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It Takes Three Receptors to Raise a B Cell

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Abstract

As the unique source of diverse immunoglobulin repertoires, B lymphocytes are an indispensable part of humoral immunity. B cell progenitors progress through sequential and mutually exclusive states of proliferation and recombination, co-ordinated by cytokines and chemokines. Mutations affecting the crucial pre-B cell checkpoint result in immunodeficiency, autoimmunity, and leukemia. This checkpoint was previously modeled by the signaling of two opposing receptors, IL-7R and the pre-BCR. We provide an update to this model in which three receptors, IL-7R, pre-BCR, and CXCR4, work in concert to coordinate both the proper positioning of B cell progenitors in the bone marrow (BM) microenvironment and their progression through the pre-B checkpoint. Furthermore, signaling initiated by all three receptors directly instructs cell fate and developmental progression.

Mammalian B Cell Development in the Bone Marrow

BM-derived lymphoid progenitor cells commit to the B cell lineage in response to signals from the BM microenvironment. The hallmark of B lymphopoiesis is the sequential rearrangement of the locus encoding immunoglobulin heavy chains (IgH/Igμ) followed by loci encoding immunoglobulin light chains (IgL/Igκ; followed, if necessary, by Igλ). Rearrangement of *Igm* (Igμ) starts with diversity (D) and joining (J) gene segments in pre-pro-B cells. Subsequent recombination of variable (V) to rearranged D(J) is completed in pro-B cells, at which point cells become committed to the B lineage [1,2]. Developing B cells are then driven to proliferate by interleukin-7 receptor (IL-7R) signaling in response to IL-7 secreted by BM stromal cells [1,2]. The successfully rearranged Igμ then associates with the surrogate light chain (SLC: λ5 and VpreB) and the signaling molecules Igα and Igβ to form the pre-B cell receptor complex (pre-BCR) that is expressed on the cell surface of pre-B cells [1,2]. Pre-B cells then undergo a proliferative burst associated with both pre-BCR and IL-7R signaling [3]. Subsequently, large pre-B cells exit the cell cycle and initiate IgL recombination, attempting recombination first at the Igκ locus (*Igk*) [3,4]. This occurs in resting small pre-B cells where there is concurrent repression of pre-BCR expression [1,5]. The rearranged Igκ light chain then associates with the Igμ heavy chain to form the B cell receptor (BCR) on the surface of immature B cells [6]. Autoreactive early immature B cells bearing an Igκ light chain can diminish autoreactivity by consecutive rearrangements of available Vκ and Jκ gene segments at the *Igk* locus and subsequent Vλ–Jλ joining [6,7].

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This process of **receptor editing** (see Glossary) helps to ensure a diverse peripheral repertoire that is tolerant to self, as extensively reviewed elsewhere [6,7]. The selected immature B cells then migrate from the BM to the spleen and lymph nodes as mature B cells to become organized in B cell follicles [7,8].

During the developmental progression of B cells, the pre-B cell stage is a crucial developmental checkpoint. The SLC probes Igμ fitness, and the expression of the pre-BCR enables the pre-B cell pool to proliferate in an IL-7-dependent manner [1,9]. Proliferation and DNA rearrangement are strictly segregated processes because concurrent replication and introduction of double-strand breaks during recombination would compromise genomic integrity [10]. Mutations or alterations that affect this checkpoint can result in the development of pre-B cell leukemias, primary immunodeficiencies, and systemic autoimmunity [1,8,9,11]. In this review we highlight a central mechanism regulating the pre-B cell developmental checkpoint, and we provide a novel model for how the dynamically regulated receptors expressed on progenitor B cells balance proliferation and differentiation by integrating cell-intrinsic and microenvironmental cues.

IL-7R Signaling in Mammalian B Cell Development

IL-7 is a crucial cytokine secreted by BM stromal cells that plays an important role in B cell lineage commitment and development [8]. The IL-7R is expressed on early B cell progenitors and is composed of the IL-7Rα chain (CD127), which confers specificity to IL-7, and the common γ-chain receptor (γc, also known as CD132) [12]. Mice lacking IL-7R have a severe impairment in B lymphopoiesis [13]. IL-7R is essential for the growth, proliferation, and survival of all the progenitor stages from **common lymphoid progenitors** (CLPs) to large pre-B cells [1,3,13]. Although B cell development is thought to be less dependent on IL-7R signaling in humans, most of the regulatory responses downstream of IL-7R are similar in mice and humans [14,15]. Indeed, many patients with IL-7Rα mutations have low titers of serum immunoglobulins, suggesting defective peripheral B cell function [1,14,15]. Furthermore, later studies examined the role of IL7R signaling in human B cell development using *in vivo* xenograft models, and concluded that B cell development in humans is absolutely dependent on IL7R signaling [16,17].

IL-7 binds to the IL-7Rα chain and induces dimerization of the α and γ chains, bringing associated Janus kinases (JAK1 and JAK3) together, and stimulating their transphosphorylation and activation [18–20]. Activated JAK kinases phosphorylate a tyrosine residue (Y449) on the IL-7Rα chain, allowing the recruitment of the transcription factors **STAT5A/B** [18,19]. The development of B cells is arrested at the pre-pro-B cell stage in *Stat5a/b*$^{-/-}$ mice [18,19], and constitutive STAT5 expression can compensate for many functions of IL-7R signaling even in cultured progenitor B cells derived from human cord blood [19,20]. In addition to the JAK–STAT pathway, IL-7R signaling also activates the phosphoinositide-3 kinase (PI3K)–protein kinase B (PKB or AKT) pathway [13,19–21] (Figure 1). Indeed, *in vivo* deletion of the PI3K regulatory subunit p85α, or the catalytic subunits p110α and p110δ, impairs mouse B cell development [22,23].

The Role of IL-7R in Survival and Proliferation

One of the major functions of activated STAT5 is to ensure the survival of pro-B cells (Figure 1) [18,19]. STAT5 activates the prosurvival factors myeloid cell leukemia sequence 1 (MCL1) and B cell lymphoma 2 (BCL2) in both mice and humans [19,24,25]. Indeed, constitutive expression of BCL2 in *Stat5*$^{-/-}$ mice partially rescues pro-B cell development [24,25]. In addition, the activation of the PI3K–AKT pathway phosphorylates and promotes the nuclear export of the forkhead box protein O (FOXO) family of transcription factors that induce proapoptotic protein BCL2 interacting mediator of cell death (BIM; encoded by *Bcl211b*) in mouse and human cells lines [26]. AKT also directly phosphorylates and inactivates the proapoptotic factor BCL2 antagonist of cell death (BAD) in mouse BM B cells [27]. Therefore, IL-7R signaling promotes the survival of pro-B cells by both upregulating survival signaling and repressing apoptotic signaling. In addition, STAT5 enhances the proliferation of B cell progenitors by inducing cyclin D3 (encoded by *Ccnd3*), which is required for the proliferation of both pro-B and pre-B cells in mice [28,29] (Figure 1). In contrast to Stat5, deficiency of both the catalytic subunits (p110α and p110δp) of PI3K, or deficiency of PI3K negative regulator phosphatase and tyrosine homolog (PTEN), in mice does not impair pro-B cell proliferation, suggesting that the PI3K–AKT pathway only plays a role in the survival (and not in the proliferation) of developing B cells [21,22,30].

IL-7R Signaling Regulates Metabolism by Promoting Glycolysis

Increased glucose uptake and glycolysis is a signature of the proliferative pro-B cells and large pre-B cells, whereas resting small pre-B cells have a higher oxidative phosphorylation to glycolysis ratio [31–33]. In IL-7-responsive B cell progenitors, IL-7R signaling promotes glycolysis via AKT and MYC [1,32]. IL-7R signaling also stimulates the phospholipase Cγ–diacylglycerol (PLC-γ–DAG) pathway for subsequent activation of **mTOR** which, together with MYC, supports anabolism by upregulating glycolysis [1,32,34,35]. The regulation of *Myc* by IL-7R signaling is evident from observations that withdrawal of IL-7 in mouse pre-B cell cultures reduces *Myc* expression, and that the overexpression of *Myc* prevents exit from the cell cycle in response to IL-7 withdrawal [36]. Although the signaling pathways leading to increased MYC expression in proliferating large pre-B cells are not known, candidates include PI3K, extracellular signal-regulated kinase (ERK), and nuclear factor-κB (NF-κB) [32,37,38]. Of note, B-lymphoid transcription factors such as PAX5 and IKAROS (encoded by *Ikzf1*) can function as metabolic gatekeepers by limiting the amount of cellular ATP to concentrations that are insufficient for malignant transformation [33].

Regulation of Immunoglobulin Recombination by IL-7R

IL-7R signaling is crucial for preventing premature *Igk* recombination [21,25,29]. The expression of *Rag1* and *Rag2* is absolutely necessary for immunoglobulin gene recombination [39]. IL-7R signaling inhibits RAG expression in mice via PI3K–Akt-mediated phosphorylation and inactivation of the transcription factors FOXO1 and FOXO3A that directly activate *Rag* expression [21,40,41]. Two other downstream effectors induced by IL-7R signaling, STAT5 and cyclin D3, are also essential for inhibiting premature *Igk*

recombination in mice [21,30,42,43] (Figure 1). They intricately coordinate the epigenetic landscape of the *Igk* locus, making it inaccessible to the RAG enzymes while the cell is proliferating [42,43]. IL-7-mediated suppression of premature *Igk* rearrangement is also the most definitive function so far described for IL-7 in human B cell development [44]. A more extensive discussion of the epigenetic regulation of *Igk* recombination is given in Box 1.

It remains unclear how Igμ recombination occurs in pro-B cells which are highly dependent on IL-7R signaling. The pro-B cell pool in mice is heterogeneous for IL-7R surface expression, which positively correlates with active STAT5 and negatively correlates with FOXO1 and *Rag* expression in mice [45]. Therefore, there could be two pro-B cell states: one that proliferates in response to IL-7 receptor signaling, and one that undergoes IgH recombination. However, FOXO1 facilitates **IgH locus contraction** and enhances V_H gene accessibility [46], whereas active STAT5 is recruited to V_H genes to promote local active **histone acetylation** that facilitates distal V_H gene recombination in mouse pro-B cells [47]. Therefore, in pro-B cells, both IL-7R high and low expression might enable IgH recombination [45,47].

Pre-BCR Signaling

Successful assembly of the V-D-J gene segments in pro-B cells results in the expression of the Igμ heavy chain (μHC) and surface expression of the pre-BCR. Pre-BCR-mediated signaling (Figure 2A) is activated in a cell-autonomous manner, and pre-BCR surface expression seems to regulate both cell proliferation and survival [48,49]. The pre-BCR is composed of two identical membrane-anchored μHC subunits and two SLC subunits (SLC, a non-covalent complex of λ5 and VpreB molecules) covalently bound to each of the μHCs and the signaling subunits Igα and Igβ [50]. B cells do not develop beyond the progenitor B stage in *Rag1*/*Rag2*-deficient (lacking μHC) [51,52] and *Igα*- and *Igβ*- deficient mice [53,54]. However, deficiency of the SLC components in mice results in a partial developmental block at the pre-B cell stage [54]. Albeit inefficiently, relative to wild-type (WT) mice, SLC deficient mice produce elevated titers of antinuclear antibodies (ANAs) in their serum, and show evidence of negative selection escape of pre-B cells expressing prototypic autoantibody heavy chains, leading to mature autoantibody secreting B cells in the periphery [55]. This suggests that one function of the pre-BCR is to select against autoreactivity, making the pre-B cell stage a tolerance checkpoint. In human B cell development, the SLC appears to play a more crucial role because no mature B cells are generated in the absence of Λ5 expression, as has been observed in patients carrying λ5 mutations [56,57].

Pre-BCR Mediated Regulation of the Cell Cycle

Generation of pre-BCR-expressing large pre-B cells is characterized by an initial proliferative phase *in vivo* with four to five rounds of cell division [58]. Although mouse pre-B cells require both pre-BCR and IL-7 for expansion, this proliferative burst is largely attributed to the PI3K–AKT pathway and to MYC-dependent induction of cyclin D3, which are activated downstream of IL-7R signaling [1,3,8,21,29,36]. The proliferative pre-B cell pool does not expand and survive well without IL-7 in culture, even though the cells express

pre-BCR [21,29,43]. Thus, although pre-BCR expression is associated with a proliferative burst in large pre-B cells, it is unclear whether this burst is directly mediated by the pre-BCR.

Igk recombination requires exit from the cell cycle to maintain genomic integrity, prompting extensive study of cell-autonomous pre-BCR signaling and the gene regulatory mechanisms that limit proliferation [1,4,8,10]. Based on the observation that pre-BCR signaling silences the expression of the SLC, and subsequently the expression of the pre-BCR itself [59], loss of SLC expression via multiple cell divisions was postulated to be responsible for attenuated pre-BCR-mediated proliferative signaling and subsequent exit from the cell cycle [59,60]. However, constitutive expression of SLC in mouse pre-B cells *in vivo* demonstrated that downregulation of the pre-BCR is not required for cell-cycle exit, but instead that pre-BCR expression and downstream signaling are necessary to cease proliferation and to prevent constitutive B cell activation [5].

Initially, pre-BCR signaling (Figure 2A) activates **SRC kinases** such as LYN, FYN, and BLKthat phosphorylate **immunoreceptor tyrosine-based activation motifs** (ITAMs) on Igα and Igβ cytoplasmic tails, facilitating the intracellular recruitment and activation of spleen tyrosine kinase (SYK) [61,62]. SYK signaling leads to induction of B cell linker protein (BLNK, also known as SLP65), Bruton tyrosine kinase (BTK), and PLC-γ2. Loss of SYK, BLNK, BTK, or PLC-γ2 results in a block in early B cell development in mice [63–65]. Thus, the central event of pre-BCR signaling is the activation of the SYK–BLNK module, which eventually functions to cease cell proliferation downstream of the pre-BCR [21,64,65].

The SYK–BLNK axis also represses the PI3K–AKT pathway, which is upregulated by IL-7R signaling and actively represses PAX5 [21]. Inhibition of PI3K–AKT derepresses PAX5 and FOXO1, both of which are necessary for optimal expression of SYK and BLNK [21]. Moreover, BLNK induces the activation of the mitogen-activated protein kinase p38, which phosphorylates and augments FOXO1 activity in mice [21]. FOXO1 and PAX5 are also necessary for the induction of interferon-regulatory factor 4 (IRF4) expression through SYK–BLNK signaling in mice [21]. IRF4 together with its binding partner IRF8 negatively regulates pre-B cell proliferation by inducing the expression of transcription factors IKAROS and AIOLOS (encoded by *Ikzf3*) [66]. IKAROS and AIOLOS directly suppress c-Myc expression and induce expression of the cell-cycle inhibitor p27 to downregulate cyclin D3 in cultured mouse pre-B cells, thereby facilitating exit from the cell cycle [36]. In addition, the low-energy state generated by extensive proliferation activates **AMP-activated protein kinase** (AMPK) to antagonize mTORC1 and restrict protein synthesis [35,67]. Furthermore, mutation of the gene encoding the AMPK binding partner folliculin-interacting protein 1 (FNIP1) in mice, leads to an excessive cell growth-mediated block in differentiation at the large pre-B cell stage [35]. AMPK can also directly phosphorylate and activate recombinant RAG1 in human and mouse cell lines, and likely contributes to *Igk* recombination [67].

Regulation of *Igk* Recombination by the Pre-BCR

Cell-cycle exit of pre-B cells is necessary, but not sufficient, to induce *Igk* recombination [68]. Constitutive expression of the pre-BCR results in induction of *Igk* recombination [5]. In mouse *Rag2*$^{-/-}$ pro-B cells, transgenic expression of μHC induces *Igk* locus accessibility, which is required for efficient *Igk* recombination [69]. Furthermore, deficiency of one or more pre-BCR signaling components enhances proliferation and reduces *Igk* recombination, suggesting that the pre-BCR plays an active role in light-chain recombination [65,69,70].

Downstream of the pre-BCR, the RAS–ERK pathway plays an important role in *Igk* recombination by inducing the expression of *Rag1/2* [29,71,72] (Figure 2A). In addition, this signaling increases the expression of the transcription factor E2A (encoded by *Tcf3*) while repressing its inhibitor ID3, thus enhancing the availability of free E2A, which then activates *Igk* enhancers, regulates *Igk* locus accessibility, and upregulates *Igk* germline transcription in mice [29,73]. Binding of E2A at the intronic enhancer of the *Igk* locus (iEκ) activates this regulatory element and allows recruitment of the cotranscriptional activators CBP and p300 to decorate H3 histones flanking Ck and Jk regions with acetyl groups (**histone H3 acetylation**, H3Ac), thereby making the region accessible to the recombination machinery in mouse pre-B cells [29,73,74]. Furthermore, the RAS–ERK pathway directly induces phosphorylation of serine 10 in histone H3 (H3S10p) which, in combination with E2A-mediated acetylation of H3K9 and H3K14 (H3K4AcK14Ac), sets up a specific epigenetic landscape (H3K9AcS10pK14Ac) to recruit the epigenetic reader BRWD1 (Bromodomain and WD repeat-containing protein 1) at the putative recombination center at *Jk* [75,76]. BRWD1 then repositions nucleosomes relative to GAGA motifs (where ERK can also be directly recruited [77]) to recruit RAG complex binding, thus making *Jk* segments accessible for recombination in mouse cells [75].

In addition to E2A, pre-BCR signaling induces the expression of IRF4 and IRF8, that are required for *Igk* recombination, thus silencing pre-B cell proliferation and suppressing the SLC [66,68]. In mice, IRF4 and IRF8 also complement the function of E2A by binding to and activating *Igk* enhancers: iEκ, the 3’ enhancer located 9 kb downstream of iEκ [68]. Therefore, with attenuated IL-7 culture conditions, pre-BCR signaling sets up several feedforward and feedback regulatory loops that are necessary for successful *Igk* recombination. The significance of reduced IL-7R signaling in pre-BCR-mediated *Igk* recombination is discussed in detail in Box 2.

Importance of the BM Microenvironment in B Cell Development

The dominance of IL-7R signaling over pre-BCR signaling prompts the question of how pre-B cells are able to overcome IL-7R signaling. The answer to this question lies in the localization of progenitor B cells within the BM microenvironment where B cells develop (Figure 3A, Key Figure). The BM microenvironment provides the extracellular cues necessary to determine cell fate [78–80], either by defining a specific signaling program or by changing the signaling threshold for a differentiation event. Therefore, positioning away from IL-7^{high} microniches within the BM was postulated to reinforce the switch from IL-7R signaling to pre-BCR signaling to allow progression through development [1,21,68,78]. In

support of this model, downstream of the pre-BCR, IRF4 was found to induce the expression of the CXC chemokine receptor 4 (CXCR4) in mouse pre-B cells, conferring responsiveness to CXC-chemokine ligand 12 (CXCL12) [21,68].

A previous study of the BM microenvironment suggested that IL-7-producing stromal cells are distinct and spatially distributed away from the CXCL12-producing stroma [78]. However, later studies using mouse BM showed that most of the stromal cells that highly express IL-7 also express CXCL12 [80,81]. A recent mouse study of BM stromal cells at single-cell resolution further showed that the fraction of mesenchymal stromal cells that highly express both *Il7* and *Cxcl12* is relatively small (~14%), but most cells that highly express *Cxcl12* express *Il7* only at a low level [82]. Consistent with these observations, using high-power confocal microscopy, our study identified three distinct major populations of stromal cells in mice: IL-7$^{neg/low}$CXCL12^{high}, IL-7-^{int}CXCL12^{high}, and IL-7^{high}CXCL12^{low} [86]. Examination of whole BM single planes revealed a widespread distribution of each cell type, and indicated that the BM is a mosaic of IL-7- and CXCL12-producing cells with varying degrees of IL-7 and CXCL12 expression, creating microniches that are relatively high in IL-7 and/or high in CXCL12 expression [82,83].

There is a significant difference in the localization of pro-B and pre-B cells in mouse BM niches [80,81,83]. Pro-B cells, which express higher amounts of **focal adhesion kinase** (FAK) and the integrin very late antigen 4 (VLA4) than pre-B cells, are more adherent to vascular cell adhesion molecule 1 (VCAM-1) in IL-7^{high} stroma [79]. High-power microscopy of mouse BM revealed that proliferating large B cells mostly localize to high IL-7^{high} microniches [83]. By contrast, small pre-B cells specifically reside in niches enriched in IL-7$^{neg/low}$CXCL12^{high} stromal cells [83]. However, both IL-7R and CXCR4 were downregulated in immature B cells [83]. This is consistent with the flow-cytometric observation that the cell-surface expression levels of IL-7R and CXCR4 are reciprocally regulated during developmental progression from pro-B, to large pre-B, to small pre-B cells in mouse BM [83]. Indeed, intravital two-photon microscopy in the calvarial mouse BM showed that pro-B cells are non-motile and pre-B cells are mostly motile [79,81]. Although pro-B and large pre-B cells can migrate along an IL-7 gradient *in vitro* [83], direct comparison of *in vitro* chemotaxis revealed that both large and small pre-B cells are highly responsive to CXCL12-mediated migration [68,83]. Large pre-B cells showed the strongest chemotaxis to CXCL12, whereas small pre-B cells – that have even higher CXCR4 cell-surface densities – were found in intimate contact with the IL-7$^{neg/low}$CXCL12^{high} stroma [83]. This repositioning of pre-B cells in CXCL12^{high} microniches was severely impaired in mice when *Cxcr4* was conditionally deleted in large pre-B cells (*Cxcr4*$^{fl/fl}$*Mb1*$^{Cre/+}$) [83]. Therefore, CXCR4 is necessary to shift signaling from IL-7R to the pre-BCR, positioning small pre-B cells away from IL-7^{high} niches, thus enabling these cells to escape IL-7 mediated proliferation and initiating *Igk* recombination [83].

CXCR4 Signaling

The movement of pre-B cells away from IL-7^{high} microenvironments by CXCR4 can explain the initiation and subsequent dominance of pre-BCR signaling over IL-7R signaling. However, several questions remain unanswered. First, given that activation of pre-BCR

signaling is associated with concurrent repression of pre-BCR expression, the model fails to explain how the pre-BCR provides continuous signals for *Igk* recombination in small pre-B cells. Second, it is unclear whether initial transient pre-BCR signaling is sufficient to execute the entire developmental program in small pre-B cells, or whether other signals are required.

Withdrawal of IL-7 *in vitro* was thought to be sufficient for pre-BCR activation, cell-cycle exit, and subsequent *Igk* recombination, both in mice and humans [21,29,43,44,68]. However, most of these experiments were performed using the stromal feeder cell line OP9, which expresses CXCL12, thus obscuring the exact role of CXCR4 signaling [84]. The aforementioned recent study found that most small pre-B cells are in tight contact with IL-7$^{neg/low}$CXCL12^{high} stromal cells, with high local accumulations of extracellular CXCL12 [83] (Figure 3A inset). Furthermore, because small pre-B cells harbor CXCL12 in their cytoplasm, this suggests that CXCL12 may have been recently internalized in the process [83]. This observation prompted the idea that CXCR4 might have an additional signaling role beyond modulating the localization of pre-B cells.

B cell progenitors express CXCR4 in various amounts during all stages of B cell development in the BM: from hematopoietic stem cells (HSCs) to mature B cells, and CXCR4 also plays a major role in B cell precursor homing (Figure 3B) [1,78,83]. In addition, CXCR4 has been associated with various cellular processes such as embryonic development, organogenesis, hematopoiesis, neuronal development, organogenesis, vascularization, and malignancies when these processes are deregulated [78,85–87].

Embryonic lethality in CXCR4- and CXCL12-deficient mice, that have impaired hematopoiesis, has made it difficult to understand the specific role of CXCR4 in the development of pre-B cells [86,87]. Early studies of CXCR4 deficiency in B cells using either fetal liver *Cxcr4*$^{-/-}$ chimeras [86] or B lineage-specific deletion of the *loxP*-flanked (floxed) *Cxcr4* locus (*Ccxr4*$^{fl/fl}$) by CD19–Cre [88] revealed developmental arrest at the pro-B/pre-B cell stages. However, the *Cxcr4*$^{-/-}$ fetal liver chimeras were limited by poor generation of B cells, and deletion by CD19–Cre was incomplete in early B cell populations, where up to 60% of *Cxcr4*$^{fl/fl}$ × *Cd19–cre* mice expressed CXCR4 in pre-B cells [88]. To address this issue, *Cxcr4* was conditionally deleted via *Mb1–cre* which provided near-complete deletion in all committed B cell progenitors from the large pre-B cell stage [83]. This study revealed that CXCR4 is necessary to generate small pre-B cells, but not earlier progenitors [83]. In addition, the epigenetic and transcriptional signature of small pre-B cells from these CXCR4-deficient mice resembled that of WT proliferative large pre-B cells, suggesting a developmental block at this stage [83]. To understand the true effect of CXCL12, a novel *in vitro* murine pre-B cell culture requiring no stromal feeder cell line was established where IL-7 and/or CXCL12 were externally supplemented [83]. High-throughput sequencing, flow-cytometry, and immunoglobulin gene recombination assays of cultured pre-B cells from CXCR4-sufficient and -deficient mice, with or without IL-7, and with or without CXCL12 *in vitro*, confirmed the direct role of CXCR4 in exiting the cell cycle and initiating *Igk* recombination at the small pre-B cell stage of development [83].

A Positive Feedback Loop Drives CXCR4 Signaling

Successful μHC recombination and the expression of pre-BCR leads to the upregulation of the transcription factor IRF4, which in turn activates CXCR4 expression [68,83] (Figure 2A). Once active, CXCR4 acts on IRF4 and increases its expression gradually from large to small pre-B cells [83]. The pre-BCR induces an IRF4/CXCR4 feedforward loop, driving cells to enter a motile phase by turning IL-7-responsive cells into CXCL12-sensitive cells. Thus, even in the presence of IL-7, CXCL12 expression appears to predominate and causes developing B cells to be positioned in CXCL2-rich areas within the murine BM [83], thus escaping the proproliferative effects of IL-7, and activating the CXCR4–CXCL12 signaling axis that is necessary to start *Igk* recombination [1,21,29,43,83].

CXCR4 Signaling in Cell-Cycle Exit and Survival

The CXCR4/CXCL12 signaling pathway serves to promote cell survival, as well as to support the role of the pre-BCR in inhibiting proliferation. CXCR4 induces the expression of NF-κB, promoting cell survival [7,83]. In addition, it represses the proapoptotic proteins BIM and BID. CXCR4 also aids the pre-BCR in promoting cell-cycle escape by amplifying a feedforward loop of ERK activation in mice [83]. This induces AIOLOS, which is necessary to cease cell-cycle progression via repression of cyclin D3. Overall, CXCR4/CXCL12 signaling in pre-B cells complements almost all of the functions initiated by the pre-BCR, including survival, by maintaining MCL1 amounts, and the continuation of differentiation programs, even when pre-BCR surface expression is attenuated by CXCR4 [83]. As a result, the initiation of complex CXCR4-dependent programs provides an extended time-window for completion of IgL recombination and for the surface expression of the BCR in newly formed immature B cells.

CXCR4 Promotes *Igk* Recombination and Late B Lymphopoiesis

The CXCR4–CXCL12 signaling pathway (Figure 2A) also coordinates the induction of both transcription factor networks and chromatin-remodeling complexes that dictate which regulatory sites are open to transcription factor binding at late stages of B lymphopoiesis in mice [1,83,89]. It induces the expression of IRF4 and NF-κB – both are crucial for late B lymphopoiesis [7,83,89]. In addition, CXCR4 signaling enhances chromatin accessibility to sites bound by multiple mediators of late lymphopoiesis, including IRF4, IRF8, E2A, SPIB, PAX5, and FOXO1 [21,43,75,83]. Conversely, sites bound by early mediators of B cell development, such as MYC and STAT5, become inaccessible upon CXCR4 signaling [21,25,43,75,83]. Because CXCR4 induces the expression of BRWD1, it is likely that at least some CXCR4-dependent changes in chromatin accessibility are mediated by BRWD1, which both opens enhancers of late lymphopoiesis and represses early developmental programs [75,89]. In addition to promoting late B lymphopoiesis, CXCR4 induces the transcription of *Rag1/Rag2* and is essential for productive *Igk* recombination [39,76,83]. The induction of *Rag1/Rag2* expression is partly dependent on ERK activation [29,72,83], which is also involved in most of the transcriptional program downstream of CXCR4 [83]. Furthermore, ERK activation downstream of CXCR4–CXCL12 signaling in mouse small pre-B cells is necessary to repress cell-cycle genes completely and to induce transcription of

Igk and other late B cell differentiation-associated genes [75,83]. In addition, CXCR4 induces NF-κB in small pre-B cells, which has been shown to facilitate Igλ recombination [7,83]. These observations therefore suggest a so far undefined role of CXCR4 in receptor editing. Altogether, CXCR4 signaling takes over the signaling previously initiated by the pre-BCR, and is essential for the development of immature B cells with a functional surface BCR.

Concluding Remarks

Previous models of late B cell development were developed with a two-receptor system in which IL-7R drives proliferation in pro-B and large pre-B cells, whereas expression of the pre-BCR in small pre-B cells directs cell-cycle exit and *Igk* recombination when IL-7 signaling is attenuated [1]. Now, by combining previous knowledge of B cell development, the visualization of B cell progenitors at different developmental stages in specific BM microenvironments, and a detailed understanding of CXCR4 signaling in late B lymphopoiesis, a new model of B cell development has emerged in which the coordinated signaling of three receptors, IL-7R, pre-BCR, and CXCR4, dictates differentiation from the pro-B to immature B cell stages, at least in mice. In brief, IL-7R signaling drives proliferation, maintains a pro-proliferative metabolic state, represses RAG protein expression, and genetically and epigenetically prevents premature IgL recombination [21,28–34,40–43,68]. Pre-BCR signaling, instead of promoting transit across a discrete ‘checkpoint’, initiates a complex CXCR4-dependent program that guides B cell progenitors through a precise epigenetic and transcriptional program. Finally, CXCR4–CXCL12-dependent signaling takes charge of pre-BCR signals and completes differentiation of small pre-B cells accompanied by downregulation of pre-BCR expression to generate functional immature B cells [83]. CXCR4 signaling further ensures the completion of *Igk* recombination by favoring the induction of *Rag* expression and maintaining the activity of the non-homologous end-joining (*NHEJ*) repair pathway versus homologous recombination (*HR*) DNA repair [83,90]. Dysregulation of IL-7R, pre-BCR, and CXCR4 signaling is associated with many human diseases. Therefore, it is the constant interplay and integration of environmental cues via appropriate receptors that determines cell fate and orchestrates B lymphopoiesis. A series of feedforward and feedback loops between the signaling cascades of IL-7R, pre-BCR, and CXCR4 ensures the dominance of one receptor at any one time. These mechanisms reveal the regulatory logic of the pre-B cell developmental checkpoint but raise several unanswered fundamental issues (see Outstanding Questions).

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Glossary

AMP-activated protein kinase (AMPK)

an enzyme that helps to maintain homeostasis of cellular energy by activating glucose and fatty acid uptake and oxidation

Common lymphoid progenitors (CLPs)
can give rise to B cells, T cells, dendritic cells, and natural killer cells, but lack myeloid and erythroid potential

Focal adhesion kinase **(FAK)**
a cytoplasmic tyrosine kinase that contributes to integrin-mediated signal transduction and migration by regulating actin cytoskeleton remodeling

Histone acetylation
a dynamic epigenetic modification of chromatin in which lysine residue(s) of histone molecules are reversibly acetylated by histone acetyttransferases; acetylation is generally associated with permissive activation of gene regulatory regions

Histone H3 acetylation (H3Ac)
a post-translational histone modification of a lysine residue on the amino-terminal tail of histone 3; H3Ac is considered to be a mark of transcriptionally active chromatin

IgH locus contraction
the process of large-scale contraction of the immunoglobulin heavy-chain (*IgH*) locus in developing B cells that are poised to undergo IgH V(D)J recombination. This contraction brings the recombining V, D, and J loci into close 3D proximity

Immunoreceptor tyrosine-based activation motifs (ITAMs)
conserved sequences of four amino acids in the cytoplasmic tails of non-catalytic tyrosine-phosphorylated receptors. They are important for signal transduction in immune cells

mTOR
a serine/threonine protein kinase and member of the PI3K-related kinase family that regulates cell growth, proliferation, motility, survival, transcription, and protein synthesis

Receptor editing
antibody gene rearrangement in a B lymphocyte to replace an already existing antigen receptor to mitigate autoreactivity

SRC kinases
a family of non-receptor tyrosine kinases that includes nine members: SRC, YES, FYN, FGR, LCK, HCK, BLK, and LYN

STAT5A/B
two highly related proteins. Once phosphorylated and activated, STAT5 proteins dimerize and bind to DNA response elements, inducing transcription of their target genes

Transcription factories
transcription foci or discrete sites where transcription occurs in the nucleus; in transcription factories several RNA polymerases are thought to be associated with the nuclear matrix

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Box 1.

The Epigenetics of *Igk* Recombination in Mouse Progenitor B Cells

Epigenetic regulation is essential for coordinating the timing of recombination at the *Igk* locus (Figure 2B). Before recombination, while developing B cells are actively proliferating, it is important to prevent cleavage of the *Igk* locus by RAG proteins to avoid genomic instability. Although it drives proliferation, IL-7R signaling also leads to the phosphorylation and activation of STAT5, which then directly binds within the *Igk* intronic enhancer (iEκ) as a tetramer, and recruits the Polycomb repressive complex 2 (PRC2), which includes the methyltransferase subunit Enhancer of Zeste homolog 2 (EZH2)[25,43]. EZH2 then modifies nucleosomes at the iEκ, Jκ, and Cκ regions with the histone H3 lysine 27 trimethylation (H3K27me3) mark. This is a repressive histone mark that silences gene regions by condensing chromatin and making DNA less accessible for transcription factor binding [109]. This mechanism of repression does not extend to Vκ regions, which are devoid of either activating or repressive histone marks [1,42]. Instead, another downstream target of IL-7R signaling, cyclin D3, serves as a potent repressor of Vκ transcription [30]. The unique action of cyclin D3 on Vκ appears to be due to the differential compartmentalization of cyclin D3 with Vκ genes within the nuclear matrix. By an unknown mechanism, the capture of Vκ gene-containing topologically associating domains by **transcription factories** is prevented by cyclin D3, resulting in transcriptional repression [42].

In pro-B cells, STAT5-mediated silencing of target genes is usually stable through subsequent stages of B lymphopoiesis via the persistence of the H3K27me3 repressive mark [43,109]. However, two STAT5 target genes, *Igk* and *Brwd1*, are immediately and strongly induced upon transition to the small pre-B cell stage [75]. BRWD1 is a histone lysine-acetylation reader and a member of the dual bromodomain and WD40 repeat protein families that associates with the SWI/SNF chromatin-remodeling complex [43,75,110]. BRWD1 is rapidly induced after escape from IL-7R signaling and is then recruited to Jκ. Binding of BRWD1 at Jκ leads to both increased local chromatin accessibility and repositioning of nucleosomes relative to DNA GAGA motifs [75]. This exposes the recombination signal sequence (RSS), enabling RAG recruitment and subsequent *Igk* recombination [75]. In addition, BRWD1 inhibits proliferation by coordinately repressing *Myc* and MYC downstream targets [89]. Thus, two downstream effectors of IL-7R, STAT5 and cyclin D3, set off an intricate system of epigenetic regulation of the *Igk* locus, temporally closing and then opening the locus to recombination.

Box 2.

Significance of Reduced IL-7R Signaling in Pre-BCR-Mediated Initiation of Igκ Recombination in Mice

A series of feedforward and feedback regulatory loops between IL-7R and pre-BCR signaling ensure the dominance of one receptor at a given time. However, numerous reports have demonstrated that IL-7R signaling dominates over pre-BCR signaling when both are present [21,29,40,41,43,68]. The reasons behind this include the following:

- **i.** IL-7R induces STAT5 activation, which drives a proliferative burst of pre-B cells by upregulating cyclin D3. To maintain genomic integrity, the cells are unable to recombine their *Igk* loci while cycling [3,11,19,28,29].
- **ii.** STAT5 and cyclin D3 directly repress Jκ and Vκ gene segments, respectively, by physically binding to those regions to epigenetically silence *Igk* recombination [25,30,42,43].
- **iii.** IL-7R-mediated induction of MYC, which promotes growth and expansion, is turned off to reach a quiescent pre-B state for *Igk* recombination [31–33,75,89].
- **iv.** STAT5-mediated repression of BRWD1 via H3K27me3 marks prevents the epigenetic set-up required for *Igk* recombination. BRWD1 is necessary to restrict *Igk* recombination in a lineage- and developmental stage-specific fashion [43,75,89].
- **v.** IL-7R destabilizes FOXO1 and FOXO3 via activation of the PI3K–AKT pathway, thus repressing the expression of *Rag1* and *Rag2* – that are absolutely necessary for recombination [40,41].
- **vi.** IL-7R-mediated inactivation of FOXO1 and PAX5 activity cannot fully induce the SYK–BLNK signaling module required for cell-cycle cessation and *Igk* recombination [21,63–65].
- **vii.** In the presence of IL-7R signaling, pre-BCR-mediated induction of the RAS–ERK pathway is not sufficient to provide the necessary epigenetic signature to recruit essential transcription factors such as E2A and IRF4, and the chromatin remodeler BRWD1, to *Igk* loci for recombination [29,66,73–75].

Clinician's Corner

The process of B cell development, and more specifically the production of a diverse repertoire of B cells, is essential for the proper functioning of a healthy adaptive immune system. Defects in the production of antibodies can lead to immunodeficiency or autoimmunity. In addition, if proliferation and gene recombination processes are not properly segregated during development, genomic instability and oncogenic transformation can occur. Thus, it is not surprising that defects in the expression or function of IL-7R, pre-BCR, and CXCR4 receptors, which are important for the regulation of such processes, have been associated with immunodeficiency, autoimmunity, and cancer in humans [11,14–17,56,91–95].

Mutations in components of both the pre-BCR and IL-7R can lead to immunodeficiency [14,15,56,91]. WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome is a congenital human immunodeficiency caused by a gain-of-function mutation in CXCR4 [92,93,96].

Several IL7R single-nucleotide polymorphisms (SNPs) have been associated with autoimmune conditions in genome-wide association studies in humans [97–100]. In addition, aberrant expression of CXCR4 has been implicated in the pathogenesis of systemic lupus erythematosus in humans [101].

IL-7R, pre-BCR, and CXCR4 all play important roles in the development of some leukemias and lymphomas, as do their upstream and downstream effectors. IL-7R (CD127) is abundantly expressed on adult human pre-B cell acute lymphoblastic leukemia (pre-B-ALL), and correlates with the expression of proliferation and survival markers [94,102]. High IL-7R expression has been associated with relapse in pediatric B-ALL [94,95]. Several large studies of pre-B-ALL in humans found that most ALL cells carrying a *BCR–ABL1* gene rearrangement failed to express a functional pre-BCR [103–105] and lacked pre-BCR signaling [106,107]. Finally, in a related cancer, Waldenström macroglobulinemia, whole-genome sequencing revealed that the second most frequent somatic mutation was in *CXCR4*, occurring in ~30% of cases [108].

Further studies assessing the interplay of IL-7R, pre-BCR, and CXCR4 might lead to the development of new potential targets and therapeutic strategies for some of these conditions.

Outstanding Questions

The role of CXCR4 in directing late B lymphopoiesis warrants reconsideration of the functions of the pre-BCR. The principal function of the pre-BCR is to induce the IRF4/CXCR4 feedforward loop. Therefore, what is the function of the pre-BCR in B lymphopoiesis when initiating a complex CXCR4-dependent program, other than promoting transit across a discrete 'checkpoint'?

CXCR4 exclusively downregulates HR to favor NHEJ DNA repair. Repression of the HR pathway may be mediated by cessation of cell-cycle progression induced by CXCR4 signaling. Alternatively, CXCR4 signaling might directly downregulate HR genes. This is relevant because this process has been linked to the pathogenesis of some leukemias. Therefore, how does CXCR4 affect the DNA repair process to favor recombination? Does it act directly or indirectly?

CXCR4 induces the expression of NF-κB, an important transcription factor for the development of Igλ$^+$ B cells [7,83]. Furthermore, self-reactive early immature B cells bearing an Igκ light chain can diminish autoreactivity by subsequent rearrangement of the *Igl* locus provided that CXCR4 surface expression remains high [6,7]. In addition, although the Igκ:Igλ ratio is 95:5 in mice, the ratio is 50:50 in humans. Therefore, what is the role of CXCR4 in Igλ recombination that determines receptor editing and central tolerance?

By controlling exposure to IL-7, CXCR4 is thought to control the balance between IL-7R and pre-BCR signaling. However, CXCR4 regulates known oncogenes, including RAS–ERK, and has been implicated in the pathogenesis of cancer through multiple processes including invasion, epithelial–mesenchymal transition, and proliferation. Thus, why might CXCR4 function differently in normal versus leukemic B cells? In addition, during leukemic progression, what is the effect of CXCR4 on normal B cell migration and localization in the bone marrow?

Highlights

The generation of a diverse antibody repertoire is crucial for the adaptive immune system and is accomplished through sequential V(D)J recombination of loci encoding the immunoglobulin heavy (IgH) and light chain (Igκ and Igλ).

The tight regulation and separation of proliferation and differentiation processes during B cell development is crucial for avoiding genomic instability.

Previous models identified two receptors, IL-7R and the pre-BCR, as being essential in the regulation of the pre-B cell checkpoint. However, new research has brought to light the crucial role of a third receptor, CXCR4, in mouse models.

CXCR4 drives the positioning of developing B cells within specific microniches in the murine BM.

CXCR4 then mediates a feed-forward loop initiated by the pre-BCR that coordinates cell-cycle exit and pre-BCR repression. CXCR4 is also crucial for IgK recombination and for inducing the pathways to recruit the required transcription factors and chromatin remodeling complexes.

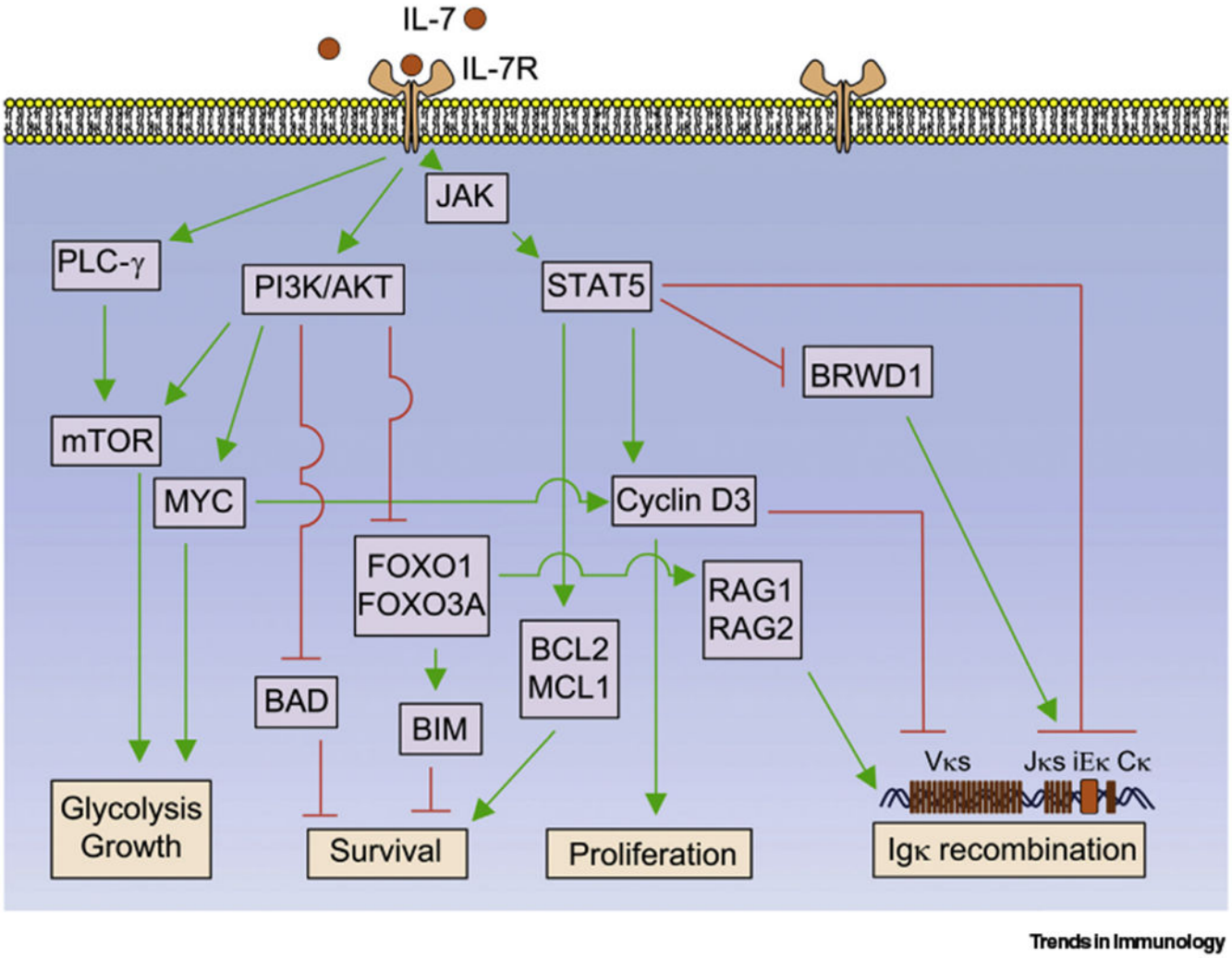


Figure 1. IL-7R Signaling Drives Proliferation and Survival while Preventing Premature *Igk* Recombination Programs During B Cell Development in Mice.

The stages of B cell development represent sequential phases of proliferation and differentiation. The survival and proliferation of pro-B and large pre-B cells is driven by signaling from the IL-7R receptor. IL-7R activates two major signaling pathways, JAK–STAT5 and PI3K–AKT. JAK kinases phosphorylate STAT5, which then stimulates transcription of cyclin D3, that promotes cell proliferation, and BCL2/MCL1 that promote cell survival. In addition, STAT5 also represses BRWD1 and *Igk* accessibility and recombination. The PI3K–AKT pathway inhibits FOXO1 and 3A, which induce the recombination-activating gene products RAG1 and 2 that are essential for recombination. The PI3K–AKT pathway, in addition to repressing recombination, plays a crucial role in cell survival by repressing the proapopotic proteins BAD and BIM. Finally, IL-7R signaling serves to promote cell growth and glycolysis via PI3K–AKT signaling, which upregulates mTOR and MYC; these in turn drive glycolysis and cell growth. mTOR is also upregulated by PLC-γ, another target of IL-7R signaling [1,31–34].

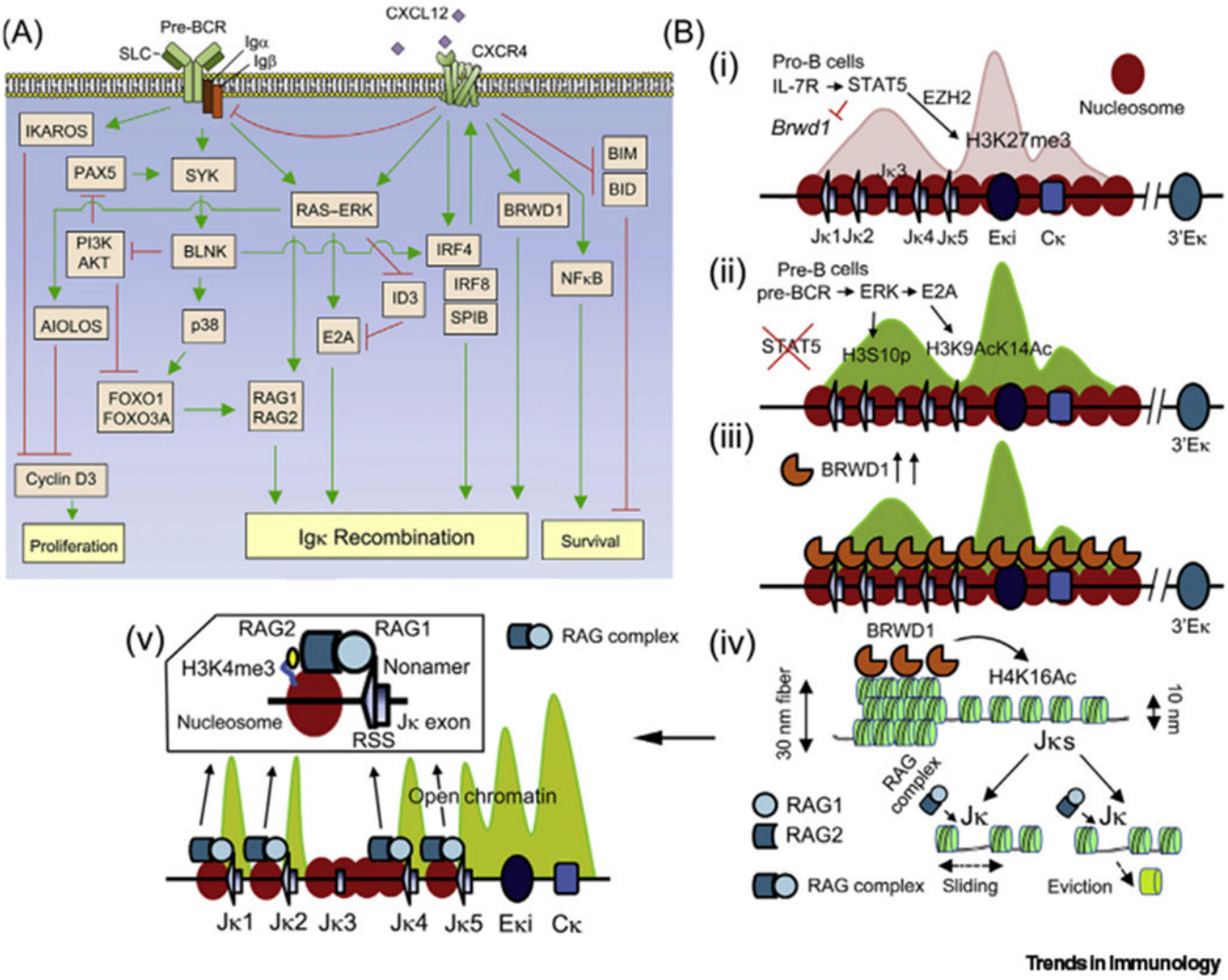
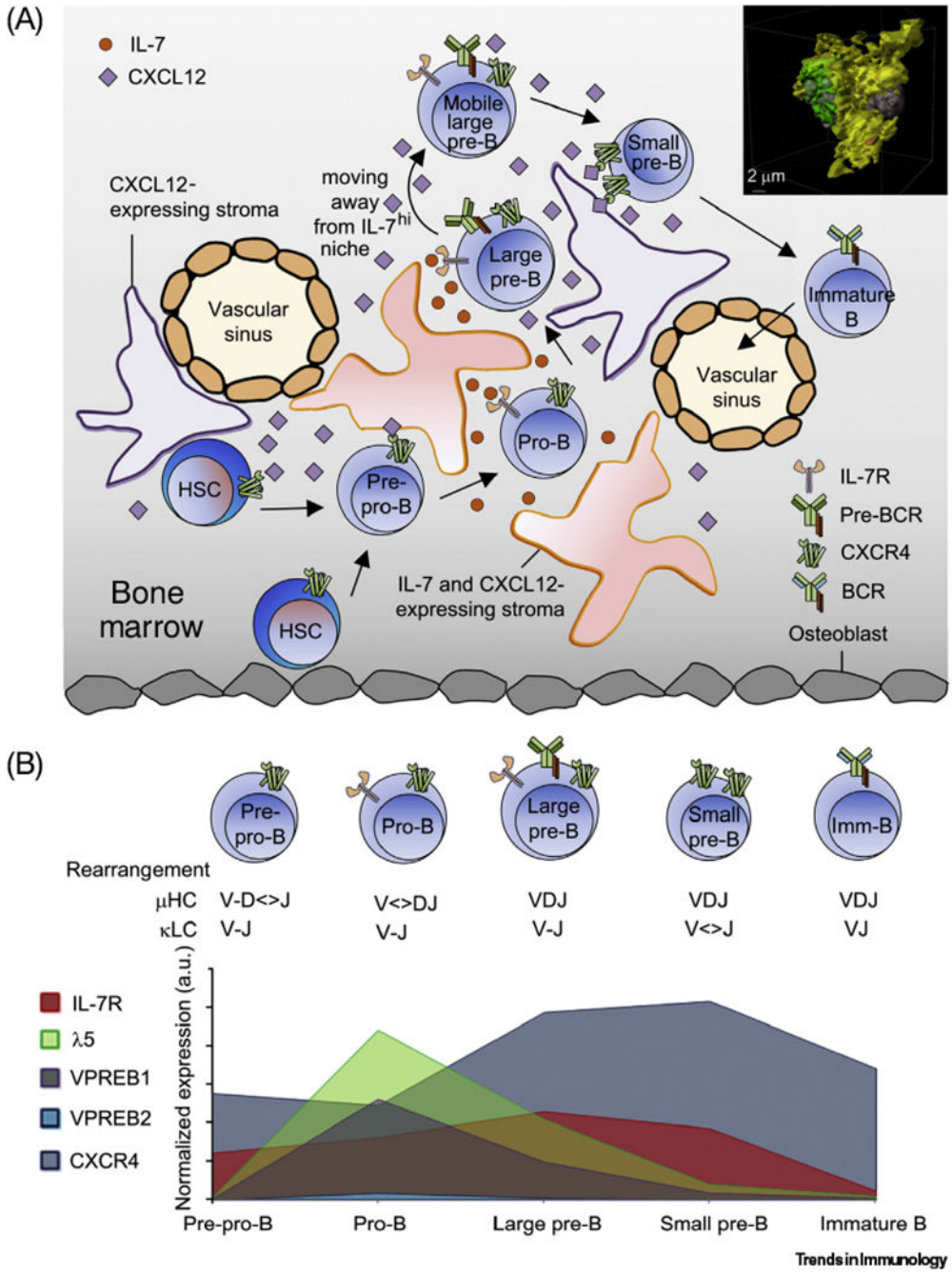


Figure 2. The Pre-BCR and CXCR4 Receptors Cooperate to Exit the Cell Cycle and Initiate Immunoglobulin Light-Chain Recombination in Mice.

(A) The pre-BCR serves to counteract the proliferative effects of IL-7R signaling by recruiting spleen tyrosine kinase (SYK), which phosphorylates B cell linker protein (BLNK), inhibiting PI3K–AKT signaling. The pre-BCR further depresses proliferative signaling by upregulating IKAROS and AIOLOS (via RAS–ERK), both of which repress cyclin D3. The pre-BCR also induces Igκ recombination by both derepressing and activating FOXO1, which then induces RAG1/2 and the transcription factor IRF4. RAS/ERK signaling is activated by the pre-BCR, which also serves to upregulate E2A and RAG1/2, both of which are required for Igκ recombination. Signaling from CXCR4, activated in response to binding its ligand CXCL12, supports and perpetuates signaling from the pre-BCR. CXCR4 helps to drive Igκ recombination by upregulating the RAS–ERK signaling module, BRWD1, and IRF4/IRF8/SPIB. In addition, CXCR4 signaling supports cell survival by repressing BIM/BID and upregulating NF-κB [7,83]. (B) During the transition from the pro-B to differentiating pre-B cell stage, significant epigenetic remodeling occurs in the *Igk* locus: (i) at the pro-B and large pre-B cell stages, IL-7R-driven STAT5 signaling represses *Brwd1* and

upregulates EZH2, which lays down repressive H3K27me3 histone marks; (ii) in differentiating pre-B cells, when ERK signaling is activated, repressive histone marks are replaced by H3S10p (directly via ERK) and H3K9AcK14Ac epigenetic marks (via E2A); (iii) BRWD1 is then recruited by this specific epigenetic landscape to the Igκ recombination center; (iv) BRWD1 regulates the positioning of nucleosomes; (v) this allows the recruitment of the RAG complex and subsequent recombination [43,75]. Abbreviations: H3K9/K14Ac, histone H3 acetylation on lysine 9/14; H3K27me3, histone H3 lysine 27 trimethylation; H3S10p, histone H3 phosphorylation on serine 10; RSS, recombination signal sequence.



Key Figure, Figure 3. The Murine Bone Marrow Microenvironment is Crucial for Early B Cell Development and for the Activation of Essential Receptors

(A) The ability of pre-BCR signaling to overcome IL-7R signaling is highly dependent on the CXCL12- and IL-7-rich microniches within the bone marrow (BM). Based on mouse models, pre-pro-B cells migrate to IL-7^{high} microniches from $CXCL12^{high}$ niches and differentiate into pro-B cells [78,80,81]. Pro-B cells are largely nonmotile and reside in IL-7^{high} niches where they upregulate IL-7R signaling and proliferate [78,79,81]. Large pre-B cells then upregulate CXCR4 and become increasingly sensitive to CXCR12. These highly motile large pre-B cells [81] undergo chemotaxis away from IL-7^{high} niches toward

IL-7$^{-/low}$CXCL12^{high} niches [83], where they activate pre-BCR and CXCR4 signaling and are able to escape from the cell cycle and recombine the immunoglobulin light-chain genes (*Igk* and *Igl*) [21,68,83]. Newly generated immature B cells downregulate CXCR4 and exit the BM for peripheral development [88]. Ligand–receptor contact in the figure depicts active signaling of that receptor. High-power confocal microscopy with 3D reconstruction showed that small pre-B cells (green) are in tight contact with CXCL12 (yellow), and with high local accumulations of extracellular CXCL12 (inset). (B) Graphical representation of the expression of the three essential early B cell development receptors discussed in mice: IL-7R, pre-BCR (represented by its components λ5 and VPREB1/VPREB2), and CXCR4. Expression of IL-7R peaks at the large pre-B cell stage, and is gradually downregulated during later developmental stages; IL-7R signaling is dependent on the presence of the IL-7 ligand in the BM microniche. Expression of the pre-BCR components spike at the pro-B cell stage; however, V_H to D_HJ_H recombination is ongoing at that stage (denoted by <>). As a result, the functional pre-BCR complex is formed at the large pre-B cell stage when the rearranged heavy chain is available to make the pre-BCR. The expression of the third receptor, CXCR4, increases in the large pre-B cell stage where it helps the motile large pre-B cells to escape IL-7R signaling. Subsequently, CXCR4 downstream signaling arrests cell proliferation and completes *Igk* recombination as well as repressing pre-BCR components to generate immature B cells with a functional BCR. Abbreviations: a.u., arbitrary units; HSC, hematopoietic stem cell; imm, immature.