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Review Article

Mesenchymal lineage cells and their importance in B lymphocyte niches

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ABSTRACT

Early B lymphopoiesis occurs in the bone marrow and is reliant on interactions with numerous cell types in the bone marrow microenvironment, particularly those of the mesenchymal lineage. Each cellular niche that supports the distinct stages of B lymphopoiesis is unique. Different cell types and signaling molecules are important for the progressive stages of B lymphocyte differentiation. Cells expressing CXCL12 and IL-7 have long been recognized as having essential roles in facilitating progression through stages of B lymphopoiesis. Recently, a number of other factors that extrinsically mediate B lymphopoiesis (positively or negatively) have been identified. In addition, the use of transgenic mouse models to delete specific genes in mesenchymal lineage cells has further contributed to our understanding of how B lymphopoiesis is regulated in the bone marrow. This review will cover the current understanding of B lymphocyte niches in the bone marrow and key extrinsic molecules and signaling pathways involved in these niches, with a focus on how mesenchymal lineage cells regulate B lymphopoiesis.

Contents

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1. Introduction

Bone is a dynamic organ that is constantly being broken down and replenished in a process known as bone remodeling. Bone remodeling is carried out by two cell types on the bone surface; osteoclasts which remove bone via resorption and osteoblasts that subsequently replace bone by forming a new bone matrix [1]. Bone remodeling is influenced by many cell types within the bone marrow, most obviously including mature osteoblasts and osteoclasts, but also other cell types including matrix-embedded osteocytes, osteoblast progenitors, osteoclast precursors, macrophages, T lymphocytes [1,2] and B lymphocytes [3]. A large focus in bone biology is the coupling of osteoblasts and osteoclasts in regulating bone formation. However, osteoblasts have also been shown to be key regulators of other hematopoietic cells. Of interest in this review is the contribution of mesenchymal lineage cells, including osteoblasts, to the regulation of B lymphopoiesis in mice.

2. B lymphopoiesis

B lymphocytes, commonly called B cells, are antibody-producing white blood cells that primarily function as a part of the adaptive immune system. B lymphopoiesis is the process of mature B lymphocyte formation, which in sequential stages take part in the bone marrow and spleen in adult mice. The tightly regulated process of B lymphopoiesis is reliant on intrinsic and extrinsic stimuli, the latter predominantly being produced by non-hematopoietic microenvironmental cells.

There are multiple distinct types of B cells that have different origins in the immune system. The most primitive B cells (B-1 cells) primarily originate from fetal liver and are sustained through self-renewal in the periphery [4]. In comparison, conventional B-2 cells (the focus of this review) mature in the bone marrow and rely greatly on the bone marrow (BM) microenvironment [5–8].

Conventional B lymphopoiesis of B-2 cells begins in the bone marrow from hematopoietic stem cells (HSCs). HSCs are rare, multipotent cells that self-renew to produce more HSCs and have the capacity to differentiate to form all mature blood cells [9]. The fate of HSCs is regulated by cell intrinsic mechanisms (e.g. transcription factors and cell cycle regulators) and additionally influenced by extrinsic factors (e.g. cytokines, growth factors, cell-cell interactions and extracellular matrix components) produced in their microenvironments. With respect to lymphopoiesis, HSCs differentiate into common lymphoid progenitors (CLPs) capable of forming T and B lymphocyte lineages, natural killer cells, innate lymphoid cells and dendritic cells. B lymphocytes continue to develop in the bone marrow followed by the spleen.

B lymphopoiesis occurs through distinct stages of differentiation: in the bone marrow, CLPs commit to B-cell-biased lymphoid progenitors (BLPs), that form early B lymphocyte precursors known as pre-pro-B lymphocytes, followed by pro-B lymphocytes, pre-B lymphocytes and then immature B lymphocytes, these then migrate from the bone marrow and mature in the spleen [5]. In secondary lymphoid organs (spleen and lymph nodes) B cells can be activated, stimulating their differentiation into memory B cells and end-stage B lymphocytes known as plasma cells; plasma cells can then return to the bone marrow [5] (Fig. 1). In the bone marrow, early B lymphopoiesis is reliant on factors expressed by non-hematopoietic cells, with different cell types orchestrating progression through each stage of early B lymphopoiesis and the homing of terminally differentiated plasma cells back to the bone marrow. (See Fig. 2.)

The first specified B lymphocyte progenitor that is almost exclusively committed to the B lymphocyte lineage is the BLP, which are Ly6D + CLPs that express lower levels of c-kit compared to the CLPs that generate all lymphoid progenitors (ALPs) [10]. The transcription factor E2A has been shown to be essential to the generation of BLPs (and subsequently all B lymphocytes) from ALPs [10]. The expression of B220 identifies pre-pro-B cells that arise from BLPs [11,12] (Fig. 1). Progression to the pro-B lymphocyte stage is induced by expression of the transcription factor paired box protein 5 (PAX5), which causes irreversible commitment to the B cell lineage, and expression of CD19 [12,13]. As committed B cells develop, variable (V), diverse (D) and joining (J) gene segments of the immunoglobulin (Ig) heavy and light chain loci are shuffled to create functional B cell antigen receptors (BCRs) and enhance antibody diversity [14]. The first Ig loci recombination occurs at the pro-B cell stage where expression of recombination-activating genes (RAG1 and RAG2) initiates Ig heavy chain rearrangement [14]. In large pre-B lymphocytes the recombined Ig heavy chain pairs with a surrogate light chain to form the pre-B cell antigen receptor (pre-BCR) which is then trafficked to the cell surface [15]. Antigen-independent signaling through the pre-BCR is crucial for further development, stimulating downregulation of RAG1/2, inducing a proliferative burst allowing clonal expansion of large pre-B cells [14]. Progression to the small pre-B cell stage is marked by downregulation of the surrogate light chain, and upregulation of RAG1/2 to commence light-chain rearrangement. Assembly and expression of the recombined Ig light chain leads to pairing with the rearranged heavy chain and transport to the cell surface of immature B cells, forming the BCR complex with CD79a (Igα) and CD79B (Igβ). Immature B cells then go through central tolerance to ensure they are non-autoreactive [14]. Immature B cells with autoreactive BCRs can be negatively selected and undergo apoptosis or receptor editing. Positive selection occurs for immature B cells with unliganded BCRs by phosphoinositide 3-kinase (PI3K) signaling, allowing progression to a transitional B cell. Transitional B cells then migrate to the spleen but are sensitive to antigen-induced apoptosis during this phase [16].

3. Microenvironment regulation of hematopoiesis

Over the last two decades there has been increasing interest in the role of cells within hematopoietic microenvironments (also known as niches) in nurturing healthy [17–21] and malignant [22–25]

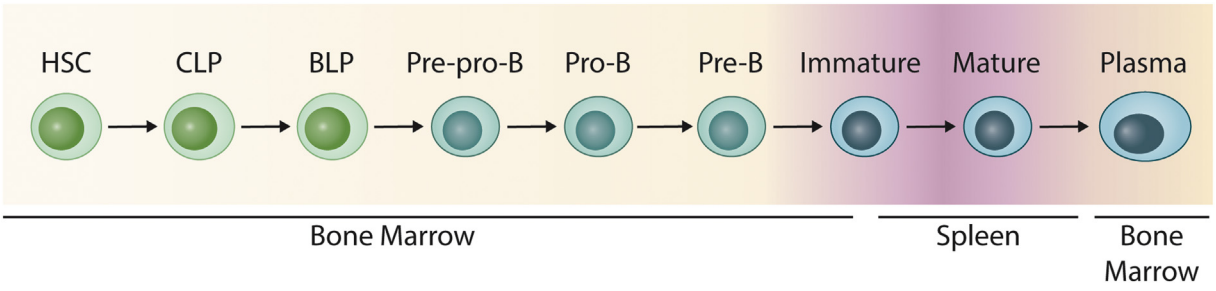


Fig. 1. Simplified schematic of B lymphopoiesis. Hematopoiesis arises in the bone marrow from hematopoietic stem cells (HSCs). In the lymphoid lineage, HSCs give rise to common lymphoid progenitors (CLPs) which form T lymphocytes, B lymphocytes, natural killer cells, innate lymphoid cells and dendritic cells. Expression of Ly6D on CLPs commits the CLP to the B-cell-biased lymphoid progenitor (BLP). B lymphocytes continue developing in the bone marrow through pre-pro-B, pro-B and pre-B lymphocyte stages, immature B lymphocytes then exit the bone marrow and mature in the spleen. End stage plasma cells can return to the bone marrow.

hematopoiesis and bone remodeling [1,2]. 'Microenvironments' or 'niches' are compartments that function to regulate the numbers and types of hematopoietic cells that are developing in the microenvironment by influencing their development into distinct hematopoietic cell lineages. Disruption to the function of microenvironment cells can have dire consequences for hematopoiesis and bone remodeling and, in turn, can cause serious health implications.

Hematopoietic niches are intricate and unique, operating to integrate the complicated processes involved in differentiated blood cell production to meet the organisms' needs. Different niches located throughout the bone marrow are responsible for regulating different stages of hematopoiesis. Exactly how these distinct niches are organized still requires further investigation, although numerous cell types known to play important roles have been identified. These include (but are not

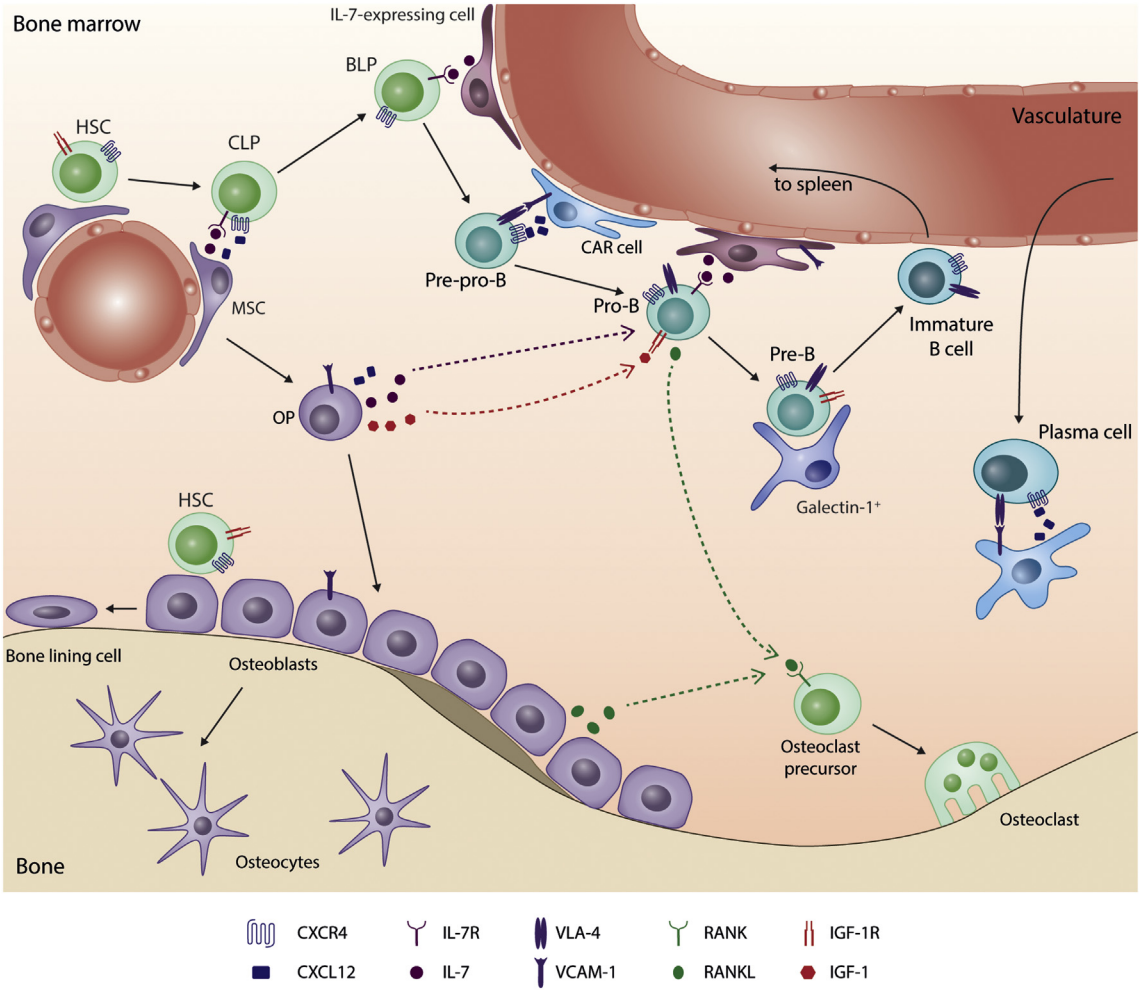


Fig. 2. The bone marrow is the site of early B lymphopoiesis. Early B lymphopoiesis occurs in the bone marrow. Hematopoietic stem cells (HSCs) differentiate to common lymphoid progenitors (CLPs) and B lymphoid-biased progenitors (BLPs) and then become the earliest committed B cell precursor, the pre-pro-B cell. Pre-pro B cells localize next to CXCL12-abundant reticular (CAR) cells, then as they differentiate into pro-B cells they localize adjacent to IL-7-expressing stromal cells. Pre-B cells localize near Galectin-1-expressing cells within the bone marrow. Immature B cells migrate from the bone marrow to mature at the spleen. End stage plasma cells then return to the bone marrow. Osteoblast lineage cells are known to play essential roles in regulating B lymphopoiesis; perivascular mesenchymal stem cells (MSCs) differentiate into osteoblast progenitors (OPs) and both of these cell types express factors that regulate B lymphopoiesis. B lymphocytes can in turn regulate bone by altering osteoclastogenesis via production of RANKL, which binds to RANK on the surface of osteoclast precursors. Mature B lymphocytes have also been shown to be a major source of OPG, thereby inhibiting osteoclastogenesis (not shown).

restricted to); hematopoietic cells (e.g. HSCs, B lymphocytes, T lymphocytes, dendritic cells, macrophages, megakaryocytes), mesenchymal stromal and perivascular cells (e.g. *Prrx1*$^{+}$ limb bud-derived mesenchymal cells [26], nestin-expressing perivascular cells [27,28] leptin receptor (LepR)-expressing mesenchymal stromal cells [29,30]), osterix (Osx)-derived osteoblast progenitor cells and osteoblasts [20,21,26], CXC-motif chemokine ligand 12 (CXCL12) abundant reticular (CAR) cells [31,32] and endothelial cells [29,30] (Table 1).

3.1. Transgenic mouse models as a tool for understanding the bone marrow microenvironment

The use of transgenic mice with specific promoter driven Cre-recombinase expression, targeting specific microenvironmental cells (Table 1) has greatly enhanced our understanding of the impact of these different cell types on distinct hematopoietic cell types. Such studies have utilized cell-specific deletion of genes such as *Cxcl12* and *Scf* to uncover which cell types are important in regulating hematopoiesis via these microenvironmental factors. While transgenic models are very useful, it is always important to consider their limitations. For instance, *Prrx1-Cre* targets limb-bud derived mesenchymal cells, hence it will target cells expressing *Prrx1* but also any cells that are derived from *Prrx1*-expressing cells, including osteoblasts, chondrocytes and adipocytes. Thus phenotypes could result due to alterations to the original cell targeted or any progeny of that cell, which could include alterations to numerous cell lineages when primitive cell types are targeted. In addition, *Prxx1-Cre* targets primitive cells in the limb bud mesenchyme, craniofacial mesenchyme and flank mesoderm [33]. Hence mesenchymal cells in the limbs, some calvarial bones and the sternum will be targeted, but not the vertebrae and thus any local effects will not be apparent in the spine.

Many of the Cre transgenic mice are also not entirely specific to the cell lineage they are often used to study. For example, *Dmp1-Cre* is used to target late osteoblasts and osteocytes but also targets skeletal muscle fibres, some cells in the brain and mesenchymal cells in the intestine and stomach [34]. While this may not have a direct impact on the bone microenvironment, changes to skeletal muscle could easily influence mechanical loading and thus have indirect effects on the bone, being a mechano-sensitive organ. *Col2α1-Cre* is used to study chondrogenic lineage cells but it has also been shown to target some osteoblastic cells. Seven-day-old *Col2α1-Cre:Rosa26LacZ* mice exhibited LacZ expression in synovial fibroblasts [35] and at embryonic day 16.5 LacZ was detected in the periosteum and primary spongiosa in the appendicular and axial skeleton [36]. Thus, despite the fact that neither the Col2α1 gene [35] nor Col2α1-Cre [36] are expressed in osteoblasts, these osteoblastic cells can arise from a cell that once expressed Col2α1-Cre. Conversely, cathepsin K- (Ctsk-) Cre is used to target osteoclasts, but also targets mesenchymal progenitors in the perichondrial Ranvier's groove [37]. Phenotypes attributed to osteoclast function might instead be due to alterations in chondrocytes or osteoblasts.

3.2. Cellular localization of B lymphocyte niches

The microenvironment in the bone marrow is known to be essential for commitment to B lymphopoiesis and the sequential stages of early B lymphopoiesis. From HSC to immature B lymphocytes ready to exit the bone marrow, each stage is regulated by unique supportive niches. How these niches are organized for the distinct stages of B lymphocyte development have not yet been completely defined. We focus here on the regulation of B lymphopoiesis from the CLP stage onwards.

CLPs have been shown to lose the potential to generate all lymphoid cell types when they express Ly6D and become BLPs, producing almost exclusively B cells [10]. After commitment to the B lymphocyte lineage, early B lymphocyte progenitors progress through distinct niches in the bone marrow. Studies using *Cxcl12:GFP* [56] knock-in mice have shown that pre-pro-B lymphocytes localize near CXCL12-abundant reticular (CAR) cells. These cells differentiate into pro-B lymphocytes and together with pre-B I cells [57,58], localize near Interleukin 7 (IL-7)-secreting cells [57,58]. The IL-7-producing cells have been described as being spindle-shaped reticular cells that usually reside in close contact with vessels and express VCAM-1 (CD106), PDGFRα, CD54 (ICAM-1), and BP-1 but not CD31 [57], suggesting these cells are of the mesenchymal lineage. While the nature of these reticular cells have not yet been determined, it is possible that they are *Osx-Cre*-targeted osteoblast progenitor cells, which have been shown to express IL-7 [59].

Pre-B II lymphocytes then migrate away from IL-7-expressing cells [57,58] to stromal cells that are scattered throughout the bone marrow. Studies in which pre-B II cells were injected into hydroxyurea-treated mice suggest that the pre-B II cells home to cells that are located throughout the bone marrow and express Galectin-1 (GAL1) [57]. Immature B lymphocytes localize near endothelial cells lining blood vessels [60,61] prior to these cells exiting the bone marrow into the circulation. Egress of immature B cells is partially reliant on expression of sphingosine 1 phosphate receptor 1 (S1PR1) expression by immature B cells and can be impaired with a small molecule inhibitor of S1PR1 (FTY720) [62] or deletion of S1PR1 from B lymphocytes using CD19-Cre [63] or *Mb1-Cre* [62], reviewed in detail by Lim et al. 2017 [64].

The migration of the developing B lymphocytes through the bone marrow to the distinct B lymphocyte niches relies on the expression of different chemokines (such as CXCL12) and adhesion molecules, which are required during different stages of B cell development and are discussed further below. A deregulation of these factors and other B lymphocyte regulatory factors, including cytokines, that are expressed by distinct B lymphocyte niche cells may result in changes in BM B lymphopoiesis. Such alterations may include either retention at the niche, or extrinsically-mediated alterations to genes that are required for B cell development. There are numerous excellent reviews focusing on the intrinsic regulators of B cell development, in particular the role of transcription factors that are essential for B lymphopoiesis [12,65,66]. Here we focus on known extrinsic regulators of B lymphopoiesis, with a particular focus on the roles of mesenchymal-derived cells in these processes.

4. The mesenchymal niches for B lymphopoiesis

Mesenchymal cells, including osteoblast lineage cells, are crucial regulators of the B lymphocyte lineage, influencing both commitment to the B lymphocyte lineage and the progression of the immature B cells through the early stages of B lymphopoiesis. The importance of osteoblast lineage cells in B lymphopoiesis has been made apparent by their ability to support all stages of differentiation from HSCs to immature B cells in vitro, and the impaired formation of B lymphocyte precursors when osteoblasts are depleted from the bone marrow in vivo. It is important to note that the term “osteoblast” has been used to describe a range of mesenchymal cell types that give rise to the osteoblast lineage [67]. Furthermore, as discussed above, the use of different mesenchymal cell lineage Cre strains does not necessarily identify the exact nature of the mesenchymal cell type involved in a given phenotype due to the potential targeting of downstream mesenchymal lineage cells in the Cre-targeted strains.

The production of macrophages, granulocytes, megakaryocytes and erythrocytes from HSCs can occur readily in vitro when permissive cytokines are provided, however, stromal cells are required as feeder layers for the optimal production of lymphoid cells in vitro. Whitlock et al. [68] were the first to demonstrate that B lymphocytes could be produced in cultures containing adherent bone marrow cells, these cultures are now commonly known as Whitlock-Witte cultures. A range of osteoblast lineage primary cells and cell lines such as S17 and OP9 cells have since been shown to support the production of B lymphocyte lineage cells in co-culture in vitro assays [69,70]. The OP9 stromal cell line is a cell line that was derived from the newborn calvaria of *op*/*op* mice, which

lack functional macrophage colony-stimulating factor-1 [(M-CSF, also known as colony-stimulating factor-1, CSF-1) [70,71]]. The OP9 cell line was originally developed to improve methods for the differentiation of embryonic stem cells into erythroid, myeloid and B lymphoid lineages in vitro [70]. The OP9 cell line (and a variant that overexpresses delta-like 1, OP9-DL1 [72]) have since become routinely used cell lines for in vitro assays of B and T lymphopoiesis, respectively, from ES cells, adult HSCs and more committed hematopoietic progenitor cells. While the exact nature of the OP9 cell line is unclear, it can readily give rise to adipocytes [73], chondrocytes [74] and osteoblasts [75] when cultured with the appropriate differentiation-inducing media. Furthermore, the immunophenotype of OP9 cells [75] is similar to that of the population derived from bone marrow that expresses PDGFRα and Sca-1 (PαS) cells and gives rise to osteoblasts, adipocytes and chondrocytes in culture [76]. While these cells are often described as mesenchymal stem cells, their potential to form other mesenchymal lineages has not been shown, hence they are best termed skeletal stem cells [67].

Furthermore, co-cultures of primary calvaria-derived osteoblasts or the stromal cell line S17 with hematopoietic stem and progenitor cells (HSPCs) supported the formation of $B220^+ CD19^+$ B lymphocyte precursors [69]. Zhu et al. [69], isolated HSPCs ($GFP^- Lin^- Sca\text{-}1^+ c\text{-}Kit^+$; LKS^+) from the bone marrow of RAG2 GFP NG BAC (B6) mice (which express GFP in committed early B lymphocyte precursors). When these LKS^+ cells were co-cultured with the primary osteoblast lineage cells, 15% of the progeny of the LKS^+ cells became GFP^+, indicating lymphocyte commitment. In comparison, no GFP^+ cells were produced when LKS^+ cells were co-cultured with the MS-1 endothelial cell line [69]. Stimulation of the osteoblast lineage cells with PTH for 3 days led to elevated levels of IL-7 and SDF-1 (CXCL12) expressed by the osteoblast lineage cells. Inhibition of IL-7, SDF-1 or thymic stromal cell-derived lymphopoietin (TSLP) during PTH stimulation reduced the number of $B220^+$ B lymphocytes. Furthermore, inhibition of integrin α4 or VCAM-1 prevented the formation of $B220^+$ cells. This indicates that osteoblast lineage cells support B lymphopoiesis through the secretion of IL-7, SCF-1 and TSLP and that VCAM-1/integrin α4 signaling is also important for osteoblastic-induction of B lymphopoiesis [69]. The calvarial-derived cells were shown to express Sca-1, CD61, ICAM-1 and VCAM-1 but lacked expression of endothelial and hematopoietic cell markers. These cells also expressed osteopontin in culture [69]. The expression of Sca-1 by these cells suggests that they are also akin to skeletal stem cells [26,41,76]. Hence, while these calvarial-derived cells are clearly of mesenchymal origin, the stage of osteoblast lineage maturation of the cells used in the cultures remains unclear.

Zhu et al., found that ablation of Col1a1–2.3 kb (Col2.3)-targeted osteoblasts in vivo using a thymidine kinase suicide gene in vivo depleted early B lymphocytes in the bone marrow [69]. When Col2.3-thymidine kinase mice were treated with ganciclovir, the thymidine kinase converted ganciclovir to an inhibitor of DNA polymerase, ganciclovir triphosphate, which is toxic to cells, killing those downstream of Col2.3. At 8 days after ganciclovir administration, osteocalcin-positive osteoblasts were shown to be depleted. This was accompanied by dramatic reductions in pre-pro-B (64%) and pro-B lymphocytes (81%) with modest reductions in pre-B and immature B lymphocytes [69]. In contrast, the numbers of LKS^+ cells (containing a mix of HSCs and multipotent progenitor cells) were unaltered at this time point (Table 2). The numbers of B lymphocyte precursors continued to decline 28 days after ganciclovir treatment, at which point LKS^+ cells were reduced and the hematopoietic cellularity in the bone marrow was dysregulated [69,77]. This indicates that osteoblasts directly regulate early B lymphopoiesis in the bone marrow, and that this occurs prior to the loss of HSPCs.

A similar study ablated osteoblast-lineage cells using an inducible diphtheria toxin receptor to induce cell death in Osx1-GFP::Cre-targeted cells following administration of diphtheria toxin [78]. This resulted in a reduction in small pre-B II lymphocytes and mature B lymphocytes in the bone marrow and an accumulation of pro-B cells and large pre-B II cells, with no changes to the more primitive B lymphoid-lineage cells. The pre-B lymphocyte defect could be rescued with administration of insulin-like growth factor 1 (IGF-1), whereas IL-7 administration increased the numbers of pro-B cells but had no effect on pre-B cells, implicating IGF-1 in the regulation of pre-B cells [78].

A range of different targeted knockout mice using mesenchymal lineage cell specific-Cre models have also been described to have defects in B lymphopoiesis, which are further described below.

4.1. Osteoclasts – direct or indirect regulators?

B lymphopoiesis can also be regulated by the activity of osteoclasts. Administration of the anti-resorptive agent, zoledronic acid, led to reduced production of CXCL12 and IL-7 from stromal cells and a reduction in early B lymphocyte precursors from the pro-B lymphocyte stage in mice [79]. The reduction in B lymphocytes was a consequence of reduced production of CXCL12 and IL-7 from the mesenchymal cells. Zoledronic acid did not have a direct effect on B lymphopoiesis or alter stromal cell production of *Cxcl12* or *Il7* in vitro [79]. This implicates osteoclast activation can indirectly regulate B lymphopoiesis through altering bone formation from osteoblast lineage cells.

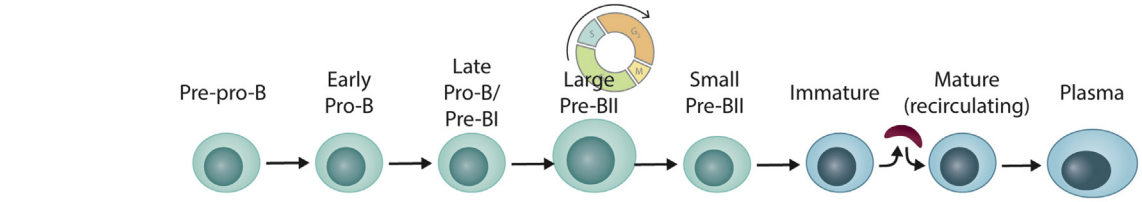
5. Extrinsic factors regulating B lymphopoiesis

Chemokines, adhesion molecules and various cytokines expressed by stromal cells in the microenvironment are essential for the regulation of early B lymphopoiesis as either positive or negative regulators. These factors are known to govern specific stages of B lymphocyte development and must be delivered to the appropriate B cell precursor at the appropriate stage of development to enable healthy B cell maturation. While it is recognized that these factors are essential for the development of B lymphocytes, how B lymphocyte niches are integrated still requires further investigation. For example, the distinct stages of B lymphopoiesis are known to involve different cell types [58], however the exact nature of each of the cell types, and whether or not each B lymphocyte niche consists of one or more microenvironment cell type currently remains unclear. Furthermore, the different functional roles of individual cell types within these niches have not been fully characterized. We describe below known B lymphopoiesis regulatory factors that have been shown to be expressed by mesenchymal lineage cells. Fig. 3 summarizes the effects of each of these on B lymphopoiesis.

5.1. Directly-acting, positive extrinsic factors

5.1.1. CXCL12

CXCL12 (also known as stromal cell-derived factor 1 or SDF1) is a chemokine produced by numerous microenvironment cells that is a key regulator of hematopoiesis. CXCL12 signals through its receptor, CXC-chemokine receptor 4 (CXCR4), which is expressed by a range of developing hematopoietic cell lineages [80]. CXCL12 is essential for regulating early B lymphopoiesis, at least in part by guiding the different B lymphoid progenitors to their correct niches [81], and also regulates quiescence and the maintenance of HSCs in the bone marrow [26]. In addition, CXCL12 is a key component involved in the homing of end stage B plasma cells to the bone marrow [26]. Early studies of $Sdf^{-/-}$ ($Cxcl12^{-/-}$) mice found that CXCL12 expression was essential for the development of pre-pro-B lymphocytes [82]. B lymphocytes express CXCR4 at different levels throughout differentiation, and this affects the chemotactic effect of CXCL12 [83]. This means that CXCL12-stimulated chemoattraction is strong in early B lymphopoiesis, is weakened in mature B lymphocytes as CXCR4 expression is suppressed and is then re-acquired by bone marrow-homing plasma cells [84]. The variation in CXCR4 expression is due to coupling of different signaling cascades following CXCR4 activation during B lymphopoiesis [84]. Disruptions to CXCR4-CXCL12 signaling, by use of a CXCL12 blocking



	Fraction	A	B	C	C′	D	E	F	
Surface antigens for subset identification	B220	+	+	+	+	+	+	++	
	IgM	-	-	-	-	-	+	+	
	CD19	-	+	+	+	+	+	+	
	CD43	+	+	+	+/low	-	-	-	
	CD24	-/low	+	+	++	+	+	-/low	
	BP1	-	-	+	+	+	-	-	
	IgD	-	-	-	-	-	-	+	
Receptors & adhesion molecules	CXCR4	+	+	+	+	+	-/+low	+	+
	IL-7R	+	+	+	+	-	-	-	
	Flt3	+	-	-	-	-	-	-	-
	cKIT	-	+	+	-	-	-	-	-*
	IGF-1R		+	+	+	+	+	+	+
	VLA-4	+	+	+	+	+	+	+	+
Extrinsic regulators	CXCL12	++	+	+	+	+			++
	IL-7		+	+	+	+			
	Flt3l	+							
	KITL (SCF)		+	+					
	RANKL					+	+		
	IGF-1					+	+	+	
	VCAM-1	+					+	+	+
	OSF-5				-	-	-		

Fig. 3. Identifiers of B lymphocyte subsets and extrinsic regulators of B lymphopoiesis. Expression of cell surface antigens allows for identification of B lymphocyte subsets in the bone marrow. The purple panel indicates expression of B220, IgM, CD19, CD43, CD24 (HSA), BP-1 (6C3) and IgD through differentiation from pre-pro-B lymphocytes to immature B lymphocytes and in recirculating mature B lymphocytes plus the corresponding fraction [93]. Expression as determined by flow cytometry is indicated as positive (+), positive-bright (++) or negative (−). The green panel indicates positive (+) and negative (−) expression of receptors and adhesion molecules involved in B lymphopoiesis. The blue panel indicates extrinsic factors expressed by stromal cells in the microenvironment that are important in the development of early B cell precursors and the specific stages they regulate in the bone marrow and are discussed in this review. Extrinsically produced factors can either positively (+ to ++) or negatively (−) regulate each stage of B lymphopoiesis as indicated. *cKIT (CD117) is upregulated on malignant plasma cells in some multiple myeloma patients [104].

antibody [85], or deletion of CXCR4 in *Cd19-Cre*-targeted B lymphocytes [86], impacted B lymphocyte motility in the bone marrow and led to excessive mobilization of B lymphocytes from the bone marrow.

The importance of microenvironment-produced CXCL12 in the initiation of B lymphopoiesis was recently demonstrated by Cordeiro Gomes et al. [81]. Deletion of the receptor for CXCL12, *Cxcr4* in *Flk2*Cre-targeted multipotent progenitor cells (MPPs) resulted in striking reductions in all blood cell lineages downstream of the MPPs. The impact on B lymphopoiesis was the most profound, with striking reductions in the early B lineage-committed Ly6D+ CLPs and all maturing B lymphocyte populations. Similar reductions in B lymphopoiesis were observed when *Cxcr4* was deleted in *Il7ra*-expressing CLPs. Intriguingly, however, when the *Cxcr4*-deficient CLPs were cultured on OP-9 stromal cell layers (which express CXCL12) and the culture media was supplemented with IL-7, the *Cxcr4*-deficient CLPs differentiated into B cells as efficiently as the wildtype CLPs [81]. Elegant imaging studies showed that loss of

Table 1
Transgenic Cre-recombinase strains used to target mesenchymal cells in the bone marrow microenvironment cells.

Cre	Cell type targeted in bone marrow microenvironment	Other targets outside the bone marrow	Ref
Prrx1	Limb bud-derived mesenchymal cells	None reported, but note that the vertebrae are not targeted by Prrx1	[33]
Nestin	Neural crest-derived perivascular mesenchymal cells	Yes, strain-dependent, reviewed in [17]	[38–40]
LepR	Perivascular mesenchymal stromal cells	Some brain cells, some hematopoietic cells	[41–43]
Gremlin1	Osteochondroreticular (OCR) stem cells	Intestinal reticular stem cells	[44]
Mx1	Osteolineage-restricted stem/progenitor cells and HSCs	Heart, kidney, liver	[45,46]
Osx	Osteoprogenitors, CAR cells, hypertrophic chondrocytes, adipocytes	Olfactory glomerular cells, some gastric and intestinal epithelial cells	[47–49]
Col1α1 2.3 kb (Mouse)	Osteoblasts	None reported	[50]
Col1α1 3.6 kb (Rat)	Osteoblasts	Tendon, fascia fibroblasts, some growth plate chondrocytes and some articular cartilage cells.	[51]
Col2α1	Chondrocytes, synovial fibroblasts, periosteum and primary spongiosa in the appendicular and axial skeleton	None reported	[35,36]
Tagln	Osteoblasts, majority of CAR cells and sinusoidal and arteriolar pericytes	Smooth muscle cells	[52,53]
Osteocalcin	Mature osteoblasts, most CAR cells and arteriolar pericytes		[54]
Dmp1 (9.6 kb)*	Late osteoblasts and osteocytes, subset of CAR cells	Skeletal muscle, brain, intestine, stomach	[34,52,55]

* The 8 kb Dmp1-Cre strain also targets some brain cells, reviewed in [17], which also discusses many of the strains listed above in more detail.

Table 2
Conditional models targeting cells in the bone marrow microenvironment and the effects on the B lymphocyte lineage.

Genetic model	HSPCs	Progenitors	B220+	Pre-pro-B	Pro-B	Pre-B	Ref
Cxcl12							
Prrx1-Cre^{+}*:Cxcl12* $^{\Delta/-}$	↓ HSCs (almost complete lack of quiescent HSCs), ↓ MPPs, ↓ repopulating activity	↓ CLPs, BLPs, CMPs	↓	↓	↓	↓	[26,29]
Nestin-Cre^{+}*:Cxcl12*$^{\Delta/\Delta}$	normal[a,b]	normal		normal	normal	normal	[29]
LepR-Cre^{+}*:Cxcl12*$^{\Delta/\Delta}$	↑ mobilization	normal		normal	normal	normal	[29]
Osx1-GFP::Cre^{+}*:Cxcl12* $^{\Delta/-}$	Normal HSC function, constitutive mobilization	normal	↓	↓	ND	ND	[26]
Col1a1(2.3 kb) -Cre^{+}*:Cxcl12* $^{\Delta/\Delta}$	normal[a,b,c], ↓ MPPs	↓ CLPs		normal	normal	normal	[29]
Osteocalcin-Cre^{+}*:Cxcl12*$^{\Delta/-}$	normal[a,b]	normal	normal	normal	ND	ND	[26]
Tie2-Cre^{+}*:Cxcl12*$^{\Delta/\Delta}$	↓ HSCs, ↓ long-term repopulating activity	normal		normal	normal	normal	[26,29]
Il7-Cre:Cxcl12 $^{\Delta/\Delta}$	↓ HSCs, ↓ MPPs	↓ CLPs, ↓ BLPs		ND	normal	↓	[81]
Il7							
Prrx1-Cre^{+}*:Il7*$^{\Delta/-}$	normal[a]	Normal CLPs, BLPs not assessed	ND	ND	↓	↓	[81]
LepR-Cre^{+}*:Il7*$^{\Delta/-}$	normal[a]	↓ BLPs	ND	ND	↓	↓	[81]
Col1a1(2.3 kb) -Cre^{+}*:Il7* $^{\Delta/-}$	normal[a]	normal	ND	ND	normal	normal	[81]
Tie2-Cre^{+}*:Il7* $^{\Delta/-}$		normal	ND	ND	↓	↓	[81]
Scf (Kitl)							
Il7-Cre:Scf $^{\Delta/\Delta}$	↓ HSCs, ↓ MPPs		ND	ND	normal	normal	[81]
Nestin-Cre^{+}*:Scf*$^{\Delta/-}$	normal[a,b]	ND	normal	ND	ND	ND	[30]
Nestin-CreER^{+}*:Scf*$^{\Delta/\Delta}$ + tamoxifen	normal[a,b]	ND	ND	ND	ND	ND	[30]
LepR-Cre^{+}*:Scf*$^{\Delta/-}$	↓ HSPCs	ND	ND	ND	ND	ND	[30]
Col1a1(2.3 kb) -Cre^{+}*:Scf* $^{\Delta/-}$	normal[a,b]	ND	normal	ND	ND	ND	[30]
Tie2-Cre^{+}*:Scf*$^{\Delta/-}$	↓ HSPCs, ↓ long-term repopulating activity	ND	ND	ND	ND	ND	[30]
Gsα							
Osx1-GFP::Cre^{+}*:Gsα*$^{\Delta/\Delta}$	ND	ND	↓	normal	↓	↓	[59]
Dmp1-Cre^{+}*:Gsα*$^{\Delta/\Delta}$	normal[a]	normal	normal	ND	ND	ND	[87]
Pth1r							
Col1a1(2.3 kb)-Cre^{+}*:Pth1r* $^{tg/tg}$ (constitutively active)	↑HSPCs	ND	ND	ND	ND	ND	[20]
Osx1-GFP::Cre^{+}*:Pth1r*$^{\Delta/\Delta}$	ND	ND	↓	normal	↓	↓	[7]
OC-GFP::Cre^{+}*:Pth1r*$^{\Delta/\Delta}$	ND	ND	↓	normal	normal[d]	normal[d]	[7]
Dmp1-GFP::Cre^{+}*:Pth1r*$^{\Delta/\Delta}$	ND	ND	↓	normal	normal	normal	[7]
Rarg							
Nestin-Cre^{+}*:Rarg*$^{\Delta/\Delta}$	normal[a,b]	normal	↓	normal	normal	↓	[28]
Osx1-GFP::Cre^{+}*:Rarg*$^{\Delta/\Delta}$	normal[a,b]	normal	normal	normal	normal	normal	[28]
IGF-1							
Osx1-GFP::Cre:Igf1$^{\Delta/\Delta}$	ND	ND		normal	↑	↓	[78]
Wntless							
Nestin-Cre^{+}*:Wls*$^{\Delta/\Delta}$	normal[a]	normal	↓	ND	↓	↓	[88]
Col1a1(2.3 kb)-Cre^{+}*:Wls*$^{\Delta/\Delta}$	normal[a]	normal	↓	ND	↓	↓	[88]
Osteoblast ablation							
Osx1-GFP::Cre:iDTR + diphtheria toxin	normal[a,b]	↑ GMP		normal	↑[e]	↓	[78]
Col1a1(2.3 kb)-Cre^{+}*: ΔTK + ganciclovir*	↓ HSPCs at day 21	ND	↓ day 8	↓ day 8	↓ day 8	↓ day 8	[69]

Recombined floxed allele (Δ), null allele (−), transgenic allele (tg). Significant changes compared to appropriate controls are identified by: elevated function or numbers (↑), lower function or numbers (↓), normal or not determined (ND). Hematopoietic stem cell (HSC), multipotent progenitor (MPP), B lymphoid-biased progenitors (BLPs), common lymphoid progenitor (CLP), lymphoid-primed multipotent progenitor (LMPP), inducible diptheria toxin receptor (iDTR) and thymidine kinase (TK).

[a] Normal HSC frequency.
[b] Normal repopulating activity.
[c] Data were based on FACS-isolated HSC transplants, however transplants using whole bone marrow cells showed significantly impaired lymphoid repopulation.
[d] Pro-B and pre-B frequencies were transiently increased in BM at 3 weeks, and normal by 12 weeks of age.
[e] Fraction B was normal and Fraction C′ and C″ were increased.

Cxcr4 in the MPPs resulted in defective CLP localization to IL-7-expressing microenvironment cells [81].

5.1.1.1. B lymphocyte phenotypes reported in different mesenchymal-specific Cxcl12 knockout mice. The role of CXCL12 in the microenvironment has recently been studied extensively by a number of investigations that involved deleting *Cxcl12* in different cell types using transgenic Cre-recombinase (Cre) mouse strains (Table 2). Deletion of *Cxcl12* in *Prrx1*-targeted limb bud-derived mesenchymal stromal cells led to a substantial reduction in pre-pro-B lymphocytes, CLPs and HSPCs, with an almost complete loss of quiescent HSCs and a loss of HSC repopulating activity [26]. When *Cxcl12* was deleted in *Osx*-targeted osteoprogenitors HSC function was normal, however the mice exhibited constitutive HSPC mobilization and reduced numbers of B lymphoid progenitors [26]. Col1α1–2.3 kb (*Col2.3*)-*Cre* deletion of *Cxcl12* had no effect on B lymphocytes but the mice exhibited reduced numbers of CLPs [29]. In comparison, deletion in *Tie2*-targeted endothelial cells and *LepR*-targeted stromal cells had no effects on B lymphopoieisis [29]. Interestingly, however, reduced HSPC numbers [29] and repopulating activity [26] was observed when *Cxcl12* was deleted in endothelial cells and HSC mobilization occurred when *Cxcl12* was deleted in *LepR*-targeted cells [29]. Deletion of *Cxcl12* in *Nestin*-targeted perivascular cells [29] and in *Osteocalcin*-targeted mineralising osteoblasts [26] did not alter HSPCs or B lymphopoiesis. Thus expression of *Cxcl12* in cells expressing *Prrx1* or *Osx* and their progeny have important regulatory functions in B lymphopoiesis through the action of CXCL12.

These studies support the theory for the existence of distinct niches regulating hematopoiesis in different ways. *Osx*-targeted cells provide a

supportive niche for B lymphoid progenitors and also maintain HSPCs in the bone marrow. In contrast, CXCL12 produced from perivascular mesenchymal cells and endothelial cells appear to be more important in the regulation of HSC number and function. The hematopoietic phenotypes of these mice, and that of other genetically modified mice discussed in this review, are summarized in Table 2.

As mentioned above, it is important to consider that in these models, CXCL12 is not only deleted from the cell in which the Cre is expressed but also all of their progeny. Thus deletion of *Cxcl12* using *Prrx1-Cre* will delete *Cxcl12* from more microenvironmental cell types compared to when deletion is restricted to cells in the late stages of differentiation, e.g. *Osteocalcin-Cre*. Thus the presence of more profound phenotypes when deleted in more primitive cells, such as *Prrx1*-targeted cells, could be due to the lack of CXCL12 in larger numbers of microenvironmental cell types rather than the phenotype that would occur if the loss of CXCL12 was restricted only to cells that express Prrx1. Furthermore, there are often direct and indirect consequences of deletion of genes in a given cell type, hence this may also indirectly influence B lymphopoiesis in any of these mice.

5.1.2. IL-7

IL-7 plays an essential role in B lymphopoiesis [89] and influences osteoclastogenesis and hence bone mass [90]. IL-7 signals through the IL-7 receptor (IL-7R), which is expressed by B lymphocyte precursor populations until the large pre-B cell stage, with subsequent B cell populations lacking expression of the IL-7R [91,92]. IL-7 has been shown to induce the proliferation of pro-B cells, but not pre-pro-B cells, in vitro [93]. In accordance with this, deletion of either IL-7 or IL-7R led to reductions in pro-B and pre-B lymphocytes with no change to pre-pro-B lymphocytes [92,94,95]. Female $Il7^{-/-}$ mice also exhibited lower trabecular bone volume and increased osteoclastogenesis [90]. The bone and B cell phenotypes in $Il7^{-/-}$ mice can be rescued by overexpression of IL-7 in osteoblasts [96].

The mechanism of action of IL-7 in B lymphopoiesis is discussed in detail in a review by Clark et al. [89] and it functions to promote the proliferation and survival of B cell precursors [97]. This occurs through the activation of JAK/STAT5 signaling stimulating expression of Cyclin D3 and proliferation of pro-B and pre-B cells [98,99], and expression of pro-survival genes including myeloid-cell leukemia sequence 1 (MCL1) [100], B cell lymphoma 2 (BCL2) and BCLXL [89]. The pro-survival and proliferation effects of pro-B cells are positively reinforced by early B cell factor 1 (EBF1) through further promotion of pro-survival genes and activation of IL-7R [101,102].

Elegant lineage tracing studies where *Il7*-Cre transgenic mice were crossed to $Rosa26^{EYFP}$ mice demonstrated that the IL-7-expressing cells in the bone marrow were predominantly bipotent CAR cells that expressed LepR and could give rise to both adipocytes and osteoblasts in vivo [81]. Deletion of *Cxcl12* in *Il7*-Cre-targeted cells resulted in a significant loss in HSCs, CLPs, and developing B cells in the bone marrow. Interestingly, the numbers of these cells were normal in the spleen, hence increased mobilization to the spleen was an unlikely cause of the reduced bone marrow cells [81].

5.1.2.1. IL-7-dependent B lymphocyte phenotypes that have been reported in different mesenchymal cell-specific knockout mice. IL-7 production by osteoblastic cells has been shown to be important for B lymphopoiesis via the regulatory actions of Gsα, a downstream mediator of PTHR1 signaling. Deletion of Gsα in *Osx-Cre*-targeted osteoblast progenitors leads to severe osteoporosis and reduced B lymphocyte precursors in the bone marrow [59,103]. *Osx-GFP::CreGsα*$^{fl/fl}$ (Gsa^{OsxKO}) mice exhibited significantly reduced proportions of pro-B and pre-B lymphocytes, with no change to pre-pro-B lymphocytes in their bone marrow [59]. The Gsα-deficient osteoblast lineage cells expressed significantly reduced levels of IL-7. Administration of IL-7 into Gsa^{OsxKO} mice or transplant of Gsa^{OsxKO} bone marrow into wild type mice was able to rescue the B lymphocyte defects [59]. Thus IL-7 produced by *Osx*-targeted cells regulates pro-B and pre-B lymphopoiesis.

In their recent study, Cordeiro Gomes et al. [81] deleted *Il7* in a range of mesenchymal cell types and determined the subsequent impact on B lymphopoiesis. Deletion of *Il7* in *Lepr*-Cre-targeted cells resulted in a significant reduction in BLPs in the mice. This was accompanied by significant reductions in all downstream B lymphocyte populations, including significantly reduced peripheral blood B lymphocytes. The numbers of pro—B, pre-B and B220 + IgM + B lymphocytes in the bone marrow were also significantly reduced in mice lacking *Il7* in *Prrx1*Cre-targeted cells. In contrast, when *Il7* was deleted in mature osteoblasts using *Col2.3*Cre, no hematopoietic phenotype was observed. Interestingly, when *Il7* was deleted in *Tie2*-Cre-targeted cells (which deletes in endothelial and hematopoietic cells [17]), there were small, but significant reductions in the numbers of pro-B and pre-B cells in the bone marrow.

5.1.3. FLT3L

FMS-like tyrosine kinase 3 ligand (FLT3L) is a growth factor that binds to FLT3 (also known as CD135), which is expressed on the cell surface of lymphoid-primed multipotent progenitor cells, CLPs and pre-pro B lymphocytes. It is important for early B lymphocyte commitment [5], and acts synergistically with IL-7 to promote B lymphopoiesis [105,106]. FLT3L has been shown to be produced by a range of hematopoietic cell types [107], but is also produced by bone marrow stromal cells. The nature of the latter cell type(s) is unclear.

Mice deficient in FLT3 or FLT3L exhibited reduced numbers of pre-pro-B and pro-B lymphocytes in their bone marrow [108,109]. Furthermore, mice deficient in FLT3L also had a marked reduction in bone marrow pre-B lymphocytes [109]. FLT3 is expressed by pre-pro-B lymphocytes until their irreversible commitment to the B lymphocyte lineage, when it is repressed by the expression of Pax5 [110]. Thus FLT3L is an important regulator of the earliest stages of B lymphopoiesis.

5.1.4. SCF

Stem cell factor (SCF, also known as KIT ligand; KITL) is a cytokine that signals though the c-kit receptor (also known as CD117), which is a receptor tyrosine kinase that is expressed on the cell surface of many developing hematopoietic cell types, including HSCs. SCF is primarily expressed by perivascular cells and exists as membrane-bound or secreted forms [5,30,91]. SCF synergizes with IL-7 in vitro to induce the proliferation of pro-B cells [111]. Furthermore, loss of *Scf* in *Il7-Cre* transgenic mice resulted in a significant reduction in HSCs and MPPs in the bone marrow of the mice [81].

Neonatal mice deficient in c-kit (termed *W*/W) do not display any B lymphocyte defects, however *W*/*W* mice die within 1 week of birth due to anemia [112]. When the anemic phenotype was rescued by administration of erythropoietin, it was found that pro-B and pre-B lymphocyte numbers decreased with age in these mice [113]. This indicates that SCF is important for B lymphopoiesis in adults.

A range of mice in which *Scf* was deleted in microenvironment-specific cell types have recently been reported [30] (Table 2). These studies focused on the HSC phenotypes of the mice, however, and to date only the B lymphopoiesis phenotypes have been reported for the mice in which *Scf* was deleted in Nestin-expressing or Col2.3-expressing cells. Both mouse strains had normal numbers of B220 + cells in their bone marrow [30], Table 2.

5.1.5. IGF-1

IGF-1 is expressed by Osx1-GFP::Cre-targeted cells and binds to the IGF-1 receptor (IGF-1R) on B lymphocytes to promote transition from the pro-B to the pre-B lympohocyte stage [78]. *Igf1* levels were reduced in the bones of *Osx1-GFP::Cre:iDTR* mice after administration of diphtheria toxin to ablate *Osx1-Cre*-targeted cells and these mice exhibited defective B lymphopoiesis [78]. Mice with targeted deletion of IGF-1 using *Osx1-GFP::Cre* exhibited reduced numbers of pre—B, immature and

mature B lymphocytes in the bone marrow, but increases in cells in fractions B, C, C′ and C″ identifying pro-B and earlier pre-B lymphocytes [78,114]. This indicates that IGF-1 derived from *Osx1-Cre*-targeted cells is important in regulating the later stages of B lymphopoiesis within the bone marrow from fraction D small pre-BII cells.

5.1.6. VCAM-1

Integrins are transmembrane receptors that mediate cell-cell and cell-matrix adhesion. In particular integrin α4, which heterodimerizes with β1 and β7 to form VLA4 or LPAM1, respectively, is required for the normal development of bone marrow B cell precursors [115]. Together with VCAM-1, VLA-4 mediates the adhesion of human B cell precursors to adherent bone marrow cells [116]. VCAM-1 is expressed on osteoblast lineage cells [69,117], and is essential for the support of B lymphocyte differentiation on cultured osteoblasts [69]. VCAM-1 is also expressed on endothelial cells [114,118], and conditional ablation of VCAM-1 in hematopoietic and some microenvironment cells, including endothelial cells and stromal cells targeted by *Mx1*Cre, resulted in the mobilization of immature B cells from bone marrow into peripheral blood [119–121]. VLA-4/VCAM-1-mediated adhesion of B cell progenitors in the bone marrow is regulated by CXCL12/CXCR4 signaling via activation of focal adhesion kinase [58,122,123]. VCAM-1 expression on non-hematopoietic stromal cells is also regulated by canonical Wnt signaling [124] and inflammation [125]. Not surprisingly, the interaction of myeloma cells with stromal cells is also mediated by VCAM-1 [126,127].

5.1.7. Galectin-1

Galectin-1 also binds to integrins (including $\alpha_4\beta_1$, $\alpha_5\beta_7$ and $\alpha_4\beta_7$) that are expressed on the cell surface of a range of cell types [128]. Developing B lymphocytes express $\alpha_4\beta_1$ (VLA-4), which also binds to other cell adhesion molecules including VCAM-1, as discussed above.

Galectin-1 was shown to be expressed in mature osteoblasts in addition to cells located throughout the bone marrow [57]. The Galectin-1$^+$ cells obtained from flushed bone marrow were shown to express CD54$^+$ but did not express VCAM-1, PDGFRα, BP-1, Sca-1 or Nestin. Interestingly, most Galectin-1$^+$ cells obtained from flushed bone marrow were CD31$^+$, but did not express CD34 or Tie2, suggesting that they were a subset of endothelial cells. In IL-7-GFP reporter mice, Galectin-1 was not co-expressed with GFP, indicating that IL-7-expressing cells are a separate cell type to Galectin-1-expressing cells [57].

Galectin-1-deficient mice had normal numbers of the different B lymphocyte populations in vivo in the steady state. In contrast, however, the recovery of pre-B II cells was significantly delayed in *Gal1*$^{-/-}$ mice after treatment with hydroxyurea [128].

5.2. Indirect, positive extrinsic factors

5.2.1. RANKL

Receptor activator of nuclear factor kappa-B ligand [RANKL; also known as osteoprotegrin ligand (OPGL), encoded by the *Tnfsf11* gene] is a protein with important functions in osteoclastogenesis and B lymphopoiesis. RANKL signals through RANK on osteoclasts, and together with macrophage colony stimulating factor (M-CSF), stimulates the expression of osteoclastic transcription factors, including nuclear factors of activated T cells c1 (NFATc1), microphthalmia associated transcription factor (MITF), PU.1 and activator protein 1 (AP-1) [129,130]. This induces myeloid precursors to differentiate into mononucleated osteoclasts, which form multinucleated osteoclast by fusing and finally activate becoming polarized osteoclasts that resorb bone [129,130].

RANKL is produced by a range of osteoblast lineage cell types [131], in addition to B lymphocytes [132]. Mice deficient in RANKL (*opgl*$^{-/-}$) exhibited severe osteopetrosis due to impaired osteoclastogenesis and had reduced numbers of B lymphocyte precursors [133]. When immunodeficient recombination-activating gene 1 null (*Rag1*$^{-/-}$) mice (which lack mature B and T cells) were reconstituted with *opgl*$^{-/-}$ fetal liver cells (the predominant site of hematopoiesis in embryos), there was a reduction in pre-B lymphocyte and immature B lymphocytes in the recipients compared to mice transplanted with *opgl*$^{+/+}$ bone marrow [133]. Interestingly, when *opgl*$^{-/-}$ mice were transplanted with wild type bone marrow, the mice exhibited normal B cell development. This suggests that loss of RANKL in non-hematopoietic microenvironment cells was not sufficient to cause the defects in B lymphopoiesis.

Similarly, when *Rank* was deleted in mice, severe osteopetrosis was accompanied by significantly reduced bone marrow hematopoiesis (<10% of normal) [134]. The spleens of *Rank*$^{-/-}$ mice were enlarged, with the spleen-to-body weight being twice the size of wildtype littermates. Despite this, there was a 50% reduction in B cells in the *Rank*$^{-/-}$ spleens, whereas all other hematopoietic cell lineages were normal (T cells, macrophages) or elevated (granulocytes, erythrocytes). Note, however, that *mb1*-Cre deletion of *Rank* in B lymphocytes from the pro-B cell stage onwards did not result in any defect in B lymphopoiesis in the conditional knockout mice, suggesting that any effect of perturbing RANKL/RANK signaling on B lymphopoiesis is indirect [135]. Given that both the *opgl*$^{-/-}$ and *Rank*$^{-/-}$ mice had significant osteopetrotic phenotypes, it is likely that the subsequent changes in the bone marrow microenvironment contributed to the B lymphocyte defects in these mice.

Mice deficient in the decoy receptor for RANK, OPG, also exhibit B cell defects. *Opg*$^{-/-}$ mice have enhanced osteoclastogenesis leading to severe osteoporosis [136–138] and elevated numbers of pro-B lymphocytes in the bone marrow and mature B cells in the spleen and lymph nodes [138]. *Opg*$^{-/-}$ pro-B cells also exhibited enhanced proliferation when stimulated with IL-7 compared to *Opg*$^{+/+}$ pro-B cells [138]. The cell type(s) responsible for the OPG-stimulated changes to B lymphopoiesis are currently unclear.

5.3. Negative extrinsic regulators

5.3.1. OSF-5

It is evident that there are many different positive extrinsic regulators of B lymphopoiesis that are derived from mesenchymal lineage cells (summarized above and in Fig. 3). Less is known about the negative extrinsic regulators of B lymphopoiesis. A recent study showed that osteoblast stimulating factor 5 (OSF-5) was highly expressed by a range of mesenchymal stromal cell lines including OP9 and MS5 cells. OSF-5 was also expressed by some maturing primary hematopoietic cells, but not HSCs, immature progenitors or developing B lymphocyte populations [139]. Transgenic mice overexpressing OSF-5 under the control of the endogenous immunoglobulin κ light chain had significant reductions in the numbers of pre-B and immature B cells in their bone marrow [139]. This was shown to be due to the secreted form of OSF-5.

When OSF-5 was knocked down in OP9 cells (OP9-KD), significantly increased numbers of pre-B cells were produced in co-cultures of the OP9-KD cells with either 2E8 pre-B cells or murine primary bone marrow cells compared to OP9 co-cultures with these cell types. Supernatant obtained from the OP-KD cells enhanced the numbers of pre-B colonies formed in vitro, whereas supernatant collected from OSF-5-overexpressing 293T cells inhibited the numbers of pre-B colonies in the same assay system. OSF-5 was shown to inhibit the proliferation of the pre-B cells, an effect that was also observed for human pre-B cells [139]. It was not clear how OSF-5 inhibited pre-B cell proliferation, but it appeared to be independent of IL-7Rα expression on the pre-B cells [139].

Each of these factors produced extrinsically to B lymphocytes is important for healthy B lymphocyte production. Yet how each niche is organized to regulate distinct functions and the specific cell types involved in each process requires further investigation. Further insights have been gained by understanding the influence on B lymphopoiesis of signaling pathways of importance for regulating bone. Given that the majority of investigators who study bone biology do not examine hematopoiesis (and vice versa), it is likely that there are numerous

mouse models that will have bone and hematopoietic (including B lymphocyte) defects that have not yet been identified. We discuss below a few such examples.

6. Signaling pathways involved in microenvironment regulation of B lymphopoiesis by mesenchymal cells

6.1. PTH

Signaling through the parathyroid hormone (PTH) receptor (PTH1R) in bone is crucial for skeletal development and mineral homeostasis, and intermittent activation of PTH1R with either recombinant PTH [1–34] (teriparatide) or modified recombinant PTH-related peptide (PTHrP [1–34], abaloparatide) are now FDA-approved treatments for osteoporosis [140,141]. PTH has significant effects on the hematopoietic niche via its actions on bone in mice and humans [20,142,143]. The PTH1R is a G protein-coupled receptor, and the G stimulatory protein G_s is a major mediator of the anabolic effects of PTH in bone [103,144]. Deletion of either the $G_s\alpha$ subunit or PTH1R in osteoblast progenitors results in severe osteoporosis with normal pre-pro-B cell numbers but loss of pro-B and pre-B cell precursors, due at least in part to decreased expression of IL-7 by osteoblast progenitors; B cell precursor numbers were rescued by exogenous IL-7 administration [7,59]. Of note, removal of PTH1R from more mature osteoblasts and osteocytes did not affect bone marrow B cell precursor populations, pointing to a crucial role for osteoprogenitors [7]. In PTH1R conditional knockout mice, an unexpected accumulation of IgM^+/IgD^+ and IgD^+ B cells in the bone marrow was seen due to aberrant retention mediated by increased expression of VCAM-1 on both osteoblast lineage and hematopoietic cells [7]. Treatment with an anti-VCAM-1 neutralizing antibody was able to mobilize immature B lymphocytes into the periphery. Whether VCAM-1 is a direct target of PTH1R signaling is unknown, but both decreased canonical Wnt signaling and increased inflammation seen in PTH1R conditional deficient mice could have contributed to the increased VCAM-1 expression [7]. Conversely, intermittent PTH decreases *Vcam1* mRNA levels in osteoblasts [145].

6.2. Wnt signaling

Canonical Wnt signaling is an important determinant of bone mass and sclerostin, an inhibitor of Wnt signaling, is a therapeutic target for the treatment of osteoporosis [146,147]. Mice deficient in sclerostin exhibited a depletion of B cells in their bone marrow, with increased apoptosis at all stages due to an impaired microenvironment [148]. The actions of Wnt ligands on B cell precursors and non-hematopoietic stromal cells have not been fully delineated in the bone marrow. However, Wnt3a inhibited the differentiation of human B progenitor cells co-cultured with stromal cells, an effect that could be reversed by the Wnt antagonists, secreted frizzled protein 1 (sFRP1) or Dikkopf1 (Dkk1) [149], and transduction of OP9 stromal cells with Wnt3a blocked B lymphopoiesis from HSPCs in co-cultures [150]. Dkk1 transgenic mice have reduced trabecular bone volume accompanied by reduced numbers of osteoblasts [151]. They also have increased numbers of CLPs, however the numbers of all other B lymphocyte populations were reported to be normal [152].

Reducing the secretion of various Wnt ligands by deletion of *Wntless* (*Wls*) has also been shown to affect bone and B lymphopoiesis. Deletion of *Wls* in *Col1 (3.6 kb)*-Cre-targeted osteoblasts (*Col1*-Cre;$Wls^{c/c}$) resulted in postnatal lethality of the mice by two weeks of age [153]. The mice were markedly smaller than their $Wls^{c/c}$ controls and exhibited severe osteopenia, with significant reductions in trabecular bone and cortical bone, and reduced numbers of osteoblasts per trabecular bone area. An impairment in osteoblast proliferation and increased apoptosis were attributed to the reduced osteoblast numbers in these mice [153]. Due to the postnatal lethality, hematopoiesis was assessed at P7 in these mice. The *Col1*-Cre;$Wls^{c/c}$ mice had significant reductions in the numbers of pro—B, pre-B and immature B lymphocytes in their bone marrow, but normal numbers of CLPs (data for pre-pro-B cells were not provided) [88]. The B lymphopenia was accompanied by significant reductions in *Il7* expression in calvarial osteoblasts. Furthermore, there were significantly reduced levels of IL-7 and Wnt5a in extracellular fluid obtained from flushed bone marrow from *Col1*-Cre;$Wls^{c/c}$ mice compared to $Wls^{c/c}$ controls [88]. The mice exhibited other hematopoietic defects (reduced thymi, increased numbers of bone marrow näive T cells and granulocytes, but normal numbers of HSCs) [88].

Deletion of *Wls* in *Nestin*-Cre-targeted cells also resulted in mice that were significantly smaller than the $Wls^{c/c}$ controls, however the bone phenotype was not thoroughly assessed in this study [88]. The *Nestin*-Cre;$Wls^{c/c}$ mice had a slightly longer lifespan (18 days postnatal), and had similar reductions in the numbers of pro—B, pre-B and immature B cells in their bone marrow, however the numbers of CLPs were not significantly altered. A more profound reduction in the thymus size was observed in *Nestin*-Cre;$Wls^{c/c}$ mice, and, similar to the *Col1*-Cre;$Wls^{c/c}$ mice, there were increased numbers of näive T cells in their bone marrow.

6.3. Estrogen

Estrogen deficiency is associated with significant bone loss in peri- and postmenopausal women [154]. Women are more susceptible to autoimmune diseases, the incidence of which declines after menopause, suggesting a role of estrogen on lymphocyte function [155]. Several studies have demonstrated decreased numbers of B lymphocytes in postmenopausal women [156–158]. However, whether these effects are due to the loss of estrogen action directly on B lymphocytes or indirectly via osteoblasts and osteoclasts remains unknown. In mice, estrogen deficiency is associated with a transient increase in B lymphopoiesis that is reversed by estrogen treatment [159,160], and may be mediated by the expression of RANKL in osteocytes [161]. Long-standing estrogen deficiency and the resultant osteoporosis may also decrease the B lymphocyte support by osteoblasts [162].

6.4. Retinoic acid receptors

Retinoic acid receptors (RARs) are expressed by many cells in the bone marrow microenvironment, and regulate hematopoiesis (including B lymphopoiesis) [28,163–166] and bone remodeling [167–171] through direct and indirect means. Canonical RAR signaling occurs through nuclear RARs of which there are three subtypes; α, β and γ [168]. RARs form heterodimers with retinoid X receptors (RXRs) and associate with retinoic acid response elements (RAREs) where they regulate gene transcription. The effects of retinoids on hematopoiesis depends on the cell types that are exposed and the RAR subtype targeted.

The role of RARs in microenvironment regulation of hematopoiesis has been studied using global and conditional deletion of RARs in mice. $Rara^{-/-}$ mice do not exhibit defects in bone [169] or hematopoiesis [165,172]. $Rarb^{-/-}$ and $Rarb2^{-/-}$ mice appear normal and have no reported skeletal abnormalities [173,174], although these have not been extensively investigated in adult mice, nor have the hematopoietic phenotypes of these mice been reported. In comparison, $Rarg^{-/-}$ mice have significantly reduced trabecular bone [169]. Furthermore, $Rarg^{-/-}$ mice have significant reductions in pro-B and pre-B lymphocytes [28] and have reduced levels of *Il7* mRNA in their bone marrow [169]. $Rarg^{-/-}$ mice also develop a myeloproliferative-like syndrome (MPS) exhibiting increased granulocyte/macrophage progenitors and granulocytes in bone marrow, peripheral blood, and spleen [166]. Bone marrow transplant studies revealed that the defect in B lymphopoiesis and the MPS-like syndrome were due to extrinsic loss of RARγ from non-hematopoietic cells. Transplantation of wild type bone marrow into $Rarg^{-/-}$ mice led to development of the hematopoietic defects, whereas these

defects were resolved when *Rarg*$^{-/-}$ bone marrow was transplanted into wild type mice.

The hematopoietic phenotypes have recently been assessed in mice with conditional deletion of *Rarg*$^{-/-}$ in nestin and osterix-targeted microenvironmental cells [28]. Deletion of *Rarg*$^{-/-}$ in *Osterix*-Cre-targeted osteoblast progenitor cells produced no hematopoietic phenotype and B lymphopoiesis was normal [28]. In contrast, mice with deletion of *Rarg* in *Nestin*-Cre-targeted cells had normal numbers of pro-B lymphocytes but significantly reduced numbers of bone marrow pre-B lymphocytes and immature B220 + IgM + B lymphocytes, in addition to significantly reduced numbers of peripheral blood B lymphocytes [28]. The effects on early B lymphocytes were not as profound in *Nestin*-Cre^{+}*:Rarg*$^{\Delta/\Delta}$ mice as those observed in *Rarg*$^{-/-}$ mice, suggesting that additional cell types in the bone marrow are likely involved in the regulation of B lymphopoiesis by RARγ. The mechanism by which impaired B lymphopoiesis occurred in the *Nestin*-Cre^{+}*:Rarg*$^{\Delta/\Delta}$ mice has not been determined.

6.5. Collagen X

Transgenic mice with dominant interference collagen X (Col X) mutations developed significant skeletal defects, including reduced trabecular bone and growth plate defects due to perturbed endochondral ossification [175]. These mice were subsequently revealed to have significant reductions in bone marrow and spleen B lymphocytes which persisted throughout the lifespan of the mice [176]. The Col X knockout mice were predominantly shown to have subtle bone and growth plate defects [177,178], however, more severe, perinatal lethal mutants were reported for both the Col X transgenic and knockout models, and these had even more profound hematopoietic and bone phenotypes [175,177,179].

There were significantly reduced numbers of CD19^{+} cells and increased production of Gr-1^{+} granulocytes in co-culture assays of lineage negative bone marrow cells with trabecular bone-derived osteoblasts from the Col X knockout mice compared to wildtype controls [178]. Cytokine profiling studies revealed decreased production of a range of cytokines important for B lymphopoiesis, including SCF, IL-7, CXCL12 and FLT3-L, in the Col X knockout co-cultures compared to the wildtype co-cultures. The addition of SCF, IL-7 or CXCL12 to the Col X knockout trabecular osteoblast co-cultures restored the production of the CD19^{+} lymphocytes to the same level as the wildtype co-cultures, and IL-7 had the most profound effect in stimulating the numbers of CD19^{+} B lymphocytes in both the Col X and wildtype trabecular osteoblast co-cultures. Finally, injection of IL-7 for four days into 7 day old Col X knockout or Col X transgenic mice increased the numbers of pro-B cells in the bone marrow of the mice comparable to wildtype mice treated with IL-7. In contrast, however, the numbers of pre-B lymphocytes remained significantly reduced in both the IL-7-treated Col X knockout and transgenic mice compared to the wildtype controls treated with IL-7, suggesting the contribution of additional B lymphocyte regulators to the B lymphocyte phenotypes observed in the Col X knockout and transgenic mice [178].

7. B lymphocyte regulation of osteoclastogenesis

This review has focused on the importance of bone and osteoblast-lineage cells in supporting B lymphopoiesis, yet it is also interesting to note that B lymphocytes are capable of influencing bone mass. B lymphocytes express both RANKL [3] and its decoy receptor OPG [180] [181], meaning that they can regulate osteoclastogenesis (and, in turn, influence the regulation of osteoblasts). B cells at different stages of development, in particular mature B lymphocytes and plasma cells, have been shown to be the major source of OPG in mouse bone marrow [180]. Mice lacking mature B cells are osteoporotic, and have reduced expression of *Tnfrsf11b* (*Opg*) in the bone marrow [180], thus allowing B cells to positively regulate osteoclastogenesis.

Interestingly, B lymphocytes also express RANKL [3] and support the formation of osteoclasts [3,182]. Studies in ovariectomized mice revealed that, along with osteoclast-driven bone loss, the mice exhibited an increase in B lymphopoiesis and an accumulation of B220^{+} cells in the bone marrow [183]. Similarly, mice treated with IL-7 to stimulate B lymphopoiesis also had enhanced osteoclastogenesis and less bone than controls and *Il7*$^{-/-}$ mice had higher trabecular bone volume [183]. Numerous cytokines that are normally suppressed by estrogen are known to contribute to ovariectomy-induced osteoclastogenesis, yet the increase in B lymphocytes drew light to the idea that B lymphocytes may be positive regulators of osteoclast formation.

B lymphocytes express RANKL [132] and deletion of RANKL from B lymphocytes using CD19-Cre protected against ovariectomy-induced trabecular bone loss by impairing osteoclastogenesis [3]. In contrast, non-ovariectomized *CD19*-Cre^{+}*:Tnfsf11*$^{\Delta/\Delta}$ mice did not exhibit any bone loss during the 7 month period of analysis [3]. Ovariectomy did not increase *Tnfsf11* expression by B lymphocytes, but increased the numbers of RANKL-expressing B lymphocytes and sRANKL levels in the bone marrow, implying a net increase in RANKL due to increased B lymphocyte numbers [3]. In B lymphocytes the RANKL/OPG axis is regulated by mechanistic target of rapamycin complex 1 (mTORC1) [184]. Deletion of a negative regulator of mTORC1, tuberous sclerosis complex 1 (TSC1), in B lymphocytes using CD19-Cre increased RANKL expression and downregulated OPG in B cells. As a result, *CD19*-Cre^{+}*:Tsc1*$^{\Delta/\Delta}$ mice had reduced trabecular bone mass and increased numbers of osteoclasts via regulation of β-catenin [184]. Under normal conditions the contribution of B lymphocyte-derived RANKL is not integral for maintaining normal bone mass, likely due to contributions of many other cell types in the bone marrow microenvironment. However, a clear role has been established for RANKL produced by B lymphocytes during estrogen deficiency, suggesting that B cells are capable of contributing to osteoclast-driven bone loss. This emphasizes the potential of B lymphocytes to regulate osteoclastogenesis, meaning that perturbed function of cells in the B cell lineage can have serious consequences for bone health. Additional examples of mice in which there are reciprocal relationships between B lymphocytes, osteoclasts and bone mass include *Cntf*$^{-/-}$ mice [185] and others that were recently reviewed by Manilay and Zouali [6].

8. Osteoblasts and multiple myeloma

Multiple myeloma is a malignancy of differentiate B cells (plasma cells) in the bone marrow that causes a destructive bone disease in up to 90% of patients [186]. Many patients with myeloma bone disease experience bone pain, hypercalcemia and pathological fractures severely reducing quality of life [187], and the bone disease increases the risk of death by 20% [188]. The bone disease is instigated by the release of factors that directly lead to an increased number and activity of osteoclasts either directly from myeloma cells or indirectly from osteoblasts following myeloma cell adhesion (e.g. RANKL, IL-6, IL-1β, IL-3, macrophage inhibitory protein 1α; MIP-1α, tumor necrosis factor α; TNFα), this results in enhanced resorption, formation of osteolytic lesions and loss of trabecular bone structure [189]. The bone disease is exacerbated by concurrent release of osteoblast inhibitory factors [e.g. dickkopf-1; DKK1, secreted frizzled-related protein 2 (SFRP2) and SFRP3], which block differentiation of osteoblasts and thus impair formation of new bone [190–192]. As bone is resorbed additional factors that further promote osteoclastogenesis are released from the bone matrix such as transforming growth factor receptor β (TGFβ), which stimulates osteoclast formation and promotes recruitment and proliferation of osteoblast progenitors but inhibits any further osteoblast differentiation [193–195]. In osteoblasts and BM stromal cells, TGFβ increases secretion of factors that stimulate MM growth (e.g. IL-6, insulin-like growth factor 1; IGF-1) [189,193], promoting tumor growth and establishing the 'vicious cycle' of myeloma progression and bone destruction.

More recently it has also been established that osteoblast-lineage cells and osteoclasts control myeloma cell dormancy [22], implicating these cells in chemotherapy evasion and relapse. Myeloma cell engagement with bone lining cells or osteoblasts induces a dormant state where cells are resistant to chemotherapeutics that target proliferating cells [22]. These dormant cells remain in patients with minimal residual disease. The dormant state can be reversed by resorbing osteoclasts which reactivate dormant myeloma cells facilitating tumor expansion [22]. Chemotherapy resistance in myeloma is also conferred through myeloma adhesion to stromal cells via VCAM-1:VLA-4 binding and expression of microRNA-15a [196,197]. While the role of osteoblasts and osteoclasts in myeloma dormancy is still under investigation, this clearly demonstrates the processes of bone remodeling and growth of this plasma cell malignancy are intricately intertwined.

9. Conclusions

Over the past two decades, the importance for the bone marrow microenvironment in facilitating healthy development of B cells has become increasingly evident. The composition of B lymphocyte niches is now known to change throughout B lymphopoiesis and has been at least partially defined, with CAR cells, IL-7-expressing cells and GAL1-expressing cells playing unique roles. Despite this, there is still a lot of confusion surrounding how much these populations overlap and what makes them distinct from one another. It also remains unclear whether these populations represent stages along the differentiation of mesenchymal stem cells towards osteoblasts, or instead are unique populations with specialized roles in support of B lymphopoiesis. Deletion of genes, such as *Cxcl12* and *Il7*, in different microenvironmental cell types has helped to reveal which cell types are critical producers of these factors in regard to B lymphopoiesis and which cell types are not essential, even if they can contribute. In recent years, the number of extrinsic regulators of B lymphopoiesis that have been identified and our understanding of the actions of these regulators has increased. The importance of the osteoblast lineage has been highlighted in cell ablation studies, conditional knockout models, and from the striking link between many mouse models that produce bone phenotypes that also produce B cell phenotypes (in the cases where this has been investigated). Interestingly, it has also become evident that B cells are also capable of influencing bone by modulating osteoclastogenesis, suggesting healthy B cell production is also important for maintaining bone mass. This is particularly evident in the plasma cell malignancy, multiple myeloma, where perturbed proliferation of differentiated B cells causes a destructive bone disease.

Future directions include the improvement of methods to identify and isolate different mesenchymal lineage cells to enable further study of their involvement in the regulation of B lymphopoiesis (and other hematopoietic cells). This will lead to the identification of additional positive and negative regulators of B lymphopoiesis through other techniques such as single cell RNA-seq. Advances in imaging technologies, such as the recent Opal multiplexing immunohistochemistry technology [198], which permits a minimum of 7 different antibodies to be assessed on a single section (with additional fluorochromes currently being developed for use with this technology), will facilitate in situ analysis of developing B lymphocytes in their niches in more detail than has previously been possible. Hence our ability to identify the specific niches for different stages of developing B lymphocytes, combined with improved methods to identify different niche cells will greatly broaden our understanding of how B lymphopoiesis is regulated in normal and diseased (such as multiple myeloma) states.

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