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Mesenchymal lineage cells and their importance in B lymphocyte niches

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Note spelling should be in USA English (hematopoietic, not haematopoietic etc)

Abstract

Overview

1. Introduction

Bone is a dynamic organ that is constantly being broken down and replenished in a process known as bone remodeling. Bone remodelling 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 remodelling 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 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 two 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 in the medullary cavity of bones 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 capable of forming T and B lymphocyte lineages that develop in the thymus and bone marrow and spleen, respectively. B lymphopoiesis occurs through distinct stages of differentiation: in the bone marrow, common lymphoid progenitors (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]. End-stage B lymphocytes known as plasma cells can return to the bone marrow [5] (Figure 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.

The first specified B lymphocyte progenitor 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] (Figure 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 immunoglobin (Ig) heavy and light chain loci are shuffled and mutated 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 die by neglect or undergo 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].

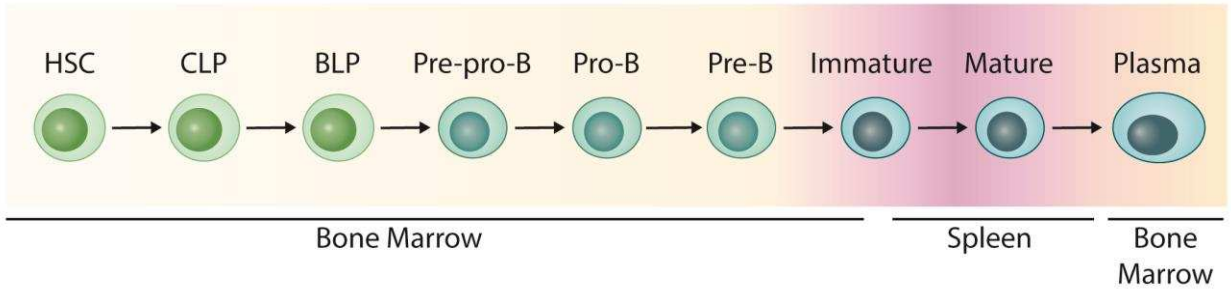


Figure 1: Simplified schematic of B lymphopoiesis

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 and natural killer cells. 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 then return to the bone marrow.

3. Microenvironment regulation of hematopoiesis

Over the recent decades there has been increasing interest in the role of cells within hematopoietic microenvironments (also known as niches) in nurturing healthy [17-19] and malignant [20-22] hematopoiesis and bone remodelling [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 organised still requires further investigation, although numerous cell types known to play important roles have been identified. These include (but are not 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 [23], nestin-expressing perivascular cells [24, 25] leptin receptor (LepR)-expressing mesenchymal stromal cells [26, 27]), osterix (Osx)-derived osteoblast progenitor cells and osteoblasts [23, 28, 29], CXC-motif chemokine ligand 12 (CXCL12) abundant reticular (CAR) cells [30, 31] and endothelial cells [26, 27]) (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 [32]. 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 [33]. 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 [34] and at embryonic day 16.5 LacZ was detected in the periosteum and primary spongiosa in the appendicular and axial skeleton [35]. Thus, despite the fact that neither the Col2α1 gene [34] nor Col2α1-Cre [35] are expressed in osteoblasts, these osteoblastic cells can arise from a cell that once expressed Col2α1-Cre.

Table 1. Transgenic Cre-recombinase mouse 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	[32]
Nestin	Neural crest-derived perivascular mesenchymal cells	Yes, strain-dependent, reviewed in [17]	[36-38]
LepR	Perivascular mesenchymal stromal cells	Some brain cells, some hematopoietic cells	[39-41]
Gremlin1	Osteochondroreticular (OCR) stem cells	Intestinal reticular stem cells	[42]
Mx1	Osteolineage-restricted stem/progenitor cells and HSCs	Heart, kidney, liver	[43, 44]
Osx	Osteoprogenitors, CAR cells, hypertrophic chondrocytes, adipocytes	Olfactory glomerular cells, some gastric and intestinal epithelial cells	[45-47]

Col1α1 2.3kb (Mouse)	Osteoblasts	None reported (the two different rat-derived Col2.3 strains have other cell targets, reviewed in [17])	[48]
Col2α1	Chondrocytes, synovial fibroblasts, periosteum and primary spongiosa in the appendicular and axial skeleton	None reported	[34, 35]
Tagln	Osteoblasts, majority of CAR cells and vinous sinusoidal and arteriolar pericytes	Smooth muscle cells	[49, 50]
Osteocalcin	Mature osteoblasts, most CAR cells and arteriolar pericytes		[51]
Dmp1 (9.6kb)*	Late osteoblasts and osteocytes, subset of CAR cells	Skeletal muscle, brain, intestine, stomach	[33, 49, 52]

*The 8kb Dmp1-Cre strain also targets some brain cells, reviewed in [17].

3.2. Cellular localisation 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 organised 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* [53] knock-in mice have shown that pre-pro-B lymphocytes localise near CXCL12-abundant reticular (CAR) cells. These cells differentiate into pro-B lymphocytes and together with Pre-B I cells [54, 55], localise near Interleukin 7 (IL-7)-secreting cells [54, 55]. 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 [54], 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 [56].

Pre-B II lymphocytes then migrate away from IL-7-expressing cells [54, 55] 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) [54]. Immature B lymphocytes localise near endothelial cells lining blood vessels [57] prior to these cells exiting the bone marrow into the circulation. Some immature B lymphocytes are retained in the bone marrow through cannabinoid receptor 2-mediated adhesion to endothelial cells lining sinusoidal vessels [57].

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, 58, 59]. Here we focus on known extrinsic regulators of B lymphopoiesis, with a particular focus on the roles of mesenchymal-derived cells in these processes.

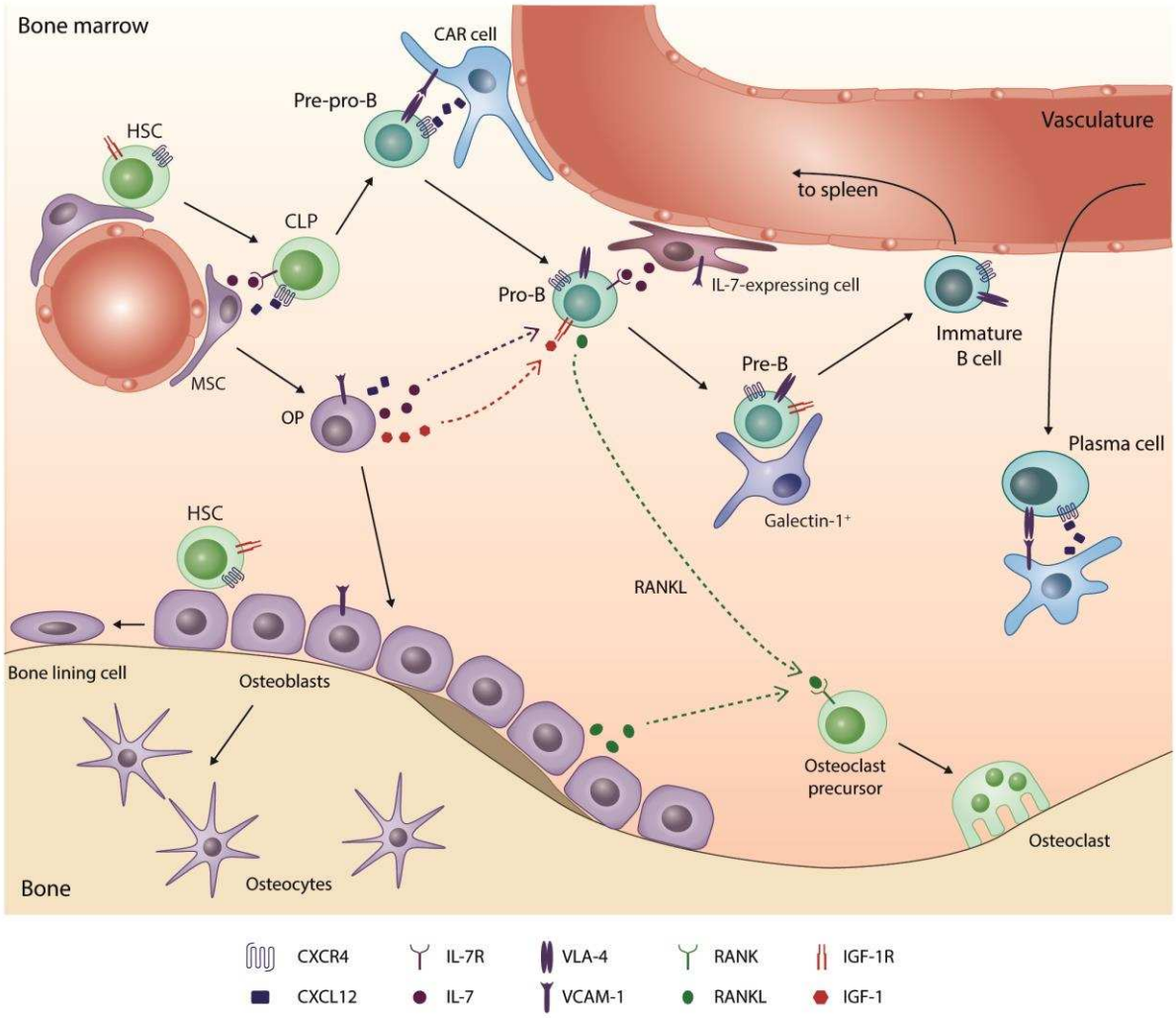


Figure 2: The bone marrow is the site of early B lymphopoiesis

Early B lymphopoiesis occurs in the bone marrow. Haematopoietic stem cells (HSCs) differentiate to common lymphoid progenitors (CLPs) and then become the earliest committed B cell precursor, the pre-pro-B cell. Pre-pro B cells localise next to CXCL12-abundant reticular (CAR) cells, then as they differentiate into pro-B cells they localise adjacent to IL-7-expressing stromal cells. Pre-B cells localise near Galectin-1-expressing cells within the bone marrow. Immature B cells migrate from the bone marrow to mature at the spleen, although some are retained through interactions with endothelial cells. End stage plasma cells then return to the bone marrow. Osteoblasts are known to play essential roles in regulating 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.

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 [60]. 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.* [61] 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 [62, 63]. 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)[63, 64]]. 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* [63]. The OP9 cell line (and a variant that overexpresses delta-like 1, OP9-DL1 [65]) 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 [66], chondrocytes [67] and osteoblasts [68] when cultured with the appropriate

differentiation-inducing media. Furthermore, the immunophenotype of OP9 cells [68] 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 [69]. 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 [60].

Furthermore, co-cultures of primary calvaria-derived osteoblasts or the stromal cell line S17 with HSCs supported the formation of $B220^{+}CD19^{+}$ B lymphocyte precursors [62]. Zhu *et al.* [62], isolated HSCs ($GFP^{-}Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$; 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 [62]. 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 signalling is also important for osteoblastic-induction of B lymphopoiesis [62]. 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 [62]. The expression of Sca-1 by these cells suggests that they are also akin to skeletal stem cells [23, 39, 69]. 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 Col1a1-2.3kb (Col2.3)-targeted osteoblasts *in vivo* using a thymidine kinase suicide gene *in vivo* depleted early B lymphocytes in the bone marrow [62]. 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 [62]. 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 [62, 70]. This indicates that osteoblasts directly regulate early B lymphopoiesis in the bone marrow, and that this occurs prior to the loss of HSCs.

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 [71]. This resulted in a reduction in pre-B lymphocytes and mature B lymphocytes in the bone marrow and accumulation of pro-B cells and large pre-B cells, with no change to 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 Pro-B cell but had no effect on Pre-B cells, implicating IGF-1 in regulation of pre-B cells [71].

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 [72]. 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* [72]. This implicates osteoclast activation can

indirectly regulate B lymphopoiesis through altering bone formation from osteoblast lineage cells. Interestingly, B lymphocytes can inhibit osteoclastogenesis (and, in turn, influence the regulation of osteoblasts) by secreting osteoprotegerin (OPG) [73]. Indeed, 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 BM [73]. In inflammatory conditions, B lymphocyte populations can also secrete RANKL [74].

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 recognised 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 [55], 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 characterised. We describe below known B lymphopoiesis regulatory factors that have been shown to be expressed by mesenchymal lineage cells.

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 [75]. CXCL12 is essential for regulating early B lymphopoiesis, at least in part by guiding the different B lymphoid progenitors to their correct

niches [76], and also regulates quiescence and the maintenance of HSCs in the bone marrow [23]. In addition, CXCL12 is a key component involved in the homing of end stage B plasma cells to the bone marrow [23]. Early studies of $SDF^{-/-}$ ($CXCL12^{-/-}$) mice found that CXCL12 expression was essential for the development of pre-pro-B lymphocytes [77]. B lymphocytes express CXCR4 throughout differentiation, although the level of expression fluctuates and this affects the chemotactic effect of CXCL12 [78]. This means 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 [79]. The variation in CXCR4 expression is due to coupling of different signalling cascades following CXCR4 activation during B lymphopoiesis [79].

The importance of microenvironment-produced CXCL12 in the initiation of B lymphopoiesis was recently demonstrated by Cordeiro Gomes *et al.* [76]. 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 [76]. Elegant imaging studies showed that loss of *Cxcr4* in the MPPs resulted in defective CLP localization to IL-7-expressing microenvironment cells [76].

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 HSCs, with an almost complete lack of quiescent HSCs and a loss of HSC repopulating activity [23].

When *Cxcl12* was deleted in *Osx*-targeted osteoprogenitors HSC function was normal, however the mice exhibited constitutive HSC mobilisation and reduced numbers of B lymphoid progenitors [23]. Col1α1-2.3kb (*Col2.3*)-*Cre* deletion of *Cxcl12* had no effect on B lymphocytes but the mice exhibited reduced numbers of CLPs [26]. In comparison, deletion in *Tie2*-targeted endothelial cells and *LepR*-targeted stromal cell had no effects on B lymphopoieisis [26]. Interestingly, however, reduced HSC numbers [26] and repopulating activity [23] was observed when *Cxcl12* was deleted in endothelial cells and HSC mobilisation occurred when *Cxcl12* was deleted in *LepR*-targeted cells [26]. Deletion of *Cxcl12* in *Nestin*-targeted perivascular cells [26] and in *Osteocalcin*-targeted mineralising osteoblasts [23] did not alter HSCs 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 HSCs 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.

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 deleting *Cxcl12* from *Prrx1* mesenchymal stromal cells will also delete in all cells derived from *Prrx1*-expressing cells including osteoblastic, adipogenic, chondrogenic lineages and other stromal populations that are derived from limb bud mesenchymal stem cells. 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.

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

Genetic model	HSCs	Progenitors	B220+	Pre-pro-B	Pro-B	Pre-B	Ref
Cxcl12							
Prrx1-Cre^{+}:Cxcl12$^{\Delta/-}$	↓ HSCs (almost complete lack of quiescent HSCs) ↓ repopulating activity	↓ MPPs, CLPs, BLPs, CMPs	↓	↓	↓	↓	[23, 26]
Nestin-Cre^{+}:Cxcl12$^{\Delta/\Delta}$	normal*^	normal		normal	normal	normal	[26]
LepR-Cre^{+}:Cxcl12$^{\Delta/\Delta}$	↑ mobilization	normal		normal	normal	normal	[26]
Osx1-GFP::Cre^{+}:Cxcl12$^{\Delta/-}$	Normal HSC function, constitutive mobilization	normal	↓	↓	ND	ND	[23]
Col1a1(2.3kb) -Cre^{+}:Cxcl12$^{\Delta/\Delta}$	normal*^#	↓ CLPs & LMPPs		normal	normal	normal	[26]
Osteocalcin-Cre^{+}:Cxcl12$^{\Delta/-}$	normal*^	normal	normal	normal	ND	ND	[23]
Tie2-Cre^{+}:Cxcl12$^{\Delta/\Delta}$	↓ HSCs, ↓ long-term repopulating activity	normal		normal	normal	normal	[23, 26]
Il7							
Prrx1-Cre^{+}:Il7$^{\Delta/-}$	normal*	normal	ND	ND	↓	↓	[76]
LepR-Cre^{+}:Il7$^{\Delta/-}$	normal*	↓ Ly6D+ CLPs	ND	ND	↓	↓	[76]
Col1a1(2.3kb) -Cre^{+}:Il7$^{\Delta/-}$	normal*	normal	ND	ND	normal	normal	[76]
Tie2-Cre^{+}:Il7$^{\Delta/-}$		normal	ND	ND	↓	↓	[76]
Scf (Kitl)							
Il7-Cre:Scf$^{\Delta/\Delta}$	↓	↓ MPPs	ND	ND	normal	normal	[76]
Nestin-Cre^{+}:Scf$^{\Delta/-}$	normal*^	ND	normal	ND	ND	ND	[27]
Nestin-CreER^{+}:Scf$^{\Delta/\Delta}$ + tamoxifen	normal*^	ND	ND	ND	ND	ND	[27]
LepR-Cre^{+}:Scf$^{\Delta/-}$	↓ HSCs	ND	ND	ND	ND	ND	[27]
Col1a1(2.3kb) -Cre^{+}:Scf$^{\Delta/-}$	normal*^	ND	normal	ND	ND	ND	[27]

Tie2-Cre^{+}:Cxcl12$^{\Delta/-}$	↓ HSCs, ↓ long-term repopulating activity	ND	ND	ND	ND	ND	[27]
Gsα							
Osx1-GFP::Cre^{+}:Gsα$^{\Delta/\Delta}$	ND	ND	↓	normal	↓	↓	[56]
Dmp1-Cre^{+}:Gsα$^{\Delta/\Delta}$	normal*	normal	normal	ND	ND	ND	[80]
Pth1r							
Col1a1(2.3kb) -Cre^{+}:Pth1r$^{tg/tg}$ (constitutively active)	↑ HSCs	ND	ND	ND	ND	ND	[28]
Rarg							
Nestin-Cre^{+}:Rarg$^{\Delta/\Delta}$	normal*^	normal	↓	normal	normal	↓	[25]
Osx1-GFP::Cre^{+}:Rarg$^{\Delta/\Delta}$	normal*^	normal	normal	normal	normal	normal	[25]
IGF-1							
Osx1-GFP::Cre:Igf1$^{\Delta/\Delta}$	ND	ND		normal	↑	↓	[71]
Osteoblast ablation							
Osx1-GFP::Cre:iDTR + diphtheria toxin	normal*^	↑ GMP		normal	↑~	↓	[71]
Col1a1(2.3kb)-Cre^{+}:ΔTK + ganciclovir	↓ day 21	ND	↓ day 8	↓ day 8	↓ day 8	↓ day 8	[62]

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). Haematopoietic 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). *Normal HSC frequency, ^normal repopulating activity. #Data were based on FACS-isolated HSC transplants, however transplants using whole bone marrow cells showed significantly impaired lymphoid repopulation. ~Fraction B was normal and Fraction C' and C'' were increased.

5.1.2. IL-7

IL-7 is also known to play an essential role in B lymphopoiesis. 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 [81, 82]. 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 [82, 83]. This suggests the role of IL-7 in B lymphopoiesis occurs subsequent to CXCL12, from the pro-B cell stage onwards.

IL-7 has been shown to induce the proliferation of pro-B cells, but not pre-pro-B cells, *in vitro*

(85). In accordance with this, *Il7* deficient mice demonstrated a marked loss in the numbers of pro-B cells and pre-B cells and their later differentiated stages (86, 87). While no changes in pre-pro B cells were observed in *Il7* knockout mice, their differentiation was severely impaired, which may have resulted in the reductions of the more mature populations (86).

The mechanism of action of IL-7 includes the induction of the expression of myeloid-cell leukemia sequence 1 (MCL1) in the developing B cell precursors to mediate their survival (88). Furthermore, it has been reported that overexpression of early B cell factor (EBF) can counteract the *Il7*-deficient B cell phenotypes (89).

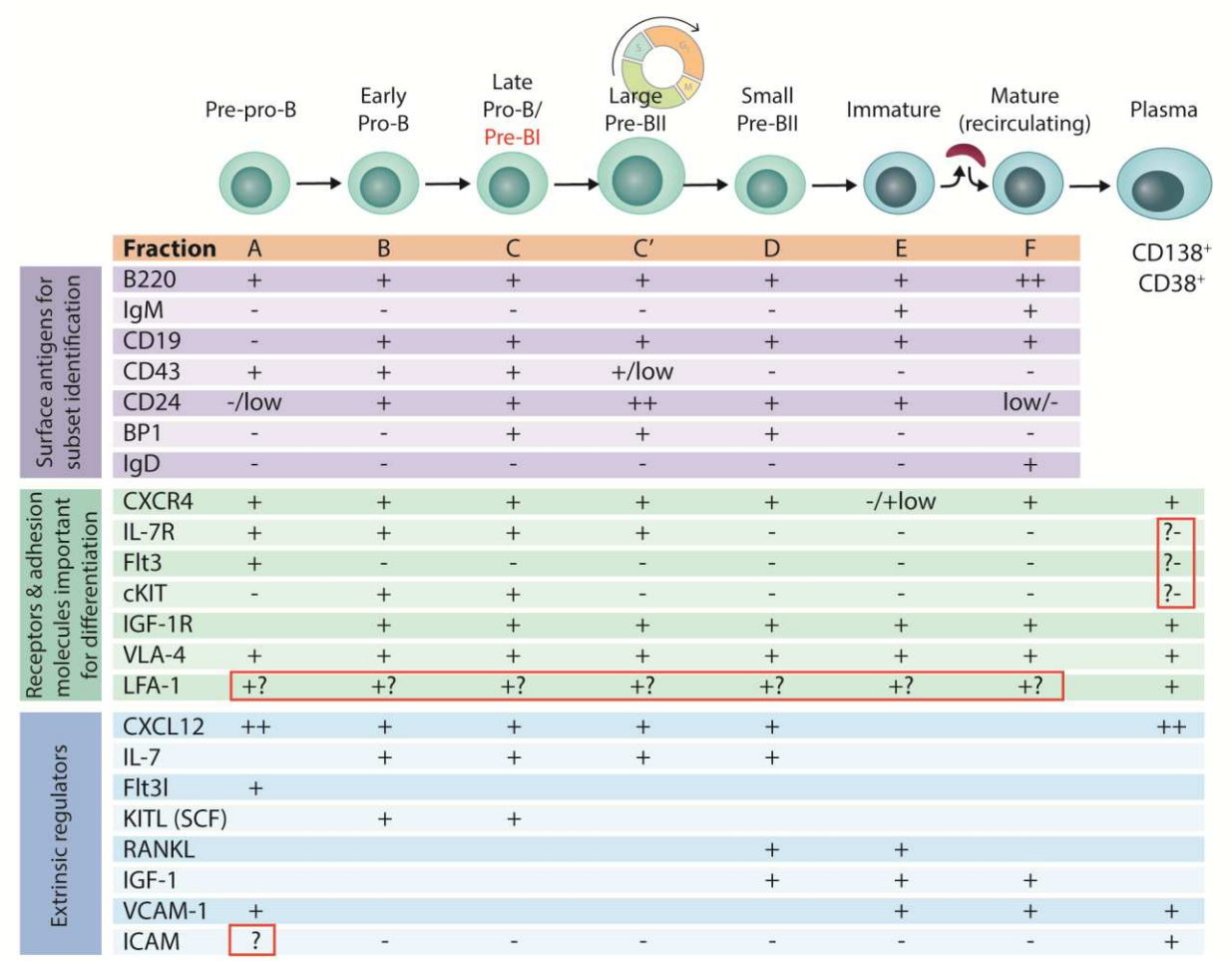
Elegant lineage tracing studies where *Il7*-Cre transgenic mice were crossed to *Rosa26*^{EYFP} mice demonstrated that the IL-7-expressing cells in the bone marrow were predominantly bipotent CAR cells that expressed LepR and could give rise to both adipocytes and osteoblasts *in vivo* [76]. Deletion 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 [76].

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 signalling. Deletion of Gsα in *Osx-Cre*-targeted osteoblast progenitors leads to severe osteoporosis and reduced B lymphocyte precursors in the bone marrow [56, 84]. *Osx-GFP::CreGsα*$^{fl/fl}$ *(Gsα*^{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 [56]. The Gsα-deficient osteoblast lineage cells expressed significantly reduced levels of IL-7. Administration of IL-7 into *Gsα*^{OsxKO} mice or transplant of *Gsα*^{OsxKO} bone marrow into wild type mice was able to rescue the B lymphocyte defects [56]. Thus IL-7 produced by *Osx*-targeted cells regulates pro-B and pre-B lymphopoiesis.

In their recent study, Cordeiro Gomes *et al.* [76] 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.

- I would include here Aguila, JBMR 2012 showing that osteoblast-specific expression of IL-7 can rescue the B cell and bone phenotype of IL-7 KO mice:
    - J Bone Miner Res. 2012 May;27(5):1030-42. doi: 10.1002/jbmr.1553.
      Osteoblast-specific overexpression of human interleukin-7 rescues the bone mass phenotype of interleukin-7-deficient female mice.
      Aguila HL[1], Mun SH, Kalinowski J, Adams DJ, Lorenzo JA, Lee SK.



	Fraction	A	B	C	C′	D	E	F	CD138⁺ CD38⁺
Surface antigens for subset identification	B220	+	+	+	+	+	+	++	
	IgM	-	-	-	-	-	+	+	
	CD19	-	+	+	+	+	+	+	
	CD43	+	+	+	+/low	-	-	-	
	CD24	-/low	+	+	++	+	+	low/-	
	BP1	-	-	+	+	+	-	-	
	IgD	-	-	-	-	-	-	+	
Receptors & adhesion molecules important for differentiation	CXCR4	+	+	+	+	+	-/+low	+	+
	IL-7R	+	+	+	+	-	-	-	?-
	Flt3	+	-	-	-	-	-	-	?-
	cKIT	-	+	+	-	-	-	-	?-
	IGF-1R		+	+	+	+	+	+	+
	VLA-4	+	+	+	+	+	+	+	+
	LFA-1	+?	+?	+?	+?	+?	+?	+?	+
Extrinsic regulators	CXCL12	++	+	+	+	+			++
	IL-7		+	+	+	+			
	Flt3l	+							
	KITL (SCF)		+	+					
	RANKL					+	+		
	IGF-1					+	+	+	
	VCAM-1	+					+	+	+
	ICAM	?	-	-	-	-	-	-	+

Figure 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 [85]. 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 stimulating or repressing B lymphopoiesis. The blue panel indicates extrinsic factors expressed by stromal cells in the microenvironment 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.

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 [86, 87]. FLT3L has been shown to be produced by a range of hematopoietic cell types [88], 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 [89, 90]. Furthermore, mice deficient in FLT3L also had a marked reduction in bone marrow pre-B lymphocytes [90]. 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 [91]. 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, 27, 81]. SCF synergizes with IL-7 *in vitro* to induce the proliferation of pro-B cells [92]. 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 [76].

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 [93]. 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 [94]. 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 [27] (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 [27].

5.1.5. IGF-1

IGF-1 is expressed by Osx1-GFP::Cre-targeted cells that binds to the IGF-1 receptor (IGF-1R) on B lymphocytes to promote transition from the pro-B to the pre-B lympohocyte stage [71]. *Igf1* levels are reduced in the bones of *Osx1-GFP::Cre:iDTR* mice after administration of diphtheria toxin to ablate *Osx1-Cre*-targeted cells and mice exhibit defective B lymphopoiesis [71]. Mice with targeted deletion of IGF-1 using *Osx1-GFP::Cre* exhibit 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 [71]. This implicates IGF-1 derived from *Osx1-Cre*-targeted cells as important in regulating the later stages of B lymphopoiesis within the bone marrow from fraction D small pre-BII cells.

5.1.6. Sclerostin

For you to consider – one paper demonstrating a B cell defect in mice lacking sclerostin, a negative regulator of canonical Wnt signalling. Potentially clinically relevant since anti-sclerostin antibody is in development as an osteoporosis medication:

> J Bone Miner Res. 2012 Jul;27(7):1451-61. doi: 10.1002/jbmr.1608. Absence of sclerostin adversely affects B-cell survival. Cain CJ[1], Rueda R, McLelland B, Collette NM, Loots GG, Manilay JO.

Osteonectin

- Luo et al 2014 – SPARC deficiency affects bone marrow stromal cell function, resulting in impaired B lymphopoiesis. https://www.ncbi.nlm.nih.gov/pubmed/24598056 → probably due to reduced osteoblasts

5.1.7. VCAM-1

Adhesion

- Tokoyoda – Cxcl12 increases VLA-4 VCAM-1 adhesiveness in pre-pro-B cells but not pro-B or pre-B cells
- Park, S. Y. et al. Focal adhesion kinase regulates the localization and retention of pro-B cells in bone marrow microenvironments. J. Immunol. 190, 1094–1102 (2013).
- Ryan et al 1999 – Vascular cell adhesion molecule-1 and the integrin VLA-4 mediate adhesion of human B cell precursors to cultured bone marrow adherent cells https://www.ncbi.nlm.nih.gov/pmc/articles/PMC295504/

5.1.8. Galectin-1

Galectin-1 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 [95]. Developing B lymphocytes express $\alpha_4\beta_1$ (VLA-4), which also binds to other cell adhesion molecules including VCAM-1.

Galectin-1 was shown to be expressed in mature osteoblasts in addition to cells located throughout the bone marrow [54]. 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 [54].

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 [95].

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), microphalmia associated transcription factor (MITF), PU.1 and activator protein 1 (AP-1) [96, 97]. This induces myeloid precursors to differentiate into mononucleated osteoclasts, which form multinucleated osteoclast by fusing and finally activate becoming polarised osteoclasts that resorb bone [96, 97].

RANKL is produced by a range of osteoblast lineage cell types [98], in addition to B lymphocytes [99]. Mice deficient in RANKL (*opgl*$^{-/-}$) exhibited severe osteopetrosis and reduced numbers of B lymphocyte precursors [100]. Differentiation of splenic osteoclast precursor cells from *opgl*$^{-/-}$ and *opgl*$^{+/+}$ mice revealed *opgl*$^{-/-}$ splenic cells displayed comparable osteoclastogenic capacity to *opgl*$^{+/+}$ cells when cultured with exogenous RANKL and M-CSF. In comparison, co-cultures of either *opgl*$^{+/-}$ or *opgl*$^{-/-}$ osteoblasts with wild type non-adherent bone marrow cells found *opgl*$^{+/-}$ osteoblasts, but not *opgl*$^{-/-}$ osteoblasts, could support osteoclastogenesis. This means the osteopetrotic phenotype in *opgl*$^{-/-}$ mice is due to impaired osteoclastogenesis caused by a lack of RANKL production from osteoblasts rather than an intrinsic loss in osteoclast progenitors.

When immunodeficient recombination-activating gene 1 null (*Rag1*$^{-/-}$) mice (which lack mature B and T cells) were reconstituted with *opgl*$^{-/-}$ foetal 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 [100]. 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) [101]. 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 [102]. 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.

- Discuss paper by Yun et al 2001 – opg-/- mice see Horowitz review
- https://www.ncbi.nlm.nih.gov/pubmed/20601290

5.3. Negative extrinsic regulators

OSF-5

- Identification of osteoblast stimulating factor 5 as a negative regulator in the B-lymphopoietic niche (Fujita et al 2015 Experimental Haematology)
  https://www.ncbi.nlm.nih.gov/pubmed/26213229

Each of these factors produced extrinsically to B lymphocytes is important for healthy B lymphocyte production. Yet how each niche is organised 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 signalling pathways of importance for regulating bone.

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

6.1. PTH

Discuss PTH in OBs and then link to Joy's publications but don't be repetitive with other sections

Further investigation into the role of PTH in osteoblasts has solidified its significance in regulation of B lymphopoiesis via IL-7. *Osx-Cre*-targeted deletion of PTH receptor 1 (PTHR1) [7] or the G_S alpha subunit ($G_S\alpha$) downstream signalling mediator [56, 84] caused reductions in bone marrow pro-B and pre-B lymphocytes and development of severe osteoporosis. This B lymphocyte phenotype was rescued by administration of IL-7 or by transplanting $G_S\alpha$-deficient bone marrow into wild type mice [56].

6.2. Estrogen

- Manolagas, S. C., O'Brien, C. A., and Almeida, M. (2013) The role of estrogen and androgen receptors in bone health and disease. Nat. Rev. Endocrinol. 9, 699–712 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3971652/

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- Masuzawa, T., Miyaura, C., Onoe, Y., Kusano, K., Ohta, H., Nozawa, S. and Suda, T. (1994) Estrogen deficiency stimulates B ymphopoiesis in mouse bone marrow. J Clin Invest. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC295170/
- Fujiwara - RANKL (Receptor Activator of NF_B Ligand) Produced by Osteocytes Is Required for the Increase in B Cells and Bone Loss Caused by Estrogen Deficiency in Mice
-

6.3. Retinoic acid receptors

Retinoic acid receptors (RARs) are expressed by many cells in the bone marrow microenvironment, and regulate hematopoiesis (including B lymphopoiesis) [25, 103-106] and bone remodelling [107-111] through direct and indirect means. Canonical RAR signalling occurs through nuclear RARs of which there are three subtypes; α, β and γ [108]. RARs form heterodimers with retinoid X receptors (RXRs) and associate with retinoic acid response elements (RAREs) were they regulate gene transcription. The effect 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 [109] or hematopoiesis [105, 112]. *Rarb*$^{-/-}$ and *Rarb2*$^{-/-}$ mice appear normal and have no reported skeletal abnormalities, although these have not been extensively investigated in adult mice, nor have the hematopoietic phenotypes of these mice been reported [113, 114]. In comparison, *Rarg*$^{-/-}$ mice have significantly reduced trabecular bone [109]. Furthermore, *Rarg*$^{-/-}$ mice have significant reductions in pro-B and pre-B lymphocytes [25] and have reduced levels of *Il7* mRNA in their bone marrow [109]. *Rarg*$^{-/-}$ mice also develop a myeloproliferative-like syndrome (MPS) exhibiting increased granulocyte/macrophage progenitors and granulocytes in bone marrow, peripheral blood, and spleen [106]. Transplant studies revealed 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 [25]. Deletion of *Rarg*$^{-/-}$ in osterix-expressing osteoblast progenitor cells produced no hematopoietic phenotype and B lymphopoiesis was normal [25]. However, 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 [25]. 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.

7. B lymphocyte stimulation 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 via stimulation of osteoclastogenesis. 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 [115]. 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 [115]. 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 [99] and deletion of RANKL from B lymphocytes using CD19-Cre protected against ovariectomy-induced trabecular bone loss by impairing osteoclastogenesis [3]. In contrast, non-ovariectomised *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) [116]. 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 [116]. 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 emphasises 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 [117] and others that were recently reviewed by Manilay and Zouali [6].

8. Osteoblasts and multiple myeloma

Multiple myeloma is a malignancy of plasma cells in the bone marrow in which the tumour cells act on cells in the bone marrow microenvironment to create a favourable niche for tumour growth. Myeloma causes a destructive bone disease in up to 90% of patients [118] resulting in bone pain, hypercalcemia and pathological fractures severely reducing quality of life [119], and increasing the risk of death by 20% [120]. The bone disease develops due to an uncoupling of bone remodelling. Factors that stimulate formation and activity of osteoclasts sare secreted directly from myeloma cells and indirectly from osteoblasts following myeloma cell adhesion (e.g. RANKL, IL-6, IL-1β, IL-3, macrophage inhibitory protein 1α; MIP-1α, tumour necrosis factor α; TNFα). This results in enhanced resorption, formation of osteolytic lesions and loss of trabecular bone structure [121]. 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 [122-124]. 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 inhibits any osteoblast differentiation [125-127]. 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) [121, 125], promoting tumour 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 [20] implicating bone 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 [20]. 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 tumour expansion [20]. Chemotherapy resistance in myeloma is also conferred through myeloma adhesion to stromal cells via VCAM-1:VLA-4 binding and expression of microRNA-15a [128, 129]. While the role of osteoblasts and osteoclasts in myeloma dormancy is still under investigation, this clearly

demonstrates the processes of bone remodelling and myeloma growth are intricately intertwined.

9. Conclusions

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