A chemoattractant-guided walk through lymphopoiesis: from hematopoietic stem cells to mature B lymphocytes

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Abstract

B lymphocytes develop from hematopoietic stem cells (HSCs) in specialized bone marrow niches composed of rare mesenchymal-lineage stem/progenitor cells (MSPCs) and sinusoidal endothelial cells. These niches are defined by function and location: MSPCs are mostly perisinusoidal cells that together with a small subset of sinusoidal endothelial cells, express stem cell factor (SCF), Interleukin-7 (IL-7), IL-15, and the highest amounts of CXCL12 in bone marrow. Though rare, MSPCs are morphologically heterogeneous, highly reticular, and form a vast cellular network in the bone marrow parenchyma capable of interacting with large numbers of hematopoietic cells. HSCs, downstream multipotent progenitor cells (MPPs) and common lymphoid progenitor cells (CLPs) utilize CXCR4 to fine-tune access to critical short-range growth factors provided by MSPCs for their long-term maintenance and/or multilineage differentiation. In later stages, developing B lymphocytes use CXCR4 to navigate the bone marrow parenchyma, and predominantly Cannabinoid receptor −2 for positioning within bone marrow sinusoids, prior to being released into peripheral blood circulation. In the final stages of differentiation, transitional B cells migrate to the spleen where they preferentially undergo further rounds of differentiation until selection into the mature B cell pool occurs. This bottleneck purges up to 97% of all developing B cells in a peripheral selection process that is heavily controlled not only by the intensity of BCR signaling and access to BAFF, but also by the proper functioning of the B cell motility machinery.

1. Introduction

As if tuned to the popular Clash song "Should I stay or should I go?" lymphocytes are constantly on the move as they develop in primary lymphoid organs and survey secondary lymphoid organs. Over the last two decades, remarkable progress has been made in the understanding of guidance cues that lymphocytes follow in peripheral lymphoid organs during homeostasis and immune responses. Seminal and elegant studies have enabled a near complete temporal and spatial understanding of where lymphocytes reside and how they interact during the course of an immune response (Pereira, Kelly and Cyster, 2010;Qi, Kastenmuller and Germain, 2014). Lagging considerably behind is an understanding of where hematopoietic stem and progenitor cells (HSPC), from which these lymphocytes develop, reside and with what cells they interact as they make the lineage commitment to the lymphoid fate.

While obvious anatomic features like a red and a white pulp, or follicular structures, characterize secondary lymphoid organs, there are no obvious landmarks in the bone marrow environment other than bone and blood vessels. Furthermore, lymphocytes can be easily distinguished by immunostaining with antibodies against single epitopes, whereas HSPCs required for many years complex combinations of antibodies against a variety of cell surface receptors in order to allow their accurate visualization by flow cytometry or microscopy. A major breakthrough was the finding that the cell surface receptor CD150 (or Signaling Lymphocytic Activation Molecule Family member 1; SLAMF1) is expressed on hematopoietic stem cells (HSCs), and can be used in combination with a cocktail of antibodies recognizing receptors associated with cell lineage differentiation to identify a reasonably pure population of HSCs in bone tissue sections using only two fluorescence channels (Kiel et al., 2005). This finding enabled researchers to identify the microenvironments inhabited by HSCs within bone marrow (often called HSC niches), and helped define the migratory and positional cues followed by quiescent and active HSCs (Boulais and Frenette, 2015;Morrison and Scadden, 2014).

Of the many chemoattractants that are produced in bone marrow (Nevius, Gomes and Pereira, 2016), CXCL12 is not only the most abundant but also the most important chemokine for regulating HSC quiescence and differentiation known to date (Nie, Han and Zou, 2008;Sugiyama et al., 2006). CXCR4, the receptor for CXCL12, is expressed by more than 95% of hematopoietic cells in bone marrow including HSCs and hematopoietic progenitors, and is a pharmacological target for HSC mobilization from bone marrow into blood, in both mice and humans (Broxmeyer et al., 2005;Liles et al., 2003). CXCL12 is expressed by heterogeneous populations of cells: Mesenchymal stem and progenitor cells and sinusoidal endothelial cells, which express the highest amounts, as well as osteoblasts and certain hematopoietic cells, which express 100-1000 fold lower amounts. CXCL12 production is regulated by a variety of external cues ranging from circadian signals such as adrenergic hormones (e.g. norepinephrine) to inflammatory mediators (e.g. cytokines, proteases). Importantly, fluctuations in CXCL12 production such as those seen during infection or sterile inflammation result in massive alterations in HSPC distribution between bone marrow and blood, and correlate with shifts in hematopoietic lineage output (Ueda, Kondo and Kelsoe, 2005;Ueda et al., 2004).

Do migratory cues regulate hematopoietic cell lineage decisions? If so, how? In this review we aim to present an integrated model of how chemotactic cues act together with differentiation, proliferation and survival factors to support the generation of mature B lymphocytes. We will start by reviewing the niches and signals regulating HSC maintenance and differentiation into lymphoid lineages in bone marrow, and then consider how cell movement within bone marrow might enable proB and preB cells to balance cell proliferation with RAG-mediated immunoglobulin V(D)J gene recombination. We will also discuss the mechanisms and routes B-lineage cells (and other hematopoietic cells) utilize for exiting bone marrow. Finally, we will review the role played by chemoattractant receptors and components of the B cell motility machinery in the differentiation of immature, transitional B cells into the mature B cell compartment.

2. Hematopoietic stem cell niches

HSCs are responsible for sustaining the lifelong production of blood cells in the process known as hematopoiesis. In order to sustain lifelong hematopoiesis, HSCs have to be maintained in a largely quiescent state in order to be protected from genotoxic harm, while dividing occasionally to self-renew or differentiate. Over the past two decades intense research has been devoted to the characterization of extracellular signals promoting HSC maintenance, bone marrow niches colonized by HSCs, and cells involved in the regulation of HSC quiescence and activation. The cells that provide essential factors for HSC maintenance constitute what is called the "HSC niche," which is defined by both location and function. The niche influence on HSCs is thought to be highly dependent on proximity and even direct HSC-niche cell interaction. This is because several signals produced by niche cells that are required for the long-term maintenance of HSCs act as short-range signals. Earlier studies suggested that HSCs tend to localize near the endosteum and are regulated by bone-producing osteoblasts (Calvi et al., 2003;Zhang et al., 2003). However, recent studies from several laboratories made compelling observations that are incompatible with a major role played by osteoblasts, and support an alternative model, which is described below. For an historical overview of the bone marrow niche hypothesis initially proposed by Schofield, and of the data in favor of and against the osteoblastic niche model, the reader is referred to other reviews that have been written on the subject (Boulais and Frenette, 2015;Morrison and Scadden, 2014).

Two factors, CXCL12 and SCF, play vital roles in the long-term maintenance of HSCs. Nagasawa and Morrison exploited this fact to identify HSC niche cells by generating CXCL12 and SCF fluorescent reporter mouse strains (Ding and Morrison, 2013;Ding et al., 2012;Sugiyama et al., 2006). This simple and elegant approach enabled the identification of a relatively rare but highly interconnected network of perisinusoidal cells of mesenchymal lineage, and sinusoidal endothelial cells, that express high amounts of SCF and CXCL12. Furthermore, the mesenchymal lineage cells also express Leptin receptor (LEPR), which allowed the use of the *Lepr*-cre transgene to enforce genetic deletion of *Scf* or *Cxcl12* from these cells. Together with the use of mice expressing cre recombinase in endothelial cells, these experiments demonstrated that HSC niche function is predominantly carried out by perisinusoidal mesenchymal cells and a small subset of sinusoidal endothelial cells (Ding and Morrison, 2013;Ding et al., 2012;Greenbaum et al., 2013), a conclusion supported by another study which used a different promoter to target mesenchymal cells (Greenbaum 2013). These results agreed with prior (and subsequent) studies showing a preferential localization of HSCs in the perisinusoidal compartment (Acar et al., 2015;Chen et al., 2016;Kiel et al., 2005;Sugiyama et al., 2006). Further elegant studies by Nagasawa and Frenette revealed that the mesenchymal-lineage cells with HSC niche activity are in fact mesenchymal stem/progenitor cells (MSPC) capable of undergoing differentiation into adipocytic, osteoblastic, and chondrocytic cell lineages (Mendez-Ferrer et al., 2010;Omatsu et al., 2010). In agreement with these findings, fate mapping of *Lepr*-Cre+ cells also revealed MSPC activity in HSC niche cells (Zhou et al., 2014). Interestingly, the MSPC differentiation potential is partly co-regulated with SCF and CXCL12 expression by the transcription factor FOXC1 (Omatsu et al., 2014).

2.1 A quiescent niche for quiescent HSCs?

In multicellular organisms, cellular differentiation is often preceded by cell division. This is certainly the case with hematopoiesis, where HSC differentiation takes place after cell cycle activation (Li and Clevers, 2010). Which signals instruct HSCs to self-renew and which promote HSC differentiation still remains poorly understood, but it has been postulated that distinct bone marrow niches might regulate distinct HSC fates. Evidence for this model came from the identification of two distinct *Nestin*-GFP positive mesenchymal lineage cell populations that differed in positional distribution, quiescence, and expression of HSC maintenance genes—an extremely rare population of *Nestin*-GFP^{bright} cells ($< 0.002\%$) that is periarteriolar and expresses pericyte markers (Neural/Glial antigen-2, NG2, and alpha smooth muscle actin), also named Nes^{peri}, and a more abundant but less quiescent population of perisinusoidal and reticular *Nestin*-GFP^{dim} cells, also named Nes^{retic}, that does not express NG2 but expresses LEPR (Kunisaki et al., 2013). In situ analyses of quiescent 5-ethynyl-2′-deoxyuridine (EdU)-retaining HSCs revealed that EdU$^+$ HSCs reside in the vicinity of Nes^{peri} cells. In contrast, active HSCs (EdU$^-$ HSCs, or that expressed Ki67) were found in non-defined areas farther away from arterioles, raising the possibility that distinct bone marrow niches control HSC quiescence and activation. In support of the model "quiescent niche for quiescent HSCs," selective ablation of the Nes^{peri} population led to a reduction in HSC number. Furthermore, nearly 50% of the remaining HSCs were in active cell cycle, suggesting a loss of quiescence. This finding is consistent with results from RNA-sequencing analysis showing that HSC maintenance genes (e.g. CXCL12, SCF) are more abundantly expressed in Nes^{peri} cells than in Nes^{retic} cells (Kunisaki et al., 2013). A careful analysis of the gene expression profile of NG2$^+$ Nes^{peri} and NG2$^-$ Nes^{retic} cells revealed, however, that NG2 (gene symbol *Cspg4*) expression is in fact higher in samples named Nes^{retic} than in those named Nes^{peri}, and reciprocally, that *Lepr* expression is considerably higher in samples named as Nes^{peri} than in those named Nes^{retic} (our unpublished analyses of gene expression dataset deposited in Gene Expression Omnibus under accession number GSE48764). This discrepancy between gene expression profile and phenotype of Nes^{peri} and Nes^{retic} cells suggests that samples named Nes^{peri} are in fact Nes^{retic} cells, and vice-versa. Furthermore, the conditional deletion of *Scf* or *Cxcl12* from Nes^{peri} cells using the *NG2-CreER* transgene did not affect HSC numbers in bone marrow (Acar et al., 2015), thus questioning the physiological relevance of SCF and CXCL12 produced by Nes^{peri} cells. Two recent studies that used mouse reporters for α-catulin (gene symbol *Ctnnal1*) and *Hoxb5*, which allowed deep imaging of HSCs in optically cleared long-bone bone marrow by confocal microscopy, have further challenged the "quiescent niche for quiescent HSCs" model. HSCs marked by α-catulin-GFP are highly enriched for long-term HSCs and when α-catulin-GFP+ and cKit+ HSCs were visualized in situ they were predominantly found within < 5 μm of both CXCL12$^+$ and Lepr$^+$ cells (Acar et al., 2015). In agreement with this study, HSCs marked by triple mCherry expression driven by *Hoxb5* regulatory elements, an HSC subset that is also highly enriched for long-term, presumably quiescent, HSCs, were also predominantly found in direct contact with bone marrow endothelial cells but without an obvious preference for arterioles (Chen et al., 2016). Hence, the exact contribution of periarteriolar pericytes to HSC maintenance remains unclear.

It is possible that the cell ablation strategy used in the Kunisaki et al. study caused indirect effects that in turn affected HSC maintenance. This could have occurred in at least three ways: 1) inflammation, 2) HSC niche alterations, and 3) changes in nutrient access or availability. 1) Tissue inflammation is likely to occur when large numbers of apoptotic cells are generated in a somewhat synchronous manner such that tissue resident phagocytes are unable to clear them effectively. Apoptotic debris is often inflammatory (Nagata, Hanayama and Kawane, 2010) as it contains a variety of ligands for cell extrinsic and intrinsic sensors, (e.g., Toll like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-, leucine-rich repeat–containing receptors, RIG I Like Receptors, and other cytosolic sensors of nucleic acids) that lead to the production of type I and type II interferons (Nathan and Ding, 2010). Given the normal physiological response of HSCs to inflammatory mediators (Baldridge et al., 2010;Essers et al., 2009), it can be difficult to deduce the role of the ablated cell population(s) in HSC maintenance simply by studying changes in HSC numbers and activation status. 2) Alterations in non-targeted HSC niches may also occur when cell ablation strategies are used (e.g. global gene expression changes may have occurred in Nes[retic] cells when Nes[peri] cells were ablated.), which in turn could affect HSC numbers in bone marrow. Finally, 3) HSC maintenance and fitness is dependent on essential amino acids, such as Valine (Taya et al., 2016), and it is at present unclear whether cell ablation strategies directly or indirectly affect access to specific nutrients in bone marrow.

2.2 HSC niche: a multilayered cellular network

Besides SCF and CXCL12, several other factors, not necessarily produced by perisinusoidal mesenchymal and sinusoidal endothelial cells, have been proposed to regulate HSC numbers in bone marrow. Glial fibrillary acidic protein (GFAP)-expressing non-myelinating Schwann cells express integrin β8, which converts latent TGFβ into active TGFβ that signals through TGFβR2 expressed on HSPCs to promote quiescence (Yamazaki et al., 2011). GFAP^{+} cells ensheath sympathetic nerves in bone marrow, are found in close association with Nes[peri] cells (Kunisaki et al., 2013), and are in direct contact with a significant proportion of HSCs (Yamazaki et al., 2011). While non-myelinating Schwann cells do not seem to express TGFβ, megakaryocytes have been identified as the main source of TGFβ for HSCs in bone marrow (Zhao et al., 2014). Besides TGFβ, megakaryocytes express platelet factor 4 (PF4, also known as CXCL4) that regulates the HSC cell cycle and acts to promote HSC quiescence and long-term maintenance, though the receptor on HSCs for PF4 remains to be identified (Bruns et al., 2014). Megakaryocytes also express Thrombopoietin (THPO), which is important for maintaining HSC quiescence, and possibly retention at HSC niches (Nakamura-Ishizu et al., 2014;Nakamura-Ishizu et al., 2015;Qian et al., 2007;Yoshihara et al., 2007). In humans, mutations in the *c-mpl* gene (encodes the THPO receptor MPL) lead to the disease congenital amegakaryocytic thrombocytopenia, characterized not only by hypomegakaryocytic thrombocytopenia, but also by a reduction in CD34^{+} hematopoietic progenitor cells in bone marrow and defective multilineage differentiation capacity (Ballmaier et al., 2003). As THPO plays an important role in megakaryocyte development it is unclear the extent to which its effects on HSC maintenance are direct or indirect. In any case, THPO, PF4 and TGFβ, likely act as short-range signals for HSC maintenance given the fact that many HSCs are often found in non-random and close proximity to megakaryocytes under homeostasis (Bruns et al., 2014;Zhao et al., 2014).

It should be mentioned, however, that several of the studies described above relied on a combination of experimental strategies that warrant concern (specifically, megakaryocyte ablation in vivo, and conditional deletion of *Tgfb1* using a *Pf4*-cre transgene). As previously discussed, cell ablation strategies may cause indirect effects that affect HSC quiescence. Furthermore, the specificity of *Pf4*-cre activity is questionable given the fact that *Pf4* expression is readily detected at high amounts in *Scf*-GFP$^+$ cells and in *Nestin*-GFP$^+$ cells (our unpublished analyses of gene expression dataset deposited in Gene Expression Omnibus under accession numbers GSE48764 and GSE33158). This raises the concern that *Pf4*-cre not only ablated megakaryocytes (or deleted *Tgfb1* in megakaryocytes) but also mesenchymal-lineage HSC niche cells.

2.3 CXCR4 as a rheostat for HSC quiescence versus differentiation

Of all extracellular signals that regulate HSC homeostasis, membrane-bound SCF (mSCF) is arguably the most important cytokine (Driessen, Johnston and Nilsson, 2003;Flanagan, Chan and Leder, 1991), and requires direct cell-cell interactions for engaging cKit on HSCs. On the other hand, secreted factors (e.g. THPO, TGFβ, PF4), while not requiring HSCs to be in direct contact with niche cells, presumably form concentration gradients due to a combination of diffusion and consumption. Hence, we argue that the function of CXCL12 is primarily to enable HSCs to position close to HSC niches in order to access critical niche factors that enforce quiescence. Based on this hypothesis, we propose a mechanism by which HSC quiescence (G0) or activation (G1 + S + G2/M), which may lead to self-renewal or differentiation, can be regulated: CXCL12 expression in bone marrow fluctuates in a circadian manner, leading to HSC release from, and homing back to the bone marrow niche (Mendez-Ferrer et al., 2008). Decreases in bone marrow CXCL12 during the day lead to HSC release from bone marrow niches and hence uncoupling from quiescence signals (G0), allowing them to enter into the G1 stage of the cell cycle, while increases in CXCL12 at the end of the day draw HSCs back to their bone marrow niches, where they return to a quiescent G0 state. In this model, an inability of HSCs to leave HSC niches can be just as detrimental as the inability of HSCs to home to the niche, as these HSCs will not be able to escape from quiescence signals to divide and differentiate. In favor of this model, patients with WHIM syndrome (Warts, Hypogammaglobulinemia, Infection, Myelokathexis), a disease caused by mutations in CXCR4 desensitization that leads to constitutive signaling, are characterized by pan-leukopenia that is only attributed to increased bone marrow retention of differentiated leukocytes (Balabanian et al., 2005;Gorlin et al., 2000;Hernandez et al., 2003). Interestingly, the loss of the heterozygous *CXCR4* mutant allele in a single HSC of a WHIM patient, in a serendipitous chromothriptic event, allowed the recovery of the patient's hematopoietic system, with the exception of lymphocytes (McDermott et al., 2015). This strongly suggested that this *CXCR4* haploinsufficient HSC gained a significant competitive advantage over *CXCR4* mutant HSCs in both self-renewal and differentiation, besides accelerated egress from bone marrow. Thus, we propose at least one additional role for the physiologic recirculation of HSCs (Wright et al., 2001)—that it enables a balance between HSC quiescence, activation, and possibly differentiation, in order to maintain lifelong hematopoiesis in organisms. This model may seem incompatible with recent observations that point to a very limited number of cell divisions that HSCs undergo during an organism's lifetime (Bernitz et al., 2016), but is consistent with the widespread notion

that more than 10% of HSCs are Ki67^{+} (and thus not in G0) at any given time (Kunisaki et al., 2013;Sugiyama et al., 2006).

Finally, it should be mentioned that besides mediating the homing, retention and maintenance of bone marrow HSCs, CXCR4 signaling itself has also been suggested to control cell cycle gene expression and in this manner to regulate HSC quiescence and proliferation. The deletion of CXCR4 in hematopoietic cells resulted in increased proliferation of HSCs in vivo (Nie, Han and Zou, 2008;Sugiyama et al., 2006), and HSCs cultured in vitro in the presence of CXCL12 were more quiescent than HSCs cultured without it (Nie, Han and Zou, 2008;Tzeng et al., 2011). In conflict with these findings, however, other studies showed that deletion of CXCL12-expressing cells in bone marrow resulted in increased HSC quiescence, while in vitro culture of HSCs with CXCL12 increased the expression of genes associated with cell cycle entry (Omatsu et al., 2010). It should be noted that in vitro studies on HSC quiescence are difficult to interpret considering the vastly different environment that tissue culture represents in contrast to the bone marrow microenvironment. Hence, it remains unclear if CXCR4 signaling itself plays any direct role in regulating HSC quiescence.

3. Hematopoietic Multipotent Progenitor niches

While there has been an abundance of studies investigating the HSC niche in bone marrow, studies looking into the niches for MPP maintenance and differentiation lag considerably behind. Early studies suggested that HSCs and their immediate progeny colonize distinct bone marrow niches upon transplantation, leading to the suggestion that separate niches might orchestrate HSC maintenance and differentiation (Czechowicz et al., 2007; Lo Celso et al., 2009). Which niches are colonized by short-term HSCs and MPPs, and what signals are provided therein, were not examined. But, like long-term HSCs, ST-HSCs and MPPs express surface receptors for the same critical signals that govern the homeostasis of LT-HSCs (e.g. cKit, MPL, CXCR4), and are likely dependent on proper signaling from these receptors for their development, maintenance and retention. Evidence for this model came from analyses of mice conditionally deficient in CXCR4 in MPP cells. Because MPPs express FLT3 (encoded by *Flk2*) while HSCs do not, MPPs and essentially all downstream hematopoietic cells can be genetically manipulated with a *Flk2*-cre transgene (Boyer et al., 2011). It was observed that when MPPs lack CXCR4 they were significantly reduced in bone marrow, and differentiated poorly into downstream hematopoietic cells (Cordeiro Gomes et al., 2016). The numerical reduction of MPPs observed in bone marrow was probably a consequence of reduced retention and reduced access to niche signals. Indeed, MPPs reside in very close proximity to mesenchymal lineage cells marked by IL-7 expression, a subset of LEPR^{+} cells that not only expresses significant amounts of mSCF but also the highest amounts of CXCL12 in bone marrow. This subset of IL-7-expressing LEPR [+] cells also exhibits multilineage differentiation potential (e.g. osteoblasts, osteocytes and adipocytes) in vivo, giving strength to the model proposed by Frenette and colleagues, and discussed earlier in this chapter, that the HSC niche is a partnership between two types of stem cells (Frenette et al., 2013). Besides MPPs, HSCs could also be found in significant proximity to IL-7^{+} cells, and occasionally an MPP and an HSC were found in the vicinity of the same IL-7^{+} cell (Cordeiro Gomes et al., 2016). Both HSCs and MPPs are dependent on

the short-range signals, mSCF and CXCL12 provided by IL-7$^+$ cells for their maintenance, suggesting that overlapping niches control HSC maintenance and multilineage differentiation (Figure 1). Consistent with this model, MPPs require FLT3L for development, maintenance, or multilineage differentiation (McKenna et al., 2000;Sitnicka et al., 2002), and although the physiological source of FLT3L for MPPs has not been defined, *Nestin*-GFP$^+$, IL-7$^+$ LEPR$^+$, and *Scf*-GFP$^+$ mesenchymal cells express *Flt3l* mRNA (our unpublished observations).

4. Bone marrow niches controlling lymphoid-lineage commitment

MPPs can give rise to the myeloid or lymphoid lineages by differentiating into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs) respectively. CLPs can be divided into Ly6D$^-$ CLPs, which give rise to all lymphoid lineages, and Ly6D$^+$ CLPs, which differentiate from Ly6D$^-$ CLPs and together with CD93$^+$ CLPs mark the earliest B-lineage committed progenitors (Inlay et al., 2009;Li et al., 1996).

4.1 IL-7 acts as B cell lineage commitment factor

In adult mice, and possibly in adult humans as well, B and T lymphocyte development are highly dependent on IL-7. The critical importance of IL-7 for B cell development can be seen by the severe lymphopenia and complete lack of B lymphopoiesis in the bone marrow of *Il7*$^{-/-}$ or *Il7ra*$^{-/-}$ adult mice (Carvalho et al., 2001;Kikuchi et al., 2005;von Freeden-Jeffry et al., 1995). The developmental block in B lymphopoiesis occurs at the Ly6D$^-$ to Ly6D$^+$ transition, as *Il7*$^{-/-}$ mice have a two-fold reduction in Ly6D$^-$ CLPs, but have an over twenty-fold reduction in Ly6D$^+$ CLPs (Tsapogas et al., 2011).

In order to commit to the B-lymphoid fate, the transcription factors PU.1, Ikaros, E2A, EBF1 and Pax5 are critical. PU.1 and Ikaros are important at the earliest stages for commitment to the lymphoid lineage, while E2A, EBF1 and Pax5 are the critical transcription factors for the commitment of CLPs to the B cell fate (Busslinger, 2004;Medina et al., 2004). E2A-deficient and EBF1-deficient mice have a very early block in B lymphopoiesis, prior to D-J rearrangement of the IgH locus, and expression of B-lineage associated genes like *Pax5, Rag1* and *mb1* in fetal liver and bone marrow was significantly reduced compared to WT (Bain et al., 1994;Lin and Grosschedl, 1995;Zhuang, Soriano and Weintraub, 1994). In addition, EBF1 seems to also play a role in maintaining B lineage commitment as deletion of EBF1 in pro-B cells led to loss of repression of genes associated with alternative lymphoid fates (Nechanitzky et al., 2013). PAX5 acts downsteam of EBF1 and E2A, and its expression and function are contingent on EBF1 (Medina et al., 2004). PAX5-deficient mice were blocked at a later stage of B lymphopoiesis, with largely normal D-J rearrangements, but no detectable V-DJ rearrangements (Nutt, Thevenin and Busslinger, 1997). PAX5 is critical for establishing B cell commitment by restricting alternative lineage choices in early progenitors and initiating the B cell transcriptional program, such as expression of CD19 and λ5, and *Pax5*$^{-/-}$ proB cells were blocked from further B lineage development, but retained ability to differentiate into alternative lineages, such as natural killer (NK) cells, macrophages, and granulocytes (Nutt, Rolink and Busslinger, 1999;Nutt, Thevenin and Busslinger, 1997).

Ebf1 and *Pax5* expression are highly dependent on IL-7 and intact IL-7Ra signaling (Dias et al., 2005;Kikuchi et al., 2005;Tsapogas et al., 2011), and the few remaining Ly6D$^+$ CLPs in *Il7*$^{-/-}$ mice retain NK and dendritic cell (DC)-differentiation potential but have severely reduced B-cell differentiation potential (Dias et al., 2005). Importantly, overexpression of *Ebf1* rescues the B cell differentiation defect in *Il7*$^{-/-}$ CLPs (Dias et al., 2005), and constitutive activation of STAT5, a downstream target of IL-7R signaling, restores *Ebf1* and *Pax5* expression (Kikuchi et al., 2005). Hence, it appears that IL-7R signaling acts to drive *Ebf1* expression, which then activates *Pax5* expression to reinforce commitment to the B cell fate. EBF1 also drives increased expression of *Il7ra* through FOXO1 (Dengler et al., 2008), and therefore perpetuates a feedforward cycle to enforce the B cell fate. IL-7 is therefore an instructive signal that controls differentiation of CLPs to the B cell fate.

4.2 IL-7-producing cells in bone marrow

Given the critical importance of IL-7 in commitment to the B cell fate, cells that form the bone marrow niche for early B lymphopoiesis must express IL-7. Multiple studies have suggested that osteoblasts and/or osteolineage cells form the niche for B lymphopoiesis in bone marrow. Ablation of osteoblasts in mice expressing herpes virus thymidine kinase under control of the 2.3Kb rat collagen α1 type 1 promoter (Col2.3) by treatment with ganciclovir resulted in significant reductions in B cell precursors in bone marrow (Zhu et al., 2007), while ablation of *Osx-cre*-expressing osteolineage cells resulted in significantly reduced CLP numbers in bone marrow (Terashima et al., 2016). Furthermore, deletion of CXCL12 from osteoblasts using Col2.3-driven cre recombinase (Ding and Morrison, 2013), or IL-7 using tamoxifen-inducible *Osx*-creER and *Ocn*-cre (Terashima et al., 2016), also resulted in a significant reduction in the number of bone marrow CLPs. Deletion of the small G alpha stimulatory (Gαs) protein from osteolineage cells using Osx-cre also resulted in significant reductions in total B cells in bone marrow (Wu et al., 2008).

It has to be noted, however, that many of the cell targeting strategies used in these studies may have targeted MSPCs in addition to osteoblasts. Firstly, Col2.3-driven cre recombinase may be active in a small percentage of MSPCs as *Col1a1* transcripts are abundant in MSPCs. Second, tamoxifen-induced *Osx*-driven creER recombinase activity marks MSPCs in both fetal and perinatal bone marrow, but is restricted to osteoblasts in adult mice treated with tamoxifen at 8 weeks of age (Mizoguchi et al., 2014). Importantly, *Osx*-driven creER recombinase activity is barely detected in osteoblasts in mice treated with tamoxifen at 6 weeks of age (Yu et al., 2016). Therefore, deletion of Gαs from *Osx*-cre expressing cells would have deleted Gαs in MSPCs, while tamoxifen-induced *Osx*-driven creER-mediated deletion of *Il7* in 6 week old mice is also likely to have deleted *Il7* from MSPCs. Furthermore, *Ocn*-cre has also been shown to target about 70% of CXCL12-expressing cells in bone marrow (Zhang and Link, 2016), and this undesired effect most likely explains the phenotype seen in mice carrying a conditional *Il7* deletion in *Ocn*-expressing cells (Terashima et al., 2016). Finally, as discussed before, the reductions in bone marrow B cells upon osteoblast ablation could be the result of inflammatory signals due to synchronized death of large populations of cells. Furthermore, inflammation is known to inhibit B cell development in bone marrow (Ueda et al., 2004).

Of all the hematopoietic lineages, B lymphocytes seem to be the most critically dependent on CXCR4 and CXCL12 for efficient development in bone marrow. Inducible deletion of *Cxcr4* in HSCs and downstream differentiated hematopoietic cells led to a profound reduction in B cell production (Nie, Han and Zou, 2008;Sugiyama et al., 2006). This defect was presumably at an early developmental stage given the fact that conditional *Cxcr4* deletion in proB and subsequent developing B cell stages did not cause major differences in total B cell production (Nie et al., 2004). Evidence for this model came from a recent study that looked at the effects of *Cxcr4* deletion at the CLP stage using the *Il7ra*-cre transgene. Deletion of CXCR4 at the CLP stage caused a profound reduction in Ly6D$^+$ CLPs in bone marrow, which resulted in a significant reduction of B, T and NK cells in the periphery (Cordeiro Gomes et al., 2016), a phenotype that remarkably resembles that of IL-7-deficient mice, with the exception of NK cell reduction. Although these results raised the possibility that CXCR4 deficiency prevents CLPs from gaining sufficient access to IL-7, a previous study examining IL-7 and CXCL12 expression in situ suggested that distinct stromal cells either expressed IL-7 or CXCL12, but not both (Tokoyoda et al., 2004). Thus, to gain further insight into CLP positioning in bone marrow, the positioning of CXCR4-sufficient and –deficient CLPs relative to IL-7-expressing cells in bone marrow was analyzed. CXCR4-sufficient Ly6D$^+$ CLPs in bone marrow were found in close proximity to IL-7-expressing cells, while CXCR4-deficient Ly6D$^-$ CLPs were positioned further away. Similarly, treatment with the CXCR4 antagonist AMD3100 shifted Ly6D$^+$ cells away from IL-7-expressing cells. In support of these findings, phosphorylated STAT5α, a downstream mediator of IL-7R signaling, was reduced in *Cxcr4*$^{-/-}$ Ly6D$^+$ CLPs compared to WT Ly6D$^+$ CLPs (Cordeiro Gomes et al., 2016). Furthermore, when *Il7*$^{GFP/+}$ mice were crossed with *Cxcl12*$^{DsRed/+}$ mice, it was observed that the IL-7-expressing cells comprise the highest producers of CXCL12 in vivo. Although we could not detect IL-7-expressing cells in situ using an anti-IL-7 antibody (a polyclonal antibody that blocks the biological activity of IL-7), detection of *Il7*-GFP$^+$ cells was possible albeit difficult, and required signal amplification with anti-GFP antibodies, most likely because IL-7 is expressed in very low amounts in vivo, and is likely rapidly consumed by IL-7R-expressing cells. Hence, positioning close to cellular sources of IL-7 is critical for CLPs to receive sufficient IL-7R signaling. Therefore, the role of CXCL12 and CXCR4 in early B lymphopoiesis appears to be for the guidance of CLPs to IL-7-expressing cells in bone marrow (Figure 1), in order for IL-7R signaling to drive lymphoid precursor commitment to the B cell fate. As previously discussed, NK development is independent of IL-7 but requires IL-15 (Kennedy et al., 2000). Of note, CXCR4-CXCL12 are required for NK cell development presumably because CXCL12-producing mesenchymal cells express IL-15 (Noda et al., 2011). Similar to IL-7 and SCF, IL-15 also acts in a short-range manner given that its biological effects require trans presentation through binding to IL15Ra (Dubois et al., 2002).

5. Transition from proB to preB to immature B cell

5.1 ProB and PreB cell niches in bone marrow

As B cells develop from CLPs they undergo several differentiation stages that are characterized by the cell cycle activity, the status of RAG-mediated V(D)J recombination, and cell surface receptor expression. Over the past several decades, a few studies examined

the positional distribution of developing B cell subsets in the bone marrow. Landmark studies by Osmond and Batten using in vivo perfusion of radiolabelled antibodies against IgM showed that about 70% of IgM$^+$ cells were located inside the bone marrow parenchyma and that 30% resided in intravascular niches in bone marrow (Batten and Osmond, 1984;Osmond and Batten, 1984). Subsequent studies used immunofluorescent microscopy to characterize the distribution of proB, preB and immature B cells by tracking cells that express nuclear terminal deoxynucleotidyl transferase (TdT, which are mainly proB), or that express the immunoglobulin (Ig) heavy but not the kappa light chain (mostly preB cells), or that express heavy and kappa light chains (a combination of immature and mature B cells). It was noted that all TdT+ proB cells and preB cells resided in the parenchyma, with an apparent enrichment near the sub-endosteal area (Hermans, Hartsuiker and Opstelten, 1989). In contrast, immature and mature B cells were more centrally located near the large collecting central sinusoid, and up to 30% of these cells were inside bone marrow sinusoids, the latter observation in agreement with Osmond and Batten's observations (Hermans, Hartsuiker and Opstelten, 1989).

5.2 Control of RAG gene expression by bone marrow niches

As studies on molecular requirements for B cell development led to the finding that IL-7 is an essential cytokine for B and T lymphopoiesis (Grabstein et al., 1993; Namen et al., 1988a;Namen et al., 1988b;Peschon et al., 1994;von Freeden-Jeffry et al., 1995), and that ProB cells are particularly dependent on IL-7 for survival and proliferation (Milne and Paige, 2006), it led to studies on the positional distribution of proB and preB cells in relationship to IL-7-producing cells in bone marrow. Taking advantage of a mouse reporter for CXCL12, Nagasawa and colleagues identified two types of stromal cells that either expressed IL-7 or CXCL12 but not both. While proB cells identified by co-expression of B220 and cKit were mostly found in proximity to IL-7$^+$ cells, preB cells identified by B220 and IL-7R expression were found predominantly away from both IL-7$^+$ cells and CXCL12$^+$ cells (Tokoyoda et al., 2004). This model of a stage-dependent niche for B lymphopoiesis gained further traction with the finding that in preB cells, high levels of IL-7 receptor signaling negatively regulated RAG-mediated Ig light chain gene recombination. As preBCR signaling induced activation of IRF4 and CXCR4 expression, it was postulated that an increase in CXCR4 would allow preB cells to migrate away from IL-7 producing cells and possibly towards CXCL12 producing cells (Johnson et al., 2008;Ochiai et al., 2012).

Although structural studies predicted that the preBCR is unable to recognize antigens (Bankovich et al., 2007), a study proposed that the preBCR can physically interact with the stromal-associated lectin Galectin-1 and activate signal transduction (Elantak et al., 2012;Gauthier et al., 2002). In situ analyses of sort-purified preB cells that were transferred into recipient mice showed a close association between preB cells and Galectin-1+ stromal cells that did not express IL-7 but expressed CXCL12 in bone marrow (Mourcin et al., 2011). However, the strategy used by the Tokoyoda study for distinguishing proB (B220 and cKit) from preB (B220 and IL7R) cells would hardly resolve between these two subsets because proB cells express significantly higher amounts of IL-7R than preB cells. Furthermore, given the heterogeneity, broad distribution, and highly reticular morphology of IL-7 and CXCL12 expressing cells it is unclear whether proB and preB cell proximity to

these mesenchymal cells is random or specifically enforced by chemotactic and adhesion mechanisms. This is an especially relevant concern given the finding that ~89% of randomly selected cells in bone marrow parenchyma are within < 5μm from the nearest *Cxcl12*-dsred[Hi] cell (Acar et al., 2015). Finally, as mentioned previously, the large majority of IL-7^{+} cells express the highest amounts of CXCL12 in bone marrow (Cordeiro Gomes et al., 2016), and so it would seem plausible that a preBCR-driven increase in CXCR4 expression would enforce preB cells to position in the vicinity of IL-7^{+} cells.

5.3 Is there a bone marrow niche for preB cell leukemias?

The strict regulation of RAG expression and activity likely protects developing lymphocytes from the potentially catastrophic genomic instability that can be brought about from genome-wide RAG activity (Teng et al., 2015). As IL-7R signaling suppresses *Rag1* and *Rag2* gene expression (Amin and Schlissel, 2008;Herzog et al., 2008;Llorian et al., 2007;Verkoczy et al., 2007), it suggests that preB cell interactions with bone marrow niches may act as a rheostat for the regulation of *Rag1* and *Rag2* expression. The transcription factor Ikaros is a major regulator of B cell development with a strong impact at the preB to immature B cell transition. Ikaros regulates the expression of several genes required for Ig light chain gene recombination, including the expression of genes in IL-7 receptor and preBCR signal transduction pathways (Heizmann, Kastner and Chan, 2013). Ikaros also regulates the expression of a variety of genes involved in cell migration and adhesion to the extracellular matrix, such that Ikaros-deficient B lineage cells are unable to migrate towards CXCL12 and are aberrantly adherent to VCAM-1-coated surfaces in vitro (Joshi et al., 2014;Schwickert et al., 2014). The impact of aberrant communication between early B-lineage cells and bone marrow niches is unclear, but is particularly relevant given the recent findings of RAG-mediated oncogenic translocations driving the development of preB acute lymphoblastic leukemias (Nussenzweig and Nussenzweig, 2010;Swaminathan et al., 2015), as it suggests that certain bone marrow niches may favor the onset of some preB acute lymphoblastic leukemias. In support of this model, an association between *Cxcr4* polymorphisms and chronic lymphocytic leukemia has been proposed (Crowther-Swanepoel et al., 2009). Thus, understanding the positional distribution of Rag-expressing proB and preB cells in relationship to bone marrow mesenchymal-lineage cells is of utmost importance as it remains controversial and largely unresolved.

6. Egress from bone marrow

6.1 Retention mechanisms

Developing B cells, and most prominently immature B cells, are highly dependent on integrin α4β1-mediated adhesion to VCAM-1 (and possibly fibronectin) for movement within parenchyma and retention inside bone marrow sinusoids (Beck et al., 2014). Integrin α4β1 requires chemokine receptor signaling for inside-out integrin transactivation, and in developing B cells this is predominantly controlled by CXCR4 signaling (Glodek et al., 2003). CXCL12 induces integrin α4β1 transactivation and promotes strong adhesion to VCAM-1, possibly through phosphorylation and activation of focal adhesion kinase (FAK) (Glodek et al., 2003). FAK is a cytoplasmic protein tyrosine kinase that accumulates within the cell at sites of attachment to the extracellular matrix and its phosphorylation and

activation are predominantly mediated by integrin signaling (Schaller, 2001). In B cells, FAK deficiency causes a mild reduction of developing B cell subsets in bone marrow (Park et al., 2013), a phenotype that is somewhat reminiscent of that seen in mice lacking integrin β1 in developing B cells (Pereira et al., 2009a). Within the femur, proB cells (identified by a non-selective method that relied on co-expression of B220 and CD43) were found enriched in the metaphysis, while in the diaphysis proB cells tended to localize in proximity to the endosteal region (Park et al., 2013). Such bone-proximal positioning was dependent on B-cell-intrinsic FAK (Park et al., 2013) and differed from the perisinusoidal distribution of mature B cells (Cariappa et al., 2005;Park et al., 2013). In rats, TdT-expressing cells also localized in sub-endosteal niches in bone marrow (Hermans, Hartsuiker and Opstelten, 1989), which suggests a non-random localization of proB cells near bone, possibly due to differential expression of α4β1 integrin ligands. A study suggested that Suppressor Of Cytokine Signaling-3 (SOCS3) associates with and negatively regulates CXCR4 signaling in a human B cell line (Soriano et al., 2002). Furthermore, short-term treatment with CXCL12 promoted the association between SOCS3 and FAK, and SOCS3 over-expression in a B cell line prevented CXCR4-mediated chemotaxis (Le et al., 2007). These studies suggested a model in which CXCR4-FAK-α4β1pathway is negatively regulated by SOCS3. In vivo, conditional SOCS3 deletion in a variety of cells, including B-lineage cells, resulted in an accumulation of immature B cells in bone marrow parenchyma, which lent support to that model (Le et al., 2007). However, two independent studies on the effects of B-lineage cell intrinsic deficiency in SOCS3 failed to detect measurable alterations in B cell development. Furthermore, careful analyses of B-lineage positioning, migration, or egress from bone marrow failed to reveal significant differences between *Socs3*-deficient and wild-type B cells (Jones et al., 2011;Nadrah, Beck and Pereira, 2015).

As developing B cells reach the immature IgM-expressing stage they continue to downregulate CXCR4 expression and reduce their motility within bone marrow, a process that is prevented by BCR signaling (Beck et al., 2014). This change in CXCR4 expression and dynamic behavior alters the B cell morphology from an amoeboid and elongated form to a rounded cell that is often seen loosely adherent to the bone marrow extracellular matrix. BCR signaling can also induce FAK phosphorylation (Mlinaric-Rascan and Yamamoto, 2001), thus implicating a direct relationship between BCR signaling and developing B cell motility in bone marrow. Therefore, attenuation of BCR signaling with concomitant downregulation of CXCR4 expression, and possibly FAK phosphorylation, are the most likely explanation for reduced adhesion and motility of late-stage immature B cells within bone marrow. This form of integrin-dependent motility, however, also informs about the biophysical properties of the bone marrow microenvironment. When leukocytes are exposed to shear stress, such as when facing fluid flow as they move along the lumen of endothelial barriers, cells become highly dependent on integrin-mediated adhesion (also described as haptokinetic motility). In contrast, cell motility in shear-free environments (e.g. densely packed niches in secondary lymphoid organs) is largely independent of integrins and is predominantly controlled by coordinated actin-based leading edge protrusion and myosin-regulated rear end contraction. Thus, developing B cells and possibly hematopoietic cells are exposed to significant shear stress that is presumably caused by plasma exudation from fenestrated sinusoidal endothelium. Consistent with this possibility, during the course of

extensive intravital microscopy studies on B cell migration within bone marrow we observed intense leakage of intravascular dyes of high molecular weight (ranging between 500-2,000 KDa) into the bone marrow parenchyma within minutes after injection. An estimate of the interstitial fluid flow rate (calculated as the rate of fluorescent voxel accumulation from vascular dye leakage into parenchyma) suggested that the entire femur parenchymal compartment is perfused with exudate plasma in less than 1 minute (Beck et al., 2014).

6.2 S1P and S1PR pathway in lymphocyte egress from lymphoid organs

Leukocytes are genetically programmed to recirculate between lymphoid organs and circulatory fluids. While leukocyte entry into lymphoid organs has been studied extensively over the past 3 decades, the mechanisms controlling their egress into circulation have been under intense investigation over the past 10-15 years. Insight into this mechanism came from an elegant study using transgenic mice that express the catalytically active S1 subunit of pertussis toxin (PTX) driven by the *Lck* promoter specifically in T cells (Chaffin and Perlmutter, 1991). PTX is an exotoxin enzyme that catalyzes ADP-ribosylation of G alpha inhibitory (Gαi) proteins, which leaves Gαi proteins in a permanently inactive GDP-bound state (Gilman, 1987). As chemoattractant receptor signaling is dependent on intact Gαi protein-coupled receptor signaling, PTX-expressing thymocytes were effectively unable to respond to chemoattractants and to migrate out of the thymus, despite being capable of undergoing a seemingly normal developmental program. Although the upstream Gαi protein coupled receptor(s) remained unidentified for a while, insight into this question came from the discovery of an immunosupressant small molecule, FTY720 (2-amino-2-[2-(4-octylphenyl) ethyl] propane-1,3-diol hydrochloride) (Fujita et al., 1996). In vivo administration of FTY720 caused a rapid and profound lymphopenia, in which T and B lymphocyte numbers were reduced by 10-100 fold within a few hours (Budde et al., 2003;Chiba et al., 1998;Suzuki et al., 1996;Yagi et al., 2000). The bioactivity of FTY720 is dependent on its phosphorylation by sphingosine kinases, and p-FTY720 is not only structurally similar to sphingosine 1-phosphate (S1P) but also acts as a ligand for four of the five S1P receptors (Brinkmann et al., 2002;Mandala et al., 2002). B and T lymphocytes express high amounts of S1PR1, whereas S1PR2, 3 and 4 are expressed at substantially lower levels (Cinamon et al., 2004;Graler and Goetzl, 2004). S1PR1 is also abundant in endothelial cells where it plays important roles in blood vessel development and permeability (Xiong and Hla, 2014). Some studies suggested that lymphocyte egress is primarily controlled by endothelial cells at exit sites (Rosen et al., 2007). In contrast, other studies provided compelling evidence showing lymphocyte intrinsic S1PR1 signals egress from thymus and lymph nodes (Cyster and Schwab, 2011). For S1PR1 to signal lymphocyte egress, a sharp gradient of S1P between lymphoid organ interstitium, where its concentration is in the subnanomolar range, and circulatory fluids (plasma and lymph), where it ranges from hundreds of nanomolar to micromolar (Schwab et al., 2005), is required. Essentially all cells generate S1P intracellularly as an intermediate metabolite of membrane sphingolipid turnover. Sphingosine is phosphorylated by sphingosine kinase 1 and 2 to form S1P, which is subsequently converted into phosphoethalonamine and 2-hexadecanal by S1P lyase (Serra and Saba, 2010;Spiegel and Milstien, 2011). Hematopoietic cells secrete roughly 90% of plasma S1P, whereas lymphatic endothelial cells are the major producers of S1P in the lymph (Pham et al., 2010). But S1P cannot diffuse across the plasma membrane and requires

an active transport mechanism. Lymphatic endothelial cells secrete S1P via a mechanism dependent on SPNS2, a member of the spinster family of transporters, but another unidentified transporter is required for plasma S1P (Fukuhara et al., 2012;Hisano et al., 2012;Mendoza et al., 2012). However, the S1P gradients formed across lymphoid interstitium and circulatory fluids are generated through active degradation mediated by Lipid phosphate phosphatase 3 and S1P lyase (Breart et al., 2011;Schwab et al., 2005). While all leukocytes produce S1P, not all leukocytes express S1P degrading enzymes. Erythrocytes lack all S1P degrading enzymes and are, thus, the major contributor of plasma S1P (Ito et al., 2007;Pappu et al., 2007). Even though S1P is abundant in the cytoplasm of cells that also express S1P receptors, it is only the extracellular S1P that acts as a ligand for S1P receptors.

In thymus or in the lymph node, lymphocytes express high amounts of S1PR1 on the surface, but after exiting into circulatory fluids S1PR1 is desensitized within few minutes of exposure to high concentrations of S1P (Lo et al., 2005). S1PR1 desensitization is nearly complete, such that it can no longer be detected on the surface of T and B lymphocytes in the blood or lymph (Pappu et al., 2007). This unique feature of S1PR1 has been exploited as a read-out of the abundance of S1P in the interstitium of various lymphoid organs. Mature naïve T cells in the thymus exhibit the highest amount of S1PR1, whereas it is detected at lower but still high levels in naïve T cells in the spleen or the lymph node. Surface S1PR1 expression inversely correlates with mass spectrometry measurements of S1P in interstitium, where it is nearly undetectable in the thymus, and increasingly detected in lymph node and spleen (Schwab et al., 2005). The bone marrow is perhaps the organ containing the highest concentration of interstitial S1P of all lymphoid organs (Jenne et al., 2009), likely as a consequence of its highly fenestrated sinusoidal endothelium that allows free passage of plasma constituents into the parenchyma and abundance of red blood cells that cannot degrade interstitial S1P.

Even though immature B cells already express high amounts of the chemoattractant receptor Sphingosine 1-phosphate (S1P) receptor-1 (S1PR1), their dependency on S1PR1 and S1P for exiting the bone marrow is relatively minor. Indeed, in mice that cannot express S1PR1 in developing B cells, or that lack S1P, there is only a 2-3-fold reduction in immature B cell egress from bone marrow (Allende et al., 2010;Pereira, Cyster and Xu, 2010). These findings were consistent with a mild egress defect in mice deficient in the S1P transporter SPNS2 (Fukuhara et al., 2012). This minor dependency on the S1P pathway for bone marrow exit contrasts with the dependency on S1P and S1PR1 for T cell egress from thymus, and T and B cell egress from lymph nodes, where S1P or S1PR1 deficiency essentially blocks cell egress thus resulting in 100-1000 fold reduction in lymphocyte numbers in blood and peripheral lymphoid organs (Matloubian et al., 2004;Pappu et al., 2007). Such strong dependency on the S1P pathway for cell egress from essentially all lymphoid organs with the exception of bone marrow is consistent with a subtle S1P gradient between the bone marrow parenchyma and sinusoids, as measured by surface S1PR1 expression (Jenne et al., 2009;Matloubian et al., 2004;Pappu et al., 2007).

Besides developing B lymphocytes, other hematopoietic cells also use the S1P pathway for exiting from bone marrow. NK cells express a dedicated S1P receptor (S1PR5) that

facilitates egress from bone marrow and is essential for lymph node egress (Jenne et al., 2009;Walzer et al., 2007). Eosinophil egress from bone marrow is also partially sensitive to FTY720, likely through effects on S1PR1, S1PR3 and S1PR4 (Sugita et al., 2010). Monocytic cells expressing MCSF receptor and that contain osteoclast differentiation potential are also sensitive to FTY720 treatment, but utilize S1PR1 for bone marrow egress (Ishii et al., 2009). This defect in egress, even though partial, is sufficient to accelerate monocyte/osteoclast precursor differentiation into bone resorbing mature osteoclasts (Ishii et al., 2009), likely as a result of increased chemoattraction to osteoclastogenic niches in the endosteum mediated by oxysterols and EBI2 (Nevius, Gomes and Pereira, 2016;Nevius et al., 2015). At later stages of monocytic cell differentiation, inflammatory monocytes are guided out of the bone marrow mostly in a CCR2 dependent manner (Serbina and Pamer, 2006;Shi et al., 2011). It is unclear whether S1P receptors, or other receptors, synergize with CCR2 to promote monocyte egress from bone marrow. Neutrophil egress is facilitated by a balanced responsiveness to CXCR2 and CXCR4 (Eash et al., 2010), although the exact role played by CXCR4 in neutrophil retention in bone marrow is less clear (Devi et al., 2013).

6.3 Bone marrow egress: an active or a passive mechanism?

An interesting feature of all the pathways identified to date that mediate leukocyte egress from bone marrow is that none seem to be essential for egress to occur. For example, CCR2 is clearly a major regulator of monocyte egress from bone marrow but CCR2 deficiency reveals a mere 6-8 fold reduction in inflammatory monocyte numbers in blood. This contrasts with a 100-1000 fold reduction in lymphocyte numbers in blood of mice conditionally deficient in S1P or S1PR1. These observations led to experiments that aimed at testing whether leukocyte egress is an active process regulated by synergy between several Gαi protein-coupled receptors, or a passive process that can occur independently of chemoattractant receptors. Importantly, when lymphoid-lineage or myeloid-lineage cells were engineered to express PTX, leukocytes were by and large numerically reduced in the bone marrow and increased in peripheral blood, indicating that leukocyte egress from bone marrow is remarkably efficient when cells cannot follow guidance cues (Beck et al., 2014). These findings were in sharp contrast with prior observations that PTX expression in developing thymocytes led to a near complete block in thymic egress, and revealed a fundamental difference in cell export mechanisms between both primary lymphoid organs (Chaffin and Perlmutter, 1991;Willinger et al., 2014). So how can leukocytes migrate through the bone marrow parenchyma and across endothelial barriers? Insight into this question came from intravital microscopy analyses of control and PTX-expressing developing B lymphocytes using a *Rag1*-GFP reporter mouse strain. It was noted that B-lineage cells exit preferably through sinusoidal fenestrations that leaked considerable amounts of large intravascular dyes (~ 2,000 KDa). Furthermore, the few B-lineage cells that were observed in the process of exiting bone marrow were mostly characterized by a non-amoeboid, rounded morphology reminiscent of red blood cells (Beck et al., 2014). Indeed, millions of newly generated red blood cells exit bone marrow every day, and yet these cells lack intrinsic cell motility mechanisms. Early studies of erythrocyte migration across blood vessels in bone marrow by electron microscopy revealed fenestrations in the sinusoidal endothelium that were nearly as wide as erythrocytes (Hudson and Yoffey, 1966). Thus, the highly fenestrated bone marrow sinusoidal network is presumably an acquired feature that

evolved to facilitate export of a variety of hematopoietic cells with a wide range of motility capabilities, from fast moving neutrophils, to essentially non-motile erythrocytes.

Mature thymocytes gain egress competence when TCR signaling strength is reduced, which reduces AKT signaling and FOXO1 phosphorylation, increases KLF2 expression and KLF2-target genes, including S1PR1 (Carlson et al., 2006). This type of molecular circuit most likely ensures that only non-self-reactive T cells become competent for exiting the thymus, given the fact that premature S1PR1 expression in developing thymocytes caused severe autoimmunity (Zachariah and Cyster, 2010), reminiscent of that seen in Aire-deficient mice (Anderson et al., 2002). In the bone marrow, however, self-reactive developing B cells increase CXCR4 expression, which promotes their retention (Beck et al., 2014). Despite this, even when immature B cells are exposed to high affinity cognate antigens, a few cells can still readily escape the bone marrow environment and accumulate in the spleen. This suggests that CXCL12 abundance in the bone marrow parenchyma and the highly fenestrated sinusoidal network are still insufficient to prevent the escape of potentially damaging autoreactive cells. Indeed, these features are presumably similar in mice and in humans given the fact that as many as 75% of developing immature B lymphocytes express antigen receptors that are significantly autoreactive and cross-reactive (Wardemann et al., 2003). Thus, we suggest that the leaky nature of the bone marrow environment imposed additional stages of peripheral B cell development that are controlled by the quality and intensity of tonic and/or self-antigen-driven BCR signaling.

7. Transitional differentiation in periphery

Once immature B cells leave the bone marrow and enter the blood circulation, they reach the spleen where they proceed through their final developmental stages to become mature B cells. Immature B cells can either become marginal zone (MZ) B cells, resident B cells of the splenic marginal zone, which are specialized to respond quickly to blood-borne pathogens, or follicular (FO) B cells, which are circulatory cells that constitute the main B cell compartment capable of mounting T-dependent immune responses. While a detailed understanding of the migratory cues influencing mature B cell recirculation through SLOs has emerged over the last decades, there is still limited understanding of the chemoattractants and niches that regulate immature B cell differentiation into mature B cells in the spleen. Immature B cells are termed transitional (T)1 B cells once they have entered the spleen. Importantly, only about 3% of newly generated bone marrow immature B cells differentiate into circulatory mature B cells (Allman et al., 1993), which illustrates the importance of understanding where and how this bottleneck occurs.

7.1 BAFF receptor and BCR signaling mediate transitional B cell differentiation

The two fundamental and non-redundant signals required for the survival of mature B cells in the periphery are tonic BCR signaling (Kraus et al., 2004;Lam, Kuhn and Rajewsky, 1997;Torres et al., 1996) and activation of B cell activating factor receptor (BAFFR) signaling by BAFF (Gorelik et al., 2003;Mackay et al., 2010;Schiemann et al., 2001;Thompson et al., 2000). These pathways are also critically involved in transitional B cell differentiation into the mature recirculating B cell compartment (Mackay et al., 2010).

Over the past couple of decades an impressive body of work elucidated signaling molecules activated by the BCR and BAFFR, and characterized stages of transitional B cell differentiation in the spleen, and these topics have been elegantly reviewed. The focus of this chapter is on the niches that may influence BCR and BAFFR signaling under homeostasis and how movement may influence transitional differentiation.

Although it remains debatable whether tonic BCR signaling is ligand-dependent or independent (Monroe, 2004;Wienands, Larbolette and Reth, 1996;Zikherman and Weiss, 2008), there is no evidence to suggest that tonic BCR signaling is anatomically restricted to the spleen, lymph node, or any other lymphoid or non-lymphoid organ. In contrast, activation of BAFFR by BAFF is thought to be influenced by B cell access to niches with high local BAFF concentrations. The major source of BAFF in vivo is radio-resistant cells (Gorelik et al., 2003), with fibroblastic reticular cells (FRCs), perhaps, being the most important source of BAFF in lymph nodes (Cremasco et al., 2014). However, transitional B cells hardly migrate to lymph nodes and so development into the mature B cell compartment presumably requires an alternative source of BAFF. Splenic stromal cells express higher BAFF amounts than non-stromal splenocytes and may constitute a physiologically important source of BAFF for transitional B cells (Lesley et al., 2004). However, B cells lacking CXCR5, the B cell follicle homing receptor, differentiate into the mature B cell stage efficiently (Forster et al., 1996;Gunn et al., 1998), and even B cells deficient in chemokine receptors required for homing to niches within follicles and T cell zone were also competent in transitional differentiation (Gatto, Wood and Brink, 2011;Pereira et al., 2009b). Whether the stromal cells producing BAFF are in the red or white pulp of the spleen remains unclear, but genetic deficiency in chemoattractant receptors, such as CB2, CXCR4, S1PR1 and S1PR3, which can guide B cells to various niches in the red pulp or the marginal zone, also did not seem to impair transitional B cell differentiation (Donovan, Pelanda and Torres, 2010;Nie et al., 2004;Pereira et al., 2009a;Pereira, Cyster and Xu, 2010). Interestingly, peripheral B cell development has been found to depend on soluble but not membrane-bound BAFF (Bossen et al., 2011), and soluble BAFF is abundant in plasma and interstitium (Kreuzaler et al., 2012). Taken together these findings suggest that transitional B cell differentiation may occur anywhere in the body, a possibility that is consistent with the observation that transitional B cells develop into mature B cells in mice lacking essentially all secondary lymphoid organs (Cariappa et al., 2007).

7.2 Components of the cell motility machinery regulate transitional B cell differentiation

Even though transitional B cell differentiation can occur outside of secondary lymphoid organs and is largely independent of several major chemoattractant receptors, it is counter-intuitively dependent on the proper functioning of the B cell migration machinery. Cell migration is a highly coordinated process requiring the dynamic organization of the actin cytoskeleton controlled by chemoattractant receptor signaling through Gαi proteins, and small GTPases of the Rho family. B cells express Gαi2 and Gαi3 proteins, and both are required for proper B cell migration and transitional B cell differentiation (Hwang et al., 2013). The defect transitional B cell differentiation is unlikely to be due to poor access to follicular stromal cells because transitional B cell differentiation was unaffected in mice lacking CXCR5, CCR7 and EBI2, even though these mice lacked recognizable follicular

structures in the spleen (Gatto, Wood and Brink, 2011). Instead, calcium flux elicited by anti-IgM stimulatory antibodies was partially reduced in B cells lacking *Gnai2* and *Gnai3*, which suggested a direct involvement of Gαi2 and Gαi3 in BCR signal transduction (Hwang et al., 2013).

Downstream of Gαi2 and Gαi3 protein signaling, various Rho family GTPases work to promote cell migration and adhesion, in addition to other cellular functions (i.e. cell cycle, survival) (Tybulewicz and Henderson, 2009). Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form. Their activity is positively controlled by guanine nucleotide exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (GAPs). Cdc42, a major regulator of membrane trafficking and leading edge protrusion, is a prototypical member of the Rho family and is abundantly expressed in B cells beginning from early developmental stages in bone marrow. Deletion of Cdc42 in B cells causes a partial block at the T1 to T2 transition and a consequent reduction of the mature FO B cell pool (Burbage et al., 2015;Guo et al., 2009). Cdc42 is known to be required for directed movement of migratory cells (Raftopoulou and Hall, 2004), and *Cdc42*-deficient B cells were impaired in their ability to migrate within lymph nodes in vivo (Burbage et al., 2015). Besides its involvement in cell migration, Cdc42 seems also to be required for proper BCR signaling (Burbage et al., 2015;Guo et al., 2009), and possibly for BAFFR signaling (Guo et al., 2009). It remains unclear if the defect in transitional B cell differentiation was caused by defective synergy between BCR and BAFFR signaling (Stadanlick et al., 2008).

A downstream effector of Cdc42 is Wiskott-Aldrich syndrome protein (WASp). WASp is required for integrin activation, immunological synapse formation, for B cell homing to secondary lymphoid organs, and for B cell migration towards CXCL13 *in* vitro (Meyer-bahlburg et al., 2008;Westerberg et al., 2005;Westerberg et al., 2012). WASp deficiency in B cells diminishes all peripheral B cell populations, with a possible defect at the transitional to mature B cell differentiation step (Meyer-bahlburg et al., 2008). Spleens contain smaller B cell follicles and lack the marginal zone but have an otherwise preserved splenic architecture (Recher et al., 2012;Westerberg et al., 2005). However, the defect in B cell populations in Cdc42 deficiency is substantially more severe than WASp deficiency, suggesting the involvement of other downstream effectors.

The main Rho GTPases coordinating polarization and cytoskeletal rearrangements during migration are Rho and Rac proteins, which have been described to act in an antagonistic manner (Cain and Ridley, 2009). Rac1 and Rac2 are both abundant in B-lineage cells and when both are simultaneously knocked out in B cells, peripheral B cell development comes to a halt at the transitional B cell stage, due to survival defects (Henderson et al., 2010;Walmsley et al., 2003). Initially, this survival defect was thought to be caused by defective BCR-induced activation of PI3K and impaired BAFF engagement on BAFFR (Walmsley et al., 2003). However, a later study showed that reduced migratory capacity of transitional B cells in the spleen also contributes to reduced B cell survival (Henderson et al., 2010). Early transitional B cells were excluded from the white pulp, which was explained by failure to respond to PTX-sensitive Gαi protein-coupled receptors. Rac1/2 deficiency led to impaired migration towards several chemokines in vitro, and it was suggested that the

survival defect in T1 B cells was caused by exclusion from white pulp survival factors, such as BAFF.

In B and T lymphocytes, chemoattractant receptor-mediated activation of Rac1 and Rac2 is largely dependent on Dedicator of cytokinesis −2 (DOCK2) (Fukui et al., 2001). Similarly to Rac1/2 deficient B cells, DOCK2-deficient B cells are also impaired in migration towards several chemokines, and are strongly impaired in homing to and movement within secondary lymphoid organs (Nombela-Arrieta et al., 2004). Importantly, DOCK2-deficiency also causes a substantial reduction in the number of mature B cells. However, it is unclear if defective development in bone marrow, differentiation in the spleen, or simply defective B cell survival in the periphery caused this overall reduction in the B cell compartment. Combined, these studies suggest that transitional B cells depend on intact cytoskeletal organization for productive BCR signaling to allow transition through BCR-dependent checkpoints (Figure 2A). Perhaps the most tantalizing evidence supporting an intimate cross-regulation between the B cell cytoskeleton and BCR signaling is a study by Batista and colleagues showing that inhibitors of actin polymerization, such Latrunculin A and Cytochalasin D, were able to stimulate calcium flux in B cells in a manner similar to BCR stimulation (Treanor et al., 2010). Remarkably, this stimulatory effect on BCR signaling caused by inhibitors of actin polymerization was entirely dependent on cell surface expression of the BCR (Mattila et al., 2013). The B cell sensitivity to low amounts of high affinity cognate antigens was improved upon CXCR5 signaling in vitro (Saez de Guinoa et al., 2011), lending support to a model where components of the cellular motility machinery influence BCR signaling.

An alternative explanation for how the defects in B cell motility machinery described above impact B cell differentiation and homeostasis is that they all cause a substantial reduction in signaling from chemoattractant Gαi protein-coupled receptors. While it still remains possible that Gαi proteins are directly linked to BCR signaling, an alternative (but not mutually exclusive) possibility is that Gαi, Gβ and Gγ proteins activate signal transduction pathways that, in turn, not only control cell migration but also promote B cell transitional differentiation and/or survival. For example, Gαi proteins can inhibit adenylyl cyclase activity and in this manner reduce the intracellular concentration of the second messenger $3',5'$-cyclic AMP (cAMP) (Gilman, 1987). Indeed, B cells encounter ligands that signal through Gαs protein-coupled receptors in the periphery. The βeta-adrenergic receptor-2 (Adrb2), a catecholamine receptor, has been shown to enhance B cell responsiveness to chemokines, and to control B cell retention in lymph nodes, providing a link between the sympathetic nervous system and peripheral B cell recirculation (Nakai et al., 2014;Suzuki et al., 2016). Thus, a balance between inhibitory signals from Gαi proteins, and stimulatory signals from Gαs proteins may regulate adenylyl cyclase activity in lymphocytes (Figure 2B). Importantly, some evidence suggests that high cAMP concentrations are inhibitory to lymphocyte function and TCR signaling (Bourne et al., 1974;Kammer, 1988), and so it is reasonable to consider that fluctuations in cAMP production may also contribute to modulate tonic BCR signaling.

8. Concluding remarks

B lymphocytes use a variety of chemoattractants and receptors to navigate primary and secondary lymphoid organs at multiple stages of development and differentiation. Migratory cues, and most important CXCL12, play a vital role in early stages of hematopoietic progenitor differentiation, particularly in stages prior to commitment into the B lineage. CXCR4 is critically important for ensuring that MPPs and CLPs achieve sustained IL-7R-mediated STAT5 phosphorylation, and accumulate sufficient amounts of B-lineage transcription factors, such as EBF1. In subsequent stages of development, proB and preB cells remain dependent on CXCR4 signaling for retention in bone marrow, but these cells are no longer critically dependent on CXCR4 or other PTX-sensitive GPCRs to develop into the immature B cell stage. At this stage, cells undergo a bottleneck-like selection process dependent on the intensity of BCR signaling and on BAFFR signaling as they differentiate into the mature B cell stage. Paradoxically, defects in a variety of proteins involved in cell migration also perturb transitional B cell differentiation, and implicate PTX-sensitive GPCR signaling as an important and unexpected pathway in B cell homeostasis. Whether GPCR signaling components directly regulate key components of the BCR and/or BAFFR signal transduction pathway through the actin cytoskeleton, or indirectly modulate BCR and/or BAFFR signaling thresholds via second messengers (e.g. cAMP) remains to be determined.

Acknowledgments

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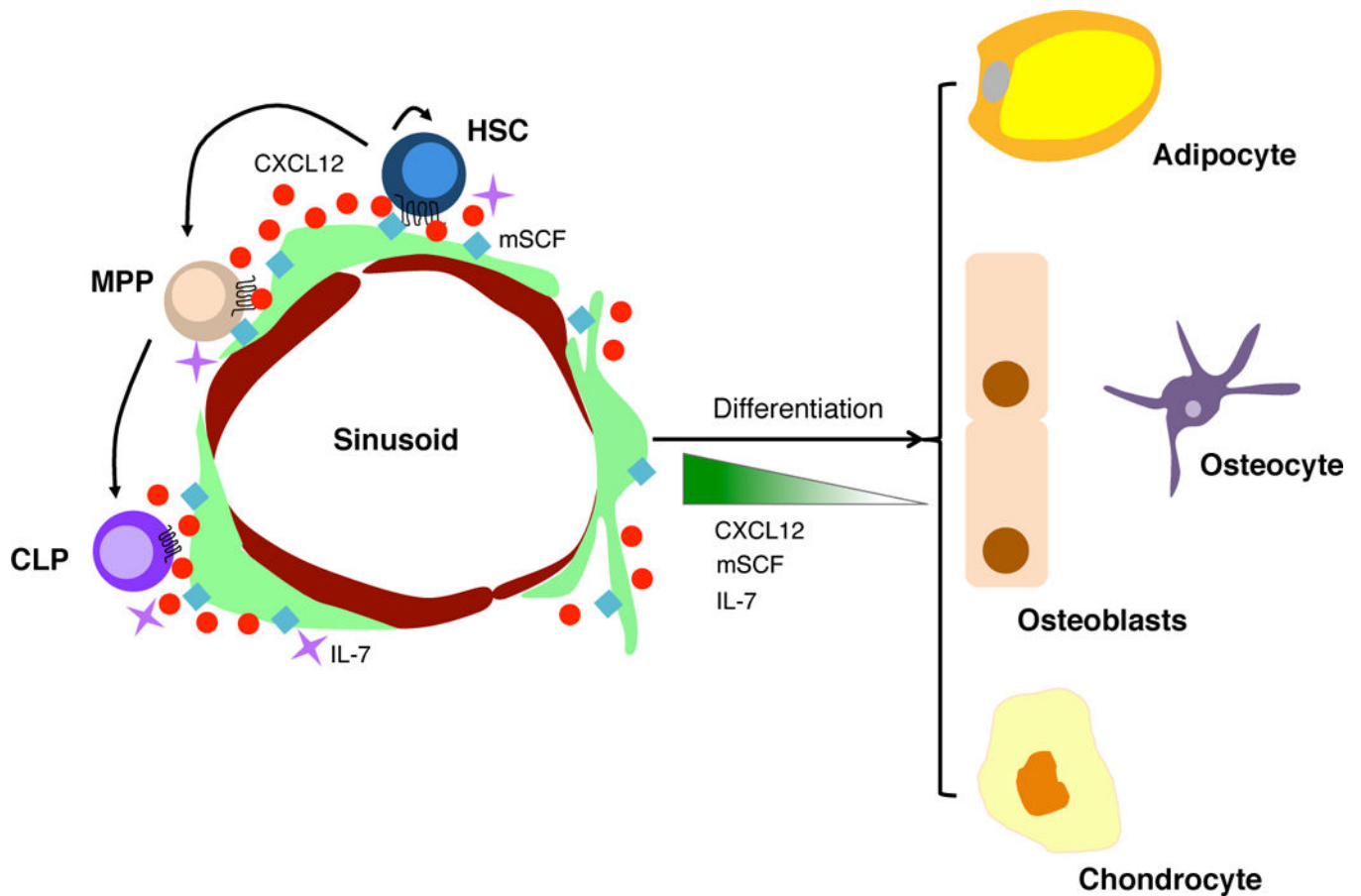
Figure 1. HSC niches in bone marrow control HSC homeostasis and lymphopoiesis
MSPCs, together with some sinusoidal endothelial cells, express soluble and membrane-bound SCF, CXCL12, and IL-7 that not only regulate HSC and MPP numbers, but also sustain lymphopoiesis. MSPCs in the bone marrow are mostly perisinusoidal cells that can differentiate into osteoblasts, osteocytes, chondrocytes and adipocytes. MSPC differentiation is coordinated with the downregulation of SCF, CXCL12 and IL-7 expression.
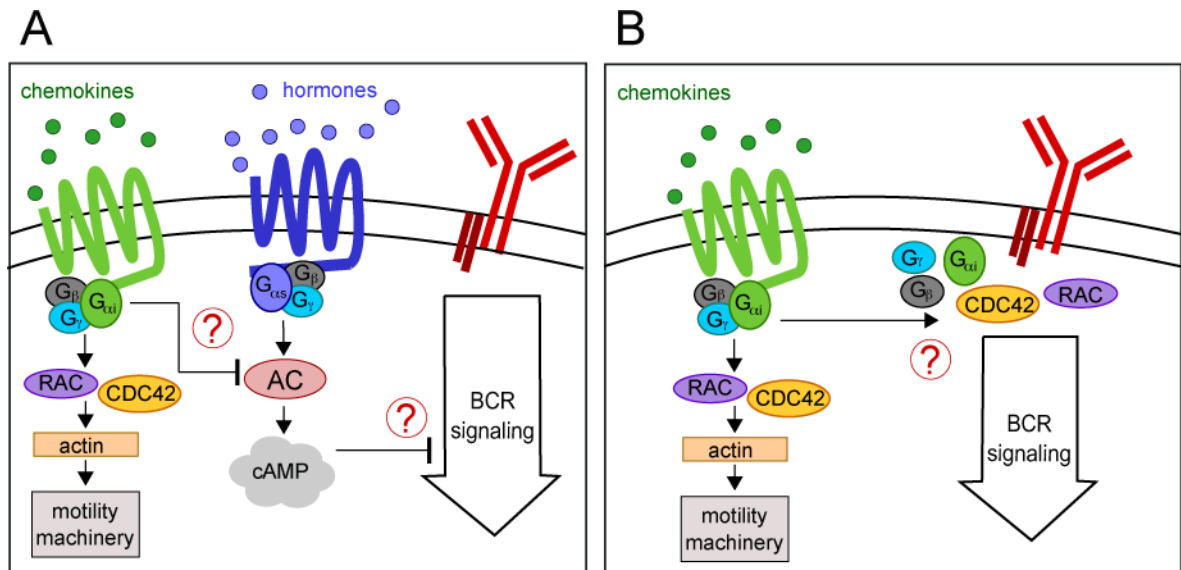
Figure 2. Interactions between Gαi protein–coupled receptors and BCR signaling
(A) Indirect regulation of BCR signaling through cAMP. Activation of Gαi proteins by chemoattractant sensing may inhibit adenylyl cyclase (AC) activity counteracting the effects of Gαs-coupled receptors and thus lower cytoplasmic concentration of the secondary messenger cAMP. In turn, by yet unidentified mechanisms, cAMP may inhibit BCR signaling.
(B) Interaction between heterotrimeric G proteins and components of the BCR signal transduction pathway (i.e. Cdc42 and RAC proteins.)