

# B Cell Signaling and Fate Decision

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## Key Words

B cell antigen receptor, calcium/NF- $\kappa$ B signaling pathway,  
microRNAs, B cell development, immune response, germinal center

## Abstract

Antigen receptors on the surface of B lymphocytes trigger adaptive immune responses after encountering their cognate antigens but also control a series of antigen-independent checkpoints during B cell development. These physiological processes are regulated by the expression and function of cell surface receptors, intracellular signaling molecules, and transcription factors. The function of these proteins can be altered by a dynamic array of post-translational modifications, using two interconnected mechanisms. These modifications can directly induce an altered conformational state in the protein target of the modification itself. In addition, they can create new binding sites for other protein partners, thereby contributing to where and when such multiple protein assemblies are activated within cells. As a new type of post-transcriptional regulator, microRNAs have emerged to influence the development and function of B cells by affecting the expression of target mRNAs.

**BCR:** B cell receptor  
**miRNA:** microRNA  
**GC:** germinal center

## INTRODUCTION

B lineage cells are the central mediators of humoral immunity. Plasma cells (PCs) are the terminal effector cells of the lineage, and they can neutralize pathogens by secreting pathogen-specific antibodies. PCs arise as a consequence of a highly regulated differentiation process that is initiated when B lymphocytes encounter antigens through their B cell receptors (BCRs) (1). However, it is clear that BCRs can also signal independently of antigen, a process termed tonic signaling, thereby regulating the antigen-independent phase of B cell development (2, 3).

For these antigen-dependent and -independent signals to be correctly interpreted, the expression of the BCR, other cell surface receptors, and intracellular molecules must be regulated correctly, depending on the developmental and activation stage of the B cells (4). In addition to the complex transcriptional programs already known to regulate the expression of genes involved in B cell signaling, microRNAs (miRNAs) have emerged as a new type of post-transcriptional regulator of gene expression.

At a given developmental and activation stage of the B cells, in response to extracellular microenvironmental cues, intracellular molecules must be integrated into a signaling complex dictated by temporal and spatial demands. Over the past few years, investigators have better appreciated the requirements for the proper localization of signaling molecules at a given time. Changing the localization and activation status of these molecules is frequently mediated by reversible covalent modifications. These post-translational modifications (5) include phosphorylation, methylation, acetylation, ubiquitylation, and sumoylation. In this review, we summarize the major advances in our understanding of the mechanisms by which transcriptional, post-transcriptional, and post-translational modifications interact spatiotemporally and how B cells integrate these interactions to make the correct cell fate decisions during development and humoral immune responses.

## miRNA-MEDIATED POST-TRANSCRIPTIONAL REGULATION

miRNAs, which investigators initially discovered from a curiosity in nematodes, have recently been recognized as important mediators of post-transcriptional gene regulation in higher metazoans, including mammals. In this section, we discuss the cellular function of miRNAs, focusing specifically on the role of miR-150 and miR-155 in regulating B cell development and immune responses.

At present, biochemical and genetic studies have provided a basic understanding of the biogenesis of miRNAs (6–9). The processes involved appear to be tightly regulated, depending on the developmental and activation stages of B cells. For instance, as discussed below, miR-150 is expressed selectively in mature B cells but not in pro-/pre-B cells (10). In the case of miR-155, its expression increases after BCR, LPS, or CpG stimulation, probably contributing to the fact that it exerts its function in germinal center (GC) responses (11, 12). Such differential expression of miRNAs is likely to be regulated at various points in their biogenesis, including the transcriptional and post-transcriptional levels; however, the molecular mechanisms underlying these regulations remain unclear.

The usual result of miRNA-mediated gene regulation is a reduction in the amount of target protein that is synthesized. Some, but not all, of the mechanisms by which miRNA achieves this outcome have been clarified. In most cases, miRNAs repress target gene expression by inhibiting translation, so that the mRNA levels remain constant while the encoded protein's level declines. Less frequently, miRNAs repress target gene expression by triggering the degradation of target mRNAs, using the same mechanisms as siRNA and shRNA to silence gene expression. Compared with other gene-regulatory mechanisms (such as chromatin modification and transcriptional controls occurring in the nucleus), miRNA-mediated gene regulation typically occurs during the step directly before protein synthesis and thus

is highly suited for fine-tuning gene expression. Moreover, at the molecular level, one species of miRNA can regulate the expression of multiple genes simultaneously, thereby more broadly impacting biological processes. For instance, miR-181a controls the T cell receptor (TCR) signaling threshold and strength in part by simultaneously dampening the expression of multiple phosphatases, such as SHP2 and PTPN22, which are negative regulators of the TCR signaling cascade (13).

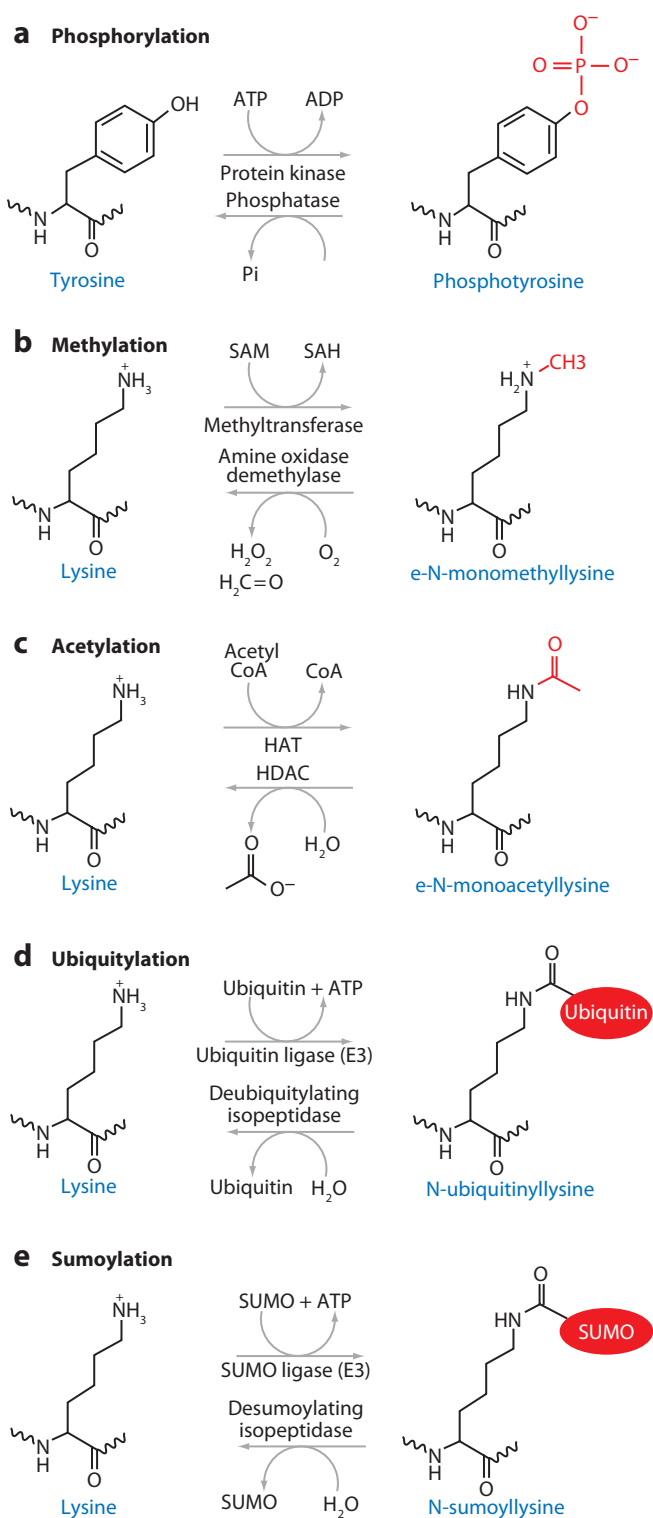
The function in B cell differentiation of two types of miRNA, miR-150 and -155, has been well elucidated. As mentioned above, miR-150 expression cannot be detected in pro-/pre-B cells, but it increases in mature B cells. Xiao and colleagues (10) recently demonstrated the importance of miR-150 in B cell formation and humoral immune responses using gain- and loss-of-function mouse models. *miR-150* knockout mice have increased numbers of B-1 cells and augmented T-dependent (TD) immune responses. In terms of miRNA target validation, bioinformatics analyses suggested that c-Myb could be a miR-150 target in this system. To test this possibility directly, Xiao and colleagues generated mice that ectopically express miR-150, in which the expression level of miR-150 in pro-/pre-B cell stages was comparable with that in mature B cells. B cell development in these miR-150 transgenic mice was blocked at the pro- to pre-B cell transition. This phenotype is similar to that previously seen in *c-Myb*-deficient mice, indicating that the enforced expression of miR-150 probably suppressed c-Myb in pro-/pre-B cells. Furthermore, in vitro analysis using a reporter assay showed that miR-150 directly inhibits c-Myb expression by binding to the 3' untranslated region of the *c-Myb* mRNA.

miR-155 is overexpressed in certain human B cell lymphomas, including diffuse large B cell lymphoma, Hodgkin's lymphoma, and Burkitt lymphoma (14), suggesting that it might participate in B cell growth. Mice carrying mutated *miR-155* are less responsive to immunization. Indeed, two groups elegantly demonstrated that miR-155 regulates the formation and response

of GC B cells (11, 12). The transcription factor PU.1 has been validated as one of the crucial targets of miR-155-mediated inhibition (11), and thus its level is downregulated in wild-type B cells. Accordingly, some phenotypes observed in *miR-155* knockout mice, such as fewer IgG1-bearing B cells, are recapitulated when PU.1 is ectopically overexpressed in a wild-type background. The activation-induced cytidine deaminase (AID) has been demonstrated recently to be another miR-155 target (15, 16), and mutation of the AID miR-155 target site resulted in increased AID expression, thereby causing enhanced class switching and defective affinity maturation (15). As the mutated AID did not change the frequency or overall pattern of Ig variable region gene mutations in GC B cells, a certain narrow range of AID expression is probably required for the in vivo positive selection of high-affinity B cells, and this optimal level is apparently titrated by miR-155 and possibly other miRNAs.

## POST-TRANSLATIONAL MODIFICATIONS

The cellular response to changing environmental conditions is mediated frequently by the reversible covalent modifications of preexisting molecules, a process termed post-translational modification. One mechanism by which post-translational modifications can alter a protein's function is by directly inducing a new conformational state. A classic example is the phosphorylation of the activation loop of protein kinases (**Figure 1a**). Protein kinases have a common "on" state, in which they are phosphorylated on one or two serine, threonine, or tyrosine residues of a key segment known as the activation loop. Activation-loop phosphorylation stabilizes the assembly of an enzymatic site that catalyzes the  $Mg^{2+}$ -dependent transfer of the  $\gamma$ -phosphoryl group of the ATP to the substrate protein (17). Such conventional allosteric regulation alone, however, cannot adequately explain why distinct signaling complexes are formed and how the activation status of these complexes is spatiotemporally



regulated. Therefore, the notion that post-translational modifications create binding sites for specific protein-interaction domains, which in turn contribute to the formation of signaling complexes, has strong appeal. A single modification cannot guarantee specificity; therefore, a combination of various post-translational modifications (including phosphorylation, methylation, acetylation, ubiquitylation, and sumoylation) is thought to be required for the selective assembly of proteins into signaling complexes and for their subsequent activation and cellular location (**Figure 1**). In the following section, we discuss the common mechanisms by which post-translational modifications regulate protein-protein interactions and subsequent signaling processes.

## Protein Phosphorylation

Protein tyrosine kinases (PTKs) can activate intracellular pathways through the inducible recruitment of proteins with SH2 domains. SH2 domains typically bind to pTyr-containing peptide motifs of five to eight residues in a manner that depends on ligand phosphorylation and the identity of the flanking amino acids (5). The biological importance of this type of association is typically exemplified by the interaction between the two SH2 domains of Syk and the doubly phosphorylated (dp) immunoreceptor tyrosine-based activation motifs (ITAMs) of Ig $\alpha$ /Ig $\beta$  (18). With the assumption that Syk has a structural organization similar to the T cell PTK, Zap-70, recent crystallographic analysis has provided significant insight

**Figure 1**

Examples of post-translational modification reactions. Various amino acid side chains can be modified by, for instance, (a) phosphorylation, (b) methylation, (c) acetylation, (d) ubiquitylation, and (e) sumoylation. The enzymes that are involved in the addition and removal of these post-translational modifications are shown on the reaction arrows. Adapted by permission from Macmillan Publishers Ltd., *Nature Reviews Molecular Cell Biology*, 7:474, **Figure 1**, copyright 2006.

into how Syk maintains the autoinhibited state and how this state is broken down by the binding of dp-ITAMs to the tandem SH2 domains of Syk (19). Two tyrosine residues (Tyr342 and Tyr346) in the SH2-kinase linker region of Syk are involved in aromatic-aromatic interactions that connect this linker to the kinase domain, thereby helping to keep the Syk kinase in the inactive state. Binding of the SH2 domains of Syk to dp-ITAMs disrupts the aromatic-aromatic interactions, removing this inhibitory constraint and subsequently activating Syk kinase activity by facilitating phosphorylation on Tyr519 in the activation loop (20). Another means to disrupt the suppressive aromatic-aromatic interactions is mediated by phosphorylation on Tyr342 and Tyr346. In fact, Tyr342 and Tyr346 are phosphorylated following BCR stimulation, and this phosphorylation plays a positive role for BCR signaling (18). Assuming that this post-translational modification is maintained even after disengagement of the dp-ITAMs and Syk, it could keep Syk activated persistently. Therefore, the phosphorylation status of Tyr342/Tyr346 could potentially impact how strongly Syk activity is maintained and for how long during and after BCR signaling. These structural and functional studies clearly demonstrate that the dp-ITAMs overcome the inherent promiscuity of Syk SH2 domains, making the recruitment of Syk to the BCR complex highly specific. In addition, this binding releases Syk from its autoinhibited state, thereby facilitating the activation of its kinase domain and probably restricting the location of the activated form of Syk to the vicinity of the BCR.

Ser/Thr phosphorylation is more prevalent than Tyr phosphorylation, and there is a correspondingly larger array of domains that selectively bind to pSer/pThr sites. Many of these associations can be discerned for pSer/pThr-binding proteins, such as the ubiquitously expressed 14-3-3 proteins (21). These proteins form noncovalent homo- and heterodimers, which can consequently bind to two pSer/pThr-containing peptides that have an appropriate consensus sequence. As a result,

14-3-3 proteins can regulate the conformation and catalytic activity of the associated phosphorylated enzymes and can control their interactions and localizations.

## Protein Methylation and Acetylation

Peptide motifs that contain Lys residues can be methylated or acetylated (**Figure 1b,c**), which can lead to their recognition by chromodomains (CDs) or bromodomains, respectively (5). These modifications are not restricted to nuclear proteins such as histones; for instance, a recent study showed that the type I interferon receptor on the cell surface undergoes acetylation mediated by the coactivator p300/CBP (CREB-binding protein), which possesses histone acetyltransferase activity, and that this post-translational modification is crucial for subsequent antiviral gene expression (22). Nevertheless, acetylation and methylation are particularly prominent features of the flexible N- and C-terminal tails of histones and are important for coupling histones to changes in chromatin organization and the epigenetic control of gene expression. The methylation of histone 3 at Lys9 (H3K9) serves as the prototype for the regulation of histone function. Di- or trimethylation of H3K9 creates a binding site for CD-containing proteins of the heterochromatin protein 1 family, and this binding is speculated to lead to gene repression via changes in higher-order chromatin structure (23).

Indeed, histone methylation/acetylation may control *Ig* gene locus accessibility to  $V_H(D)J_H$  recombination. In early developing bone marrow B cells, *D* to *J\_H* rearrangement precedes  $V_H$  to  $DJ_H$  joining. Prior to *D* to *J\_H* recombination, histone H3 acetylation is abundant within a 120-Kb region that encompasses the *D* gene segments and extends to the  $C\mu$  exons. Then the hyperacetylated domain spreads into the distal  $V_H$  gene region in an interleukin (IL)-7-dependent manner accompanied by  $V_H$  to  $DJ_H$  recombination (24, 25). However, acetylation alone appears to be insufficient to allow recombination factors access to the DNA template. H3K9

**DUB:**  
deubiquitylating  
enzyme

methylation correlates inversely with the efficiency of  $V_H(D)J_H$  recombination (24). Recombinase access requires the removal of this repressive methylation mark, a process regulated by the transcription factor Pax5 (26). The repressive role of methyl H3K9 in  $V_H(D)J_H$  recombination has been supported further by the observation that *TCR $\beta$*  mini-locus recombination is inhibited when the DNA substrate is tethered to the histone H3K9 methyltransferase G9a (27).

## Protein Ubiquitylation and Sumoylation

Many proteins are modified by ubiquitin (Ub) or the Ub-like protein SUMO (**Figure 1d,e**). Ubiquitylation occurs through a three-step process involving Ub-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3) enzymes (28, 29). The pathway of sumoylation is mechanically analogous to ubiquitylation; however, SUMO conjugation requires a set of enzymes that is distinct from that acting on Ub (30). Ub is a 76-residue protein that is most often linked to the Lys side chains on target proteins. The types of Ub modifications are diverse. In the simplest form, a single Ub molecule is attached, which is defined as monoubiquitylation. Because Ub itself contains several Lys residues, Ub molecules can form different types of chains on the target proteins in an iterative process called polyubiquitylation. All seven Lys residues in Ub can potentially be involved in chain formation in vivo, but Ub chains linked via Lys48 or Lys63 are the best characterized. Lys48-linked poly-Ub chains clearly represent a signal for the degradation of the modified substrate by the proteasome. However, other types of Ub conjugates (e.g., those that are Lys63-linked) are involved in the regulation of different processes, such as the facilitation of signaling and intracellular trafficking by the formation of macromolecular complexes. Ub is essentially a transferable interaction domain, which, following its attachment to target proteins, is recognized by proteins that contain specialized interaction modules

(Ub-binding domains). So far, at least 11 families of Ub-binding domains have been identified (31). They are structurally different but generally bind with relatively low affinity to the same hydrophobic patch on Ub, centered on Ile44. Many Ub-binding proteins possess several Ub-binding domains and can therefore be coupled to distinct Ub signals simultaneously. This feature seems ideally suited for the rapid assembly and disassembly of multiple proteins through Ub-based protein-interaction networks.

Ubiquitylation is now known to be a reversible reaction in which the Ub chains are deconjugated by deubiquitylating enzymes (DUBs) (32). Similar to the E3 Ub ligases, DUBs have a certain degree of substrate specificity, which appears to be created in at least three ways. First, DUBs often contain protein-interaction domains, which enable them to bind specific target proteins. Second, some DUBs have a preference for specific Ub branches, such as Lys48- or Lys63-linked chains. Third, the mode of expression and the subcellular localization of DUBs could contribute to their in vivo functions, although this aspect has not been studied extensively.

This paradigm apparently also holds for sumoylation. For example, SUMO-binding proteins contain a SUMO-interacting motif, which forms a  $\beta$  strand that binds in a parallel or antiparallel orientation to the  $\beta$ 2 strand of SUMO (33, 34). A sumoylated Lys residue therefore recruits a different set of effectors than if the same residue was ubiquitylated. For instance, the sumoylation of proliferating cell nuclear antigen (PCNA), a polymerase processivity factor that forms a sliding clamp around DNA, at Lys164 promotes high-fidelity replication by recruiting a helicase (Srs2) that contains a SUMO-interacting motif to replication forks, thereby preventing inappropriate recombinational repair. By contrast, in the case of DNA repair, DNA damage promotes the monoubiquitylation of PCNA at the same Lys residue (Lys164), recruiting preferentially damage-tolerant polymerase  $\eta$  and mediating post-replicative lesion bypasses (35, 36).



## B CELL RECEPTOR SIGNALING PATHWAYS

The earliest biochemical events of the BCR signal have been well characterized. However, these previous analyses have two main weaknesses. First, because they required a relatively large number of B cells, experiments usually have been conducted using antigen stimulation. As discussed below in more detail, in addition to ligand-induced signaling, ligand-independent signaling also plays a critical role. For instance, the cell surface expression of the pre-BCR, *per se*, has been thought to be sufficient for inducing biological outcomes. Because of the difficulty of analyzing such cell surface expression-dependent signaling, our understanding of the biochemical nature of ligand-independent signaling has been limited, and models for this type of signaling are often speculative extrapolations from studies of ligand-induced events. Second, because biochemical analyses rely on the examination of populations of cells, these studies cannot provide quantitative data at the single-cell level or on the dynamic localization of membrane receptors and signaling molecules. Thus, to overcome this second limitation, investigators have employed a new technology using supported planar lipid bilayers, which are thought to mimic the recognition of membrane-tethered antigens on the surface of an antigen-presenting cell. This approach is combined with advanced imaging techniques such as total internal reflection fluorescence and Förster resonance energy transfer (FRET), providing insight into novel aspects of the spatio-temporal dynamics of receptor signaling. Therefore, the following section discusses new ideas generated by such imaging studies and the progress made in understanding signaling events through biochemical and genetic studies.

### Early B Cell Receptor Signaling Events

The ligand-binding subunit of the BCR complex consists of the transmembrane form of

Ig, composed of two identical heavy chains (IgH) and two identical light chains (IgL) in covalent association. BCR signal transduction capability is provided by a noncovalently associated heterodimer of Ig $\alpha$ /Ig $\beta$ . BCR oligomerization induced by multivalent antigen binding was thought to be sufficient to initiate the signaling event (18, 37). The question then arose about what structural changes, particularly in the cytoplasmic domains of Ig $\alpha$  and Ig $\beta$ , take place after BCR oligomerization. Pierce and colleagues (38) addressed this question by monitoring FRET between individual BCR subunits. After binding of multivalent antigen, the cytoplasmic domains of the BCR components also cluster. Then the Ig $\alpha$  and Ig $\beta$  cytoplasmic domains are separated by a considerable distance, transitioning from a closed to an open conformation. The open conformation is dependent on ITAM phosphorylation, most likely by the action of Lyn, one of the Src family PTKs. Supporting Lyn's involvement in the transition to this open conformation, the BCR selectively and transiently associates with Lyn after antigen engagement, followed by induction of the open conformation (39). Thus, imaging analysis using the FRET methodology supports the model previously proposed based on biochemical and genetic analyses; namely, the phosphorylation of ITAMs located in the cytoplasmic domains of Ig $\alpha$  and Ig $\beta$ , mediated by Lyn, leads to an open conformation, thereby recruiting other signaling molecules, including Syk.

Although B cells do respond to soluble antigen *in vitro*, some proportion of the antigens encountered *in vivo* are in a membrane-associated form. For instance, in the interaction between follicular dendritic cells (FDCs) and B cells, FDCs hold large amounts of intact antigens on their surfaces by virtue of antigen binding to the FDC Fc and complement receptors. In this physiologically relevant context, B cells will recognize cognate antigens in a cell-cell contact-dependent manner. Batista and colleagues (40) have analyzed the molecular events that follow this type of recognition by using TIRF microscopy and

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**IgH:** immunoglobulin heavy chain

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**IP<sub>3</sub>:** inositol-1,4,5-trisphosphate

**SOCE:** store-operated Ca<sup>2+</sup> entry

planar lipid bilayers containing ICAM-1 and antigens.

During the early stages of membrane-bound antigen recognition, B cells spread out over the antigen-bearing membranes and then contract, collecting bound antigen in a central aggregate. This response is concomitant with the formation of the immunological synapse, which is similar to that originally described for T cells (41, 42). This structure is characterized by the central accumulation of BCR and antigen, termed the central supramolecular activation cluster (c-SMAC), surrounded by a ring of adhesion molecules (peripheral SMAC, or p-SMAC). Intracellular signaling molecules such as Syk, phospholipase C $\gamma$ 2 (PLC $\gamma$ 2), and Vav1 are colocalized with the initial BCR-containing microclusters (43). After maximal spreading (approximately 3 min), these BCR clusters started to translocate to form c-SMACs. However, unlike the BCR, clusters including Syk (referred to here as microsignalosomes) did not move to the c-SMACs and appeared to dissociate from the BCR clusters. Similarly, after the recognition of membrane-bound antigens, CD19 was transiently recruited into the BCR clusters. This recruitment is functionally important for amplifying BCR signaling because CD19-deficient B cells exhibited a decrease in early signaling events, including calcium mobilization and tyrosine phosphorylation (44). These observations suggest that the microsignalosomes (probably including CD19, Syk, PLC $\gamma$ 2, PI3K, and Vav) are a critical site for the initiation of BCR signaling. At a later time point (15 min after stimulation), the microsignalosomes dissociate from the BCR clusters that reside in the c-SMACs, suggesting that the Ig $\alpha$ /Ig $\beta$  signaling components of the BCR inside the c-SMACs might be dephosphorylated. Given the evidence that the nonphosphorylated form of the Ig $\alpha$  is preferentially endocytosed (45), the non-tyrosine-phosphorylated BCR in the c-SMACs may no longer be involved in signaling, but instead would be internalized for subsequent antigen presentation on the B cell surface.

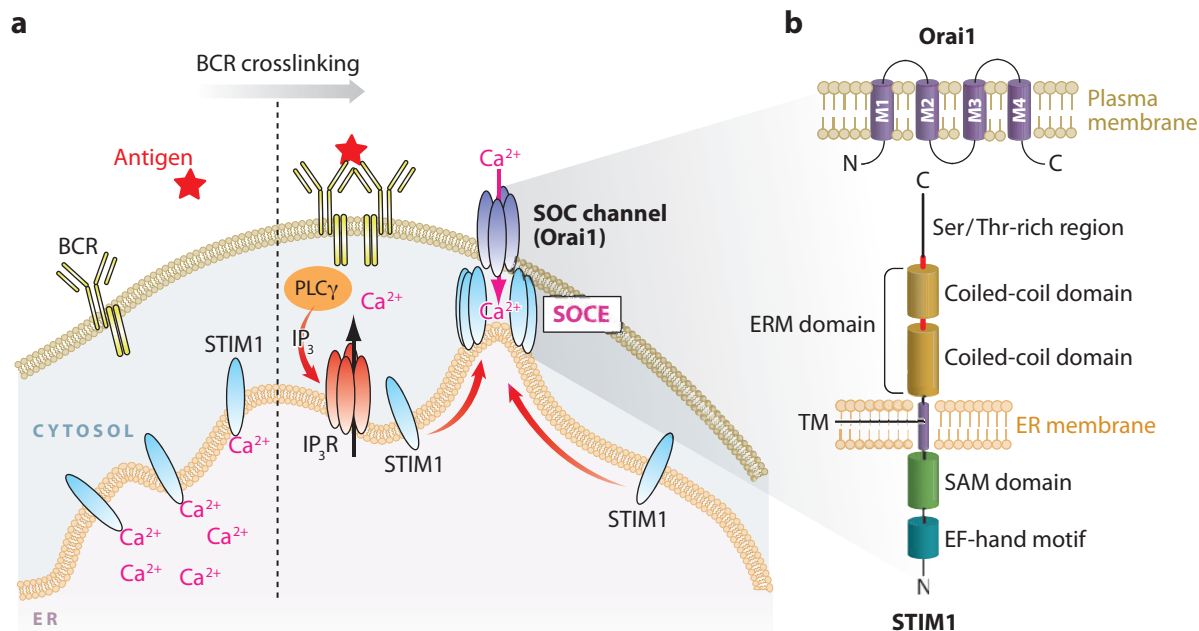
## Effector Systems

During BCR signaling, PLC $\gamma$ 2 and PI3K are crucial lipid metabolizing effector enzymes that generate key second messengers, which in turn activate I $\kappa$ B kinase (IKK) and extracellular signal-related kinase (ERK). In the following section, we discuss how the activation status of these important enzymes is regulated and how these activities are translated into biological outcomes.

**PLC $\gamma$ 2/calcium/NFAT pathway.** Activation of PLC $\gamma$ 2 by Syk, Btk, and BLNK results in production of the second messenger, inositol-1,4,5-trisphosphate (IP<sub>3</sub>), and diacylglycerol (DAG) (see Reference 46 for a detailed discussion of PLC $\gamma$ 2 activation mechanisms). BCR stimulation induces a dramatic increase in the intracellular Ca<sup>2+</sup> concentration, which is derived from two main sources: Ca<sup>2+</sup> release from the intracellular Ca<sup>2+</sup> stores, mainly from the endoplasmic reticulum (ER), and Ca<sup>2+</sup> entry from the extracellular space. The binding of IP<sub>3</sub>, a product of PLC $\gamma$ 2 activity, to IP<sub>3</sub> receptors in the membrane of the ER results in a rapid and transient release of Ca<sup>2+</sup> from the ER Ca<sup>2+</sup> stores into the cytosol (47). The subsequent reduction of Ca<sup>2+</sup> within the ER lumen triggers a sustained influx of extracellular Ca<sup>2+</sup> across the plasma membrane. More than 20 years ago, Putney (48) proposed the so-called store-operated Ca<sup>2+</sup> entry (SOCE) model, which postulated that the Ca<sup>2+</sup> influx across the plasma membrane results from emptying of the ER Ca<sup>2+</sup> stores (49). The recent identification of two molecular players, STIM1 (49, 50) and Orai1 (51, 52), and analysis of their knockout cells have identified the SOCE mechanism as the predominant effector in immune cells, including B cells (**Figure 2**).

STIM1, a type I ER membrane protein, was originally identified as a molecule on stromal cells that support bone marrow B cell differentiation (53). STIM1 has turned out to be the ER Ca<sup>2+</sup> sensor and SOCE activator, but not a channel by itself, as demonstrated by





**Figure 2**

Functional coupling between STIM1 and Orai1 in BCR-mediated store-operated Ca $^{2+}$  entry (SOCE) activation. *(a)* BCR stimulation causes PLC $\gamma$ /IP $_3$ /IP $_3$ R-mediated Ca $^{2+}$  release and store depletion followed by the relocation of STIM1 into puncta. The decrease in luminal ER Ca $^{2+}$  results in the dissociation of Ca $^{2+}$  from the EF-hand motif, which induces STIM1 aggregation and redistribution in the ER in close proximity with the plasma membrane. Here STIM1 functionally associates with the Orai1 channel in the plasma membrane, leading to SOCE. *(b)* A schematic representation of the functional domain of STIM1 and Orai1 proteins. The STIM1 protein contains an EF-hand motif (Ca $^{2+}$ -binding site), a sterile  $\alpha$  motif (SAM) domain, a transmembrane (TM) domain, coiled-coil domains, an ERM domain, and a Ser/Thr-rich region. The Orai1 protein contains four TM domains (M1–M4).

knockdown experiments in fibroblasts, T cells (49), and endothelial cells (50) and by knock-out studies in mast cells (54), T cells (55), and platelets (56). STIM1 also functions in SOCE activation in B cells, as shown by analysis of gene-targeted DT40 B cells (57) and primary B cells (Y. Baba & T. Kurosaki, unpublished results). STIM1 contains several conserved domains, including the EF-hand motif, sterile  $\alpha$  motif (SAM), and coiled-coil domains. In resting B cells, STIM1 looks to move dynamically in a tubulovesicular way on the ER, along with microtubules (57). This behavior is mediated through the coiled-coil domain and Ser/Thr-rich C-terminal region, which are located in the cytosolic side. After BCR-induced store depletion, Ca $^{2+}$  dissociates from the EF-hand motif located in the ER lumen, which may result in

a conformational change in STIM1 to form a homomultimer. STIM1 is then rapidly redistributed into discrete puncta that are located underneath the plasma membrane and associate with store-operated Ca $^{2+}$  channel components both physically and functionally (57–59). The SAM, coiled-coil domains, and Ser/Thr-rich domains are essential for the store-depletion-induced cluster formation of STIM1 and subsequent SOCE activation (57).

STIM2, a related homolog of STIM1, is also located in the ER membrane, but its precise role remains unclear. Consistent with a slight reduction of SOCE in STIM2 knockdown fibroblasts (49), SOCE is also moderately inhibited in STIM2-deficient DT40 B cells (Y. Baba & T. Kurosaki, unpublished results). Although possessing similar conserved domains,

functional differences between STIM1 and STIM2 have been proposed. Unlike STIM1, STIM2 plays a more dominant role in regulating basal cytosolic and ER  $\text{Ca}^{2+}$  concentrations; STIM2 can induce SOCE following a smaller reduction in ER  $\text{Ca}^{2+}$  content than STIM1 (60). Thus, STIM2 appears to be more sensitive to  $\text{Ca}^{2+}$  store depletion than does STIM1.

The molecular identification of the store-operated  $\text{Ca}^{2+}$  channel has been advanced by an analysis of T cells derived from severe combined immunodeficiency syndrome patients deficient in SOCE. A linkage analysis and RNAi-based screening identified *Orai1* (also known as *CRACM1*) as the gene mutated in these patients (51, 52). The coexpression of Orai1 and STIM1 apparently results in synergic effects on SOCE (61, 62). A direct physical association between STIM1 and Orai1 has been proposed but is still controversial; however, these two molecules clearly colocalize after  $\text{Ca}^{2+}$  store depletion. Thus Orai1 is currently the best candidate for the store-operated  $\text{Ca}^{2+}$  channel or a subcomponent of this channel, which is a target of STIM1. The Orai family consists of Orai1, Orai2, and Orai3 (58, 59). In chicken DT40 B cells, Orai1 and Orai2 have a redundant role for STIM1-mediated SOCE (Y. Baba & T. Kurosaki, unpublished results). One study of Orai1-deficient mice demonstrated that SOCE was abolished in mast cells but not in T cells (63), whereas another report showed that Orai1-deficient T cells have severe defects in SOCE, similar to the human severe combined immunodeficiency case mentioned above (64). Recent reports suggest that the store-operated  $\text{Ca}^{2+}$  channels are composed of the Orai family and the C-type transient receptor potential family, and that STIM1 is not a component of the channels but is essential for their activation (65, 66).

In most cells (including B cells), calmodulin is a cytosolic  $\text{Ca}^{2+}$ -binding protein that functions as a calcium sensor by binding  $\text{Ca}^{2+}$  with its two EF hands. Calmodulin bound with  $\text{Ca}^{2+}$  activates several molecules, and one important target is calcineurin phosphatase, which is a

heterodimer composed of a catalytic subunit ( $\text{A}\alpha$ ,  $\text{A}\beta$ , and  $\text{A}\gamma$ ) and a regulatory subunit (B1 and B2). Nuclear factor of activated T cells (NFAT) is a well-studied calcium-dependent transcription factor downstream of calcineurin (67). NFAT is a cytosolic protein and requires a continuous increase in  $\text{Ca}^{2+}$  to remain in the nucleus, wherein it activates transcription of target genes. B cells express three NFAT family members, NFATc1 (alternatively named NFAT2 or NFATc), NFATc2 (NFAT1 or NFATp), and NFATc3 (NFAT4 or NFATx) (68), which are components of the BCR signaling cascade. Although mice deficient in each NFAT member have been reported (69–71), these reports have not addressed the B cell-intrinsic phenotype caused by NFAT inactivation. As demonstrated by the B cell-specific ablation of the regulatory B1 subunit of calcineurin (CnB1) (72), this phosphatase is not essential for the development of mature B2 B cells, although it is required for B1 B cell development. However, B cell-specific CnB1-deficient mice have reduced PC differentiation, which in turn leads to decreased antigen-specific antibody responses to TD antigens. Because interferon regulatory factor 4 (IRF4), a critical transcription factor for the differentiation of B cells into PCs (73, 74), cannot be induced properly after *in vivo* as well as *in vitro* antigen stimulation in these knock-out B cells, IRF4 induction is regulated by the calcineurin-NFAT pathway, possibly explaining the phenotype of B cell-specific CnB1-deficient mice. Indeed, the IRF4 promoter has several NFAT consensus binding sites.

**PI3K pathway.** CD19 is a B cell-specific cell surface molecule expressed from the pro-B cell stage to the plasmablast stage (75). On BCR ligation, the cytoplasmic tail of CD19 can be phosphorylated on multiple tyrosines by Lyn, providing binding sites for the SH2 domains of the p85 subunit of PI3K, Vav, and Lyn (76–78). As discussed above, the importance of CD19, particularly after the recognition of membrane-bound antigens, can be accounted for by its association with Lyn and its role in further

amplifying Lyn activity in the context of BCR signaling. Indeed, the SH2-dependent association of Lyn with tyrosine-phosphorylated CD19 was reported to release the autoinhibition of Lyn, thereby enhancing its enzymatic activity (77).

Although CD19's involvement in the BCR-induced activation of PI3K is clear, this mechanism does not fully account for the activation of PI3K. Investigators have reported that the cytoplasmic adaptor molecule BCAP (B cell adaptor for PI3K), which possesses binding sites for the SH2 domains of the p85 subunit (YxxM motifs), is tyrosine phosphorylated and is required to target PI3K to the plasma membrane in DT40 B cells (79, 80). Recently, *CD19<sup>-/-</sup>BCAP<sup>-/-</sup>* primary immature B cells were found to have almost complete abrogation of BCR-mediated PI3K activation, supporting overlapping functions for BCAP and CD19 in this activation (81). Furthermore, the YxxM motifs in BCAP and CD19 are critical for the development of immature B cells, as well as for PI3K activation (81).

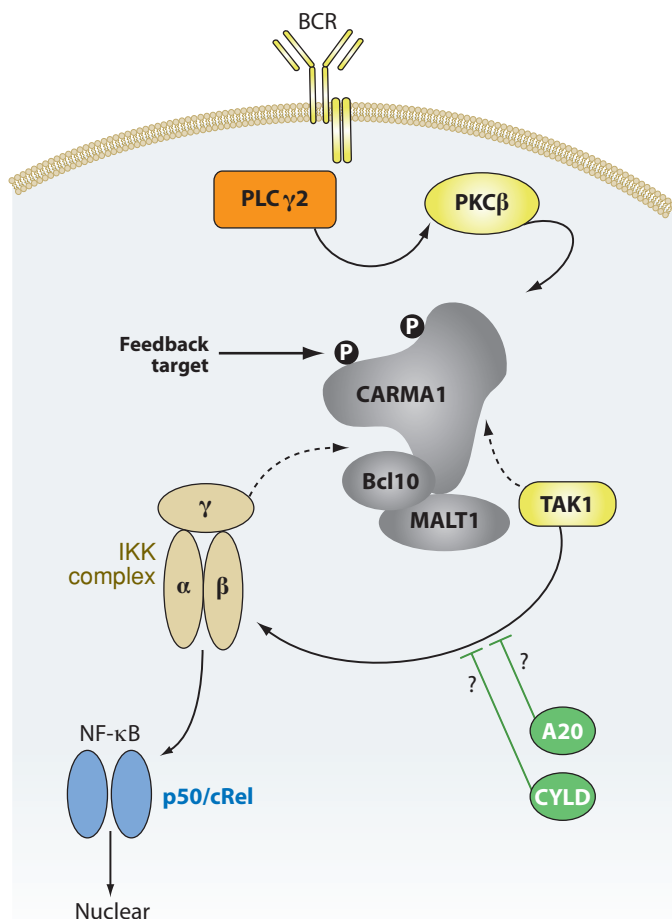
Three developmental defects in *CD19<sup>-/-</sup>* mice are prominent: the loss of peritoneal B1 lineage cells, spleen marginal zone (MZ) B cells, and spleen CD1d<sup>hi</sup>CD5<sup>+</sup> B cells. Although investigators have not addressed directly the requirement of PI3K activation in the development of CD1d<sup>hi</sup>CD5<sup>+</sup> cells, the development of B1 and MZ B cells clearly requires PI3K activation (82, 83). PI3K p110 $\delta$ , the predominant isoform activated downstream of the BCR, is also required for B1 and MZ B cell development (84). Moreover, the defective development of MZ B cells in *CD19<sup>-/-</sup>* mice is restored in *CD19<sup>-/-</sup>PTEN<sup>-/-</sup>* mice (85). PTEN and SHIP hydrolyze PIP<sub>3</sub> (a product of PI3K action) to PI(4,5)P<sub>2</sub> and PI(3,4)P<sub>2</sub>, respectively. The CD1d<sup>hi</sup>CD5<sup>+</sup> B cells appear to be the B cell subset that is responsible for evoking IL-10 production, which in turn can alleviate autoimmune diseases in experimental autoimmune encephalomyelitis, collagen-induced arthritis, and inflammatory bowel disease (86). Thus, in addition to a positive role in inducing humoral TD immune responses, CD19 exerts a negative

role, namely suppressing TD inflammatory responses by participating in the development of the CD1d<sup>hi</sup>CD5<sup>+</sup> subset.

PTEN is thought to be constitutively active, although various mechanisms have been reported to regulate its activity. In contrast, SHIP is activated specifically by the coligation of Fc $\gamma$ R2 and the BCR and, to a lesser extent, by BCR ligation alone. Analysis of B cell-specific *PTEN*-deficient mice has revealed that optimal PI3K activity is required for two aspects of B cell biology during TD immune responses (87). First, hyper-PI3K activation inhibits class switch recombination. Second, this activation also enhances antibody-secreting cell (ASC) formation. Therefore, mice lacking B cell-specific *PTEN* generated large numbers of IgM-ASCs but failed to produce IgG1 ASCs in TD immune responses.

**IKK/NF- $\kappa$ B pathway.** Recent elucidation of the connecting mechanism between BCR and IKK has inspired the notion that covalent modifications (phosphorylation and ubiquitylation) take place coordinately and affect the localization and activation status of signaling molecules, thereby changing their transcriptional activities (**Figure 3**). As many excellent reviews describe the role of NF- $\kappa$ B transcription factor components (p105, p100, RelA, RelB, and c-Rel) (88–90), we focus here on how IKK activity is fine-tuned in the context of BCR signaling.

NF- $\kappa$ B transcription factors are retained in the cytoplasm by binding to the inhibitor I $\kappa$ B. Stimulation of antigen receptors, including the BCR, activates the IKK complex consisting of IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  (alternatively named NEMO), thereby phosphorylating I $\kappa$ B and subsequently inducing its degradation (91). In B cells, protein kinase C $\beta$  (PKC $\beta$ ) is activated by DAG and Ca<sup>2+</sup>, both of which are generated by PLC $\gamma$ 2 activation. Ablation of PKC $\beta$  leads to the defective activation of the canonical NF- $\kappa$ B pathway, which in turn results in defects in B cell activation and maturation (92). In the signaling pathway between PKC $\beta$  and IKK activation, the formation of



**Figure 3**

A model for BCR-mediated IKK activation. Stimulation of BCR leads to the activation of phospholipase Cγ2 (PLCγ2) through the proximal protein tyrosine kinases and the subsequent activation of protein kinase Cβ (PKCβ). Activated PKCβ phosphorylates CARMA1 (S668), directly or indirectly, which results in recruitment of TAK1 to the phosphorylated CARMA1. Meanwhile, the IKK complex is recruited, probably through the Bcl10/MALT1 complex, to the phosphorylated CARMA1. These interactions (CARMA1-IKK and CARMA1-TAK1) allow access of two key protein kinases, TAK1 and IKK, leading to the activation of the IKK complex. IKKβ also facilitates the formation of the CARMA1/Bcl10/MALT1 complex by phosphorylating S578 of CARMA1 (feedback target), thereby optimizing the strength and duration of the NF-κB signal. The CYLD and A20 deubiquitinases negatively regulate the signals that lead to IKK and NF-κB activation, but their exact targets are unknown.

a macromolecular NF-κB signaling complex is required. This complex is composed of adaptor molecules: CARMA1, Bcl10, and MALT1 (93). Both *MALT1* and *Bcl10* are targets of chromosomal rearrangements associated with

the formation of MALT lymphomas, and oncogenic *CARMA1* mutations have been found recently in human diffuse large B cell lymphoma (83, 94–100). The fine-tuned regulation of the NF-κB signaling pathway imposed by CARMA1/Bcl10/MALT1 is crucial to maintain normal B cell proliferation/survival and differentiation.

PKCβ phosphorylates CARMA1 on Ser668, which is essential for subsequent CARMA1/Bcl10/MALT1 association, as well as IKK activation (101, 102). In addition to this critical phosphorylation site on CARMA1, Ser/Thr phosphorylation of CARMA1/Bcl10 on other sites may contribute to the formation of a positive and negative feedback regulation of IKK activation (102–104), thereby fine-tuning the NF-κB pathway. As a positive feedback, the activated IKKβ phosphorylates CARMA1 on Ser578, which in turn facilitates or stabilizes its association with Bcl10/MALT1, thereby enhancing IKK activation. As a negative feedback, the activated IKKβ also phosphorylates Bcl10, thereby inducing its degradation and the disengagement of Bcl10 and MALT1. Thus, the timing of these IKKβ-mediated phosphorylation events and the lag time between phosphorylation and degradation are key determinants in fine-tuning NF-κB activity.

MALT1 is a caspase-like protein related to the so-called paracaspases found in metazoans and *Dictyostelium* (98). The importance of its proteolytic function has been appreciated in T cells. Rebeaud et al. (105) demonstrated that the proteolytic activity of MALT1 is required for optimal TCR-mediated NF-κB activation. Recently, the importance of the MALT1 proteolytic activity toward its substrate, A20, has been suggested (106). A20 inhibits NF-κB signaling by deubiquitinating its substrates (K63-linked chains) on target molecules including TNF receptor-associated factor (TRAF)2, TRAF6, RIP1, and IKKγ (32, 107). This K63-linked ubiquitination has been thought to play a positive role in canonical NF-κB activation. By cleaving and inactivating A20, MALT1 contributes to IKK activation. A similar mechanism is likely utilized by B cells, making clarifying

how and when paracaspase activity is regulated during BCR activation important.

The dynamic balance between K63-linked ubiquitylation and deubiquitylation thus plays a crucial role in BCR-mediated IKK activation. The CARMA1/Bcl10/MALT1 complex is also associated with the ubiquitylating enzymes UBC13 (Ub-conjugating enzyme 13), UEV1A (Ub-conjugating enzyme E2 variant 1A), and TRAF6 (Ub E3 ligase), possibly triggering the recruitment of IKK and TAK1 and inducing K63-linked ubiquitylation of IKK $\gamma$  and TAK1 (108–111). Counteracting this ubiquitylation is a DUB, cylindromatosis (turban tumor syndrome) (CYLD), which plays an important role in regulating NF- $\kappa$ B activity (112–116). Indeed, CYLD deficiency in B cells results in the constitutive activation of the canonical NF- $\kappa$ B pathway, leading to an enlarged B cell compartment (117, 118). The B cell targets of CYLD are unknown, but based on T cell data, it seems reasonable to propose that the K63 ubiquitylation of IKK $\gamma$  and TAK1, both of which are critical for IKK activation in the BCR signaling context, is a likely candidate.

Because the Ser residues in the activation loop of IKK $\alpha$  and IKK $\beta$  are not in the context of a PKC $\beta$  consensus phosphorylation site, it has been thought that PKC $\beta$  mediates phosphorylation of IKK indirectly through an intermediate kinase, which then directly phosphorylates the Ser residues on IKK $\alpha$ /IKK $\beta$ . TAK1, a member of the MAP3K family, corresponds to this IKK kinase (111). Indeed, *TAK1*-deficiency in DT40 B cells resulted in the complete abrogation of BCR-mediated IKK activation (101). Moreover, immunoprecipitated TAK1 can phosphorylate activation-loop Ser residues of IKK $\beta$  in vitro. Studies with *TAK1* conditional knockout mice using CD19Cre to delete the gene in B cells have shown that TAK1 is dispensable for NF- $\kappa$ B activation induced by BCR engagement (119). However, these conflicting results between primary and transformed B cells may be due to incomplete deletion of *TAK1* in the conditional knockout mice. Indeed, in another study using a different B cell-specific Cre (mb1-Cre), BCR-mediated

IKK activation was markedly reduced (H. Shinohara & T. Kurosaki, unpublished results).

**ERK pathway.** Similar to other cell types, B cells activate Ras following BCR stimulation. The introduction of a dominant-negative Ras inhibits BCR-mediated ERK activation in DT40 B cells, as well as in primary B cells, demonstrating that Ras functions as an upstream regulator for subsequent ERK activation (4, 120). Investigators have assumed for some time that Sos, a nucleotide exchange factor for Ras, participates in BCR-mediated Ras activation (18). However, data emerging from several laboratories indicate that RasGRP3, another Ras exchange factor, plays a more important role than Sos in coupling the BCR to Ras activation (121–123). Ras activation occurred normally even in *Sos1/Sos2* double-deficient DT40 B cells, whereas this activation was inhibited, although not completely, in *RasGRP3*-deficient DT40 B cells. The necessity of RasGRP3 in Ras activation appears to explain the previous findings that PLC $\gamma$ 2 was required for BCR-mediated Ras activation (124). Because the C1 domain of RasGRP3 binds to DAG (a product of PLC $\gamma$ 2 action), PLC $\gamma$ 2 may participate in the recruitment of RasGRP3 in a DAG-C1-dependent manner. Indeed, RasGRP3 with a C1 domain deletion does not move to the plasma membrane and thus fails to activate Ras in BCR signaling. Moreover, wild-type RasGRP3 cannot move to the plasma membrane in *PLC $\gamma$ 2*-deficient DT40 B cells (121). In addition to RasGRP3's recruitment to the membrane, the phosphorylation of Thr133 on RasGRP3 is also important for its full activation (125, 126). This is mediated by activated PKC $\beta$ , thereby leading to the increased enzymatic activity of RasGRP3. Thus, a new model has been proposed in which DAG generated by PLC $\gamma$ 2 facilitates the recruitment of both PKC $\beta$  and RasGRP3 to the plasma membrane, where PKC $\beta$  phosphorylates Thr133 on RasGRP3, which is crucial for RasGRP3's full activation. Once activated, this GTP-bound Ras binds directly to Raf-1, the MAP3K in the ERK pathway. Activated Raf-1 and B-Raf phosphorylate



and activate MEK1/MEK2, which both in turn phosphorylate ERK1/ERK2. Phosphorylated ERKs form dimers, a step required for nuclear translocation and the subsequent phosphorylation of transcriptional regulatory proteins, including Fos, Jun, and Ets family members (127).

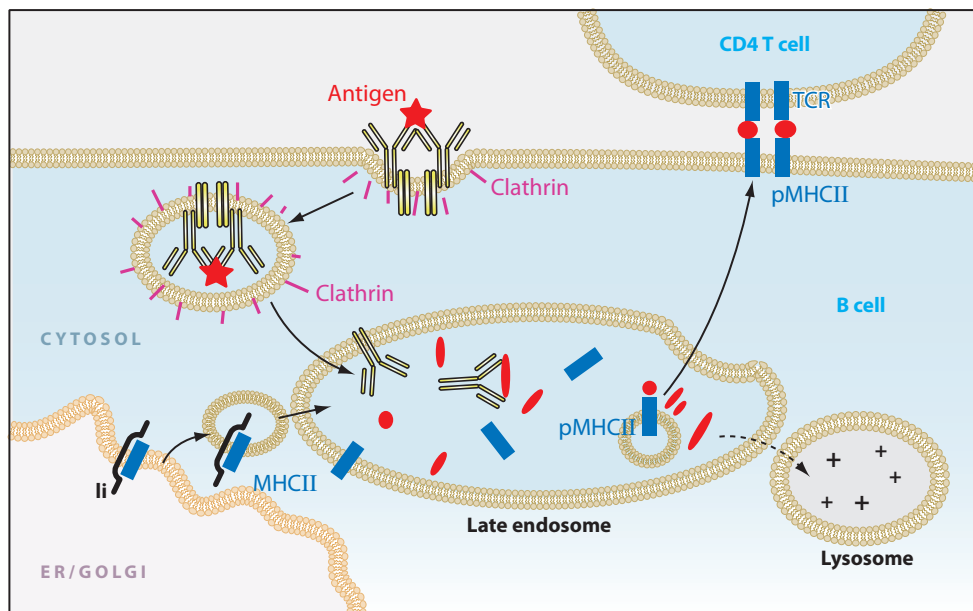
## Antigen Presentation

In addition to the above-mentioned signaling events, the recognition of antigen by BCR induces the rapid internalization of engaged receptors, which is required for the effective presentation of antigen-derived peptides to major histocompatibility complex (MHC) class II-restricted T cells (128–130). The signaling and internalization processes are interconnected. For instance, assuming that signaling takes place predominantly on the plasma membrane, internalization would eventually negatively regulate signaling because crosslinked BCRs are ultimately degraded following their uptake. For example, BCR signaling was augmented and prolonged in mice that carry Igβ<sub>AA</sub>, a mutant form of Igβ lacking phosphorylation and internalization motifs (37). Conversely, endocytosis might also play a positive role in receptor-mediated cellular activation. Indeed, some receptors such as the epidermal growth factor receptor continue to signal in endosomes (131), amplifying their impact after internalization and before degradation. Signaling cross talk also should be considered. The innate immune receptors, Toll-like receptor (TLR)7 and TLR9, are predominantly expressed in endosomes but not on the plasma membrane. In this setting, B cells can recognize DNA- or RNA-containing antigens through their cell surface BCRs, which in turn deliver the DNA/RNA ligands to TLR9/7-containing compartments (132, 133). In this scenario, the BCR functions as a transfer vehicle for inducing TLR9/7 signaling. Thus, BCR internalization can affect several distinct signaling outcomes.

With regard to antigen presentation, successful antigen processing in B lymphocytes relies on the following directional membrane trafficking events: (a) antigen internalization

through BCR and targeting into multivesicular late endosomes (alternatively named MHC class II-containing endosomes), presumably through early endosomes; (b) MHC class II complexes, proteases, and H2-DM convergence toward this incoming pool of antigen-BCR complexes; and (c) the export of MHC class II-peptide complexes to the cell surface (Figure 4) (129). The initial internalization of antigen-bound BCRs requires clathrin, but not Igβ ubiquitylation (134), but further trafficking to late endosomes requires Igβ ubiquitylation, which appears to be mediated by the E3 ligase Itch (135).

In addition to antigen capture, the generated BCR signal has been thought to be necessary to bring resting B cells into an activated state for efficient antigen presentation. The requirement of Syk in antigen presentation may be due to its participation in the enhancement of the above-mentioned directional membrane trafficking events through the reorganization of the actin cytoskeleton (136). In the transition of immature dendritic cells (DCs) to their mature professional state, two mechanisms have been well recognized. First, upon maturation, intracellular cystatin C, an inhibitor of cathepsin S, is decreased, thereby enhancing cathepsin S proteolytic activity, which in turn facilitates the degradation of the invariant chain and subsequent peptide loading into class II molecules. Second, before maturation, MHC class II accumulates in lysosomes, where it is constitutively degraded, whereas the cell surface expression of MHC class II is upregulated upon maturation (137). Similar mechanisms are likely to operate in B cells. Indeed, a few hours after BCR engagement, cathepsin S activity is increased. With regard to the second mechanism, the importance of membrane-associated ring finger (MARCH)-1 has been highlighted recently. Knockout of *MARCH-1*, an E3 polyubiquitin ligase, resulted in constitutive high expression of MHC class II on B cells (138). More importantly, expression of MARCH-1 is downregulated upon BCR crosslinking, which may be involved in terminating constitutive MHC class II degradation in vivo as well (M. Hoshino,



**Figure 4**

Antigen presentation by B cells. B cells can capture soluble or membrane-bound antigen through their BCRs. Whether the BCR-antigen complex traffics through early endosomes before its arrival at late endosomes is not clear and might depend on the type of B lymphocyte. MHC class II molecules associate with their chaperone molecule invariant chain (li) in the ER. In the antigen-processing late-endosomal compartment, the antigen and li are proteolyzed. The cysteine protease CatS is specifically required for a late step of li cleavage and allows for peptide exchange by H2-DM. By removing the cytosolic tail of li, this proteolytic event liberates the li motif for endosomal retention and permits mature peptide complexes (pMHC) to be exported to the cell surface for interaction with cognate T cells. TCR, T cell receptor.

Y. Aiba, T. Kurosaki & S. Ishido, unpublished data).

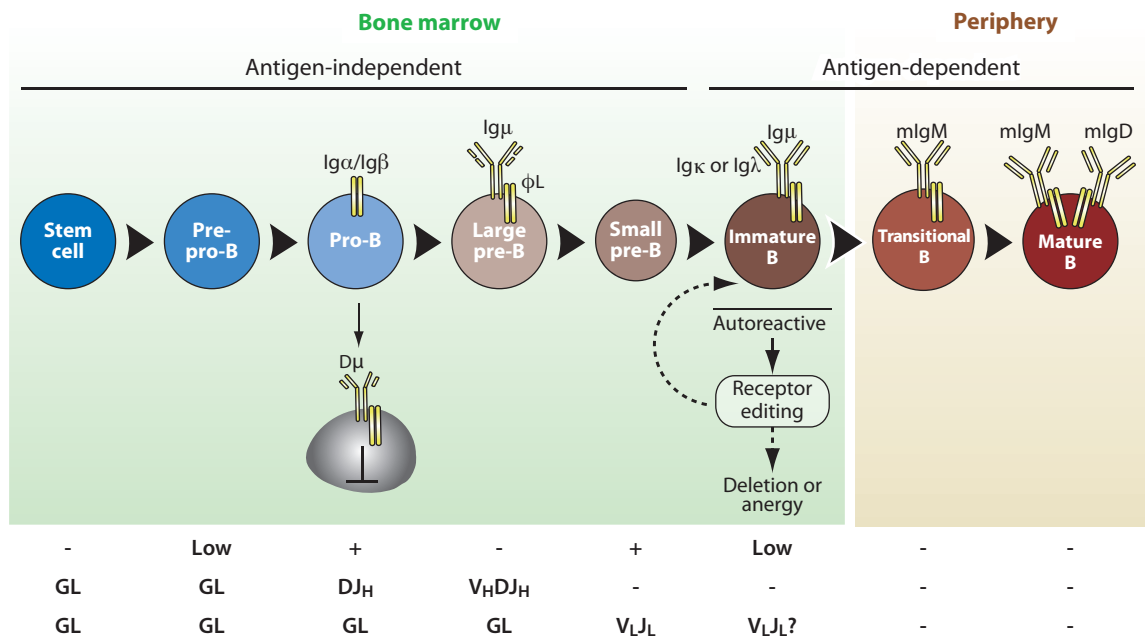
## B CELL DEVELOPMENT

Important insights concerning the requirement of receptor and signaling components in B cell development have been obtained using conventional knockout mice. However, this approach has at least two potential problems. First, if early B cell development were arrested by the simple gene knockout mice, the function of these genes in later phases of development cannot be addressed using these mice. Second, if B cell outcomes are dictated by combined effects of both B cell-intrinsic and -extrinsic factors, this approach cannot be used to dissect these two factors. These problems are now closer to resolution through spatiotemporal gene deletion.

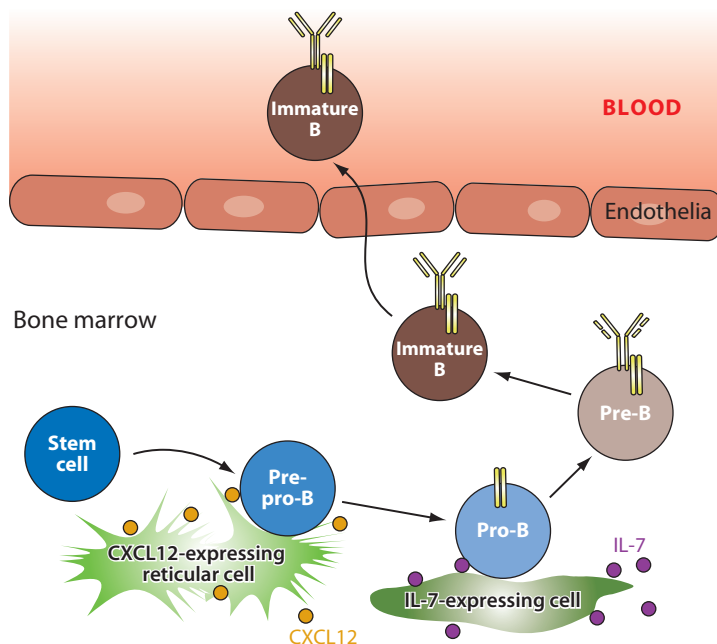
## Pre-Pro-B to Pro-B Cell Stages

Pre-pro-B cells have low expression of the recombination activating genes (*Rag1* and *Rag2*). Their *IgH* and *IgL* genes are in the germline, unrearranged configuration, so no Ig is produced in these cells (**Figure 5a**) (139). Pro-B cells are distinguished from earlier precursors because  $V_H(D)J_H$  recombination is initiated at this stage. Pro-B cells also differ from pre-pro-B cells in that they are the first B lineage cells to express  $Ig\alpha$ ,  $Ig\beta$ , and cell surface calnexin (pro-BCR) (4). Ig gene recombination begins with diversity (*D*) to joining ( $J_H$ ) segment rearrangements. After formation of  $DJ_H$  rearrangements,  $V_H$  genes become accessible to the  $V_H(D)J_H$  recombinase, and complete heavy-chain transcription units are assembled. The switch from  $DJ_H$  to  $V_H(D)J_H$  recombination in pro-B cells is probably

**a**



**b**



regulated at the level of  $V_H$  gene accessibility and appears to require the cytokine IL-7, the transcription factors Pax5 and YY1, and high levels of Rag1/Rag2 (13, 140–143). The requirement for IL-7 in  $V_H(D)J_H$  recombination fits well with recent observations that pro-B cells are in contact with IL-7-expressing stromal cells in bone marrow microenvironments. In bone marrow, several types of stromal cells (at least three types: osteoblasts, CXCL12<sup>hi</sup> reticular cells, and IL-7-expressing cells) have been proposed to function as specific cellular niches for promoting early B cell development in a stepwise manner (**Figure 5b**) (144). Most pre-pro-B cells are in contact with CXCL12<sup>hi</sup> reticular cells, whereas pro-B cells move away from CXCL12<sup>hi</sup> reticular cells and instead associate with IL-7-expressing stromal cells.

$V_H$  gene accessibility is dependent on transcriptional regulatory elements, including the IgH chain enhancer, and is associated with the onset of transcription of germline  $V_H$  gene segments. Indeed, it has been proposed that transcription per se renders the  $V_H$  gene segments accessible to the recombinase (145). Both sense and antisense germline  $V_H$  transcripts may impact the accessibility of these segments. Recent data have shown that antisense transcripts are generated in the vicinity of nonrearranged  $V_H$  gene segments and that they are found in pro-B cell populations that are actively undergoing  $V_H$  to  $DJ_H$  joining (146). A second possibility is that *cis* regulatory elements in *IgH* promoters and enhancers recruit factors that remodel chromatin domains and make  $V_H$  genes accessible for recombination independently of transcription. For instance, YY1, a zinc finger protein, binds to the intronic enhancer (*iE $\mu$* ) of the *IgH* locus, where it is essential for distal  $V_H$  to  $DJ_H$

recombination (147). Finally, histone modifications by acetylation and demethylation, as discussed above, contribute to  $V_H$  gene accessibility in an IL-7- and Pax5-dependent manner, respectively (148).

Although the chromatin state of the *IgH* locus is a main determinant of Rag-mediated recombination, another mechanism is the transcriptional regulation of the components of the recombination machinery. In fact, regulation of the *Rag* genes by Foxp1 has been demonstrated recently (149). Forkhead (Fox) transcription factors are proteins that share a winged-helix DNA-binding domain and regulate diverse biological processes. Foxp1 regulates the B cell-specific expression of *Rag1* and *Rag2* in pro-B cells by binding directly to sequences in the *Erag* enhancer, located near the 5' end of the *Rag2* gene. This *Erag* enhancer is known to be a critical sequence for the lineage-specific expression of *Rag* genes in B cells, but not in T cells.

## Pre-B Cell Stage

The expression of  $\mu$ HC results in the assembly of the pre-BCR and marks the transition to the pre-B cell stage. The pre-BCR is composed of the transmembrane form of  $\mu$ HC (mIg $\mu$ ), the surrogate light chains ( $\lambda 5$  and VpreB), Ig $\alpha$ , and Ig $\beta$ . Its expression on the cell surface, which occurs after the cell has made a successful  $V_H(D)J_H$  rearrangement, is a key checkpoint regulator in B cell development (**Figure 5a**). The primary functions of the pre-BCR are to trigger clonal expansion, heavy-chain allelic exclusion, and further differentiation (150).

Whether pre-BCR crosslinking by an unknown ligand or by simple surface expression

## Figure 5

(a) B cell differentiation scheme and (b) a model of the movement of early B cells as they develop in the bone marrow. (a) Developmental stages of B lymphopoiesis, RAG expression, and rearrangements on both heavy (*IgH*) and light (*IgL*) chain loci. (GL: *IgH* or *IgL* loci in the germline configuration.) (b) Pre-pro-B cells move toward CXCL12<sup>hi</sup> reticular cells and associate with them, whereas pro-B cells move away and instead adjoin IL-7-expressing cells. Subsequently, pre-B cells leave the IL-7-expressing cells. B cells expressing IgM exit the bone marrow and enter the blood to reach the spleen, where they mature into peripheral mature B cells that coexpress IgD. Not illustrated here is the extensive proliferation that occurs at the large pro-B and pre-B cell stages.

is sufficient to trigger these biological consequences is still debated. However, receptor self-aggregation likely is required for signaling and subsequent cellular responses because pre-B cell development cannot occur in the absence of ITAMs in the cytoplasmic domains of Ig $\alpha$  and Ig $\beta$  (151). With regard to the self-aggregation property of the pre-BCR, Ohnishi & Melchers (152) provided an intriguing model in which the unique non-Ig-like regions at the N terminus of  $\lambda 5$  and the C terminus of VpreB mediate constitutive pre-BCR aggregation on the cell surface via their homotypic ionic interactions, which in turn are critical for inducing signaling and internalization. This model has been verified by recent crystallographic and electron microscopic analysis, revealing the existence of pre-BCR dimers, consisting of two pre-BCR monomers connected by a flexible hinge between the tips of the variable domains (153). As predicted by the previous report (152), this hinge region has turned out to be formed by the unique non-Ig-like regions of  $\lambda 5$  and VpreB (153). Functional data also support this concept. Surrogate light-chain expression strongly enhances the autonomous ability of mIg $\mu$  to induce calcium flux irrespective of additional receptor crosslinking (154).

The pre-BCR transmits a signal to induce several rounds of cell division and eliminate the dependence of developing B cells on IL-7. Because of its analogy to BCR signaling, Src family and Syk family PTKs had been considered critical for transmitting the pre-BCR signal for cell expansion. Indeed, three Src kinases (Lyn, Fyn, and Blk) contribute to pre-B cell expansion in a redundant fashion (155). Because pre-BCR-mediated NF- $\kappa$ B activation was abolished in *Lyn/Fyn/Blk* triple knockout pro-B cells, this pathway has been proposed to be critical for pre-BCR-mediated expansion; however, direct evidence for the involvement of the NF- $\kappa$ B signaling pathway in pre-B cell expansion is still lacking. In contrast, the mechanisms by which Syk family kinases participate in pre-BCR-mediated expansion are becoming clearer. Downstream of Syk and Zap-70, ERK1 and ERK2 are activated through Ras in

a pre-BCR signaling context and are required for pre-BCR-mediated cell expansion through the activation of the transcription factors Elk1 and CREB (156). In line with this model, mice expressing a constitutively activated Ras mutant transgene on a *Rag*-null background bypass the pre-BCR checkpoint; conversely, mice expressing a transgenic dominant-negative Ras, H-RasN17, show a developmental arrest at the pro- to pre-B cell stage (120, 157). Moreover, mice overexpressing a dominant-negative form of CREB lacking the ERK phosphorylation site (Ser119 to Ala mutation) in a B cell-specific manner have the same developmental block as mice lacking *ERK1* and *ERK2* (158).

Ig HC allelic exclusion, the process by which successful rearrangement of one *IgH* allele inhibits further rearrangement of the other allele, remains an enigmatic aspect of pre-BCR signaling. Because of allelic exclusion, rearrangement of the endogenous *IgH* genes is normally inhibited in wild-type mice carrying a prerrearranged  $\mu$ H chain transgene. However, *Syk*<sup>-/-</sup> *Zap70*<sup>-/-</sup> double mutants cannot allelically exclude the endogenous *IgH* genes in the presence of a  $\mu$ H chain transgene (159). Downstream of Syk and Zap-70, the above-mentioned Ras-ERK pathway appears not to be involved in this process because deletion of both *ERK1* and *ERK2* does not break allelic exclusion (T. Yasuda & T. Kurosaki, unpublished data). In contrast, PLC $\gamma$  is critical; *PLC $\gamma$ 1*<sup>+/-</sup> *PLC $\gamma$ 2*<sup>-/-</sup> mice on an *IgH* chain transgenic background fail to induce allelic exclusion (160). The mechanism for the involvement of PLC $\gamma$  in allelic exclusion is not understood, but it is possible that protein kinase D (PKD), which is downstream of PLC $\gamma$ , can function to connect the cytoplasmic and nuclear events required for allelic exclusion. Indeed, in T cells, PKD1 is known to bind DAG, a product of PLC $\gamma$ , and mice expressing cytosol-targeted active PKD1 can suppress *V $\beta$*  to *DJ $\beta$*  rearrangements of the *TCR $\beta$*  locus (161, 162).

After the clonal expansion of mIg $\mu$  producers, pre-B cells arrest in G1 and become small pre-B cells. Because pre-BCR signaling terminates the transcription of the genes encoding  $\lambda 5$  and VpreB, it had been proposed that



this transition to small pre-B cells is caused by the transcriptional silencing of surrogate light chain genes. Indeed, *Btk*<sup>-</sup>, *BLNK*<sup>-</sup>, and *IRF4/IRF8*-deficient mice have impaired pre-BCR downregulation and manifest a hyperplastic pre-B cell phenotype (163, 164). The resulting chronic pre-B cell proliferation seems to allow for secondary genetic mutations, with pre-B cell lymphomas developing in these mice within weeks (165). However, the enforced expression of surrogate light chains beyond the large, cycling pre-B cells turn out to have no apparent effect on pre-B cell proliferation and subsequent differentiation, despite the high expression of the pre-BCR (166), thereby calling the above transcriptional model into question. We propose that instead of the regulation of pre-BCR expression, pre-BCR signaling mediates rapid and transient expression of key transcription factors for proliferation, which might explain why pre-B cells become arrested in the G1 state. According to this model, when the amount of these factors drops below a critical level, expression of proliferative genes is downregulated, and that of antiproliferative genes is possibly upregulated, together leading pre-B cells into a quiescent state. The downregulation of IL-7 responsiveness in pre-B cells might also contribute to this quiescence. In this regard, pre-B cells are in the process of moving away from IL-7-expressing stromal cells in the bone marrow (**Figure 5b**), which in turn might contribute to conferring attenuated IL-7 responsiveness on pre-B cells.

Upon the cessation of pre-B cell proliferation, the small pre-B cells induce *Igk* locus activation and *V<sub>k</sub>* to *J<sub>k</sub>* recombination, resulting in the synthesis and cell surface expression of the IgM BCR (167). Signaling by the pre-BCR and loss of IL-7 signaling together enhance the accessibility of the *Igk* locus and its recombination. These two pathways act via distinct mechanisms to regulate such processes. The *Igk* locus contains two distinct transcriptional enhancers, the intronic enhancer (*iEκ*) and the 3' enhancer (*3'Eκ*), which function to regulate recombination. The expression of IRF4, a regulatory transcription factor, is induced by signaling through

the pre-BCR, and together with another transcription factor, PU.1, it interacts with the *3'Eκ* (164, 168). Increased binding of these proteins to the *3'Eκ* is associated with its increased accessibility to restriction endonucleases, as well as increased association with acetylated histones in B cells. Indeed, the overexpression of IRF4 in an *IRF4*<sup>-/-</sup>*IRF8*<sup>-/-</sup> background resulted in high levels of *Igk* germline transcripts and preferentially stimulated histone H4 acetylation at the *3'Eκ*. In contrast, lowering the IL-7 signaling preferentially induces histone acetylation at the *iEκ* (169). Although the mechanisms are not entirely clear, the lowered IL-7 signaling appears to facilitate binding of the transcription factor E2A to the *iEκ*, which in turn is required for *Igk* recombination in pre-B cells. Thus, IL-7 signaling impacts Ig gene rearrangement in two opposite ways, depending on the B cell differentiation states: IL-7 signaling in pro-B cells enhances histone acetylation at distal *V<sub>H</sub>* genes (as discussed above), whereas it inhibits histone acetylation at *iEκ* in pre-B cells.

Similar to the FoxP1-mediated regulation of *Rag1/Rag2* transcription in *IgH* chain recombination, Foxo1 appears to be involved in *IgL* chain gene recombination through regulating, in part, the transcription of *Rag1/Rag2* (170, 171). Foxo1 has an interesting mode of regulation, which may dictate its stage-specific role in Ig gene rearrangement. The activation of PI3K and subsequent Akt activation lead to the phosphorylation of Foxo1 and to its degradation. This precludes the accumulation of Foxo1 protein in the nucleus, thereby blocking its function. Hence, although not completely proven, Foxo1's involvement in *IgH* chain gene recombination is unlikely (172), given that PI3K and Akt are highly activated by IL-7R signaling in pro-B cells, which are actively undergoing *V<sub>H</sub>* to *DJ<sub>H</sub>* recombination. In contrast, in the case of the pre-B to immature B cell transition, the expression of pre-BCR on large cycling pre-B cells induces PI3K-Akt activation, turning off Foxo1 function. When this proliferation ceases and the cells become small pre-B cells, PI3K is no longer activated, thereby releasing the Akt-mediated inhibition on Foxo1 and allowing it

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**iEκ:** the intronic enhancer

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to participate in *IgL* chain gene recombination (172).

To maintain the one cell–one antibody rule, allelic exclusion must be imposed on light as well as heavy chain genes (2). One of the mechanisms that contributes to light-chain allelic exclusion is asymmetric demethylation of the *Igκ* alleles. Asymmetric demethylation is thought to render one of the two *Igκ* alleles preferentially accessible for *VJκ* rearrangement.

### Immature B Cell Stage

Immature B cells are the first B lineage cells to express surface BCR, and they display surface IgM but little or no IgD (**Figure 5a**). B cells remain in the immature compartment for 3.5 days on average.

Similar to the pre-BCR, the BCR on the B cell surface is thought to be capable of ligand-independent signaling (termed the tonic BCR signal), based on inducible BCR-ablation studies together with biochemical evidence that expression of the BCR, per se, induces tyrosine phosphorylation (4, 173, 174). The data from genetic manipulations of the signaling subunit  $Ig\alpha$ - $Ig\beta$  also support this concept. In the absence of the  $Ig\alpha$  cytoplasmic domain, immature B cell development was perturbed (175, 176). Conversely, expression of a plasma membrane-targeted  $Ig\alpha$ - $Ig\beta$  chimeric protein (considered a gain-of-function mutant of  $Ig\alpha$ / $Ig\beta$ ) in BCR-deficient ( $\mu$ MT-derived) bone marrow progenitor cells was sufficient to generate mature follicular CD23<sup>+</sup> B cells (177).

Several recent studies have provided fresh insight into the nature of the BCR tonic signal. Using an in vitro culture system, Keren et al. (178) showed that a reduction in the level of tonic signaling (achieved using PI3K inhibitors) resulted in increased Rag2 levels and new *IgL* gene recombination. Similarly, Tze et al. (179) examined the effect of the loss of the BCR on the differentiation state of B cells using an inducible Cre-loxP system to eliminate the BCR. Ablation of BCR expression resulted in B cells reverting to a less-differentiated earlier phenotype, as evidenced by the reexpression of several

pro- and pre-B cell-associated genes, including *Rag1*, *Rag2*,  $\lambda 5$ , and *VpreB*. Furthermore, under conditions in which BCR expression was retained, the addition of PTK and PI3K inhibitors attenuates the BCR-mediated tonic signal, thereby promoting the accumulation of new *IgL* gene recombination, too (179). These studies, together with the previous suggestion that a lack of BCR tonic signaling traps developing B cells in a compartment in which they would continue to undergo secondary recombination (180, 181), substantiate the concept that the strength of BCR-mediated tonic signaling is a critical sensor for B cells in their fate decision between pre-B and immature B cell stages. Although transient inhibition of PI3K activity in immature B cells can drive B cells to attempt more rounds of *IgL* rearrangements (see above), continued PI3K inhibition eventually halts immature B cell development, probably because of the lack of vital survival signals. Indeed, *BCAP*<sup>-/-</sup> *CD19*<sup>-/-</sup> mice have severe defects in the generation of immature and mature B cells, as well as in PI3K activation, and this developmental defect was partly relieved by the introduction of a constitutively active form of PI3K (81). Mice deficient in the *p85α* subunit of PI3K also displayed a defect in immature B cells.

Cell surface expression of an autoreactive BCR on immature B cells leads to the internalization of the self-antigen-BCR complexes and to the activation of intracellular signals that upregulate Rag1/Rag2 expression, halts differentiation, and initiates secondary *Vκ* rearrangements that replace the original *VκJκ* rearrangement. In contrast to B cell anergy and deletion mechanisms, this type of tolerance mechanism, termed receptor editing, spares autoreactive B cells by replacing their receptors and is therefore an example of molecular selection (**Figure 5a**). Amin & Schlissel (170) have proposed that regulation of Foxo1 participates in the receptor editing process. The binding of self-antigen can probably induce antigen-BCR internalization, thereby facilitating nuclear localization of Foxo1 and reinducing Rag1/Rag2 expression and *Igκ* rearrangements.

## B Cell Development in the Periphery

In the case of conventional B2 B cells, immature B cells that emigrate from the bone marrow to the periphery are referred to as transitional 1 and 2 (T1 and T2) B cells (**Figure 5a**). Developmentally, T1 B cells appear to precede T2 B cells, which, in turn, are considered as the immediate precursors of naive mature B cells. T1 and T2 B cells are short-lived, and only 10–30% of these cells enter the long-lived (15–20 weeks in the case of mice) mature peripheral B cell compartment. In addition to a functional BCR, immature B cells require BAFF-mediated survival signals for full maturation. Indeed, B cell maturation in *BAFF*-deficient mice was impaired beyond the T1 stage. Current evidence indicates that all three BAFF receptors (BAFFR, BCMA, and TACI) are expressed on B cells at differing levels, depending on their maturation and/or activation stage (182). For instance, BCR ligation upregulates BAFFR expression on B cells, which promotes increased sensitivity to BAFF-mediated survival signals. Among BAFFR, BCMA, and TACI, BAFFR is the key receptor that triggers BAFF-mediated survival of immature B cells, as mice deficient in this receptor phenocopy the *BAFF*-null mice (183). Furthermore, in vitro and in vivo experiments demonstrate that T2 B cells are critical targets for BAFF effects on B cell development.

Recent investigations have elucidated the pathways that mediate BAFFR's prosurvival signaling. The major pathway involves activation of the so-called noncanonical NF- $\kappa$ B pathway (the processing of NF- $\kappa$ B2 and the nuclear translocation of the p52/RelB heterodimer) (184, 185), although weak activation of the canonical NF- $\kappa$ B pathway (nuclear translocation of the p50/RelA or p50/c-Rel heterodimer) occurs to some extent (186–189). The BAFFR has only a single TRAF-binding site, which is specific for TRAF3 (190). Unlike other TRAFs (such as TRAF2, TRAF5, and TRAF6), TRAF3 does not activate the canonical NF- $\kappa$ B or JNK pathway (191). Instead, TRAF3, in the resting state, suppresses the noncanonical NF- $\kappa$ B pathway by binding NIK and targeting

it for proteosomal degradation (192). NIK is responsible for directly phosphorylating and activating IKK $\alpha$  and the subsequent activation of the noncanonical pathway (193, 194). Activated BAFFR recruits TRAF3, allowing B cells to terminate TRAF3-mediated degradation of NIK. NIK then accumulates and activates IKK $\alpha$  and the downstream noncanonical pathway. Indeed, B cells that lack TRAF3 show greatly elevated levels of NIK and NF- $\kappa$ B2 processing (195). More significantly, the B cell developmental defect in *BAFF*<sup>-/-</sup> mice was corrected by the loss of TRAF3, formally demonstrating the importance of the BAFFR-TRAF3-noncanonical NF- $\kappa$ B pathway in the development of mature B cells. TRAF3-mediated NIK degradation is likely mediated by the TRAF2-associated Ub ligase c-IAP1, although how TRAF3 regulates TRAF2 is not clear. In this context, TRAF2, TRAF3, and c-IAP1 function as suppressors of the noncanonical NF- $\kappa$ B pathway in B cells (196). In addition to the activation of the noncanonical NF- $\kappa$ B pathway, BAFFR regulates B cell survival by regulating the nuclear localization of PKC $\delta$  (197).

BCR signaling (presumably like the ligand-independent tonic signal as mentioned above) appears to be an important determinant in the transition from T1/T2 B cells to mature B2 B cells. The loss of BCR expression by conditional IgM ablation aborts all further development beyond the transitional stage (198). In addition, many mutations that affect BCR signaling pathways also interfere with this transition. In the absence of *Btk*, *BLNK*, *BCAP*, *PLC $\gamma$ 2*, or *Vav1/Vav2/Vav3*, BCR signaling is insufficient to induce T1/T2 B cells to differentiate into mature B cells (199). One of the critical pathways downstream of these signaling molecules is the canonical NF- $\kappa$ B activation pathway. The importance of this pathway has been recently highlighted in studies using mice deficient in CARMA1 or IKK $\gamma$ ; these mice have a decreased number of mature B cells (200). Once CARMA1 is phosphorylated by BCR-activated PKC $\beta$ , this adaptor molecule forms a scaffold for other molecules including Bcl10, MALT1, and TRAF6/2, thereby activating the

IKK $\alpha$ /IKK $\beta$ /IKK $\gamma$  complex and facilitating the nuclear translocation of p50/RelA or p50/c-Rel heterodimers (canonical NF- $\kappa$ B pathway) (201). Some of the BCR signaling molecules also participate in BAFFR signaling. For example, PLC $\gamma$ 2 is known to be involved in BAFFR-mediated signaling, directly or indirectly, in addition to its involvement in BCR-mediated canonical NF- $\kappa$ B activation (202). Rac1, a downstream effector in the Vav family, mediates the BCR-induced upregulation of BAFFR, thereby augmenting the BAFFR signaling (203). Together, BAFFR and BCR likely predominantly utilize the noncanonical and canonical NF- $\kappa$ B pathway, respectively, both of which are essential to generate a sufficient number of mature B cells.

Mutations in *CARMA1*, *Bcl10*, or *MALT1* result in a relatively mild defect in conventional B2 B cell development, whereas these mutations lead to a complete loss of B1 B cells in the peritoneal cavity (93). Conversely, B1 B cell development remains unaffected by the disruption of *BAFF* or *BAFFR* (204). Given that the repertoire of B1 B cells is enriched in autoreactive BCRs (205), these observations suggest that B1 B cells require comparatively strong BCR signaling for their development and/or maintenance, which can be provided by strong BCR crosslinking induced by autoantigens. For B2 B cells, in contrast, such strong BCR crosslinking by autoantigens would lead to deletion in order to purge the autoreactive BCR repertoire. During transition of the T1/T2 to mature B2 B cells, the ligand-independent BCR-mediated survival signal provided by the canonical NF- $\kappa$ B pathway might not be sufficient by itself to generate a sufficient number of long-lived mature B cells. For ensuring such longevity, the B2 B cells additionally require a BAFFR-mediated survival signal provided by the noncanonical NF- $\kappa$ B pathway.

## B CELL FATE DECISION AFTER ANTIGEN ENCOUNTER

After naive B2 B cells bind protein antigens, they upregulate the expression of CCR7 and are

attracted by a gradient of CCL21 to the outer T cell zones of secondary lymphoid tissues, where they elicit T cell help (206, 207). In this location, B cells form dynamic conjugates with T cells, receive cognate T cell help, and differentiate along one of two pathways: a follicular pathway, which gives rise to GCs, and an extrafollicular pathway, which gives rise to short-lived PCs (208). In the extrafollicular pathway, antigen-specific B cells leave the T cell zone and localize to the splenic bridging channels or the lymph-node medullary cords, where they form extrafollicular foci of short-lived PCs that produce low-affinity antibody. The few antigen-specific B cells that seed the follicles as GC founder cells undergo intense proliferation and then differentiate into high-affinity memory B cells and long-lived PCs (209).

In the case of TI type II antigens (which are characterized by repeating determinants on a large polysaccharide backbone), the extrafollicular pathway is initiated, and the plasmablasts are generated in splenic bridging channels and the lymph-node medullary cords (210). A CD11c<sup>high</sup> DC population is found in these regions and is thought to be essential for the survival of plasmablasts by providing BAFF and APRIL (211). Under physiological circumstances, the GC reaction is believed not to occur in response to the TI type II antigens.

## Initiation of GC Responses

When an antigen-stimulated B cell meets an activated effector T cell at the outer T cell zone, the interaction between the TCR on the T cell and MHC class II–peptide complexes on the B cell is critical for providing T cell help to the B cell; however, many other molecules play essential roles in the subsequent GC formation (210, 212). The importance of the interaction between CD40L and CD40 has been well recognized; CD40 is constitutively expressed on B cells, and CD40L is upregulated on T cells following their activation by DCs. Mice deficient in either *CD40* or *CD40L* are unable to mount a sufficient primary response or a recall response to TD antigens and do not form GCs

(213). Similarly, in humans, defective *CD40L* expression results in the X-linked form of hyper IgM syndrome. B cells in these patients are unable to undergo Ig class switching due to the lack of the CD40L-CD40 interaction. In vitro, the ligation of CD40 on B cells stimulates their survival, proliferation, and differentiation; promotes Ig isotype switching; and induces the up-regulation of surface molecules involved in antigen presentation.

The CD40-associated TRAF2, TRAF3, and TRAF6 initiate the NF- $\kappa$ B (both canonical and noncanonical), JNK, and p38 pathways (213, 214). MEKK1, a member of the MAP3K family, is associated with TRAF2 in CD40 signaling and is required for the optimal activation of JNK and p38, but it is not needed for NF- $\kappa$ B activation (215). Although it has not been tested directly, TAK1, another MAP3K member, is likely involved in canonical NF- $\kappa$ B activation in CD40 signaling (119). Probably as a result of defective CD40 signaling, B cells harboring defective TAK1 or MEKK1 do not generate GC B cells and subsequent TD humoral responses. Recent studies have revealed the existence of negative signaling adaptor molecules, Act1 (216) and BANK (217), in CD40 and BAFFR signaling. As mentioned above, the ligation of BAFFR leads to TRAF3 binding to the receptor, which is required to terminate TRAF3-mediated degradation of NIK. The recruitment of Act1 to the BAFFR-TRAF3 complex may provide the negative signal for subsequent NIK activation. In contrast to Act1's involvement in both CD40 and BAFFR signaling, BANK plays a negative role only in CD40 signaling, presumably through the inhibition of CD40-mediated Akt activation (217).

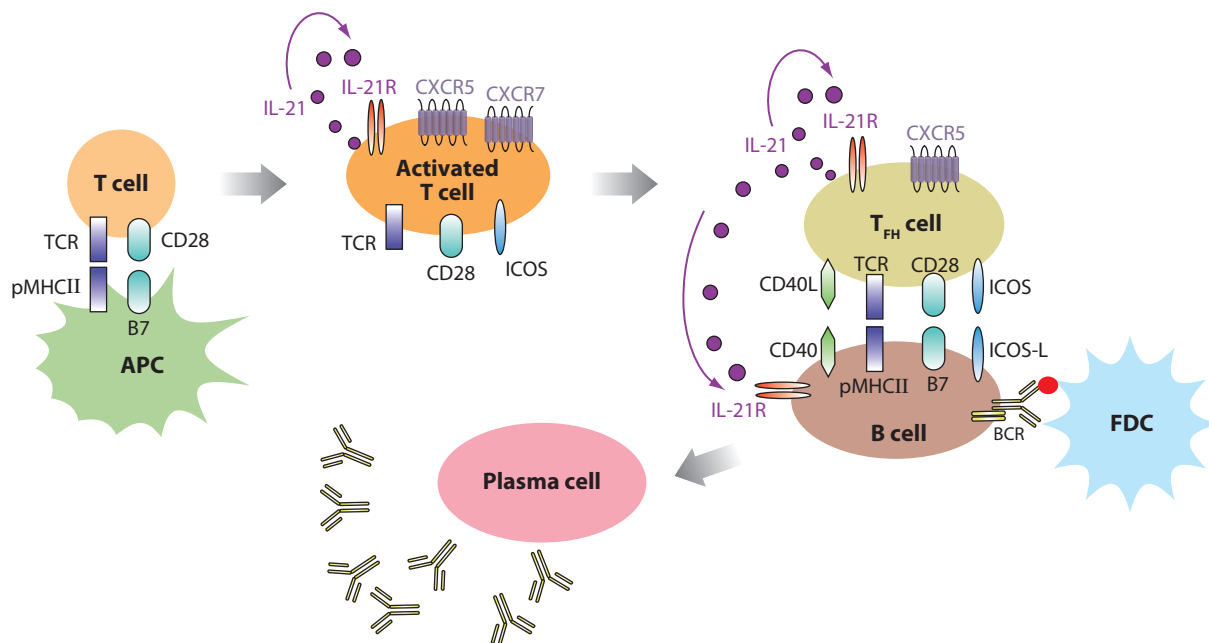
In B cells, Bcl6 is a master regulator of GC commitment, maintenance, and suppression of PC differentiation (212, 218). Thus, it is important to clarify when and how Bcl6 is turned on and off. However, these questions have not yet been adequately addressed. With regard to the mechanisms by which Bcl6 is turned off, signaling through the BCR and CD40 has been implicated. BCR stimulation may lead to Ub-mediated proteosomal degradation

of Bcl6 following its phosphorylation by ERK1/ERK2. Signaling through CD40 results in the transcriptional silencing of *Bcl6* through NF- $\kappa$ B-mediated activation of IRF4 (212, 219). Together, these observations suggest that signaling through BCR and CD40 is required for both GC development and, paradoxically, for the termination of GC reactions, too.

## Events During the GC Reaction

Proliferating GC B cells, which are known as centroblasts, undergo somatic hypermutation of their Ig variable region gene segments (212, 220). Centroblasts exit the cell cycle to become centrocytes, and if they bind antigen that is associated with FDCs, they receive survival signals. The cells can then differentiate into long-lived PCs and memory B cells or undergo further rounds of somatic hypermutation (208, 221). Previously, T helper (Th) 1 and 2 effector cells were thought to regulate B cell responses. For example, Th1-derived IFN- $\gamma$  regulates IgG2a production, whereas Th2-derived IL-4 is critical for IgG1 and IgE production in mice (208). The recent identification of a new subset of T cells, termed T follicular helper cells (T<sub>FH</sub> cells), has provided a fresh insight into how GC B cells are maintained and differentiated (218, 222–225). These T<sub>FH</sub> cells are present in GCs and are characterized by their expression of the CXCR5 chemokine receptor. Investigators now think that these cells regulate humoral immunity, especially GC reactions (**Figure 6**). Two types of cells are thought to participate in the development and/or maintenance of T<sub>FH</sub> cells: CD4<sup>+</sup>CD3<sup>−</sup> accessory cells and GC B cells. The CD4<sup>+</sup>CD3<sup>−</sup> cells present in the T cell zone–B cell follicle boundary probably provide signals through OX40 and CD30 expressed on the surface of T<sub>FH</sub> cells (226). Indeed, naive T cells that are stimulated by DCs transfected with OX40L upregulate CXCR5 expression (227), and mice that overexpress OX40L on the surface of DCs have an increased number of T<sub>FH</sub> cells (228). In the case of GC B cells, interaction between ICOS on T cells and ICOSL on GC B cells likely





**Figure 6**

Interactions between B cells and T follicular helper cells (T<sub>FH</sub> cells) during T-dependent immune responses. In the T cell zone of lymphoid tissues, mature DCs expressing B7.1 and B7.2 present peptide–MHC class II (pMHCII) ligand to the T cell receptor (TCR) of naive CD4<sup>+</sup> T cells. Activated CD4<sup>+</sup> T cells produce IL-21 and induce the expression of CD28 and ICOS. The sustained signaling of activated CD4<sup>+</sup> T cells through the TCR, CD28, and IL-21R in the T cell zone and at the T cell–B cell interface leads to the modulation of the expression of chemokine receptors such as CXCR5 and CCR7 and costimulatory receptors including ICOS, CD40L, and OX40. Although it is still unclear how T<sub>FH</sub> cells develop, the migration of T<sub>FH</sub> cells to follicles and the delivery of T cells help support the selection and differentiation of activated B cells in germinal centers. APC, antigen-presenting cell; FDC, follicular dendritic cell.

provides maintenance signals to T<sub>FH</sub> cells because B cells devoid of ICOSL cannot generate sufficient numbers of T<sub>FH</sub> cells (218).

Several molecules are crucial for mediating T<sub>FH</sub> cell help for B cells and B cell help for T<sub>FH</sub> cells during GC reactions (**Figure 6**). Although the interaction between CD40 and CD40L is essential to initiate GC reactions (as discussed above), this interaction is also important for the maintenance of GC reactions in that established GCs can be disrupted using a CD40L blocking antibody (229).

ICOS is a CD28-like molecule, and it is one of the most important molecules expressed on T<sub>FH</sub> cells for TD antibody responses. Its ligand, ICOSL, is expressed by antigen-presenting cells, including GC B cells. Mice deficient in ICOS and ICOSL cannot form GCs or undergo

Ig class switching (230–232). Conversely, when the negative regulation of ICOS by the Ub ligase Roquin E3 is impaired, there is an increased number of T<sub>FH</sub> cells and increased IL-21 production (218, 233). The connection between ICOS and IL-21 in T cells has been clarified; the ICOS–ICOSL interaction is necessary for IL-21 expression by T cells (234). Given that T<sub>FH</sub> cells can be generated in the presence of IL-21 in vitro and that T<sub>FH</sub> cells express IL-21R, it seems reasonable to anticipate that, once naive T cells are activated through their TCR and ICOS, they will secrete IL-21, which in turn may function in a paracrine fashion to regulate T<sub>FH</sub> cell development (207). Because high levels of IL-21R are also expressed on the surface of most B cells, IL-21 secreted by T<sub>FH</sub> cells also impacts B cells (234–236). Indeed,

**T<sub>FH</sub> cell:** T follicular helper cell

B cells cultured with a CD40 agonistic antibody showed enhanced proliferation and differentiation into ASCs, secreting all antibody isotypes when IL-21 was added to the cultures (237).

Signals through the BCR are thought to regulate the activity of GC B cells in several ways. One important outcome of the BCR signal is to provide survival and proliferative signals, thereby contributing to the generation and maintenance of GC B cells. Other cell surface molecules important in this process include CD45 or CD19; mice deficient in these proteins cannot generate sufficient survival signals, resulting in defective GC formation. In addition, the calcineurin-regulated transcription factor Mef2c is thought to function as a downstream target of the calcium/NFAT axis and has been shown to be critical for BCR-mediated

proliferation and survival (238). Consequently, the B cell-specific deletion of Mef2c results in decreased GC formation, leading to lower TD antibody responses (238).

## CONCLUSION

Signaling by the BCR and various coreceptors establishes a number of distinct checkpoints in the B cell pathway to ensure that B cell development occurs normally and to shape the correct naive BCR repertoire and subsequent BCR-triggered antibody responses. Additional work in this area is required to define the interconnections among the membrane, cytoplasmic, and nuclear events that set the thresholds for these checkpoints in distinct populations of developing and responding B cells.

## DISCLOSURE STATEMENT

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## Errata

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