha_4\beta_1$ with FN protects AML cells from chemotherapy and is associated with the maintenance of minimal residual disease (MRD). Treatment with a blocking antibody against $\alpha_4\beta_1$ abrogates chemoresistance and MRD in mice (98). Similarly, integrin $\alpha_4\beta_1$ interaction with VCAM-1 contributes to drug resistance by activating NF-κB pathway in BM stromal cells which is essential to promote chemoresistance in leukemic cells as demonstrated by inhibition of NF-kB signalling (99). This study illustrates the reciprocal crosstalk between LSC and stromal cells since NF-κB activation in stromal cells upregulates VCAM-1 which serves as a positive feedback loop for leukemic cell adhesion to stromal cells.

More recently, the interaction between the integrin $\alpha_2\beta_1$ and collagen has been shown to confer doxorubicin chemoresistance *via* the inhibition of Rac-1 (100). This protective effect is reversed by anti-$\alpha_2\beta_1$. Although these studies show the therapeutic potential of integrin inhibition in AML, they do not formally prove that LSC are more addicted to integrin-mediated adhesion than normal HSC. To find such differential adhesive cues, Ebert and collaborators have used results from pooled *in vivo* shRNA screens. They have found that the integrin $\alpha_v\beta_3$ is essential for leukemic initiation and maintenance but dispensable for normal HSPC activity (101). This was attributed to constitutive

TABLE 1 | Knock-out mice of Ig Sf molecules presenting haematopoietic defects.

Adhesion molecule	Year	Ligands	Altered phenotype	Haematopoietic phenotype	References
ICAM-1	1994	$\alpha_L\beta_2$	cardiovascular, cellular, digestive/alimentary, growth/size/body, haematopoietic, homeostasis, immune, mortality/aging, neoplasm, vision/eye	Expansion of Lt-HSC compartment associated with impaired quiescence and myeloid expansion	(69, 70)
VCAM-1	1995	$\alpha_4\beta_1$ $\alpha_4\beta_7$	cardiovascular, embryo, growth/size/body, homeostasis, mortality/aging, haematopoietic	Increased frequencies of circulating progenitors	(65, 71)
ESAM	2003	ESAM	cardiovascular, cellular, growth/size/body, haematopoietic, immune	Increased HSCs frequency and proliferation compared to wild-type mice	(63, 72)
ALCAM (CD166)	2004	ALCAM CD6	nervous system, vision/eye, haematopoietic	Defects in Lt-HSC engraftment although no differences in absolute numbers of HSCs were observed	(61, 73, 74)
JAM-C	2004	JAM-C JAM-B $\alpha_M\beta_2$	behaviour, cardiovascular, cellular, craniofacial, digestive/alimentary, endocrine/exocrine, growth/size/body, haematopoietic, immune, integument, mortality/aging, nervous system, reproductive, respiratory, skeleton	Increased number of CMPs	(75–77)
JAM-B	2011	JAM-C $\alpha_4\beta_1$	haematopoietic, homeostasis, mortality/aging, skeleton	Loss of quiescent HSCs and exacerbated response to mobilizing agent	(78)

activation of Syk, a candidate therapeutic target in AML, that is phosphorylated upon engagement of surface receptors including not only $\alpha_v\beta_3$ integrin, but also β_2 integrins (102, 103). In summary, integrin signalling converging toward specific activation pathway such as NF-kB or Syk may represent attractive therapeutic targets.

E-Selectin

E- and P-Selectins are constitutively expressed by bone marrow endothelial cells and play a role in HSPC rolling on micro vessels (39, 104, 105). However, they induce contrasting effects in HSPC upon interaction *in vitro* (86, 106–108). The study of early haematopoiesis in E-Selectin deficient mice (*Sele*$^{-/-}$) has revealed that inhibition of E-Selectin *in vivo* increases dormancy and self-renewal of HSC (49). This is not mediated by the conventional ligands of E-Selectin since HSC isolated from mice deficient for P-selectin glycoprotein ligand-1 (Psgl-1 encoded by *Selplg*), HCELL (*Cd44*) or both do not present increased dormancy. In contrast, LSC of AML make a different selectin receptor usage that promotes AML cell survival. Indeed, leukemic cells present alterations in glycosylation which leads to expression of fucosylated ligands such as PSGL-1 (CD162) that activate PI3K/Akt survival pathway (109, 110). Even more interesting is the fact that inhibition of E-selectin interaction with its ligands using a glycomimetic stimulates proliferation of AML blast while dampening HSC cycling. Since these finding have been confirmed in preclinical mouse models, this led to the opening of phase II/III clinical trials combining inhibition of E-selectin with conventional chemotherapy in AML (NCT03616470, NCT03701308).

Ig Sf Adhesion Molecules

Most of the Ig Sf molecules expressed by normal HSC are also expressed by LSCs in AML, however only few of them allows enrichment of cells with leukemic initiating activity associated to poor prognosis. We have shown that JAM-C is expressed by a fraction of LSCs presenting high activation of Src kinase family and enriched for leukaemia initiating activity. Increased frequencies of JAM-C expressing cells identify AML patients with poor disease outcome (111). This has been confirmed in an independent study on a large cohort of AML patients (112, 113). The "CD34$^+$ CD38^{low} CD123$^+$ CD41$^-$ JAM-C$^+$" cells are enriched tenfold for LSCs as compared to cells lacking JAM-C expression within the same compartment suggesting that JAM-C may play a cell-autonomous signalling function at the transition between healthy HSC and LSC. This would be consistent with results showing that PDX or AML cell line engraftment of JAM-C-expressing cells is only partially dependent on JAM-B expression by recipient mice and with results showing that silencing JAM-C expression is sufficient to decrease Src family kinase activation (111). This could be due to promiscuous cis-interactions between JAM-C and the integrin $\alpha_4\beta_1$ since JAM-B has been shown to bind $\alpha_4\beta_1$ when interaction is facilitated by the simultaneous engagement with JAM-C (67).

NCAM1(CD56) is another Ig Sf molecules whose expression is correlated with poor overall survival in AML with t(8;21) (q22; q22) and highly expressed by LSC in mouse AML models using MLL-AF9 or Hoxa9-Meis1 as driver translocations (114). NCAM1 expression confers drug resistance to AML cells and knockdown of NCAM1 sensitizes blasts to genotoxic agents (115). This is likely due to constitutive activation of the MEK-ERK pathway, similar to what has been reported during neural development (116). These two examples pave the way for the use of Ig Sf molecule expression to stratify patients eligible to treatments targeting downstream signalling pathways such as Src or Mek/Erk.

OUTLOOK

Recent studies have shown that HSC niches are altered during AML development with strong coordinated changes of the osteolineage and endothelial compartments, and alterations of the mesenchymal compartment occurring early during leukemic development. Whether such alterations depend on adhesive interaction of

leukemic initiating cells with BM microenvironment resulting in localization of LSCs in specific sites remain to be defined, but it seems that LSC take advantage of pre-existing adhesive pathways in the niche to maintain survival signals and dormancy that protect them from chemotherapies. Therefore, the selective disruption of LSC from their niche by targeting single adhesion molecule remains a major limitation for current therapies. A better knowledge of the differences between LSC/Niche and HSC/Niche integrated adhesive networks will help refining specificity of therapeutic strategies directed against adhesive cues.

AUTHOR CONTRIBUTIONS

JG wrote and revised the manuscript. CT, CF, and SM revised the manuscript and MA-L supervised the work. All authors contributed to the article and approved the submitted version.

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Recent Advances in Developmental Hematopoiesis: Diving Deeper With New Technologies

Bart Weijts[1‡], *Laurent Yvernogeau*[1†‡] *and Catherine Robin*[1,2*]

[1] Hubrecht Institute-KNAW (Royal Netherlands Academy of Arts and Sciences) & University Medical Center Utrecht, Utrecht, Netherlands, [2] Regenerative Medicine Center, University Medical Center Utrecht, Utrecht, Netherlands

The journey of a hematopoietic stem cell (HSC) involves the passage through successive anatomical sites where HSCs are in direct contact with their surrounding microenvironment, also known as niche. These spatial and temporal cellular interactions throughout development are required for the acquisition of stem cell properties, and for maintaining the HSC pool through balancing self-renewal, quiescence and lineage commitment. Understanding the context and consequences of these interactions will be imperative for our understanding of HSC biology and will lead to the improvement of *in vitro* production of HSCs for clinical purposes. The aorta-gonad-mesonephros (AGM) region is in this light of particular interest since this is the cradle of HSC emergence during the embryonic development of all vertebrate species. In this review, we will focus on the developmental origin of HSCs and will discuss the novel technological approaches and recent progress made to identify the cellular composition of the HSC supportive niche and the underlying molecular events occurring in the AGM region.

Keywords: hematopoietic stem cells, aorta-gonad-mesonephros, microenvironment, niche, single cell RNA sequencing, tomography sequencing, embryo, hemogenic endothelium

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****Correspondence:***
Catherine Robin
c.robin@hubrecht.eu

†Present address:
Laurent Yvernogeau, Sorbonne Université, IBPS, CNRS UMR7622, Inserm U1156, Laboratoire de Biologie du Développement, Paris, France

‡These authors have contributed equally to this work and share first authorship

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INTRODUCTION

The origin of the hematopoietic system lies within the early developing embryo (1–5). Successive waves give rise to hematopoietic stem and progenitor cells (HSPCs) with various lineage potentials and self-renewal capacities. While initially a pool of short-lived differentiated cells is formed to sustain the fast-growing embryo, multilineage and self-renewing hematopoietic stem cells (HSCs) are then produced to support long-term hematopoiesis. HSCs are first detected in the aorta-gonad-mesonephros (AGM) region prior to colonize mainly the fetal liver (and also the placenta) where they mature and expand through self-renewal. Shortly before birth, HSCs emigrate from the fetal liver and home to the bone marrow (BM) where they form the pool of adult HSCs that will participate to the replenishment of all blood lineages for the remaining life of the organism. For long, stem cells were considered as independent entities able to self-regulate their own behavior. Four decades ago, Schofield was the first to postulate that stem cells are not complete autonomous entities, as they require external signals from the local microenvironment or niche to regulate their behavior and fate decisions to either remain quiescent, to self-renew or to differentiate in response to the need of the organism (6). HSCs are in this regard unique as their formation requires sequential interactions with distinct anatomical sites throughout development and in adults, e.g. the AGM,

fetal liver and BM. While HSC niches are well documented in the adult [reviewed in (7–9)], this is far from being the case during ontogeny.

How embryonic and fetal niches exactly support and contribute to the development of HSCs and the hematopoietic system is of great interest both for our fundamental knowledge and for the clinic to advance the therapeutic application of hematopoietic (stem) cells. The increased number of diseases and disorders treated at least in part by HSC transplantations and the difficulties to find HSCs with the best donor-patient compatibility is a major issue. Decades of efforts to develop culture conditions, either to expand HSCs *ex vivo* or to generate new HSCs *in vitro*, hold great promise but success remains limited (10–13). The low production of HSC-like cells with limited multilineage and/or self-renewal properties remains a major barrier to a successful use of HSCs for transplantation and gene modification. The reconstitution of a complete microenvironment or at least some of its key components to support the generation, maintenance and/or expansion of HSCs *in vitro* will be necessary to overcome this barrier. However important clues on how HSC production is supported by successive niches *in vivo* is missing. In this review, we will focus on the developmental origin of HSPCs and will discuss the novel technological approaches and recent progress made to identify the cellular composition, the importance of cell cross-talk and the underlying molecular events involved between the HSCs and the supportive microenvironment in the embryonic aorta, the physiological cradle of the first adult-type HSCs.

HEMATOPOIETIC PRODUCTION OCCURS IN VARIOUS HIGHLY VASCULARIZED TISSUES DURING DEVELOPMENT

In mammals, the first hematopoietic cells are formed independently of HSCs. This first or primitive wave generates nucleated erythrocytes that emerge with and in close proximity to endothelial cells (14). They derive from mesodermal derivatives in blood islands of polyclonal origin, in the extra-embryonic yolk sac (YS), around mouse embryonic day (E)7.25 (15–17). Beside primitive erythrocytes, a first wave of primitive megakaryocyte and macrophage progenitors also arise in the YS blood islands (18–20). A portion of these "primitive" macrophages will persist throughout adulthood and give rise to microglia, the tissue-resident macrophages in the adult brain and central nervous system (21). Multipotent erythro-myeloid progenitors (EMPs) will then produce erythrocytes, macrophages, granulocytes and megakaryocytes (and possibly also few B lymphocytes and NK cells) through a second hematopoietic wave (also referred to as transient definitive wave or EMP wave) in the YS vascular plexus, starting at ~E8 - E8.25 (18, 22–26). The YS does not provide a competent niche for the differentiation of EMPs in mature cells, which instead occurs in the fetal liver (23). Some macrophages reside in tissues, also referred to as tissue-resident macrophages, of embryos and adults where they act as immune sentinels involved in tissue homeostasis (27, 28). The YS and the developing dorsal aorta in the para-aortic splanchnopleura (which will give rise to the AGM region) also generate lymphoid progenitors at ~E8.5 - E9.5, independently of HSCs, that are responsible for the initial immunity during development and the persistence of some immune cells (i.e. B-cells) into adulthood (29–33). The importance of these long thought short-life "primitive" hematopoietic cells, of which in fact some subsist and play important roles in late fetuses and adults, reinforces the need for a better identification of the niche-derived signals regulating their production. The 3rd or definitive hematopoietic wave leads to the formation of HSCs that can be detected as early as E10.5, in the aorta of the AGM region and in the extra-embryonic vitelline and umbilical arteries (34–36). Slightly later, HSCs are also found in other highly vascularized tissues such as the YS, and then the placenta and the fetal liver where they expand before colonizing their final destination, the BM (37–40). The impact of the microenvironment on HSC behavior is well illustrated in the fetal liver. During birth, the fetal liver undergoes dramatic changes in hemodynamic forces when the umbilical inlets are ligated. These changes trigger the transformation of arterial endothelial cells lining the portal vessels into venous endothelial cells, characterized by the loss of arterial markers (Neuropilin-1 and Ephrin-B2), acquisition of the venous marker (Eph Receptor B4) and the loss of Nestin^{+}NG2^{+} pericytes through apoptosis (41). The latter cells are critical niche components and are probably the main cause of the emigration of HSCs from the fetal liver and their homing to the BM.

Hemogenic endothelial (HE) cells are a small subset of endothelial cells (1–3% in distinct tissues), which have either an arterial or venous identity (28, 42). HE cells can transdifferentiate into hematopoietic cells through a so-called endothelial to hematopoietic transition (EHT), a highly conserved process across vertebrate species (17, 43–49). HE cells can give rise to different types of hematopoietic cells, suggesting that different types of HE cells exist (50, 51). In the floor of the dorsal aorta, HE cells give rise to both EMPs and the first adult-type HSCs (17, 43–47, 49, 52). Most HSPCs are generated from HE cells with arterial characteristics, e.g. in the AGM, vitelline and umbilical vessels and the vascular labyrinth of the placenta and YS (53, 54). However, EMPs can also be produced by venous HE cells in the plexus of the YS (55, 56) and from HE cells in the heart that have not yet acquired an arterial-venous specification (57). Macrophages can also directly derive from HE cells *via* EHT in the placenta (58). The mere presence of HE cells does not guarantee that an EHT event occurs, indicating that the EHT process is differentially steered by signals from the niche, depending on their precise anatomical location and surroundings. For example, HE cells in the aorta that are not exposed to hemodynamic forces from blood flow do not give rise to HSCs, while EMP emergence is unaffected (59–63). How precisely HE cells acquire their hemogenic potential and how this potential leads to the formation of different hematopoietic cells through an EHT event remains to date largely elusive. Nevertheless, there are strong evidences that spatial and temporal signals from the microenvironment play a major role in hemogenic specification (to acquire a hemogenic potential), EHT, IAHC formation and HSPC production. As described in this review, new single cell technologies will shed new light on

these processes to better understand how the establishment of the hematopoietic system is regulated.

After EHT, hematopoietic cells are organized in intra-aortic hematopoietic clusters (IAHCs) that remain transiently attached to the inner side of the vessels in most species (44) (**Figure 1**). IAHC-like structures have also been observed in non/less-hematopoietic sites such as the somites or the cerebrovascular sinusoids in the head (64). However, the detection of HSCs (which circulate through the blood circulation) and the presence of IAHCs in a tissue do not prove that EHT occurs *in situ*, as shown in the mouse embryonic head (65, 66). The ultimate proof that the *de novo* formation occurs in a tissue was provided for the aorta by performing confocal imaging on zebrafish embryos *in vivo* and on thick mouse embryo slices *ex vivo* (43, 67–69). The continued existence of HE cells beyond embryonic stages, leading to the formation of multipotent progenitors (MPP-3) and few HSCs in the sinusoids of the BM in fetal/neonate chicken and mouse was also reported and imaged, which could be referred to as a 4th hematopoietic wave (70).

SPATIAL AND TEMPORAL EMERGENCE OF IAHCs AND HSC ACTIVITY

Extensive research has explored the formation of the first HSCs during development, with a particular focus on IAHCs. IAHC cells express similar hematopoietic and endothelial markers as HSCs (67, 71, 72), and both are absent in *Runx1* knock-out embryos (72, 73), suggesting that HSCs are likely part of IAHCs. However, IAHCs appear earlier than HSCs in the aorta (E9.5 versus E10.5), suggesting that some IAHC cells will become HSCs through gradual specification and maturation (**Figure 1**). Indeed, it was found that IAHCs are mainly composed out of HSC precursors (pre-HSCs type I and type II) that progressively mature into functional HSCs (72, 74–76). Accordingly, IAHCs contain very few HSCs and committed progenitors (that are mainly present in the blood circulation and transiting from the YS to the fetal liver). The process of maturation begins in the aorta but mainly takes place after migration of the cells into the fetal liver and placenta in mammals or in the caudal hematopoietic tissue (CHT) in zebrafish embryos (77). Limiting dilution transplantations and statistical analyses suggest that the pool of adult HSCs in the fetal liver is formed by the pool of IAHC cells (76). This finding raises the question what the contribution of HSCs found in the YS and placenta (39) and the pre-HSCs found in vitelline and umbilical arteries (54) is. A transient production of lympho-myeloid-biased progenitors and lymphoid cells prior to pre-HSC production was also reported in IAHCs (78–80). Although the connections between these different cell types remain unclear, it underlines the heterogeneity and complexity of IAHC composition at different time points and location during development. Of note, various differentiation potentials of IAHC cells have been revealed *ex vivo*, in presence of supportive stromal cell lines (OP-9) and/or cytokines, which might not reflect the true fate of these cells *in vivo*. The EHT is orchestrated by hematopoietic transcription factors primarily driven by RUNX1 (46, 81, 82), although it was reported that *Runx1* deficiency does not preclude

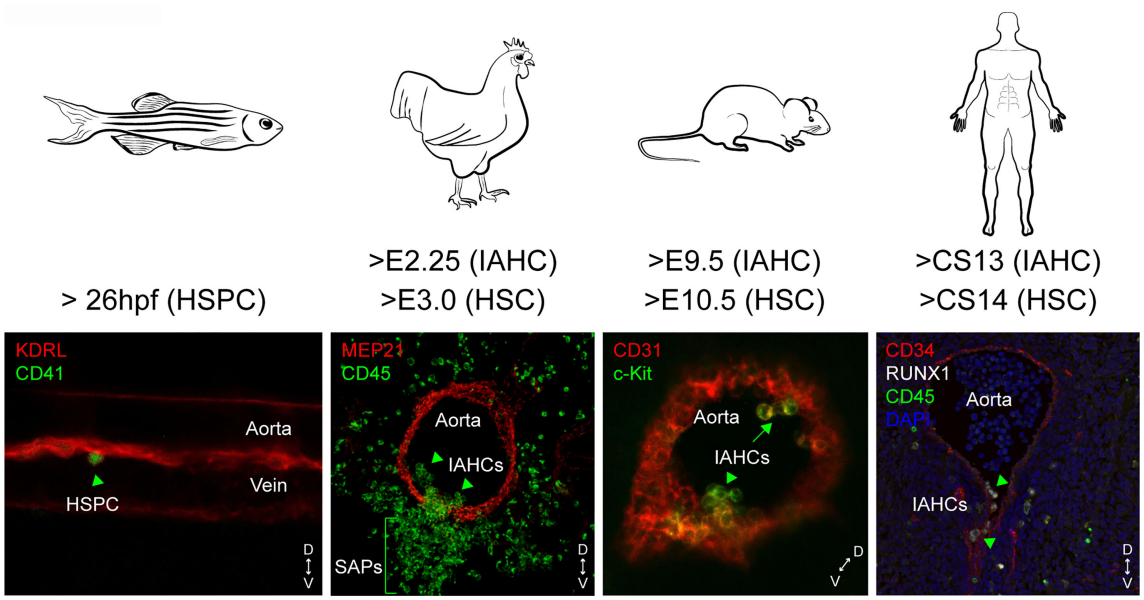

FIGURE 1 | Spatial and temporal locations of IAHCs and HSPCs in the aorta of zebrafish, chicken, mouse and human embryos. The endothelial to hematopoietic transition (EHT) leads to the production of single HSPCs in the aorta of zebrafish embryos, or clusters (intra-aortic hematopoietic clusters or IAHCs). IAHCs emerge exclusively in the ventral side of the aorta in chicken and human embryos (arrow heads). In contrast, IAHCs also emerge in the dorsal side of the aorta in mouse embryos (arrows). In chicken, IAHC cells ingress underneath the ventral aortic endothelium to form sub-aortic patches (SAPs). The starting time of IAHCs and HSPCs/HSCs detected is indicated for each embryo species. D, dorsal; V, ventral.

the formation of pre-HSCs type I but most likely blocks their maturation into pre-HSCs type II (83). Ectopic expression of Runx1 is sufficient to induce EMPs from non-HE cells (i.e. in YS, aorta, heart), but only between E7.5 to E8.5 of mouse development (82). Therefore, more complex events, such as the initiation of the blood circulation and the proper arterial–venous specialization of endothelial cells in the vascular niche microenvironment, might contribute to temporal restriction of EMPs and (pre-)HSCs appearance in IAHCs.

IAHCs originate from single HE cells that undergo 1 or 2 divisions to form a monoclonal core of IAHCs. Neighboring HE cells are then recruited into IAHCs that become thereby polyclonal while the cellularity increases (84, 85). Intra-cardiac injection of Dll4 blocking antibodies results in the enhanced recruitment of HE cells into IAHCs, suggesting that Dll4-Notch signaling in HE cells regulates IAHC cellularity (84). Unusually large IAHCs were also observed in *Svep1*$^{-/-}$ embryos that did not originate from ectopic proliferation of IAHC cells (86). *Svep1* is expressed and secreted by mesenchymal cells surrounding the aorta, highlighting the importance of microenvironmental factors (Notch independent) in determining IAHC size (86). Size and composition might also be determined by an increased proliferation during pre-HSC expansion and a decrease when cells start to acquire HSC identity (87). While slowly cycling cells are located at the base of IAHCs, more proliferating cells preferentially locate at the more apical part of IAHCs (87). Thus, IAHC size is determined by recruitment of neighboring HE cells, controlled by a combination of direct signaling between HE cells and factors derived from the microenvironment, and proliferation of the different cell types within IAHCs. Such local regulation within and between IAHCs is an important concept to further explore since HSC activity is affected by the increased cellularity in IAHCs, as shown in *Svep1*$^{-/-}$ embryos (86).

The mechanism of HSC emergence is highly conserved and regulated both in space and time in between species, with few species-specific differences most likely due to anatomical constrains (1) (**Figure 1**). The EHT is polarized and restricted to the ventral side of the aorta in chicken, zebrafish and human embryos (4, 43, 88–91). After EHT, cells form IAHCs that remain transiently attached to the endothelium before detaching and leaving *via* the circulation to colonize the fetal liver in human and mouse. In chicken, the entire floor of the aorta becomes hemogenic, forming IAHC cells that ingress, at least in part, in the mesenchyme underneath the ventral endothelium to form sub-aortic patches, a site considered as the mammalian fetal liver equivalent (89) (**Figure 1**). In zebrafish, HSPCs bud off as single cells from the floor of the dorsal aorta into the sub-aortic space, where they transiently reside and roughly half of them divide before entering the circulation via the posterior cardinal vein (43, 68, 91) (**Figure 1**). Half of these HSPCs are considered as HSCs, the rest being possibly committed myeloid progenitors (92). Although "true" HSCs exist in chicken (89), it remains unknown whether they emerge as pre-HSCs and whether maturation and/or expansion occurs in the sub-aortic patches.

HSPCs start to emerge at 26 hours post fertilization (hpf) with a peak at 40 hpf, in the aorta of zebrafish embryos. IAHCs are found between E2.25 and E5.5 (with a peak at E3) in the anterior portion of the chicken aorta, from days 27 to 42 in the middle portion of the human aorta, and between E9.5 and E14.5 (with a peak at E10.5) in the middle portion of the mouse aorta (4, 34, 43, 68, 88–90, 93, 94). In contrast to other species, the mouse embryo has the particularity to produce IAHCs also in the dorsal part of the aorta (95, 96) (**Figure 1**). RNA-sequencing (RNA-seq) comparative analyses performed on whole IAHCs isolated from the ventral or dorsal part of the aorta revealed a strong similarity at the molecular level at both E10.5 and E11.5 (97). However, dorsal IAHCs are less numerous and have a lower HSC potential (four times less) compared to ventral IAHCs, as shown by limiting dilution transplantations of the subdissected parts of the aorta (ventral [AoV] versus dorsal [AoD]) (95, 98). Using a dissociation-reaggregation culture system that recapitulates HSC development *ex vivo* (99), it was shown that the AoD tissue induces a higher HSC production in the AoV tissue isolated at E10.5 but not at E11.5. In contrast, the AoV induces the production of HSCs in AoD at E11.5 but not at E10.5 (98). Such experiments, although performed *ex vivo*, reveal that the ventral and dorsal aortic microenvironment have reciprocal effects on HSC development, depending of the developmental time, and most likely on the differential release of factors by the two regions (i.e. SCF, Shh, BMP) and the capacity of IAHC cells to respond to specific signals by expressing the right level of receptors at the right time point (75, 98, 100–102). The lack of hemogenic potential of the dorsal aortic endothelium of most other vertebrate species might also be explained by the different origin of the endothelial cells populating the ventral and the dorsal part of the aorta. In zebrafish embryos, the dorsal endothelium does not originate from the splanchnopleura (lateral plate mesoderm) but from the paraxial mesoderm (103). In the avian embryo, the dorsal endothelium that derives from the splanchnopleura is progressively replaced by paraxial mesoderm-derived endothelial cells (as the ventral endothelium), which corresponds to the end of aortic hematopoiesis (104–106).

TISSUE COLLECTIONS AND TRANSCRIPTOMIC APPROACHES TO UNRAVEL THE MOLECULAR LANDSCAPE OF THE AORTIC NICHE

Various interacting signals from unique niche populations present in the different anatomical sites, as well as biomechanical forces, form an intricate signaling network that regulates the formation of HSPCs [for reviews (2, 9, 107–109)]. These signaling events are far from fully understood and many questions remain such as the exact nature (timing and duration) of signaling interactions between the niche and HSPCs and how these interactions contribute to determine different cell fates in endothelial/HE cells and IAHCs. Single-cell (sc) qPCR analyses paved the way for the (single-cell) genomic techniques to explore HSPC development. Although sc-qPCR offers high sensitivity

and specificity, the quality of the data relies on high cell numbers and carefully selected and tested primer panels. Such approach identified important players that specify early blood formation (110) or revealed that HE cells are molecularly specified toward a hematopoietic fate two days prior the emergence of the first HSCs (111). Bulk RNA-seq, scRNA-seq and microarray analyses have then been performed to analyze the intrinsic regulation (e.g. by transcription factors) of HSPC formation by sequencing phenotypically enriched populations for arterial endothelial cells, HE cells, cells undergoing EHT, pre-HSCs and/or HSCs sorted from mouse, chicken, human and/or zebrafish embryos (78, 86, 97, 112–123). Overall, these studies highlighted important features. Among them are (i) the molecular heterogeneity of the HE, pre-HSC and HSC populations, (ii) the gene regulatory networks and trajectories involved during HSPC formation, (iii) new surface markers for a better localization and isolation of these rare embryonic cells, (iv) the identification of specific cell-cell-interactions and (v) important novel niche secreted factors. Single-cell transcriptomics of whole mouse and human embryos or organs collected at early time points of development also provided information on early mesoderm specification and important regulators of the early hematopoietic development (124–126). Several signaling molecules and pathways critical for HSC emergence or maturation have been identified by using knock-out/knock-down approaches, large drug screening (in zebrafish) or by performing mouse tissue explant or dissociation-reaggregation cultures in presence of either growth factors or stromal cell lines. Among others are Wnt, Notch, vitamin-A derived retinoic acid signals, BMP4, cytokines such as the interleukin-3 (IL-3) and stem cell factor (SCF), the catecholamines produced by the sympathetic nervous system, pro-inflammatory signals, the blood shear stress, chemokine such as Cxcl12 (SDF1), hyaluronan and extracellular matrix compounds (9, 75, 100, 109, 127–129). Overall, these approaches hardly link a regulator to a specific cell type (e.g. due to the limited purity of the cell populations tested) or to an anatomical location, particularly for the soluble factors.

In the avian model, dissection procedures that prevent the migration of the sub-aortic mesenchyme, also abolish Runx1 expression in HE cells. Subsequently, the formation of IAHCs is inhibited, proving the supportive role of mesenchymal cells in hematopoiesis in the aorta (130). Notch expression also tightly controls aortic hematopoiesis at specific time points of chicken, zebrafish and mouse development (84, 130, 131). Obtaining a global picture of all the molecular players expressed by the surroundings of the aorta during HSC emergence is still not achieved. In an attempt to identify putative molecules secreted by the HSC supportive microenvironment, several groups have compared cell lines derived from embryonic, fetal and postnatal mouse blood-forming tissues where HSCs emerge, expand or are maintained *in vivo* (e.g. from AGM sub-regions (aorta-mesonephros [AM] and urogenital ridges [UG]), the embryonic liver [EL] or the BM) (132, 133). Such cell lines have been tested and characterized for their competency to maintain/expand mouse and human HSPCs at different levels *in vitro*. Macro-array-based gene expression analyses of HSC-supportive (UG26-1B6 and EL08-1D2) versus less/non-supportive (UG15-1B7, AM20-1B4, EL28-1B3, and AM30-3F4) stromal cell lines revealed an up-regulation of fibroblast growth factor-7 (FGF-7), cathepsin K, thrombospondin 2 (TSP2), pleiotrophin (PTN), and IGFBP-3 and -4 in the supportive cell lines (134). This study demonstrated that 'niche' cells are not necessarily in direct contact nor need to be in contact with HSCs to fulfil their support capacity since secreted factors from the microenvironment are sufficient to maintain the HSC stemness properties (134). Using a similar approach, bulk transcriptome comparative analyses of AGM (UG26.1B6 [supportive] *vs* UG26.3B5 [less-supportive]), fetal liver (AFT024 [supportive] *vs* BFC012 [non-supportive]) and BM (BMC9 [supportive] *vs* BMC10 [less-supportive]) cell lines established the genetic signature of the sequential embryonic, fetal and adult HSC niches (135) (**Figure 2A**). Through comprehensive transcriptomic meta-analyses, 481 mRNAs and 17 micro-RNAs were found organized in modular networks and involved in critical signaling pathways. Beside known HSC regulators, this study also identified unexpected ones such as Pax9 and Ccdc80 that were functionally validated using morpholino injections in the zebrafish model. While these studies used solely *in vitro* cell lines, they opened the way for a better identification/characterization of the molecular landscapes of the sequential supportive HSC microenvironments.

Although very informative, studies performed on stromal cell lines that are often clonal do not recapitulate the complexity of the HSC microenvironment. Moreover, critical *in vivo* components (i.e. blood flow/shear stress, circulating cells and growth factors) as well as the spatial three-dimensional organization of the aortic surrounding tissues are missing. It is not certain that all the *in vivo* features need to be reproduced *in vitro*, since HSC development can occur *ex vivo* when AGMs are cultured after dissociation-reaggregation or as explant for few days (35, 99). Several labs explored the aortic microenvironment by dissecting intact hematopoietic organs or by dissecting defined sub-regions, based on their HSC activity (i.e. emergence, expansion). In mice, the middle third of the aorta was identified as the HSC-containing region compared to the most anterior or posterior third regions that were devoid of HSCs (93) (**Figure 2B**). A micro-array transcriptomic analyses performed on these different regions collected at E9 and E11 (before and during the acquisition of an HSC potential, respectively) identified p57Kip2 and IgF2 as important hematopoietic regulators (93). Using a more precise microsurgery, the dorsal and ventral parts of the aorta and the urogenital ridges (UGRs) were isolated from E9.5 to E10.5 since the polarity along the dorsal to ventral axis of the embryo was demonstrated as a clear demarcation of the supportive HSC niche (136) (**Figure 2C**). Bulk RNA-sequencing on these different tissues allowed to identify critical signaling pathways and several secreted molecules, including Bmper as a ventrally polarized new regulator of HSC development in the AGM region. The use of human embryos at early stages when IAHCs appear is challenging due to the difficulty to collect intact

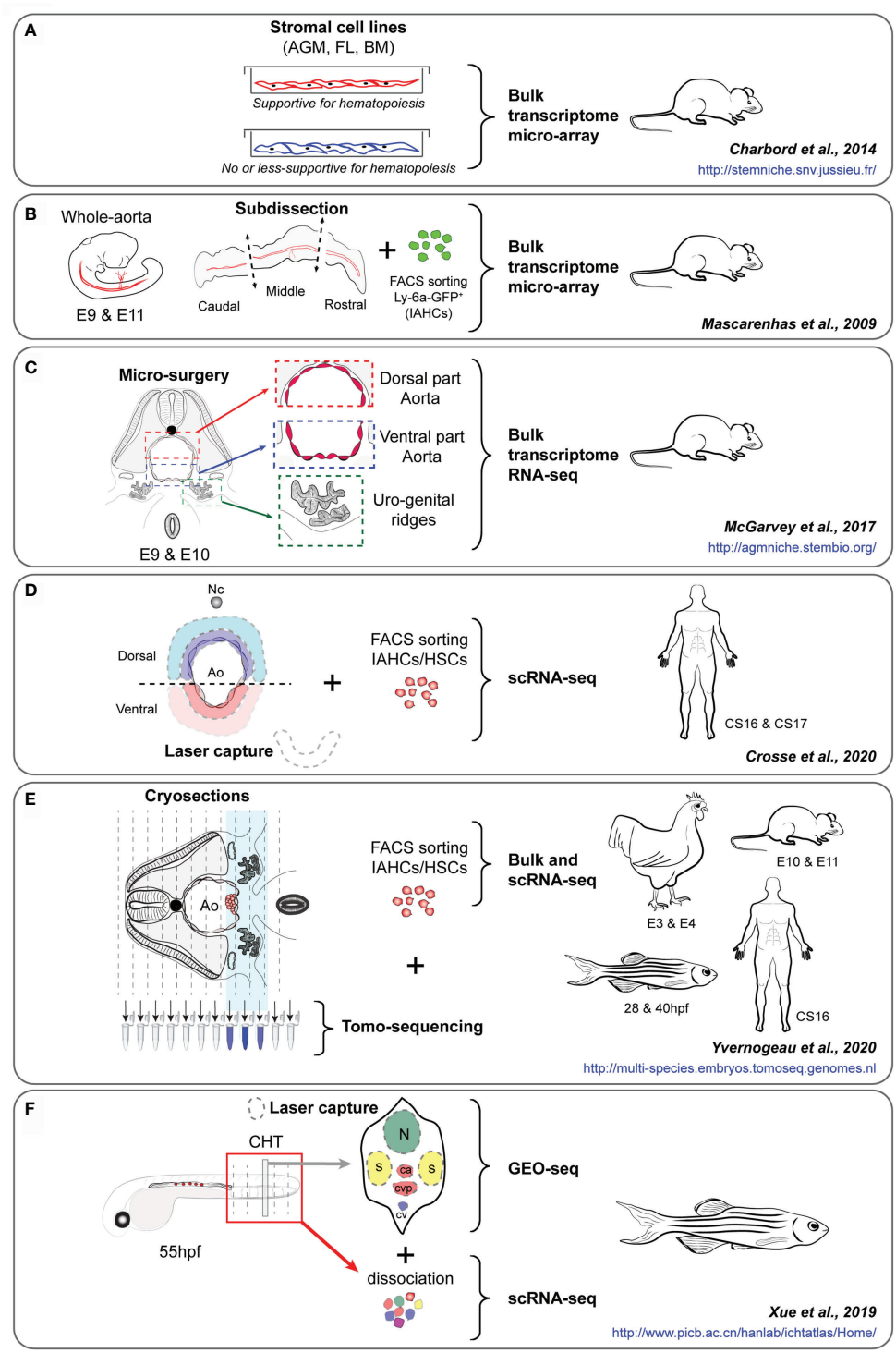

FIGURE 2 | **(A–F)** Experimental approaches of key studies to identify the HSPC supportive landscape of the embryonic aorta, i.e. the studies of Charbord et al. (135) **(A)**, Mascarenhas et al. (93) **(B)**, McGarvey et al. (136) **(C)**, Crosse et al. (117) **(D)**, Yvernogeau et al. (86) **(E)** and Xue et al. (137) **(F)**. The embryo species and stages, the type of cells/tissues analyzed, and the experimental approach used for each study are indicated. The interactives website resources provided in each study are also indicated (in blue). AGM, Aorta-Gonad-Mesonephros; FL, Fetal liver; BM, Bone marrow; E, Embryonic day; IAHCs, Intra-Aortic Hematopoietic Clusters, HSCs, Hematopoietic stem cells; Nc, Notochord; Ao, Aorta; BM, Bone marrow; CS, Carnegie stage; CHT, Caudal hematopoietic tissue; hpf, hour post-fertilization.

embryos at the time of IAHC emergence in the aorta [around Carnegie stage (CS) 13 (post-fertilization age of 30-33 days) (4)] Different layers of cells surrounding the ventral and dorsal sides of the aorta of human embryos were captured by laser dissection and sequenced (LMO-seq) (117) (**Figure 2D**). In parallel, scRNA-seq data obtained from sorted human $CD34^+$ IAHC cells were used to explore the cross-talk (e.g. involving secreted factors, cell surface receptors) occurring between IAHCs and the surroundings of the aorta. This approach allowed to highlight the cardiac epidermal growth factor (EGF) and its major receptor, endothelin 1 (expressed and secreted by endothelial cells), as a potent enhancer of HSC generation in human embryos (117).

A major drawback in single cell and bulk sequencing experiments is the lack of spatial information, which is essential to understand the interactions between niche and HSC cells. To perform RNA-seq while keeping spatial information, tomography-sequencing (tomo-seq) was developed (138). Embryonic thick slices or complete embryos are sequentially cryosectioned along the axis of the embryo, e.g. following a transversal or longitudinal orientation, and RNA from each slice is collected for sequencing. RNA expression profiles can then be visualized along the embryo axis. Genes expressed highly or solely in specific tissues/areas can be used for anatomical or orientation confirmation, e.g. *Shh* expression to locate the notochord, or *Mpl* (chicken), *Gata2* (mouse) or *Cxcl12* (zebrafish) expression for the aortic region containing IAHCs/HSPCs (86) (**Figure 2E**). Genes expressed in tissues or regions of interest can then be readily filtered out. By using this technique, thick transversal embryo slices and/or embryo trunks were collected from the 4 main species used to study developmental hematopoiesis, i.e. zebrafish, chicken, mouse and human, at two different developmental time points (beginning and time of HSPC production) (86). Genes specifically expressed in the ventral mesenchyme located underneath the aorta, and more expressed in this region than in the rest of the tissue sample, were identified. To further enhance the usability of these data, transcriptomic datasets of sorted mouse (97) and chicken (86) IAHC cells were generated and compared to the tomo-seq datasets to identify genes and pathways potentially involved in the cross-talk between IAHCs and the ventral aortic microenvironment. Known ligands and corresponding receptors in the aortic ventral microenvironment and IAHC cells were identified, validating this experimental approach and analysis. These were known to be involved in EHT and HSPC survival, attachment, maturation, and/or expansion (e.g. integrin, WNT, BMP, FGF, NOTCH, catecholamines), in inflammation, extracellular matrix organization, cytoskeleton rearrangement, in various cellular processes (protein phosphorylation, intracellular protein transduction) and specific pathways (Pi3K-AKT, MAPK, ERK, RAP1, and RAS). Molecules with unknown hematopoietic role were also identified and functionally validated *in vitro* and *in vivo* as important and conserved HSPC regulators in mouse, chicken and zebrafish embryos. These included (1): the adrenomedullin (ADM), a hypotensive and vasodilator agent and its receptor RAMP2, which regulates HSPC emergence in the aorta, and (2) SVEP1 (Sushi, Von Willebrand Factor Type A, EGF and Pentraxin Domain Containing 1), a secreted extracellular factor critical for proper lymphangiogenesis (139), that was shown to also regulate IAHC cellularity and HSPC production in the aorta (86).

The cellular and molecular mechanisms underlying HSC and multipotent progenitor expansion remain poorly understood. Zebrafish embryos have been used to generate a "3D transcriptional atlas" to characterize the spatiotemporal transcriptome during HSPC expansion in the CHT region (137) (**Figure 2F**). In this study, multi-dimensional RNA-seq approaches were used, including bulk and scRNA-seq on HSPCs isolated at 6 different time points (between 28 hpf and 3 mpf), and *in vivo* GEO-seq performed on the CHT region where six regions were collected on embryo sections by laser capture microdissection at 55 hpf. These regions included the neural tube (in the dorsal region), the muscles (left and right regions), and the caudal artery, caudal vein and caudal vein plexus (in the middle, intermediate and ventral regions, respectively). Such approach combined with functional validation allowed to reconstruct the panoramic transcriptome landscape (temporal and spatial) of the zebrafish CHT, and highlighted the integrin signaling protein Smchd1 as critical for HSPC expansion. Single-cell and spatial transcriptomics recently provided a spatio-temporal transcriptome map of the mouse fetal liver and thereby identified transcriptionally heterogeneous HSPC subsets, as well as HSC 'pocket-like' units composed of niche cells (i.e. hepatoblasts, stromal cells, endothelial cells, and macrophages), where macrophages and growth factors (MDK, PTN, and IGFBP5) played an important role in HSPC expansion (140).

TECHNOLOGICAL ADVANCES TOWARDS THE MOLECULAR AND CELLULAR DISSECTION OF THE HSC MICROENVIRONMENT

The ability to visualize HSCs in their native environment has been paramount for our current knowledge regarding HSC dynamics and behavior *in vivo* (43, 48, 67, 68, 89, 141, 142). However, immunostainings, *in situ* hybridizations and the use of transgenic reporters only allow for the simultaneous visualization of a handful of genes. Ideally, one would like to image HSCs embedded in their niche in detail, or even follow them by time-lapse imaging until they display the desired behavior, capture the entire transcriptome and map this back to the imaging data. This combination would permit the precise identification of the different (sub)types of cells, as well as their transcriptomic state. Such approach would be especially powerful to e.g. elucidate the exact composition of small and large IAHCs, the heterogeneity of various endothelium (hemogenic and non-hemogenic) and the direct interactions of HSCs with their successive embryonic, fetal and adult niches. Although laser micro-dissection allows for the isolation of relatively small sections of tissue after imaging that can be processed for transcriptomic analysis (137), current

technological advances are heading towards a more precise capture of the transcriptome of a full slide. Visium spatial gene expression by 10X Genomics permits such capture of the whole transcriptome from a tissue section. Prior capture, there is an optional step that allows for the visualization of proteins of interest by immunofluorescence to gain a deeper understanding of tissue organization or the localization of cells of interest (**Figure 3A**). This first version of Visium has 5.000 uniquely barcoded spots on 4 separate capture areas on each slide. Each spot will capture a range of about 1-10 cells, depending on the tissue thickness and tissue architecture (e.g. cell size). This capture grid will undoubtedly become smaller in future versions to reduce the number of cells captured per spot. While such approach will definitely add to our understanding of how HSCs are embedded and interacting with their microenvironment, the financial burden to systematically study HSCs in their native environment will be extremely high.

Whilst much attention goes out to transcriptomic approaches (122), recent advances in protein-based techniques should not be overlooked. Measuring proteins present in or on the cell by fluorescence-based flow cytometry has proven to be a rapid and powerful tool for isolating, sub-typing and phenotyping the cells of the immune system, including HSCs (143, 144). Multiplexing classic fluorescent and quantum dots labeled antibodies have stretched the limit of this technique up to 17 parameters (145, 146). Further expansion of fluorescent based cytometry seems unlikely due to the limitations to resolve the spectral overlap. Replacing fluorescent proteins or quantum dots with element isotopes (chelated antibody tags) dramatically reduced the cross-talk between channels and enabled the simultaneous measurement of up to 40 parameters, which is referred to as cytometry by time-of-flight (CyTOF) (147, 148). Distinct isotopes can be used to label different antibody panels that include surface markers, transcription factors as well as signaling molecules (phosphoproteins). By using CyTOF with about 31 different isotopes, functional and hierarchical maps of the immune/hematopoietic system have been drawn and show that hematopoiesis is a continuum rather than a collection of defined subsets (149–151). CyTOF was also instrumental in identifying a pro-inflammatory subset of macrophages that is involved in the development of HSCs (152). In addition, integrating CyTOF with scRNA-seq could provide additional discriminatory power for further sub-setting or functional analysis between distinct subsets of cells like HSCs and progenitors (153). Besides the precise immunophenotyping of cells in suspension, recent advances have enabled CyTOF of tissue sections or cells cultured on a slide. Aerosols of evaporated cells by laser ablation are transported to the CyTOF mass cytometry by an inert gas for detection (154). Data for each cell is then mapped based on the laser ablation coordinates to reassemble the original tissue architecture (**Figure 3B**). While the selection and availability of antibodies used is crucial to the success of this technique, it provides a new powerful way to study for example sections of the embryonic aorta to elucidate the composition of the vascular aortic, fetal liver and BM niches.

RECAPITULATING THE ENDOGENOUS HSC NICHE IN 3D-CULTURE SYSTEMS TO PRODUCE *BONA FIDE* HSCS AND OTHER BLOOD FORMING CELLS

Limitless access to different types of blood producing cells manufactured *in vitro* is the "holy grail" of regenerative medicine. This would, among others, combat the current shortage of donor HSCs by providing a readily accessible source to all blood groups (red blood cells) (155, 156) or functional T-cells that can be engineered for anti-cancer therapies (157, 158). The crux of the matter is that these *in vitro* produced cells should faithfully mimic their *in vivo* counterparts. *In vitro* production of hematopoietic progenitors and mature blood cells (e.g. red blood cells, platelets, megakaryocytes, T-cells) from pluripotent stem cells or somatic cells, through reprogramming or transgene free protocols, is achievable (159). Reprogramming by (transient) expression of transcription factors is also a very promising strategy to generate HSC-like cells *in vitro* since a decade (11, 160–166). However, these HSC-like cells are produced at a very low yield and remain limited in their capacities to self-renew and/or to replenish all blood lineages, which is an absolute requirement for therapeutic use. Moreover, the association of the reprogramming factors with the development of leukemia remains an underlying risk. Indirect reprogramming (transgene free) through co-culturing pluripotent stem or progenitor cells with supportive cells that mimic the microenvironment in combination with chemical manipulation has therefore become a more favorable option. In its simplest form, this would be co-culturing pluripotent stem cells (e.g. iPSCs, ESCs) or somatic cells with a supportive cell line (e.g. OP9-cells) and/or a cocktail of growth factors, hormones and/or cytokines. Under these conditions, the formation of HE cells and some hematopoietic progenitors was successfully obtained, while HSCs are not or very rarely produced (167–173). As discussed above, the formation of HSCs *in vivo* requires a chain of events involving a complex sequence of both cell intrinsic and extrinsic factors that are difficult to recapitulate in a "simple" *in vitro* setting. Furthermore, one of the important open standing questions is whether it is necessary to first mimic the AGM-like microenvironment, to ensure HE formation and pre-HSC production *in vitro*, and second to mimic the fetal liver microenvironment to support pre-HSC maturation and HSC expansion. Comparing the transcriptomes of *in vitro*-generated HSC-like cells to fetal liver HSCs is an interesting approach to identify transcription factors and molecular pathways that could improve the *in vitro* production of HSCs (174). However, *in vitro*-generated HSCs that faithfully mimic the functionality of *bona fide* HSCs might have a different transcriptional landscape.

Recent advances in the *ex utero* culture of post-implantation mouse embryos, enabling the development until the hindlimb formation stage (E11) (175), or in 3D-culturing systems (176, 177) might offer a more sophisticated way of producing transplantable HSCs *in vitro*, as these systems recapitulate key aspects of developmental processes or organs. Disaggregation-reaggregation assays in the 1950's showed that a suspension of chicken mesonephric cells could self-organize into the structural pattern of

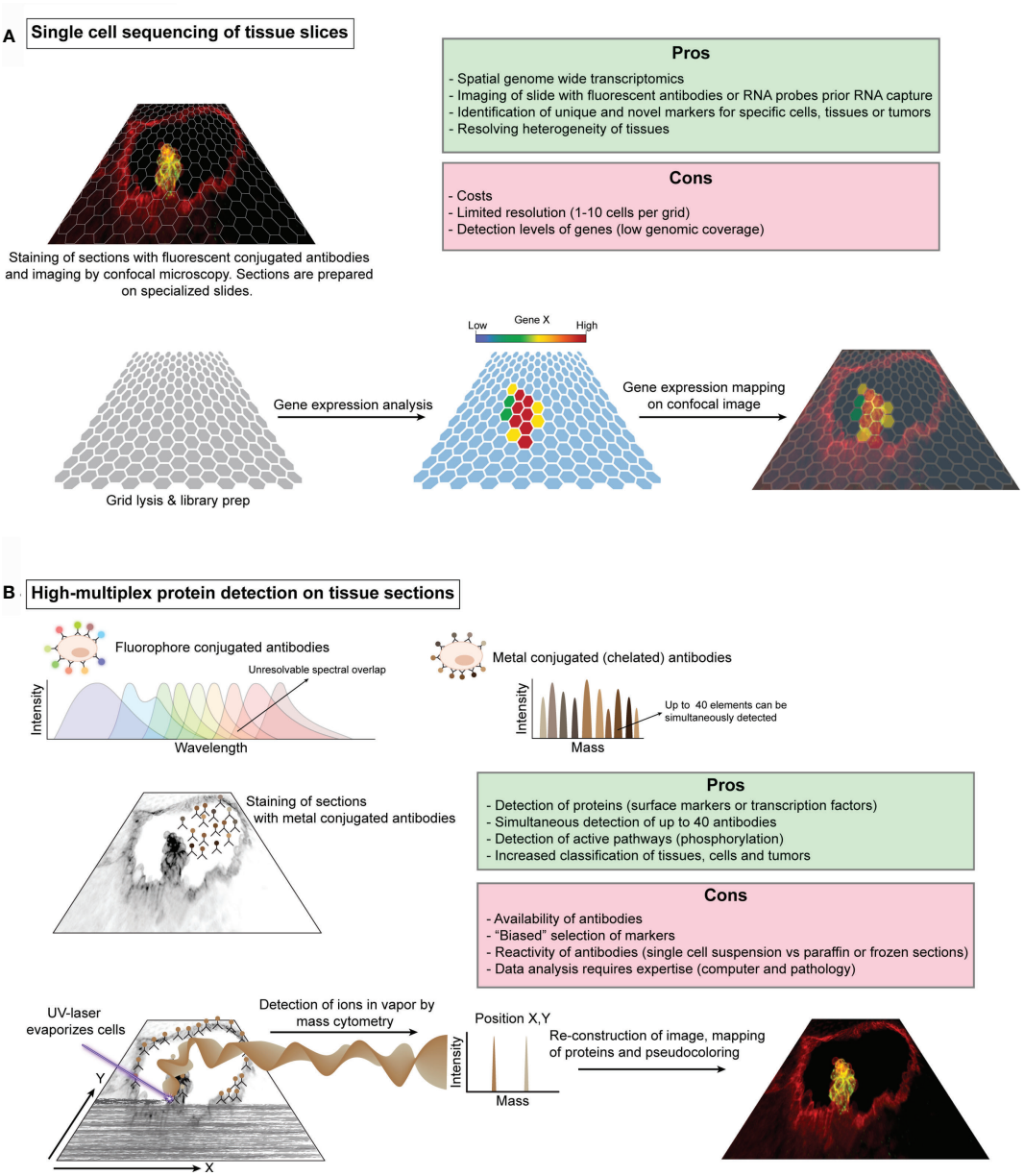

FIGURE 3 | Schematic illustration of single cell sequencing of tissue slices and high-multiplex protein detection on tissue sections. **(A)** Schematic representation of 10X Genomics Visium workflow. Fresh-frozen or paraffin embedded tissue sections are prepared on specialized slides that contain several capture areas or grid. Each grid position has a known unique barcode, which is used for reconstructing the tissue after sequencing. These tissues can be stained with fluorescent antibodies or with one of the principle tissue stains, e.g. hematoxylin & eosin, and subsequently imaged by microscopy prior RNA capture. Cells are then permeabilized and the RNA content is captured for barcoding and library preparation. The library is sequenced by standard procedures and the data can be visualized in relation to the imaged tissues. **(B)**. The use of multiple fluorescent proteins and quantum dots to simultaneously label different cell components is limited to the ability to resolve spectral overlap between the different fluorochromes. To increase the number of labels, classic fluorescent proteins have been replaced by specific elements or isotopes such as noble and post-transition metals, rare-earth elements and halogens, which can be detected by mass cytometry. Antibodies against specific proteins (e.g. receptors or transcription factors) can be tagged with these isotopes and used for staining of tissue sections and an image of the general morphology can be taken. A UV-laser evaporates the cells in a typical confocal scanning movement (X,Y) and fumes of the cells are transported by an inert gas into the mass cytometer for measurement. The measured isotopes and the corresponding X,Y coordinates can then be used to reconstruct an image which can be superimposed on top of the microscope images.

the original tissue, now generally referred to as organoids (178, 179). To date, organoid technology is mainly used to model the development of organs "in a dish", including various diseases affecting these organs. The AGM is a complex region composed of a myriad of cells, including endothelial cells, HE cells, mesenchymal cells and various immune cells and it would be a challenging task to mimic the precise organization and timing of influx of supportive immune cells, like macrophages, within organoids. Hence, reports of

successful HSC production by organoid technology are scarce, although there are some 3D iPSC-derived organoid-like culture systems that produce hematopoietic progenitors (180). The succeeding step of organoid cultures are techniques to mimic the first days of embryonic development in a dish. Pluripotent stem cells or iPSCs in a round bottom plate or in a hanging drop will aggregate into embryoid bodies (EBs) to form the three embryonic germ layers. Providing these EBs with a tailored cocktail of growth factors and cytokines at the right time will result in the production of endothelial cells, hematopoietic progenitors, erythrocytes, macrophages, neutrophils and mast cells (181–186). Until recently, this approach did not yield any transplantable HSCs. However, by extending the EB culture and optimizing the cocktail of cytokines and growth factors, which includes BMP4, VEGF, IGF1, SCF, FLT3, TPO, IL-1, -3, -6 and G-CSF, the formation of multilineage HSCs that produced myeloid, lymphoid and erythrocytes in sequential transplanted recipients was achieved (187). The success of generating HSCs from EBs might result from the extension of the EB culture time, allowing germ-layer specification and lineage commitment, or alternatively from the maturation of pre-HSCs. Reconstitution of sub-lethally irradiated recipients by these EB-derived HSCs required the injection of 400.000 cells from dissociated EBs per mouse, indicating that the production of HSCs in EBs is extremely low. One potential explanation for the low yield might be the restricted number of cells receiving the required spatial and temporal signals. This might be due to a sub-optimal organization of the different germ layers in these EBs. Interestingly, stimulating EBs with a pulse of a WNT/β-catenin signaling agonist results in the break of symmetry and subsequently leads to an anteroposterior axial organization with a bilateral symmetry similar to vertebrate embryos (188, 189). These so-called gastruloids display key features of mammalian development after implantation although tissue organization is often limited. Improvements in culturing conditions, such as embedding in Matrigel or fusion of a pulsed and non-pulsed EB, enhanced tissue organization and led to the formation of somite-like and neural structures (190–192). Single cell transcriptomics of gastruloids of different "developmental stages" and culturing methods showed different mesodermal derived populations with expression of endothelial and early blood markers like *Etv2, Kdr, Cdh5, Kit, Gata2, Runx1, Cd34* and *Itga2b* (190, 191, 193). This suggests that gastruloids produce a hemangioblast-like cell type that might differentiate into endothelial, HE and/or even early HSPCs. Conformingly, detailed analysis of endothelial and blood markers in gastruloids revealed the formation of a vascular plexus, the production of blood progenitors and erythroid-like populations (193). The presence of blood vessel like structures and expression of markers like *Kit, Itga2b* and *Runx1* is suggestive for the presence of HE cells and/or pre-HSCs, although additional functional assays are needed to confirm whether gastruloids can produce such cell types. Gastruloids are thus a promising and exciting new tool that does not need to use animals (respect of the 3R rules) and might proof valuable in understanding how extrinsic signals derived from the microenvironment instruct HE cells to undergo EHT and maybe produce (pre-)HSCs. However, the limited time when gastruloids can be cultured (equivalent to a mouse embryo around E8.5-E9.5) might preclude the formation of (pre-)HSCs.

CONCLUDING REMARKS

An increasing number of studies based on RNA-sequencing and/or spatial transcriptomics performed in different embryo species confirmed the complexity of the aortic niche. The development of new molecular approaches and the increasing power of scRNA-seq technologies now offers the possibility to go one step further in the study of HSC regulation by the surrounding microenvironment and to generate high throughput datasets with limited material. The main challenge will be to manage and integrate all RNA-seq, spatial transcriptomic datasets in a comprehensive manner to obtain a global/real picture of what is happening in the AGM region when pre-HSCs/HSCs are generated, and to identify the fine tuning of all the regulators that evolve both in time and space. Most datasets are freely accessible and several labs have invested in creating interactive websites, which makes the exploration of these data relatively easy and allows to interrogate for the expression of any mRNA and miR of interest in supportive/non-supportive cell lines (135), gene expression in different sub-dissected regions of the AGM in mouse (136) and in multiple species for comparison and conservation (86), as well as in the CHT, the HSPC expansion niche in zebrafish embryos (137) (**Figure 2**). The number of molecules and pathways identified to be involved in hemogenic specification, EHT, IAHC formation and pre-HSC maturation within the aorta are continuously increasing. However, the mechanism by which most of these molecules interact and/or interfere, directly or indirectly, to regulate HSPC fate remains to be elucidated.

The evolution and combination of sc-genomic and multiomic techniques (e.g. Scifi-seq, ASAP-seq, ECCITE-seq, Visium) and the efforts made to increase cell throughput with lower costs or the detection of rare cell populations will continue to pave the way for a better understanding of HSPC production and its fine-tuned regulation by the supportive niche. The integration of transcriptomics, proteomics, and epigenetic changes at single-cell resolution and functional validations *in vitro* or *in vivo* will be essential to understand HSPC development in physiological condition with the goal to improve cell-replacement therapy, but also in immune and blood disease conditions, e.g. in the case of childhood leukemia that originate *in utero*.

AUTHOR CONTRIBUTIONS

BW and LY contributed equally to the review. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Bone Marrow Lymphoid Niche Adaptation to Mature B Cell Neoplasms

Erwan Dumontet[1,2]*, *Stéphane J. C. Mancini*[1] *and Karin Tarte*[1,2]

[1] Univ Rennes, Institut National de la Santé et de la Recherche Médicale (INSERM), Établissement Français du Sang (EFS) Bretagne, Unité Mixte de Recherche (UMR) U1236, Rennes, France, [2] CHU Rennes, Pôle de Biologie, Rennes, France

B-cell non-Hodgkin lymphoma (B-NHL) evolution and treatment are complicated by a high prevalence of relapses primarily due to the ability of malignant B cells to interact with tumor-supportive lymph node (LN) and bone marrow (BM) microenvironments. In particular, progressive alterations of BM stromal cells sustain the survival, proliferation, and drug resistance of tumor B cells during diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and chronic lymphocytic leukemia (CLL). The current review describes how the crosstalk between BM stromal cells and lymphoma tumor cells triggers the establishment of the tumor supportive niche. DLBCL, FL, and CLL display distinct patterns of BM involvement, but in each case tumor-infiltrating stromal cells, corresponding to cancer-associated fibroblasts, exhibit specific phenotypic and functional features promoting the recruitment, adhesion, and survival of tumor cells. Tumor cell-derived extracellular vesicles have been recently proposed as playing a central role in triggering initial induction of tumor-supportive niches, notably within the BM. Finally, the disruption of the BM stroma reprogramming emerges as a promising therapeutic option in B-cell lymphomas. Targeting the crosstalk between BM stromal cells and malignant B cells, either through the inhibition of stroma-derived B-cell growth factors or through the mobilization of clonal B cells outside their supportive BM niche, should in particular be further evaluated as a way to avoid relapses by abrogating resistance niches.

Keywords: B-cell non-Hodgkin lymphomas, cancer-associated fibroblasts, extracellular vesicles, tumor microenvironment, stroma cell

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***Correspondence:**
Erwan Dumontet
erwan.dumontet@chu-rennes.fr

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INTRODUCTION

B-cell non-Hodgkin lymphomas (B-NHL) are a heterogeneous group of hematological malignancies that emerge from different stages of normal mature B-cell differentiation (1). Lymphoma evolution and treatment are complicated by a high prevalence of relapses (2) primarily due to the ability of malignant B cells to interact with protective lymph node (LN) and bone marrow (BM) microenvironments (3–5). In agreement, several studies have correlated BM involvement with worsened prognosis and impaired chemotherapeutic response in B-cell lymphomas (6–8). This review delves into the current knowledge of the BM stromal cell

modifications induced by the protumoral niche establishment in B-NHL with a specific focus on diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and chronic lymphocytic leukemia (CLL). Interestingly, these three B-NHL subtypes displayed various BM involvement, with 11%-34% of DLBCL (9, 10), 70%-80% of FL (11), and virtually all CLL cases showing BM infiltration at diagnosis (**Table 1**). Moreover, this review highlights the newly described role of extracellular vesicles (EVs) in the seeding of the BM niche. EVs are released during homeostasis and cell activation, with pleiotropic effects on signaling between cells. EV cargos are enriched in nucleic acids, proteins, and lipids. Briefly, the International Society of Extracellular Vesicles had classified EVs into three main groups: i) exosomes, the small vesicles with diameters ≤100-150 nm that are formed inside multivesicular bodies; ii) microvesicles, medium-size vesicles of plasma membrane origin with diameters of up to 1000 nm; and iii) apoptotic bodies, the large vesicles with diameters > 1000 nm that are produced by apoptotic cells (12). Excellent reviews on the biomolecular and functional characteristics of EVs as well as on the techniques used for EV isolation and characterization have recently been published (13, 14).

DLBCL is the most common aggressive B-NHL and accounts for approximatively 24% of new NHL cases (15). Gene expression analysis and study of genomic alterations have identified distinct genetic subtypes in DLBCL, reflecting differential pathogenesis, and associated with distinct clinical behavior (16–19). Interestingly, recent studies have highlighted the impact of tumor microenvironment (TME) heterogeneity on tumor B-cell biological features and on DLBCL patient outcome (20, 21).

FL accounts for about 20% of adult lymphoma and is an indolent disease characterized by prolonged periods of remissions preceding relapses and ultimate transformation into DLBCL in about 30% of cases. The genetic hallmark of FL is the t (14, 18) translocation occurring during the V(D)J recombination of immunoglobulin genes in the BM. The resulting deregulation of BCL2 provides a selective survival advantage to B cells during the germinal center (GC) reaction, triggering illegitimate recirculation of t (14, 18)^{pos} post-GC B cells detectable in most healthy individuals. Iterative (re)entry of these FL precursor cells inside GC favors accumulation of additional genetic alterations sometimes converging towards overt FL (22). Importantly, FL is the paradigm of a neoplasia fully dependent on a complex microenvironment network that coevolves with tumor B cells to create a tumor supportive niche in both LN and BM (23, 24).

CLL is the most common hematologic malignancy in adults in Western countries. CLL is preceded by a stage of monoclonal B-cell lymphocytosis and is characterized by the accumulation of mature clonal B cells resistant to apoptosis in the blood, BM, and lymphoid organs. Patients with CLL have a heterogeneous clinical course with some never needing treatment, while others require treatment immediately after diagnosis or during illness due to a more symptomatic and unfavorable clinical course. In typical CLL cases, the tumor B cell clone exhibits an abnormal expression of markers like CD5, CXCR4, and ZAP-70, that are used to stratify the disease in conjunction with the mutational status of the BCR reflecting different cell of origin (25, 26). Despite fully disseminated presentation, TME provides crucial survival signals to malignant CLL cells within the proliferation centers of LN and BM (27).

In these three mature B-cell neoplasms, specialized tumor niches support survival, proliferation, and drug resistance of tumor B cells. These highly heterogeneous niches include defective tumor immunity, due to altered recruitment and cell exhaustion of cytotoxic cells, to the amplification of immunosuppressive cells, or to immune escape mechanisms developed by tumor B cell themselves, hampering tumor recognition, immune synapse formation, or anti-tumor cell activation (23, 24, 27). Conversely, fully functional tumor permissive cells, including CD4^{pos} T cell, myeloid cell, and stromal cell subsets, could be found. The relationship between LN and BM protumoral niches and how the similarities and differences between these microenvironments could impact malignant B-cell features remains elusive. In FL, malignant B cells found in the BM are characterized by a lower cytological grade, a decreased proliferation, and a reduced CD10 expression compared with LN FL B cells (28). Moreover, their gene expression profile reflects their reduced proliferation and active metabolism (29). Finally, somatic hypermutation analysis and targeted deep sequencing demonstrate that different FL B-cell subclones could be detected within LN *versus* BM, and suggested that FL originates in the LN and infiltrates BM early in the course of the disease, allowing further accumulation of BM-specific

TABLE 1 | Key elements involved in the generation of B-cell non-Hodgkin lymphomas bone marrow supportive niches.

	DLBCL	FL	CLL
BM involvement (% of cases)	11-34%	70-80%	All
Pattern	Mixed: from localized focal infiltrates to complete disruption	Nodular aggregates admixed with lymphoid-like TME	Mixed nodular-interstitial, interstitial, and diffuse
BM stroma factors involved in B-cell homing	Unknown	CXCL12 CCL19, CXCL13	CXCL12 and VLA-4
BM stroma factors involved in B-cell survival	BAFF IL-6 IL-17A	Hehghog ligands BAFF, TGF-β, VLA-4, CXCL12	BAFF, CD44, Plexin-B1, CXCL12, C1q
Metabolic reprogramming induced by BM TME	Unknown.	BM B cells are metabolically less active than LN B cells	BM stromal cells release glutathione and trigger CLL glycolytic shift
Effects of tumor EVs on BM stromal cells	Unknown.	↗CXCL12, ↗ ANGPT1, ↗ KITLG, ↗IL-7	↗VEGF Inflammatory pro-tumoral phenotype

mutations (28, 30, 31). Besides the exact cell composition and supportive signals provided by BM niches, a major issue remains to establish how these niches evolve during tumor development, from the pre-tumoral stage to overt lymphoma, during remissions and relapses.

LYMPHOMA BM STROMAL MICROENVIRONMENT

BM constitutes the primary site for the maintenance and differentiation of hematopoietic stem cells (HSCs) and for B-cell lymphopoiesis. Different stromal cell niches dynamically control these processes. Seminal papers have recently proposed a molecular atlas of the BM stromal cells at the single cell resolution, including osteoblasts, perivascular cells, endothelial cells, and mesenchymal stromal cells, providing clues on how various stromal cell subtypes could interact with HSCs and differentiating B-cell subsets (32–34). In the context of B-NHL, dynamic interactions between BM stromal cells and tumor B cells have been described to play a key role in converting the BM TME into a tumor supportive niche (34–36). DLBCL, FL, and CLL display distinct patterns of BM infiltration (**Table 1**). DLBCL show a mixed pattern of BM involvement that can potentially range from localized focal infiltrates to complete disruption of BM by lymphoma cell proliferation (37). In contrast, FL infiltration is primarily localized to the paratrabecular regions as nodular aggregates admixed with lymphoid-like TME (38). In CLL several BM infiltration patterns can be found including mixed nodular-interstitial, interstitial, and diffuse (39). In each cases, stromal cells exhibiting specific functional phenotype support recruitment, survival, and proliferation of tumor B cells, mimicking the cancer-associated fibroblasts (CAFs) described in solid cancers.

BM Stromal Cells Support B-Cell Recruitment

BM DLBCL-CAFs have been poorly explored *in situ*. In contrast, in FL, BM-CAFs, like their LN counterparts, overexpress CXCL12 involved in the recruitment, adhesion, and activation of FL B cells (40) (**Table 1**). Moreover, they ectopically express CXCL13 and CCL19, the two lymphoid chemokines classically expressed by LN follicular dendritic cells (FDC) and fibroblastic reticular cells (FRC) respectively, thus recreating GC-like structures able to recruit and support $CXCR5^{pos}CCR7^{pos}$ FL B cells (41, 42).

CLL B lymphocytes could be attracted *in vitro* to BM stromal cells whose protective effects require close cell proximity (43–45). This colocalization of CLL tumor cells with their supportive stromal cell niche relies on the deregulation of several chemokine pathways (**Table 1**). The demonstration that the clinical efficacy of BCR inhibitors in CLL is mediated, at least in part, by the inhibition of chemokine receptor activity and the corresponding mobilization of tumor cells out of their protective niches further highlights the crucial role of stromal cell-derived chemokine in CLL survival (46). First, high expression of CXCR4 on the surface of peripheral blood CLL cells triggers their migration to BM stromal cells producing CXCL12 (45, 47–49). CXCR4 surface expression is regulated by its ligand, thus explaining the decrease in CXCR4 expression on tissue tumor B cells, while recirculating CLL B cells express high levels of CXCR4. In parallel, blood CLL cells express high amounts of CCR7 (50). Indeed, the recycling of CXCR4 and CCR7 receptors is potentiated in CLL cells and contributes to their stronger expression (51). Recently, it was shown that p66Shc (SHC-transforming protein 1), which limits the recycling of CXCR4 and CCR7 by inhibiting their de-phosphorylation, is deficient in CLL (52). Interestingly, CCR7 could also form heterodimers with CXCR4 thus disrupting the CXCR4/CXCL12 downstream signaling and reducing B-cell retention within BM (53). Furthermore, other proteins expressed by CLL cells, such as ZAP70 or CXCR7 have been shown to regulate the function of CXCR4 (54, 55). Altogether, the modulation of CXCR4 function could regulate the homing capacity of CLL cells within BM. Second, CXCR5, the CXCL13 receptor, is also expressed at high levels by CLL cells (56, 57). However, conversely to the ectopic induction of CXCL13-expressing FDC in FL BM, CXCL13 seems to be only involved in CLL B cell homing into LN and the increase of CXCL13 level in the plasma of CLL patients is correlated with LN size but not BM infiltration (58). Finally, integrin α4β1 (VLA-4) plays a prominent role in the homing of CLL cells to BM niches. VLA-4 major ligands, fibronectin and VCAM-1, are constitutively present on BM stromal cells and endothelial cells and are upregulated by inflammatory signals in a NF-kB-dependent manner (59). In mouse xenograft models, CLL cells from $VLA\text{-}4^{neg}$ patients showed significantly lower BM homing rates than those from $VLA\text{-}4^{pos}$ patients. In contrast, the spleen homing rates did not significantly differ. Clinically, the VLA-4 status directly drives in the extent of human BM infiltration (60).

BM Stromal Cells Support B-Cell Survival

In DLBCL, the upregulation of Notch-3 in tumor cells under close cell-cell contact with BM-derived stromal cells has been implicated in the development of aggressive lymphoma cells (61). In turn, such direct interaction between DLBCL cells and stromal cells mediates an increase in B-cell activating factor (BAFF) expression by stromal thus resulting in a decrease of chemotherapy-induced B-cell apoptosis (62, 63) (**Table 1**). One of the factors involved in the regulation of DLBCL B-cell interaction with the BM stromal niche is the level of Jun expression. Indeed, Jun-regulated genes mediate the interaction of malignant cells with stromal cells and extracellular matrix proteins and impact extranodal localization (64). There is also evidence for tumor permissive effects of BM stromal cells on DLBCL cells through secretion of IL-6 and IL-17A, which promote both cell proliferation and drug resistance (8). Finally, the crosstalk between malignant B cells and stromal cells in DLBCL could also impact metabolic reprogramming in DLBCL. DLBCL have been early considered as metabolically heterogeneous (65, 66). Non-malignant cells from TME including stromal cells have been proposed to contribute to DLBCL metabolism by providing metabolic intermediates (67)

but no data specifically address this issue in BM versus LN niches even if the use of specific metabolic inhibitors have been recently explored in some DLBCL subsets (68).

In FL, tumor B cells are strongly dependent on direct interactions with a microenvironment close to that of normal GC, including in particular follicular helper T cells (Tfh), myeloid cells, and lymphoid stromal cell subsets (23, 24, 69). The protumoral role of infiltrating lymphoid stromal cells has been demonstrated in particular by the identification of ectopically-induced FRC- and FDC-like cells within invaded BM (40, 70). To date the origin and heterogeneity of the stromal cells supporting FL B cells within LN and BM are not perfectly understood and it is very likely that several FL CAF subtypes co-exist and organize different cell niches with specific functions (38). Stromal cells supporting FL B cell survival have been initially identified as lymphoid-like stromal cells obtained *in vitro* by stimulation of BM mesenchymal precursors by TNF-α (TNF) and Lymphotoxin-α1β2 (LT) or by direct contact with malignant B cells (3). Interestingly, BM stromal cells obtained from FL patients display a specific gene expression profile even after *in vitro* amplification, suggesting an imprinting on these cells by the tumor context (40, 63, 71). VLA-4, which is expressed by FL-CAFs, is involved in the growth of GC lymphomas and their resistance to anti-CD20 treatments (72). *In vitro*, FL stromal cells decrease tumor B cell apoptosis through a set of partially resolved mechanisms, including the production of hedgehog ligands (Hh), BAFF and TGF-β, over- expression of ABC-type multi-drug transporters, and activation of a c-MYC/HDAC6 loop in tumor cells (24, 73). Moreover, CXCL12 contributes to FL B cell activation and synergize with BCR signaling (40). To date, the metabolism of FL remains broadly unexplored. Gene expression profile of FL B cells obtained from medullary niche reveals a decreased expression of the genes involved in of glycolysis, fatty acid synthesis, and OxPhos pathway compared to LN B cells (29). However, the role of stromal cells from BM versus LN niches in FL B-cell metabolic reprogramming remains to be evaluated.

CLL B cells could interact with stromal cells *via* different receptor/ligand couples including ICAM-1/LFA-1 (74), VCAM-1/VLA-4 (75–78), CXCR5/CXCL13 (79), BCMA/BAFF, or TACI/BAFF (80), or by transpresentation of IL-15 from stromal cells to B cells (81). Amon those, ICAM-1, VCAM-1 and BAFF have been shown to be expressed by BM stromal cells. These interactions could lead to leukemic cell survival *via* a CD44-dependent mechanism involving up-regulation of MCL-1 in CLL B cells (82), activation of NF-κB pathway (80), and result in migration and proliferation of leukemic cells. In the same way, the interaction between CD100 (on CLL B-cell surface) and Plexin-B1 (present on BM stromal cells) extends CLL B cell viability and enhances proliferation (83). The mutual activation of stromal cells and tumor cells also depends on the CLL-mediated activation of Notch2 in BM stromal cells, leading to C1q overexpression the reciprocal activation of the canonical Wnt pathway in CLL cells (84) Moreover, BM stromal cell derived CXCL12 exhibits a pro-survival effect on CLL tumor cells (44, 85, 86). BM Stromal cells may also induce protective epigenetic modifications in CLL B cells including hypomethylation of the lysine 27 of histone H3 protein subunit (H3K27me3) (87). Finally, BM stromal cells have an important role on CLL metabolism. CLL cells have a net increase of reactive oxygen species (ROS) compared to their normal counterpart and are highly sensitive to cellular antioxydants, such as glutathione, to maintain their redox balance. BM stromal cells trigger glutathione synthesis by CLL cells through cysteine release, thus protecting tumor cells from drug-induced apoptosis (88). Moreover, BM stromal cells contribute to the glycolytic shift in CLL cells, at least in part by the Notch/Myc axis, triggering an increased glycolysis associated with higher lactic acid production, glucose uptake, and glucose transportation (89, 90).

BM Stromal Cells Organize the Tumor Niche

Beyond these functions of direct B-cell support, lymphoma CAFs are thought to be the organizers of the tumor niche. A role for the composition of the stromal-cell derived extracellular matrix in the pathogenesis of DLBCL was recently identified within tumor LN, raising the question of its direct and indirect impact on tumor growth, as an example through the modulation of immune cell infiltration, within invaded BM (21).

FL-CAFs overexpress the chemokine CCL2 within invaded BM, thus triggering the recruitment of monocytes that are then converted into pro-angiogenic and anti-inflammatory macrophages (71). FL tumor-associated macrophages have been shown to play a key role in the growth of FL B cells through the transpresentation of IL-15 and the triggering of BCR-dependent signaling involving DC-SIGN-expressing macrophages and oligomannose residues introduced in FL BCR (91, 92). BM and LN FL-CAFs could also promote the recruitment and survival of pro-tumoral neutrophils through the release of large amounts of IL-8 (63). Of note, in DLBCL, tumor cells have been shown to produce themselves IL-8 involved in the recruitment of APRIL-producing neutrophils (93). Moreover, BM and LN FL-infiltrating stromal cells also overexpress the immunosuppressive molecule PGE2 (94) involved in the recruitment or activation of suppressor cells such as Tregs and MDSCs (95). Finally, CAFs have been shown in solid tumors to physically hamper the recruitment of cytotoxic T cells to the tumor and $CD8^{pos}$ T cells are retained at the periphery of FL tumor aggregates in both LN and BM, suggesting that FL-CAFs could contribute to tumor exclusion in lymphomas (96–98).

Overall, it is clear that close interactions of tumor B cells with stromal cells within the BM, together with modulation of chemokines and cytokines directly influence the growth of DLBCL, FL and CLL, providing evidence that the BM niche plays a critical role in both lymphoma survival and drug resistance. Regardless of their cell of origin, the mechanisms underlying the differentiation of lymphoma CAFs are of the utmost importance given their potential as therapeutic targets.

EMERGENCE OF THE BM LYMPHOMA STROMAL MICROENVIRONMENT

FL tumor B cells could directly contribute to the commitment of BM stromal precursors into an FRC-like phenotype overexpressing

CCL2 and IL-8 through TNF-dependent mechanisms (3, 63, 71). Moreover, even if they produce less LT than normal centrocytes, the large number of GC-like B cells ectopically found in invaded FL BM probably contributes to a local overproduction of LT that synergizes with TNF for the induction of lymphoid stroma commitment. However, surrounding non-malignant cells could also participate in the polarization of FL-CAFs. Neutrophils, recruited by IL-8-producing BM FL stromal cells, could in turn contribute to their differentiation into FRC-like cells through activation of the NFκB pathway (63). In addition, LN FL-Tfh overexpress IL-4 which induces a Transglutaminase^{hi}Podoplanin^{low} CD106^{hi}CXCL12^{hi} phenotype on human stromal cell precursors. FL-Tfh also produce high amounts of TNF and LT, which sensitize stromal cell precursors to the effect of IL-4, notably through increased expression of the STAT6 signaling molecule (40). Even if fully mature Tfh have not been detected within FL BM, IL-4 and CXCL12 have been shown to be correlated in invaded FL BM (40). Finally, some of the recurrent genetic alterations in FL regulate the re-education of the tumor niche by tumor B cells. In particular, the gain-of-function mutations of the histone methyltransferase EZH2, which occurred early in 20% to 30% of FL, are proposed to uncouple GC B cells from the critical Tfh checkpoint whereas switching them to FDC dependency (99). EZH2-mutated GC B cells downregulate many genes linked to Tfh signaling, fail to engage Tfh, thus limiting recycling toward the dark zone of GC, and survive in the light zone as proliferating centrocytes overexpressing LT, TNF, and BAFFR, all involved in GC B-cell/FDC crosstalk. HVEM loss-of-function mutations detected in about 40% of patients with FL have been associated, in a murine model of FL and in FL patients, with an amplification of Tfh producing large amounts of IL4, TNF, and LT, and able to activate FL-CAF within LN (100). No study had currently evaluated how these genetic events could impact FL TME co-evolution within BM. Even if such data are essentially lacking in the context of DLBCL, some recurrent genetic alterations have been recently associated with a specific TME pattern, with some of them related to overexpression of genes associated with GC-like stroma or extracellular matrix/FRC/CAF genes (21).

Finally, LT produced by CLL cells is involved in the polarization and/or *in situ* generation of the tumor stromal network and the secretion of CXCL13, IL-6, and IL-8 (74, 79). Moreover, the leukemic clone produces retinoic acid in the stromal microenvironment which contributes, at least in part, to the CXCL13 induction (101).

In addition to the factors described above, tumor derived EVs seem to be involved in the communication between tumor cells and their TME, in particular CAFs. Such mechanism could play a central role in triggering initial induction of tumor-supportive niche within distant sites, including BM.

ROLES OF EVS IN THE INDUCTION OF BM LYMPHOMA STROMAL NICHE

To date no study has explored the putative involvement of EVs in the induction of a BM lymphoma stromal niche in the context of DLBCL. Moreover, only few studies have investigated the involvement of EVs in the pathophysiology of FL (**Figure 1**). Recently, FL-derived EVs were shown to modulate the gene expression profile of BM stromal cells, triggering an upregulation of HSC niche factors including CXCL12, angiopoetin-1, KITLG, or IL-7, and increasing the capacity of stromal cells to interact specifically with BM FL B cells and support their survival and their quiescent phenotype (29). Interestingly, the phenotype of EV-treated stromal cells is quite different from that obtained under treatment by TNF/LT or coculture with FL B cells supporting a role of EVs in the activation of BM stromal cells before BM seeding by malignant B cells. In fact, the level of CXCL12 is increased in non-involved BM plasma suggesting that FL EVs could shape the BM stromal niche before BM infiltration by tumor cells or at distance from this BM infiltration (unpublished data). In the same way, the analysis of the gene expression profile of BM stromal cells highlights a *continuum* ranging from healthy donor BM stromal cells, to stromal cells obtained from FL patients without BM involvement, and finally from FL-invaded BM (29). Altogether these data suggest that EVs could contribute to CXCL12 upregulation in the absence of direct contact with malignant B cells and could then synergize with IL-4 produced by infiltrating T cells admixed with FL B cells to further enhance local CXCL12 production. Interestingly, BM stromal cells activation by FL-derived EVs was shown to rely on TGF-β dependent pathways something that is reminiscent of the role of TGF-β in the B-cell/stromal cell crosstalk within FL LN (42). How TGF-β and STAT6 pathways could synergize for the acquisition of FL CAF phenotype within FL BM remains to be explored.

Bidirectional crosstalk has also been reported between CLL B-cells and their surrounding stroma *via* EVs (**Figure 1**). CLL B cells release large amounts of exosomes that show strong expression of CD37, CD9, and CD63. Ibrutinib, a Btk inhibitor, significantly reduces the amount of plasma exosomes in CLL patients. Likewise, *in vitro* treatment of CLL cells with Idelalisib (a PI3K inhibitor) decreases exosome secretion, something that is not observed during treatment with fludarabine (102). This result highlights the role of the BCR-PI3K pathway in controlling exosome secretion in CLL. Besides BCR itself, CLL supportive TME produces BAFF, APRIL, CD31, and plexin B1 that all protect CLL cells from spontaneous apoptosis by synergizing with BCR signaling (44, 103) and could influence EV secretion. The comparison of the mRNA content of EVs produced by B cells from healthy donors *versus* patients with CLL, and stimulated or not through the TLR9 pathway, shows enrichment for the kinases of the BCR pathway, LYN, SYK, MAPK1, MAPK2, and the anti-apoptotic proteins BCL2 and BCL3 in CLL-derived EVs. These EVs released by tumor B cells transfer their mRNA content to non-malignant cells in the TME (104). Microvesicles derived from malignant CLL cells and detected in peripheral blood also deliver the receptor tyrosine kinase Axl into BM stromal cells leading to the activation of a AKT/mTOR/p70S6K/HIF-1α axis resulting in an increase in VEGF synthesis (105). This increase in VEGF is associated with an increased neovascularization in medullary (106) and extramedullary tissues, as well as a paracrine pro-survival stimulation of tumor B cells (107). The miRNA content of CLL B cell-derived exosomes is strongly enriched in miR-21, miR-155, miR-146a, miR-148a, and let-7g

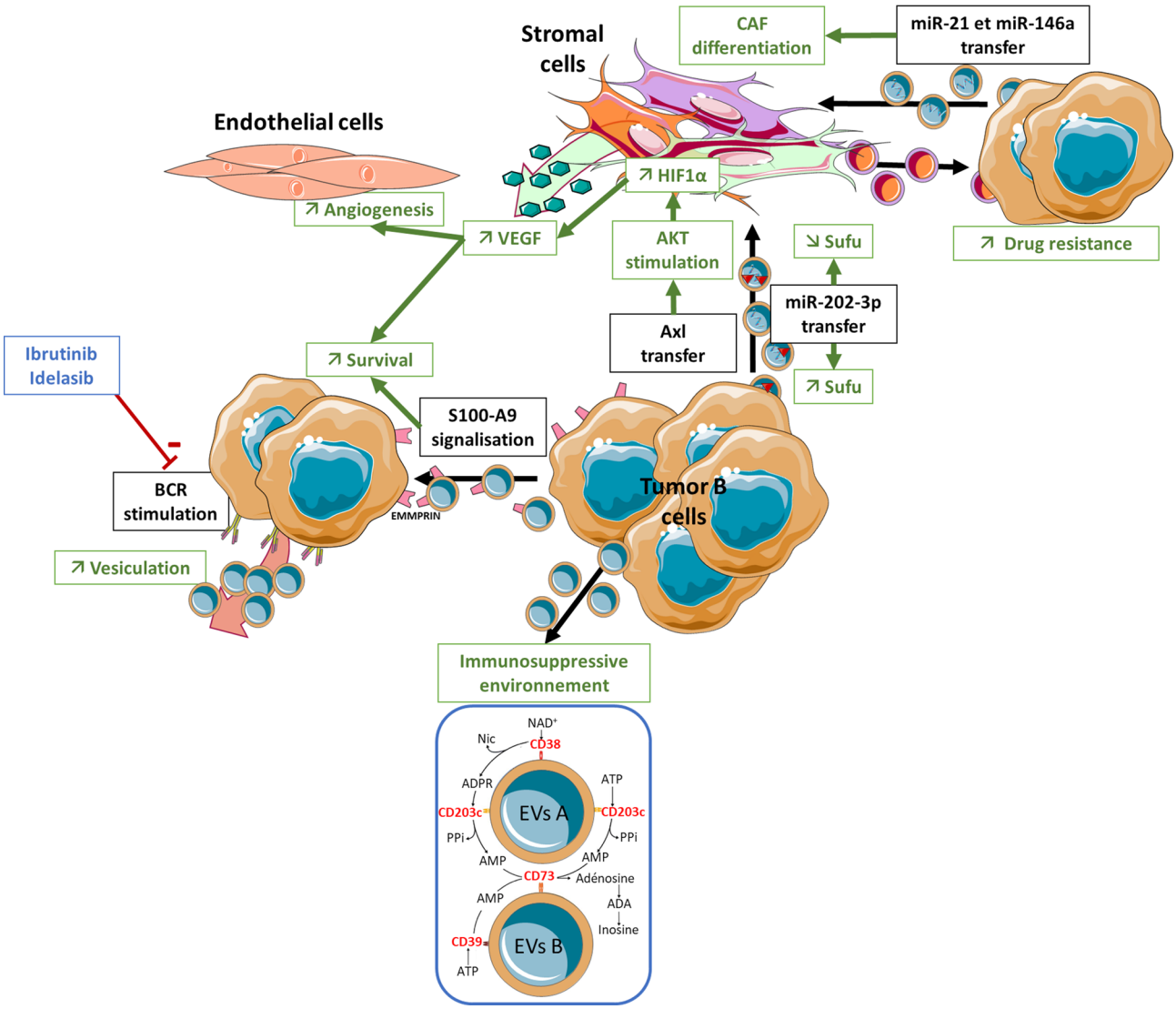

FIGURE 1 | Role of EVs in the lymphoma microenvironment structuration. A bidirectional crosstalk has been reported between CLL and FL tumor B-cells and stromal cells *via* EVs that can give rise to a highly organized pro-tumor niche. ANGPT, Angiopoietin; BCR, B Cell Receptor; BM, Bone Marrow; CAF, Cancer Associated Fibroblast; EVs, Extracellular Vesicles; TGF, Transforming Growth Factor-β; VEGF, Vascular Endothelial Growth Factor.

(108). BM stromal cells treated *in vitro* with these CLL exosomes acquire an inflammatory pro-tumoral phenotype, while endothelial cells increase their capacity for angiogenesis (108). These effects are consistent with what is known about the effect of miR-21 and miR-146a in the transition from normal fibroblast to CAFs (109–112). Indeed, CLL miR-146a^{pos} exosomes induce the transition of BM stromal precursors into CAFs showing over-expression of α-SMA and FAP (113). In addition, CLL exosomes show specific enrichment in miR-202-3p, able to decrease expression of Sufu (a component of the hedgehog pathway) in stromal cells and to trigger stromal cell proliferation (114). Finally, EVs isolated from cultures of CLL BM stromal cells induce a significant decrease in spontaneous apoptosis of tumor B cells and an increase in their chemoresistance to several drugs, including fludarabine, ibrutinib, idelalisib, and venetoclax. In addition, these EVs induce changes in the gene expression profile of CLL cells mimicking the transcriptomic signatures obtained after BCR stimulation (115).

DISRUPTING THE EV "REMOTE COMMUNICATION" TO IMPROVE LYMPHOMA PROGNOSIS

Analyzing the deregulation of extracellular proteins or miRNAs in the blood and tumor niches of patients during B cell tumorigenesis is a reliable tool for the identification of new tumor-targeted therapies. For example, the detailed mode of action of the CD30

antibody-drug conjugate Brentuximab vedotin in DLBCL is not well understood since the clinical outcome seems to be partially independent of the CD30 expression on the tumor cells. However, as $CD30^{pos}$ bystander cells are enriched in the tumor tissue in many cases of DLBCL, CD30 might be released within TME-derived EVs. Thus a model was proposed in which even in the absence of CD30 on the tumor cells, EVs can transport the targeting protein from cells of the TME to tumor cells (116). This model would explain the clinical efficacy of Brentuximab vedotin also in cases of lack of the targeting antigen on tumor cells. In the same way, DLBCL EVs carrying miR-125b-5p can reduce tumor sensitivity to rituximab by inhibiting TNFAIP3 expression and reducing CD20 expression (117). Whether the miR-125b-5p/TNFAIP3 axis can be used as a therapeutic approach for increasing DLBCL sensitivity to anti-CD20 antibodies requires further investigations.

EVs released by B cell could carry CD39 and CD73, two surface molecules known to hydrolyze ATP released by dying cancer cells into adenosine that hijacks CD8 T cell immune activity by binding the A2A adenosine receptors (118). One could speculate that B-cell-derived EVs may have a similar effect. The decrease of B-cell-derived EVs bearing CD73 and CD39 can be achieved by deregulating the docking protein RAB27A (118). This could be performed using an inactivated Epstein–Barr virus carrying siRNA, but it is also possible to generate EVs derived from cell lines producing RAB27A siRNA and to specifically deliver it to tumor cells.

Ultimately, thanks to their molecular structure mimicking the plasma membrane of the cells and their capability to reverse their cargo into target cells, exosomes could be shaped and filled of drug molecules, acting as drug-delivery systems. In fact, cancer vaccine clinical trials relying on the administration of exosomes produced by dendritic cells (Dexosomes), exploited to shuttle antigenic determinants of immune response, were conducted to immunize patients in the context of solid tumors (119–121). In the same way, systemic administrations of TNF-Related Apoptosis-Inducing Ligand (TRAIL)-armed exosomes have shown a great anti-tumor effectiveness against FL/DLBCL cell lines both *in vitro* and in a mouse model (122).

CONCLUSION

Despite very interesting recent data highlighting BM as a survival niche for lymphoma B cells, numerous controversies remain open on the role of the BM versus LN niches during the early step of lymphomagenesis or at the stage of post-treatment minimal residual disease that could generate relapse. In FL, both pre-tumoral B cells and early committed precursor cells, that will give rise to overt FL, have been shown to be enriched in BM (22). However, transformation events required iterative passages throughout the GC making it difficult to define precisely whether BM is a primary or a secondary tumor niche. The influence of tumor genetics or patient features on the capacity of tumor B cells to home and develop into BM remains completely unexplored. A major limitation for all BM-dedicated studies is the limited availability of good quality samples to perform phenotypic, transcriptomic, and functional studies and the lack of iterative sampling allowing evaluation of the impact of disease evolution or therapeutic strategies. BM aspirates are scarce and do probably not include the whole diversity of tumor/TME components, in particular stromal cells. Moreover, fixed BM biopsies are very difficult to exploit for spatial transcriptomics and even multiplex immunohistofluorescence approaches. Such technical issue hampers a precise evaluation of spatial heterogeneity in B-cell lymphomas integrating BM as a key tumor site.

Altogether, many evidence support the clinical interest of targeting the crosstalk between BM stromal cells and malignant B cells, through the inhibition of stroma-derived B-cell growth factors, the mobilization of clonal B cells outside their supportive BM niche, or the reprogramming of tumor-supportive stromal cells. Identifying the best therapeutic options, and how to combine them with tumor-targeting drugs or immunotherapy approaches will be the major challenge in the field.

AUTHOR CONTRIBUTIONS

ED wrote the paper, SM reviewed the paper, and KT supervised and wrote the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Diversity of Vascular Niches in Bones and Joints During Homeostasis, Ageing, and Diseases

Naveen Kumar[†], *Pepijn Saraber*[†], *Zhangfan Ding and Anjali P. Kusumbe*[*]

Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences (NDORMS), Tissue and Tumor Microenvironments Group, University of Oxford, Oxford, United Kingdom

The bones and joints in the skeletal system are composed of diverse cell types, including vascular niches, bone cells, connective tissue cells and mineral deposits and regulate whole-body homeostasis. The capacity of maintaining strength and generation of blood lineages lies within the skeletal system. Bone harbours blood and immune cells and their progenitors, and vascular cells provide several immune cell type niches. Blood vessels in bone are phenotypically and functionally diverse, with distinct capillary subtypes exhibiting striking changes with age. The bone vasculature has a special impact on osteogenesis and haematopoiesis, and dysregulation of the vasculature is associated with diverse blood and bone diseases. Ageing is associated with perturbed haematopoiesis, loss of osteogenesis, increased adipogenesis and diminished immune response and immune cell production. Endothelial and perivascular cells impact immune cell production and play a crucial role during inflammation. Here, we discuss normal and maladapted vascular niches in bone during development, homeostasis, ageing and bone diseases such as rheumatoid arthritis and osteoarthritis. Further, we discuss the role of vascular niches during bone malignancy.

Keywords: bone, joint, ageing, endothelial cell, vascular niche

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***Correspondence:**
Anjali P. Kusumbe
anjali.kusumbe@kennedy.ox.ac.uk

[†]*These authors have contributed equally to this work*

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INTRODUCTION

The development of the skeletal system can progress *via* intramembranous ossification or endochondral ossification. During intramembranous ossification, MSCs (Mesenchymal Stem Cells) directly differentiate into osteoblasts to support bone growth. Endochondral ossification is, however, used to generate most of the bones in the skeletal system and occurs *via* the formation of a cartilage scaffold which is later replaced with bone through invasion of osteoclasts and osteoprogenitors (1). Invasion of osteoclasts and osteoprogenitors is mediated through the ingrowth of new blood vessels by the release of proangiogenic factors such as VEGF-A (Vascular Endothelial Growth Factor A). During the later stage of endochondral ossification, an extension of blood vessels towards the epiphysis facilitates the replacement of cartilage with trabecular bone and the formation of long bones (2).

Bone participates in many physiological mechanisms due to its high degree of plasticity, which is essential for maintenance of structure, protection and locomotion (3). Bone tissue consists of osteoblasts, bone lining cells and osteoclasts in addition to mineral deposits. Subsequently, it has a

cavity filled with blood vessels and soft BM (Bone Marrow) (4). This part of the BM contains premature HSCs (Hematopoietic Stem Cells) and non-HSCs. Later these premature HSCs become mature, differentiate and get released into the vascular system. These cells interconnected with the vessel network in the BM and laid the foundation of 'niche', a dynamic environment for stem cells renewal and home to differentiated cells (5, 6).

Although bone cavity nurture HSCs, MSCs also takes part in cellular development and differentiation here. MSCs differentiate into adipocytes, pericytes and neuronal cells. These differentiations are termed as stromal network formation by a class of biologists (7, 8). Recent studies expand the knowledge of the heterogeneity of mesenchymal stem and progenitor components and their specific functions. These approaches allow us to understand their role in hematopoiesis and disease progression (9, 10). The BM contains multiple stem cell lineages, which participates in bone homeostasis and osteogenesis. These cells create a specialized local microenvironment, rich with growth factors and hormones. Due to the enriched microenvironment, the BM serves as a niche for metastatic cells, which disseminate from other organs to bone. The BM is considered a vibrant ecosystem that regulates tumour cells invasive, angiogenic and metastatic behaviour (11, 12). Incessant crosstalk between cells and vessels in the BM creates opportunities for the tumour cells to stabilize and interact with neural, mesenchymal and endothelial cells in the tissue microenvironment (13, 14).

Despite the aggressive approaches in detection and therapeutic interventions, tumour cell dissemination remains the foremost problem for cancer patients, particularly in bone metastasis. Metastatic dissemination is a well-controlled multistep process, which includes the crosstalk of tumour cells with the local microenvironment, especially within the vasculature. Human bone vasculature plays a key role in tumour progression and helps establish the secondary site for tumour development. The BM endothelial cells participate in homeostasis and help in maintaining bone integrity (15, 16). Different stress conditions modulate the bone vasculature and create a halt in blood flow in aged bones which affects bone density and BM homeostasis. Endothelial dysfunction also contributes to disease progression, especially in cardiovascular mortality.

DIVERSE VASCULAR NICHES AND PERIVASCULAR COMPONENTS OF THE BONE MARROW

The BM tissue microenvironment encompasses functional, cellular and non-cellular components including adipocytes, immune cells, pericytes and stroma (8, 17). The BM tissue is considered the most dynamic organ of the body due to its ability to create virtually all blood cell lineage throughout the entire life span of adult individuals (18, 19). The BM is an essential portion of the bone cavity to regulate bone homeostasis and facilitate the stem cell niche formation for self-renewal and differentiation of stem cells. Extensive studies have been piloted to probe the role of tissue microenvironment in homeostasis and disease progression, and interestingly, a major segment of the research ramble around the non-stromal cells. However, research signifying the role of stromal components in the fate of disease remains poorly explored. The term stromal cells is a vaguely-defined and consist of a network of neural, mesenchymal and endothelial cells with roles in homeostasis, tissue repair and diseases in every organ (20, 21). Immuno-oncologists largely term pericytes, diverse mesenchymal cells and endothelial cells as stromal cells, however, the term is loosely defined and used variably with and without the inclusion of vascular cells.

The BM stem cell niche is a very distinct site that is comprised of supporting cells and makes a promising microenvironment for cellular interactions and signalling (22, 23). The BM tissue is enriched with different cell lineages including hematopoietic and non-hematopoietic cells. All the non-stromal cells have their determined contribution in tissue development along with mesenchymal stem and progenitor cells. Interestingly, mesenchymal stem cells are being used interchangeably with mesenchymal stromal cells but a report of ISCT elaborates the differences between these two cell populations. Mesenchymal stem cells pose the ability of self-renewal and differentiation and mesenchymal stromal populations contain homing and immunomodulatory properties (24). To differentiate further, the mesenchymal stromal cells should show plastic adherence and express CD105, CD73 and CD90 markers and must not express endothelial and haematopoietic markers (25, 26).

The BM mesenchymal cells are precisely used in tissue engineering, tissue development and regeneration studies. Recently it has been observed that MSCs can be differentiated into ECs and VSMCs under mechanical stimulation (27). In another study, the combined effect of small molecule inhibitors of kinases and mechanical stimulation induces vascular cell-like phenotypic alterations in MSCs. Such inductions increase the expression of pericytes and endothelial markers *in-vitro* and also the regenerative abilities of MSCs (28, 29). On the other hand, the mesenchymal stromal network of the BM surrounds HSCs for regulatory crosstalk, which has essential relevant implications in stem cell biology and appeared as a principal regulator in bone metabolism (14, 30).

Nevertheless, the mesenchymal network is not limited to regulating the HSCs but encompasses the entire BM hematopoietic development and comprises lineage-specific differentiation, cellular trading, disease regulation and tissue structural maintenance (30, 31). As we posit that the vasculature is a completely different entity from the mesenchymal stromal components, a detailed investigation needs to be done to characterize each cell lineage. Recent technical progress expands the understanding of phenotypic characterization, anatomy, composition and unique functions of mesenchymal stromal components (32). These advancements allow us to understand the heterogeneity of the BM mesenchymal stromal components and how these multiple cell lineages orchestrate hematopoiesis and participate in malignancy (21). Interestingly mesenchymal stromal cells express similar cell surface markers as on activated ECs and mesenchymal stem cells (CD105+ CD45-). However, mesenchymal

stromal cells only possess limited pluripotent potential with differentiation directed towards osteogenic, adipogenic and chondrogenic lineages, whereas mesenchymal stem cells also can regenerate ECs (24–26, 33). Bone marrow derived mesenchymal stem cells (CD105+, CD73+, CD90+, CD166+ and CD45-) cultured in VEGF rich medium show increased levels of endothelial-specific markers such as KDR and FLT-1 (34). Subsequently, Meng *et al.* shows the differentiation of mesenchymal stem cells into endothelial cells *in-vivo* (35). Nevertheless, mesenchymal cell are of increasing interest in regenerative medicine approaches to restore worn-out or damaged tissue.

DIVERSE VASCULAR NICHES IN BONE DEVELOPMENT AND HOMEOSTASIS

Human skeleton organization is a highly dynamic system with a role in architectural support, homeostasis and blood cells formulation (36). Bone formation is a continuous process. The bone formation process in the course of early embryogenesis begins in two different modes, namely intramembranous and endochondral ossification, as discussed above (37). Pre-existing mesenchymal tissue transforms into bone tissue in both processes. Intramembranous ossification is the result of direct condensation of mesenchymal tissue in the bones. Skull, maxilla, clavicle and mostly the flat bones are the products of this process (38–40). In the mode of endochondral ossification, mesenchymal cells differentiate into intermediate cartilage, which is later replaced by bone. This process occurs in the femur and tibia, long bones of the system. Chondrocytes develop through mesenchymal aggregation during endochondral ossifications and help in the activation of osteoblast differentiation. Surrounding cells of chondrocytes formulate perichondrium, which has a quiescent state of cells and undergoes hypertrophy (41).

Recent developments in endothelial biology suggests that infiltration of vessels initiates bone formation during embryogenesis. During this process, endothelial cells vascularize the bone tissue and create a vascular bed throughout the length of the bones. The vascular bed is composed of countless capillaries, the central draining vein and arteries (42). Vascular infiltration into hypertrophic columnar cartilage is responsible for the generation of the primary ossification centre, which is eventually converted to a secondary centre during embryogenesis (36). The process of embryonic development includes vessel invasion to acquire nutrients and oxygen (**Figure 1A**). The vessel infiltration process during osteogenesis is somewhat similar to angiogenesis and directed by specialized structures in the vessels. These vessels are type-H and type-L; the distinction between these vessels is made up of comparative expression of endothelial markers, i.e., Endomucin (non-arterial vessel marker) and PECAM-1 or CD-31 (a canonical marker for endothelial cells). Type-H shows high expression, and type-L shows a low expression of these markers (43, 44). A high level of Endomucin and PECAM-1 is determined by Notch signalling, also responsible for higher expression of Kinase Insert Domain Receptor (a VEGF receptor). Blood vessel infiltration enables the enlistment of chondro-resorptive cells to disintegrate the existing cartilage and initiate osteoblastogenesis (45).

Ossification is a well-coordinated and regulated process, an essential part of homeostasis in the skeletal system. In general, bones contains three major compartments, i.e. epiphysis, diaphysis and metaphysis (46). Epiphysis contains the rounded portion of the growing end of the bones; diaphysis is the midsection of the bone, and metaphysis is the connection between epiphysis and diaphysis of the bones and is responsible for bone growth. Metaphysis contains an epiphysis growth plate and is compartmentalized in several zones based on the developmental process. Quiescent chondrocytes found in reserve zones divide rapidly in the proliferation zone and move to the epiphysis and begin to formulate hypertrophy in the hypertrophic zone. Few chondrocytes start calcification in the other zone, and the rest of the cells mature into the osteoblast and become a part of the development of the skeleton.

Embryonic osteogenesis is the outcome of the ossification process. Recent studies suggests the role of transcriptional regulation in the development of osteoblast. SOX9 is the major factor in endochondral ossification by controlling the development of the skeleton. SOX9 activates chondrogenic genes to initiate cartilage differentiation. Chondrogenic gene, i.e. *Col2a1* participates in craniofacial development and mutation in this gene can cause spondyloepimetaphyseal dysplasia. Loss of *Sox9* can hinder cartilage differentiation and lead to cell death ultimately (47–49). RUNX2 participates in skeletal development by regulating the genes in osteoblast differentiation i.e., *Spp1* and *Ibsp*. The latest outcomes suggests that deletion of *RUNX2* may lead to inhibition of osteoblast differentiation and loss of the above genes. Few studies reported that RUNX2 participates in immature osteoblast and hinder the maturation of osteoblast (50). OSX (Osterix) is another transcription factor that participates in the maturation of osteoblast and the generation of osteocytes. OSX regulates *Spp1* and *Sparc*, and inhibition of OXS resulted in irregular bone formation and accretion of abnormal cartilage (51).

Skeletal homeostasis is referred to as the dynamic balance of damage and repair of bone tissue. Bone formation and bone resorption are the two major processes of homeostasis facilitated by osteoblast and osteoclast, respectively (52). These cells are metabolically very active, and any irregularities may prime to numerous congenital disorders, deformities and bone sickness. In general, osteoblasts generate mineralized osteons, which are concealed in calcium deposition and later differentiate in osteocytes. After reaching a certain limit, osteocytes activate osteoclastic differentiation *via* RANKL, and when osteoclastogenesis leads to bone deformation, they secrete IGF to activate osteogenesis (53). Bone homeostasis is regulated by intrinsic and extrinsic factors such as mechanical stress, obesity, and senescence. A dynamic balance between osteoblast and osteoclast makes bone healthy and stronger. Recent studies show that sirtuin1 (SIRT1) participates in the differentiation of stem cells in the BM and bone-forming cells and regulate bone homeostasis. SIRT1 is a deacetylase and formulate epigenetic changes in histone or non-histone proteins (54). In bone, it is associated with bone mineralization. In mesenchymal stem cells, SIRT1 deacetylases β-catenin and prompts its

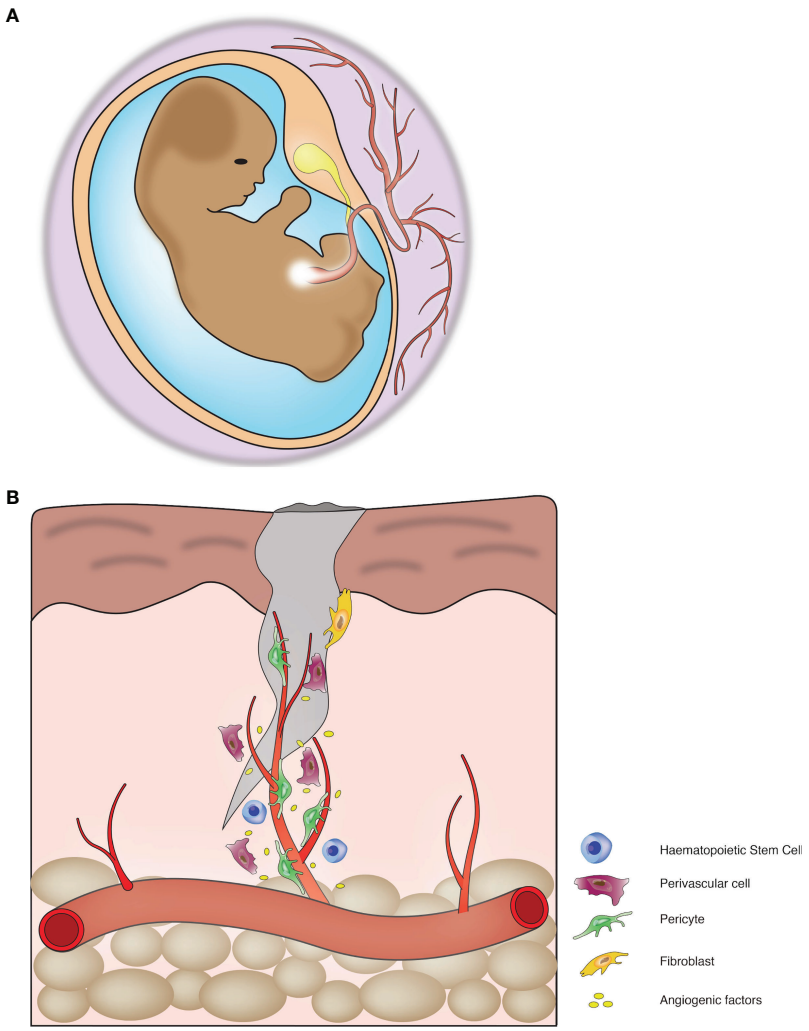

FIGURE 1 | Blood vessels mediate tissue development and regeneration: **(A)** Blood vessels play crucial roles during organogenesis. **(B)** Blood vessels play a critical role in tissue repair and regeneration.

nuclear localization, where it regulates osteogenic differentiation. It also regulates the differentiation of adipogenic tissue by inhibition of PPAγR2 (55). ATF4, a leucine zipper transcription factor activates *BGLAP2* in osteoblast and participates in terminal differentiation. Knockout studies show that it hinders bone homeostasis and osteoblast differentiation (56). AP-1, another transcriptional factor, which makes a complex with FOS, JUN and ATF, may act on osteoblastic enhancer gene and promotes osteoblastogenesis and regulate homeostasis (57, 58).

As discussed above, the vascular network is important for the development of the skeletal system; it plays a requisite role in homeostasis. Bone vasculature represents the prototypical hierarchical network of vessels and arteries, participates in paracrine signalling, blood perfusion, draining veins and interconnecting capillaries (59, 60). Here we have shown the specific role of vessel elongation required for the growth of cells during regeneration (**Figure 1B**). Long bone contains infiltrated vessels which are the source of blood, and some arteries invade the diaphysis and reach towards metaphysis near the growth plate. Type-H vessels are present in metaphysis and endosteum and are the major factor in regulating bone homeostasis. Type-H vessels are involved in the cross-talk between multiple cells in the bone and couple osteogenesis and angiogenesis (44). Hypoxia-inducible factors, especially HIF-1α, regulates type-H vessels. HIF-1α stimulates type-H vessel expansion, increases the number of osteoprogenitors and increase bone mass. Type-H vessels are also regulated by the Notch pathway. Any functional disruption in Notch signalling reduces the abundance of endothelial cells and type-H vessels in postnatal angiogenesis (61, 62).

Bone mineralization is a major factor in bone homeostasis. SOD3 (superoxide dismutase-3) regulates oxidative stress levels in cells by the formation of hydrogen peroxide from superoxide. The bone remodelling process involved resorption of the

mineralized matrix through osteoclast and replaced through osteoblast by making new bones. Recent reports explored that $SOD3^{-/-}$ mice show reduced bone strength and impaired mineralization which affects bone mass and density (63). *Tang et al.* recently explored the Role of *Runx1* in osteogenesis and homeostasis. Runx1 binds to core-binding factor β (*cbfβ*) and form a heterodimeric complex to bind the promoter complexes. *Runx1* plays a key role in mesenchymal stem cells commitments for differentiation and regulates several signalling cascades involved in bone formation, especially WNT/catenin pathways which have a significant role in osteoblast-adipocytes lineage differentiation. This study shows that knockout of *Runx1* generates osteoporosis phenotypes in mice. Runx1 binds and regulate the expressions of *Bmp7* and *Atf4* promoters and participates in postnatal bone homeostasis (64). Vasculature plays a significant role in the development of bone and maintaining homeostasis.

The skeletal system is a highly important organ responsible for the maintenance of haematopoiesis and osteogenesis. This includes the production of hematopoietic stem cells and differentiation into mesenchymal stem cells, osteoblasts and other mature functional cells (65). Maintenance of stem and progenitor cells is crucial in terms of healthy whole-body homeostasis and function (66). The vessels in the bones supply oxygen and nutrients *via* the central nutrient artery, the periosteal artery or metaphyseal-epiphyseal artery. Blood flows *via* these vessels through a densely populated capillary network and drains through the central vein (67). Recent studies shows the crucial role of the BM vasculature in regulating the fate of stem and progenitor cells *via* BM niches (68, 69). The BM niches have a distinct microenvironment that is highly complex and predominantly consists of vascular components and signals responsible for regulating stem and progenitor cell survival, quiescence, mobilization, and differentiation (70, 71). These signals consist of cell surface ligands, soluble factors, or cell-cell interactions (68, 69).

As we discussed above the BM vascular niche consists of multiple endothelial cell subpopulations, namely type-L, type-H and arterial ECs (44). Subsequently, they are physically associated with osteoprogenitors. Functional differences between type-H and other BM vessels, and secretion of angiocrine factors, regulate blood cell proliferation and differentiation and therefore maintain homeostasis and function. Different from sinusoidal ECs, arterial ECs are found to be the major source of stem cell factor (SCF) in the BM, which is crucial for HSC function (72). Sinusoidal type-L vessels are mainly supported by LepR-expressing perivascular cells, which contribute to CXCL12-abundant reticular (CAR) cells that support HSCs and contribute to the adipocyte lineage (73–75). Distinct perivascular cell types from the mesenchymal origin are found to be important for the support of the specialized vascular niches (76, 77). Type-H vessels are covered with RUNX2 and Osterix expressing progenitors (44, 78–80). Subsequently, type-H capillaries and arterioles are associated with pericytes that express NG2 and PDGFR-β receptors together with Nestin expressing mesenchymal stem and progenitor cells (MSPCs) (81, 82). Arteriolar niches play a pivotal role in maintaining HSCs quiescence and HSCs distribution between the BM niches (81, 83) and HSCs prefer to localize within the different vascular niches in the BM. Imaging of HSCs localizing in the BM shows a highly abundant presence of both dividing and non-dividing HSCs in the central diaphyseal BM around sinusoidal vessels and distant from arteriolar vessels (84). A quiescent subset of HSCs was found to mainly localize around endosteal arteriolar vessels surrounded by NG2+ pericytes. Proliferative HSCs moved away from the arteriolar vessels towards LepR+ perisinusoidal vessels (81, 82). Thus, endothelial interaction with HSCs in the distinct BM vascular niches regulate HSC quiescence and proliferation. This interaction occurs mainly *via* the secretion of certain signalling factors by the BM ECs, which is critical for HSCs homeostasis. These factors consist of HIF-α, Notch ligands, CXCL12 and SCF (76, 85). Cellular crosstalk in the bone tissue microenvironment is operated through the vasculature. Ageing of vasculature has a specific role in the functional capacity of organs. Here we are discussing vascular ageing in the skeletal system in the next section.

AGEING OF VASCULAR NICHES IN BONE

Ageing is shown to affect the skeletal system *via* loss of mineralized bone and the increase of fracture risk and subsequently increases the risk of osteoporosis (86). In the process of ageing, the BM vasculature shows both morphological and metabolic changes with a significant reduction in arteriolar vessels. The reduction of type-H endothelium causes a decline in blood flow and reduced expression of angiocrine and pro-hematopoietic factors such as HIF-α, SCF, CXCL12 and Notch (76, 85). This decrease of angiocrine factors is often associated with poor angiogenesis, bone construction and increased risk of osteoporosis (87). HIF-α is a transcription factor that is responsible for the regulation of cellular response to oxygen levels (80, 88). In terms of bone angiogenesis, HIF-α expression is increased in ECs and osteoblasts under hypoxic conditions; this promotes the formation of new bone and the growth of new vessels *via* the expression of vascular endothelial growth factor-A (VEGF-A) and other proangiogenic factors (80, 89). In the metaphysis bone area, expression of HIF-α occurs in an oxygen-independent manner by type-H vessels (44). The endothelial decrease of HIF-α that is observed upon ageing, therefore, contributes to type-H vessel decline and a reduction of osteoprogenitors, osteogenesis and bone density (44). Interestingly, the presence of sinusoidal type-L vessels remained unchanged upon biological ageing (44, 90).

As mentioned in the paragraph before, endothelial signalling in the distinct BM vascular niches *via* Notch ligands, CXCL12 and SCF pathways regulate HSC homeostasis (76, 85). In aged mice, the BM ECs show significantly lower levels of these signalling pathways when compared to young mice (91, 92). Notch signalling is one of the most critical cell-cell interaction mechanisms that control cell fate (93). Notch activation in the BM leads to ECs proliferation and the formation of type-H vessels (94, 95). Subsequently, activation of Notch enhances HSCs and both PDGFR-β+ and NG2+ perivascular cells, indicating Notch as a mediator to promote vascular niche

function (65, 76). CXCL12 is essential for HSC and lymphoid progenitor maintenance and quiescence (96). CXCL12 is expressed by BM EC, perivascular cells, osteoblasts, sympathetic neuronal cells, and Nestin+ perivascular stromal cells that are physically associated with HSCs (65, 70). Deletion of CXCL12 in ECs and MSPCs reduced HSC frequency and impaired long-term repopulation activity (75, 96, 97). Endothelial and perivascular SCF is crucial for HSC maintenance and survival (74, 98). SCF is expressed by perivascular stromal cells, arterial ECs, type-H ECs and sinusoidal ECs (65, 74, 76). Deletion of membrane-bound SCF causes the significant depletion of HSCs. Subsequently, depletion of SCF from peri-arterial mesenchymal stem cells also results in the depletion of HSCs, which indicates the importance of the mesenchymal compartment in HSC maintenance (74). Total HSC numbers increase upon ageing. However, age-related relocation of HSCs away from endosteal arteriolar niches correlates with a reduction of self-renewal and loss of quiescence (99–101). Subsequently, ageing of the BM vascular niche can induce this ageing-associated HSC phenotype. Infusion of young ECs is able to partially restore HSC function, suggesting a relationship between changes in the vascular niche and HSC ageing (85).

Ageing of the BM causes a set of complications leading to both haematological and non-haematological diseases. Haematological ageing inevitably leads to decreased functionality of the immune system, which comes with a range of complications. Impairment of the immune system increases the susceptibility for infection, autoimmune disorders, and haematological malignancies (102–104). Inflammatory responses by the immune system are an essential response to tissue injury and infection. Upon infection, ECs, MSCs and other hematopoietic and non-hematopoietic cells are activated. Activation of ECs leads to an upregulation of pro-inflammatory cytokines such as interleukins (IL) and TNF-α (105–107). In the BM this response stimulates HSC proliferation, migration, and differentiation to maintain the pool of immune cells (104). Inflammation changes both the morphology and function of the BM endothelium. These alterations show many similarities to changes that are observed in the aged BM niche (108, 109). Both inflammation and ageing induce myeloid differentiation and impair HSC self-renewal capacity (104). Subsequently, serum levels of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are upregulated in aged individuals (110, 111). The presence of these pro-inflammatory cytokines also further enhances the myeloid skewing of HSCs (112). Other complications that can occur due to ageing of the BM are numerous cancers such as acute myeloid leukaemia or osteosarcoma and osteoporosis, which is also referred to as bone loss disease. Non-haematological diseases associated with skeletal ageing are OA (osteoarthritis) and RA (rheumatoid arthritis). These conditions are characterized by bone reabsorption of osteoclasts and high levels of pro-inflammatory cytokines such as IL-6, IL-11, and TNF-a (113, 114). Vascular ageing is one of the crucial aspects of the skeletal system to grow and differentiate. It has an impact on bone joint disease and bone angiogenesis. We have covered the inflammatory status of joint synovium in the coming section.

VASCULAR NICHES IN JOINTS

Joints are built up by a series of different tissues that serve different individual functions. However, all tissues cooperate to maintain healthy joint movement and homeostasis. The diarthrodial joint is structured by the presence of muscle, bone, bursae, tendon, cartilage, joint capsule, synovial membrane, and synovial fluid.

The synovial cavity is surrounded by the joint capsule, which consists of fibrous connective tissue that is attached to both bones. The synovium, apart from diarthrodial joints, is also located in tendon sheets and bursae and is comprised of a surface layer of cells, referred to at the intima and subintima. Between all the intimal surface layers, fluid is located, which is high in hyaluronic acid and has non-adherent properties. The intima mainly contains bone-marrow-derived macrophages called type-A synoviocytes and fibroblast-like cells called type-B synoviocytes. Other than fibroblasts, B-synoviocytes express high levels of VCAM-1. The cells of the intima are responsible for the production of extracellular matrix molecules and mediation of synovial fluid clearance and production (115, 116). We have illustrated joint synovium in a healthy environment, which does not show any inflammation (**Figure 3**).

Blood vessels and lymphatic vessels are located in a mostly collagenous tissue below the intima called the sub-intima (117). The synovium can be categorized in fibrous, areolar, and adipose depending on the composition of the sub-intimal layer. The sub-intimal layer of fibrous synovium is found in locations that are exposed to high pressure and are mainly composed of large collagen fibres (118) (**Figure 2A**). The areolar synovium has fewer collagen fibres, but more interfibrillar matrix and is found in places where the synovium moves freely over the joint capsule (118). Adipose synovium is found in intra-articular fat pads (119). These three different types of synovia can also be found together in a combination (118). Synovium is highly vascularized tissue with the presence of arterioles, capillaries and venules with fenestrae to supply oxygen and nutrients (**Figure 2A**). The distribution of vessels is organized in a non-uniform manner with the difference in population density according to the level of mechanical stress. Synovium that is subjected to higher levels of mechanical stress shows long loops of arterioles to supply more blood. However, the synovium that is subjected to very high mechanical forces has few vessels due to the low mechanical stress resistance of blood vessels. Capillary density is not only related to anatomical location but also the depth beneath the synovial surface. As previously mentioned, most blood vessels are located just below the intima, placing them in the sub-intima (120–122). In the synovial joint, VEGF, angiopoietin (Ang) and PDGF-β regulate vessel stability and induce fibroblast invasion. Complementary action of VEGF and Ang is essential for vessel formation, stability, and maturation; *via* regulation of EC proliferation, migration, survival, and pericytes/EC interaction. Dysregulated expression of VEGF and Ang in synovial tissue has been associated with multiple pathogenic outcomes such as rheumatoid arthritis. As shown, stress conditions alter vascular and perivascular microenvironments in the knee joint

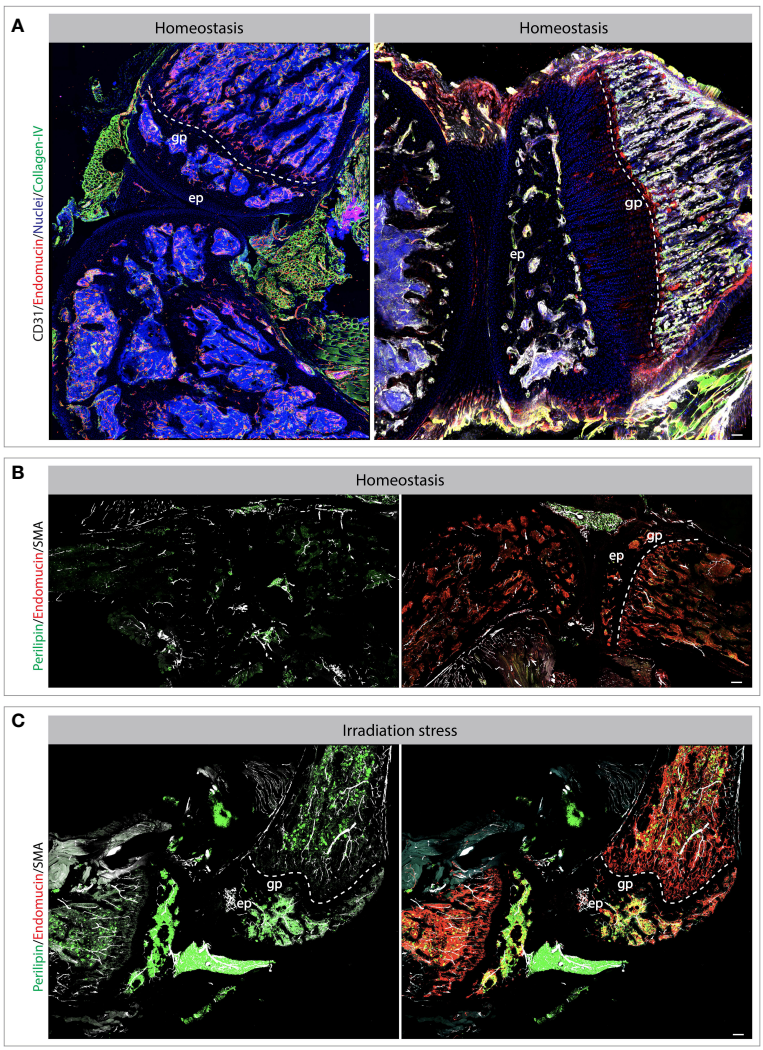

FIGURE 2 | Bone and joint vasculature and perivascular niches. Confocal images showing blood vessels and perivascular cells in bones and joints. Exemplar bones and joint sections immuno-stained for endothelial and perivascular cell markers as indicated on the images **(A–C)**. The changes and vascular cells and perivascular microenvironments can be observed during stress conditions such as radiation-induced injury. Specifically, the tile view imaged demonstrate the changes in vascular morphology and accumulation of adipocytes post radiation **(B, C)**. Scale bar represents 200 μm. gp, growth plate; ep, epiphysis.

(**Figures 2B, C**). Osteoarthritis and Rheumatoid arthritis are the major chronic diseases associated with the joint. We have covered these two interesting aspects of chronic inflammation in the next section.

DYSREGULATION OF VASCULAR NICHES IN OSTEOARTHRITIS

OA (Osteoarthritis) is a degenerative and chronic joint disease, resulting from gradual degradation of articular cartilage and underlying bone remodelling. Articular cartilage is positioned on the joint surfaces and participates in movement (123, 124). Articular cartilage is subsequently made up of chondrocytes, collagen and proteoglycans, in a combined form of a hydrated extracellular matrix. It divides into several zones based on the distribution and alignment of chondrocytes and collagen fibres. During OA, subchondral bone shows sclerosis, abnormal vasculature and formation of osteophytes. It is also assumed that subchondral bone abnormalities contribute to cartilage disintegration (125). In disease progression, blood vessels invade the cartilages tissue and prompt the release of cytokines and create a low-grade inflammatory environment. This inflammatory environment hinders the identification of the molecular mechanism of OA initiation. Increased inflammation may induce angiogenesis and promote the invasion of vasculature in cartilage tissue. Studies suggest that vascular changes are the prime factors in the disease progression as it shows reduced perfusion. It is reported

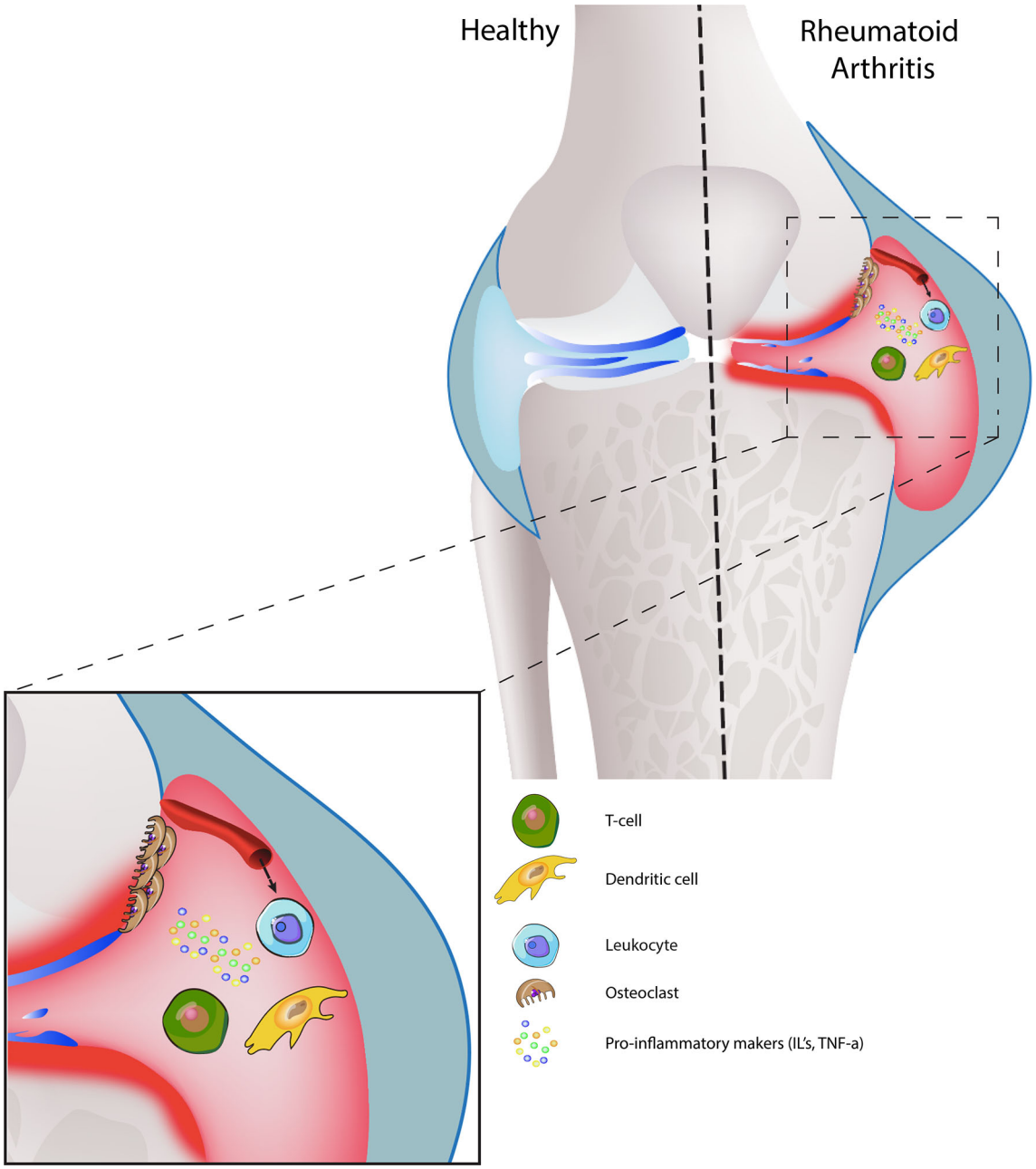

FIGURE 3 | Joint synovium in healthy and diseased condition: Healthy joint synovium displays a thick layer of cartilage, medium synovial fluid levels in the joint cavity with intact synovial membrane and a strong, smooth outer bone layer (top left). In RA, the synovial membrane is swollen and has a high presence of synovial fluid, which leads to damaged bone and cartilage (top right). On the cell-interaction level, increased leukocyte infiltration promotes inflammation, hyperplasia, and bone destruction by osteoclasts. Dendritic-T cell interactions also release pro-inflammatory markers, which further enhances disease progression.

that subchondral bone has a link with cartilage and these interactions increase during the disease progression. Overexpression of TGF-β and osteoclast contributes to OA, and expression of TGF-β increases through osteoclast induced matrix resorption by positive feedback mechanisms. Targeting TGF-β can attenuate the OA by inhibiting aberrant bone remodelling and angiogenesis (126–128).

To explore the role of the vasculature in articular cartilage destruction, studies were conducted on temporomandibular joint osteoarthritis (TMJ OA). Vascular changes were analyzed with the expression of $CD31^+$ and α-SMA^+ in human and miniature pigs. They show that change in the vasculature is linked with the bone transformation from cartilage tissue. The study also shows the transformation of chondrocytes to

osteoblast, confirmed with expression *COL2* and *RUNX2* in vascular stretches (129). In another study, a microfluidic-based *in-vitro* model presents the tissue vasculature role in OA progression. This cartilage on a chip method includes co-culture studies of primary endothelial cells with mesenchymal cell lines and investigates the osteogenic differentiation and tubes formation. Exposure of inflammatory cytokines to this model is able to attain the OA characteristics by depicting increased expression of MMP13 and ADAMTS5 (130).

The disintegration of cartilage tissue is completed by digestive enzymes. MMP and ADAMTS target their respective molecule i.e., collagen and aggrecan, respectively. Activation of these proteases is done by inflammatory cytokines secreted by chondrocytes, especially IL-1β and TNF-α (131). OA, instigates the functional and structural changes in bone and promotes catabolic protease activities. However, the role of inflammation in bone disintegration is debatable. Bone tissue undergoes plenty of modifications in OA by attaining a sclerotic phenotype. Such structural and molecular changes induced the BM lesions, which are sensitive to cardiovascular risk factors. These lesions make the way to cartilage damage and appreciate the subchondral bone changes (132). Despite the understanding of the clinical aspects of OA, the molecular basis is still vague. Recently, functional analysis of several genes explains the significance of molecular pathology. High throughput imaging analysis in mice resulted in 14 genes, and their functional role in the pathogenesis of OA and 6 out of them characterize for human pathogenesis. Based on rigorous screening, data highlighted 4 genes, namely *Bhlhe40, Pitx1, Sh3bp4 and Unk*. Reduced expression of PITX1 protein promotes subchondral bone thickness and is involved in OA pathogenesis. A detailed study in humans on gene expression shows that *Ccd6, Col4a2, Arhgap30, Gsdme, Unk, Josd1*plays a crucial role in the pathogenesis of OA (133).

Recently data shows a positive feedback mechanism that is present in bone-cartilage and vascular crosstalk. In the process of bone regeneration, type-H vessels participate in several mechanisms along with the coupling of osteogenesis and angiogenesis. This process involves type-H vessels, mTORC-1, chondrocytes and VEGF-A. This complex environment promotes activation of VEGF-A secretion and an increase in subchondral angiogenesis, resulting in OA (134). Targeting angiogenesis in the coupling of such pathways may affect the vascular invasion, blocking of VEGF induced angiogenesis shows a promising effect on cartilage destruction. Recent studies show that cytokine neutralizing antibodies are effective to show a potential effect on OA. It is observed that rapamycin can affect the pro-inflammatory cytokines i.e., IL-1. IL-6 and inhibits mTOR pathway, which may be an alternative approach to target OA in patients (135).

DYSREGULATION OF VASCULAR NICHES IN RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a common chronic inflammatory autoimmune arthritis inclined by environmental and genetic factors and resulting in inflammatory pain in the hands, feet and knees. During the disease progression, angiogenesis promotes infiltration of inflammatory leukocytes and fibroblast into the joints and leads to bone destruction and hyperplasia in synovial joints. Hyperplastic conditions may prompt synovium to bone invasion and destruction, which is in line with the help of osteoclast cells. This process also inhibits the bone-forming process and leads to RA (125, 136).

Primary endothelial cells arranged in blood vessel lining and helped in cellular trafficking. Leukocytes migrate throughout the vessels and enter the connective tissue after interaction with the endothelial adhesion molecules. The endothelial lining of vessels activated by pro-inflammatory factors lead to the expression of adhesion receptors on the luminal side of endothelial cells and promote the binding of leukocytes and fibroblast. These interactions operate through ICAM-1, VCAM-1 and E-selectin majorly (137, 138). The landing of leukocytes and fibroblasts increases the inflammatory load and affects the joints. Leukocyte trafficking starts with interaction with selectin (CD15s) and is followed by the rolling on the endothelial surface *via* VCAM-1, which helps in transmigration. It has been observed that dendritic cells attract towards joint and secrete inflammatory cytokines and contributes to RA through IFN-α, IFN-β and IL-23. Dendritic cells regulate Th-cell response in RA and create an imbalance in the cytokine secretion and inflammation (139, 140).

Vasculature changes contribute to the pathology of both conditions. In OA disrupted blood flow and ischemia in the subchondral bone reduce the nutrient supply to the articular cartilage, which lead to osteocyte cell death and articular damage (141). Subsequently, increased type-H vessel formation due to overexpression of VEGFA, PDFG-B and TGF-B induce pathological subchondral bone angiogenesis, therefore contributing to the development of OA (142–144). During RA, activated blood vessels expressing ICAM-1, VCAM-1 and E-selectin are responsible for leukocyte and fibroblast migration. Therefore, actively contributing to the progression of RA (145–148). The signalling process of joint inflammation, including the cellular cross-talk, depict the diseased conditions (**Figure 3**).

Recent reports suggest that the interaction of leukocytes with endothelial cells can increase after the TNF-α activation in endothelial cells. The level of TNF-α is found to be increased in RA pathogenesis. Few studies show that the generation of biologic DMARDs (Disease-Modifying anti-RA Drugs), which has specific targets in cytokine pathways, may affect the disease progression in RA patients (149, 150). Oxidative stress is one of the prime reasons for inflammatory activities in the joints. Interaction between immune cells and antigens create ROS in arthritis pathogenesis. Upregulation of p38 MAPK increases the ROS generation, which in turn induce the secretion of pro-inflammatory cytokines in RA. Activation of p38 contributes to cartilage damage, synovial inflammation and angiogenesis. Recent studies depict the importance of ROS inhibition in the prevention of RA (151, 152). Tissue vasculature has different properties in different organs. The specificity of each organ is supported through its specific vascular niches. We have explored this aspect of organ-specific vasculature in the following section.

TISSUE-SPECIFIC VASCULAR NICHES AND VASCULAR CHANGES

The kidney is an important organ in the health and homeostasis of the human organism due to its role in clearing the blood of toxins and waste products while maintaining haematological homeostasis *via* regulation of acid-base balance, red blood cell count and blood pressure *via* the secretion of hormones (153). The renal vasculature is highly complex and essential for renal function. Renal endothelial cells (RECs) show functional differences according to their location (154, 155). RECs can be subdivided into glomerular RECs (gRECs), medullar RECs (mRECs) and cortical RECs (cRECs). Each subtype of RECs differs from supporting the function of the renal gland. For instance, cRECs are surrounded by pericytes and smooth muscle cells, which can regulate the glomerular filtration rate (GFR) in the cortex. gRECs, on the other hand, are fenestrated to allow water passage yet restrict the passage of high-molecular-weight substances (156, 157). ScRNA-seq exposed even higher levels of heterogeneity across the RECs population with five sub-populations found in the gRECs population, nine for the cRECs population and ten for the mRECs (158). The heterogeneity of the renal vascular system may hold the potential to better comprehend and identify targets for a variety of renal conditions and disease such as chronic kidney disease (CKD). CKD is considered a major global problem, with 850 million people affected (159). Early-stage CKD already shows clear evidence of endothelial dysfunction, affecting vascular permeability, angiogenesis, inflammatory response, and immunity. Further endothelial damage leads to atherosclerosis and worsening of CKD disease prognosis with progression towards end-stage kidney disease (160, 161).

The lung has a high population of ECs that serve to maintain lung homeostasis. The vasculature of the lung can be subdivided into systemic bronchial and pulmonary circulatory systems. Quite remarkable of the lung vasculature is its capability to recruit available vessels to allow for an increase in flow with little increase of pressure during, for instance, exercise (162). The pulmonary ECs (PECs) play a key role in regulating oxygen exchange, controlling barrier function, and regulation of vascular tone *via* nitric oxide, serotonin, endothelin, and prostacyclin pathways (163). Extracellular interaction of PECs with circulating and surrounding cells is essential to maintain homeostasis by controlling thrombosis, inflammatory cell adhesion, angiogenesis, and vascular wall integrity (164, 165). Single-cell transcriptomics data of lung ECs show enrichment for immune, regulatory signatures, suggesting a role in immune surveillance. Subsequently, veins in the lung tend to have upregulated expression levels of genes involved in cAMP metabolism (166). cAMP is involved in controlling ciliary beat frequency and suppression of the pro-inflammatory activity of immune and inflammatory cells (167). Alterations of the pulmonary endothelium are involved in the pathogenesis of multiple lung diseases such as chronic obstructive pulmonary disease (COPD) or acute respiratory distress syndrome (ARDS). Lung diseases associated with pulmonary endothelial are mainly characterized by increased permeability leading to vascular leakage and oedema formation, the altered balance between vasocontraction and vasodilation, acquisition of pro-inflammatory phenotype, acquisition of pro-thrombotic phenotype and miscommunication with adjacent vascular cell wall (165, 168).

The liver is crucial for the maintenance of homeostasis due to its involvement in detoxification, immunity, metabolism, and nutrient storage. In order to fulfil these tasks, the liver is comprised of numerous different cell types apart from parenchymal hepatocytes. The non-parenchymal cells (NPCs) consist of liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs) and stellate cells (169). The nutrient-rich blood from the hepatic portal vein and oxygen-rich blood from the hepatic artery meet in the sinusoidal blood vessels (170). During this process, the LSECs of the sinusoidal vessels assist in clearing macromolecular waste and regulating hepatic vascularity (171). Other than most endothelial cells, LSECs possess a higher endocytic ability. As an example, 45% of all pinocytic vesicles are attributed to LSECs and LSECs are shown to be more efficient in absorbing/internalizing circulating antigens than dendritic cells or macrophages of the spleen and Kupffer cells and dendritic cells of the liver (172–174). LSECs are able to filter the blood *via* selective exchange of molecules in the blood and underlying stellate and hepatocytes due to their fenestrated morphology (175). Due to the lack of a basement membrane or basal lamina, there is direct access to the space of Disse (perisinusoidal space) for interaction between blood and hepatocyte or stellate cells (176). The LECs fenestrae have the ability to change their diameter according to as a response to the cellular microenvironment (177). The fenestrae are maintained by cooperative paracrine and autocrine signalling of hepatocytes and stellate cells. VEGF, NO and serotonin pathways have been shown to be involved in the maintenance and regulation of contraction or dilation of the fenestrae (178, 179). Loss or reduction fenestrae number in LSECs is referred to as defenestration (177). Defenestration leads to reduced hepatic uptake of lipoproteins which can cause hypolipoproteinemia (180). Subsequently, defenestration is involved in multiple disorders like liver fibrosis, atherosclerosis, or non-alcoholic fatty liver disease (NAFLD).

Malignancies in the kidney, lung and liver are associated with a poor prognosis due to their fast progression and metastasis. The role of vasculature is very important for the progression of metastasis from different organs towards the bone. We have created a dedicated section for bone metastasis and tumour dormancy. Across these organs, metastasis to the skeletal system is found to occur commonly (181–185). After initial tumour growth in the organ of origin, tumours cells undergo changes in cell signalling and cell-cell interactions such as reduced intercellular adhesion (186, 187). This causes the release of tumour cells in the circulatory system (188, 189). In most tissues, blood vessels only express adhesion markers such as VCAM-1 and ICAM-1 during exposure to inflammatory cytokines (190). However, the blood vessel ECs in the metaphysis of the long bones are characterized by their continuous expression of these adhesive proteins, which promote the interaction between circulating tumour cells (CTCs) (190, 191). The presence of

voluminous sinusoids reduces blood flow in the blood vessels of the metaphysis, which allows for easier docking of CTCs (192, 193). Subsequently, the BM ECs release high levels of growth factors that attract metastatic tumour cells (194). Thus, across organ vasculature supports bone metastasis by its structural, cell-interaction and growth factor releasing characteristics.

As described in the previous paragraphs, age-dependent changes of the vascular niche led to the loss of functional HSCs and osteoprogenitors. In ageing vasculature, inflammation, endothelial senescence, elevated oxidative stress, mitochondrial dysfunction, impairment of proteostasis and genomic instability is observed (195). Therefore, signalling from the microenvironment is an essential driver of stem cell and tissue ageing. Exposing the age-dependent changes of the vasculature has therefore has the potential to identify markers and targets of the ageing process across different organs and tissues. In recent years the topic of ECs heterogeneity across organs and tissues has become more of interest (166, 196, 197). Mapping of the tissue wide distribution of ECs, pericytes, mesenchymal stromal cells, and the matrix is essential to understand the age-related changes in the tissue microenvironment. Recently, loss of both vessel density and pericytes are exposed as a mark of ageing across tissue and organs (198). Ageing in organs show the specific role of vasculature (**Figure 4**) Tissues like the skin, gut and uterus who have high remodelling and regenerative capabilities (199–201) are, however, able to maintain the abundance of blood vessels and pericytes upon ageing (198). A similar phenomenon is observed in bones where the vessel density is unaffected by ageing (44). This can be explained by the relatively high regenerative capacity of bone when compared with the kidney, spleen, heart, or brain. Increased pericyte to fibroblast differentiation is observed with ageing, which could help explain the general loss of pericytes. Fibroblasts involved in joint inflammation and organ fibrosis are subsequently shown to originate from pericytes, wherein in the case of organ fibrosis, differentiated pericytes are considered a driver of fibrosis. Down-regulation of multiple signalling pathways responsible for the regulation of blood vessel maintenance and remodelling across multiple organs results in vascular attrition and the pro-inflammatory nature of ECs that is observed during ageing (**Figure 5**) (198). It is proposed that EC inflammation combined with alteration in the signalling pathways responsible for the regulation of blood vessel maintenance and remodelling, ultimately lead to loss of vasculature and accumulation of fibroblasts. Accumulation of fibroblasts *via* pericytes to fibroblast transition is known to occur in tumours and promote tumour growth and metastasis (202). To understand the details of metastasis in the bone microenvironment, we have dedicated a specific section. This will describe the tumour metastasis and dormancy in the bone microenvironment.

ROLE OF VASCULAR NICHES DURING BONE MALIGNANCY

Cancer metastasis is a distinctive mechanism of malignancy, and the invasion of bone is the most common choice of solid tumours, especially in the breast and prostate. Recent studies formulated that bone metastasis is the major cause of death in solid tumours (203, 204). Uprising clinical challenges during the dissemination lead to hyper mortality in patients. Prior to dissemination, tumour cells transition to mesenchymal to initiate the invasion through basal lamina and sustain in the circulatory system with the help of platelets. Tumour cells trigger platelets aggregation by binding to VWF (von Willebrand factor) and stimulating the VEGF secretion to support angiogenesis. Platelets thus secrete cytokines like LPA (Lipopolysaccharide) and make the molecular switch which contributes to tumour cell detachment and circulation in the bloodstream (205, 206). The mesenchymal transition of tumour cells is modulated with a few specific signalling cascades e.g., TGF-β and MAPK, and firmly associated with metastasis progression. In tumour cells, MAPK upregulation promotes MMP activation and, therefore, digestion of surrounding tissue and invasion of the tumour cells. Upregulation of MAPK shoves metastasis in a multidirectional way by activation of MMP, regulation of adhesive components and inhibition of retinoblastoma phosphorylation. Heparin-binding epidermal growth factor receptor upregulates the metastasis signalling *via* activation of MAPK in prostate cancer (207, 208). In addition to MAPK, NF-kB also participates in tumour metastasis *via* induction of EMT in tumour cells. In general, NF-kB signalling is regulated by an inhibitor of NF-kB (IkB). During cancer progression, the tumour necrosis factor-alpha receptor inhibits the IkB activity. IkB inhibition hinders the binding of NF-kB and IkB, which leads to hypoxic conditions and NF-kB mediated activation of HIF-1α. HIF-1α triggers the EMT in tumour cells and promotes metastasis (209, 210).

Although the reason for cancer metastasis is less implicit, the whole process is well coordinated. The tumour microenvironment is a major factor in metastasis as it contains multiple cell lineages, which interact with the tumour cells (211). Out of the mesenchymal stromal component, it is evident that macrophages participate in all the phases of the metastasis cascade. These macrophages are derived from monocytes and are involved in cancer progression, as explained in recent studies. Simultaneous deletion of IL-4 and CCR2 alone with monocytes add back approach, explained that bone tissue originated macrophages do not participate in tumour cells establishment in bone tissue microenvironment (11, 212).

During circulation, platelet interaction with tumour cells upregulates CCL2 expression on tumour cells and thus promotes vascular permeability. Tumour cells engaged with stromal cells *via* CCL5 metastasize and make a stable network at bone tissue (213). Bone vasculature is different from other organs due to fenestrated vessels architecture, which contributes to tumour engagement and extravasation by constitutive expression of adhesion proteins. Recent time-lapse imaging and single-cell sequencing studies show the tumour-specific blood endothelial cells, which helps in metastasis and are a part of cells that express *csf-1* (214). Type-L and type-H vessels express proteins like P-selectin, E-selectin, ICAM-1 and VCAM-1, which allow tumour cells to adhere and infiltrate to acquire extravasation (215, 216). During the extravasation, endothelial cells express and secrete growth factors, i.e. stromal cell-derived

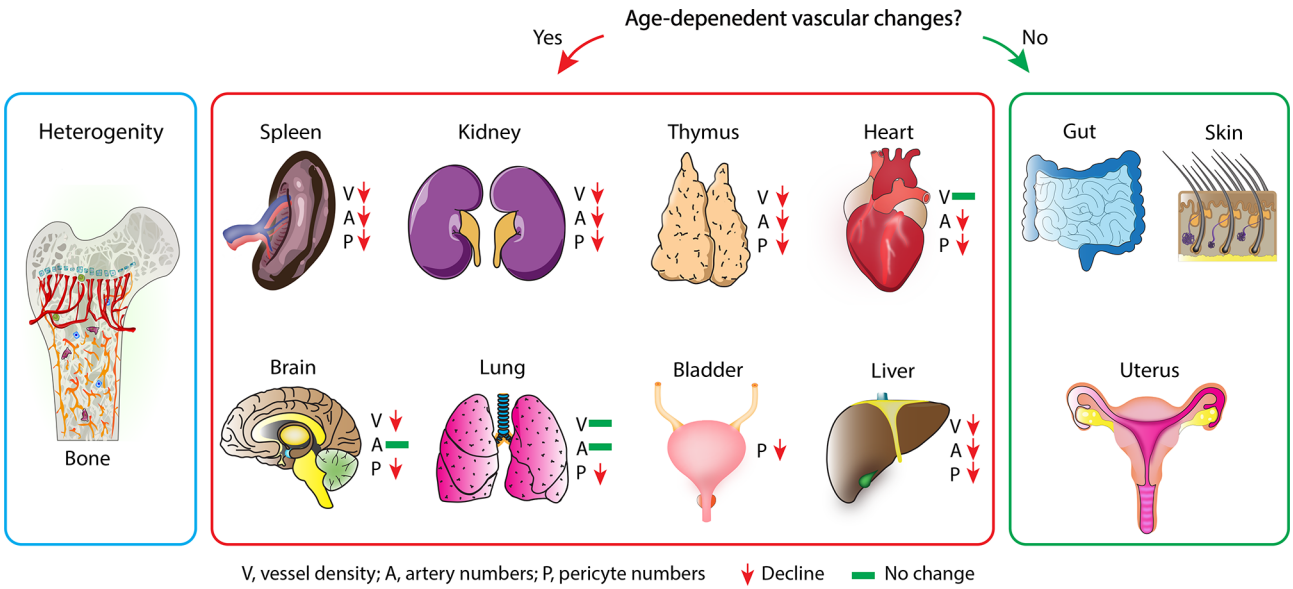

FIGURE 4 | Vascular ageing across different tissues: Vascular ageing causes the decrease in endothelial heterogeneity of the bone but not the overall vessel density. This age-dependent change of the bone marrow vasculature is hallmarked by the decrease of type-H vessels (Left). Across all organs, different vascular changes are observed during the ageing process. Decreased vessel density, artery numbers and pericytes number are observed in spleen, kidney, thymus and liver. Fewer changes are observed in heart, brain, and lung (middle). On the other hand, gut, skin, and uterus maintain their vasculature integrity during ageing (right).

factor-1 (SDF-1), which promotes tumour cells to engage and establish a cell mass through their interactions with the BM cells (217).

Recent studies show the specific role of tumour-stromal cell interactions in cancer progression and regulation. CXCL12, also known as stromal cell-derived factor-1, and its localized receptors participate in cellular interaction during cancer progression and metastasis (218). CXCL12 and its receptors can induce multiple signalling processes that control gene transcription, cellular survival and apoptosis. The binding of CXCL12 and its receptor CXCR4 initiates the membrane changes and GTP exchange, which leads to dissociation of Gα units of G proteins. Dissociation of Gβ/Gγ activates phospholipase C (PLC-β) and induce the catalysis of PIP2 into IP3 and DAG signalling and chemotaxis. Interaction between CXCL12/CXCR4 initiates the phosphorylation of CXCR4, which supports the calcium flux and activation of PI3K, MAPK signalling and thus induce cancer cell proliferation (219–221).

Interestingly, CXCL12 secretion through osteocytes functions as a chemoattractant and helps in homing and retention of CXCR4 expressed cancer cells in the BM microenvironment. Tumour cell homing requires interactions with the ECM, with a crucial role for integrins. Integrin expression on tumour cells mediate critical interaction in tumour development. Integrin α2β1 expressed on tumour cells binds with type-I collagen, utmost ample protein available in bone. Studies show that binding of integrin with collagen type-I activates the integrin associated kinases and induce cytoskeleton rearrangement through activation of Rho pathway. Activation of RhoC GTPase is known to be a prominent factor for metastasis, and it helps in the invasiveness of tumour cells and metastasis to bone (222, 223). Recently, it is suggested that once the tumour cell attains bone proximity, plenty of them undergo apoptosis, and few of them survive. Tumour cells bump into the unreceptive milieu, which hinders the instant tumour growth in the bone environment. The BM niche produce multiple factors like Annexin A2, TGF-β, CXCL12 and IGF (insulin-like growth factors), which allows tumour cells to remain in the dormant stage (223, 224). Studies observed that tumour cells express a high level of Axl during dormancy. Axl controls cellular proliferation, EMT and innate immune response in general. Osteoblasts secrete GAS 6 and activate Axl receptors in tumour cells. Co-culture studies of tumour cells with pre-osteoblastic cells show that osteoblasts reduced the proliferation of tumour cells, which can be regulated by low expression of Axl *via* targeting of TGF-β and TGFBR2. The expression of TGF-β and TGFBR2 is elevated in co-culture studies and thus contributes to tumour cell dormancy. The BM vasculature niche provides stability to tumour cells and supports the tumour dormancy due to the low sinusoidal blood flow and large vessel diameter (225).

Dormancy of tumour cells depicts the progression stages of cell cycles, i.e. G0/G1. Such cells remain dormant for many years until activation occurs. Dormant tumour cells show higher expression of p38 MAPK signalling and downregulation of ERK MAPK signalling pathways. It is reported that p38 controls the grid of quiescent transcription factors, responsible for cellular growth/arrest and self-renewal genes. TGF-β also contributes to cancer cell dormancy *via* the regulation of activation of p38 signalling and quiescence. These specific regulators in disseminated tumour cells marks as a dormant signature in cancer cells. In addition to the

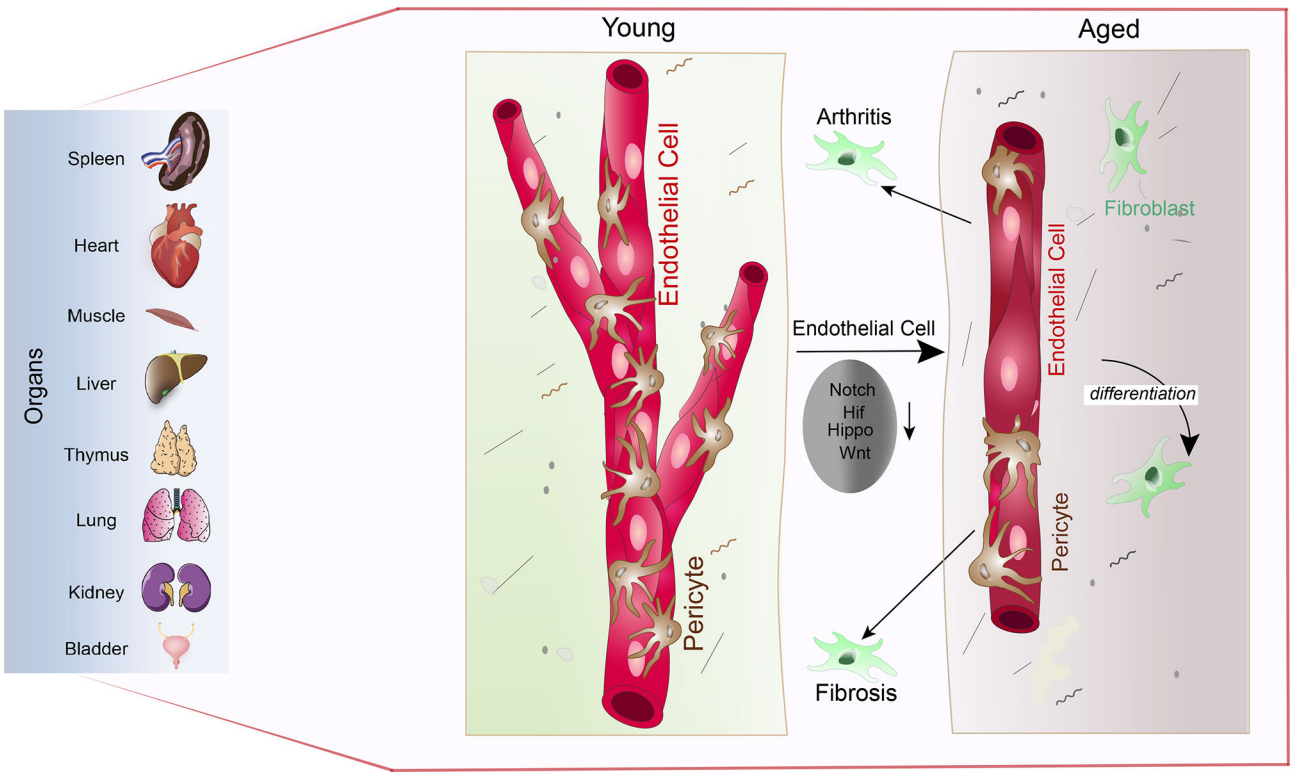

FIGURE 5 | Vascular attrition is a primary hallmark of ageing: Pericytes abundantly cover the endothelial lining throughout the spleen, heart, muscle, liver, thymus, lung, kidney, and bladder (left) in young mice (young). Upon ageing, reduced Notch, Hif, Hippo and Wnt signalling in endothelial cells leads to pericyte loss *via* pericyte to fibroblast differentiation (aged). Loss of pericytes and accumulation of fibroblasts subsequently supports arthritic and fibrotic processes.

predominance of p38 activity, NR2F1 also regulates tumour cell dormancy. NR2F1 is a nuclear hormone receptor that regulates induced pluripotent reprogramming and also neural cell crest differentiation. NR2F1 arrest the cell growth in disseminated tumour cells *via* the regulation of SOX9, NANOG, SOX2 and RARβ (226–229).

Disseminated tumour cells get support from osteocytes and start a positive feedback mechanism that initiates differentiation of osteocytes in osteoblast and/or osteoclast. Tumour cells recognize secretary molecules i.e. CCL5 and CXCL12 released from stem cells and osteocytes (230). Due to the positive feedback mechanism, this recognition prompts the osteocytes to release growth derived factors (GDF 10 & 15) and secretion of PTHrP (parathyroid related hormone protein) from tumour cells. PTHrP has a specific receptor PTH1R on the surface of osteoblast cells (231). After ligand interaction with the receptor, osteoblasts secrete RANKL (receptor activator of NF-KB ligand). RANKL binds on the RANKL receptor on osteoclast and induces osteoclastogenesis (232). Recently it has been found that EZH2, a transcriptional factor, play a significant role in bone metastasis. EZH2 promotes PTHrP expression *via* integrin β1 and the knockout of *EZH2* inhibit breast cancer-induced bone metastasis (233). However, RUNX2, a transcription factor, also participates in osteoclastogenesis. Phosphorylation of RUNX2 by integrin $\alpha_v\beta_3$/sma5 cascade or integrin $\alpha_v\beta_3$/src/rac1 cascade activates Akt pathway and leads to upregulate NF-kB expression, activation of RANKL, resulting in osteoclastogenesis (234–236).

This process induces bone resorption and secretion of growth factors from osteoclast cells. All the growth factors maintain the function of osteoblast and osteoclast, along with tumour proliferation. This process disturbs the homeostasis and lead to the formation of bone lesions and release the growth factors, which in turn promotes tumour growth and increase bone resorption. This feedback loop, named as "vicious cycle", amplified the metastatic lesion formation in bone and ultimately progressed towards bone fracture and hypercalcemia (237, 238).

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NK and PS wrote the review. All authors contributed to the article and approved the submitted version.

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