

B lymphocyte antigen receptor signaling: initiation, amplification, and regulation

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Abstract

B lymphocytes and their differentiated daughters are charged with responding to the myriad pathogens in our environment and production of protective antibodies. A sample of the protective antibody produced by each clone is utilized as a component of the cell's antigen receptor (BCR). Transmembrane signals generated upon antigen binding to this receptor provide the primary directive for the cell's subsequent response. In this report, we discuss recent progress and current controversy regarding B cell receptor signal initiation, transduction and regulation.

Introduction

B cells are central components of adaptive immunity, responding to pathogens by proliferation, differentiation and production of antibodies. B cells also function in presentation of antigen to T cells and secretion of regulatory cytokines and chemokines. Like T cells, they express highly variable and clonally restricted antigen-binding receptors, the specificity of which is determined by immunoglobulin variable region gene rearrangement. The development and function of the B cell is primarily affected by signaling via their B cell antigen receptor (BCR).

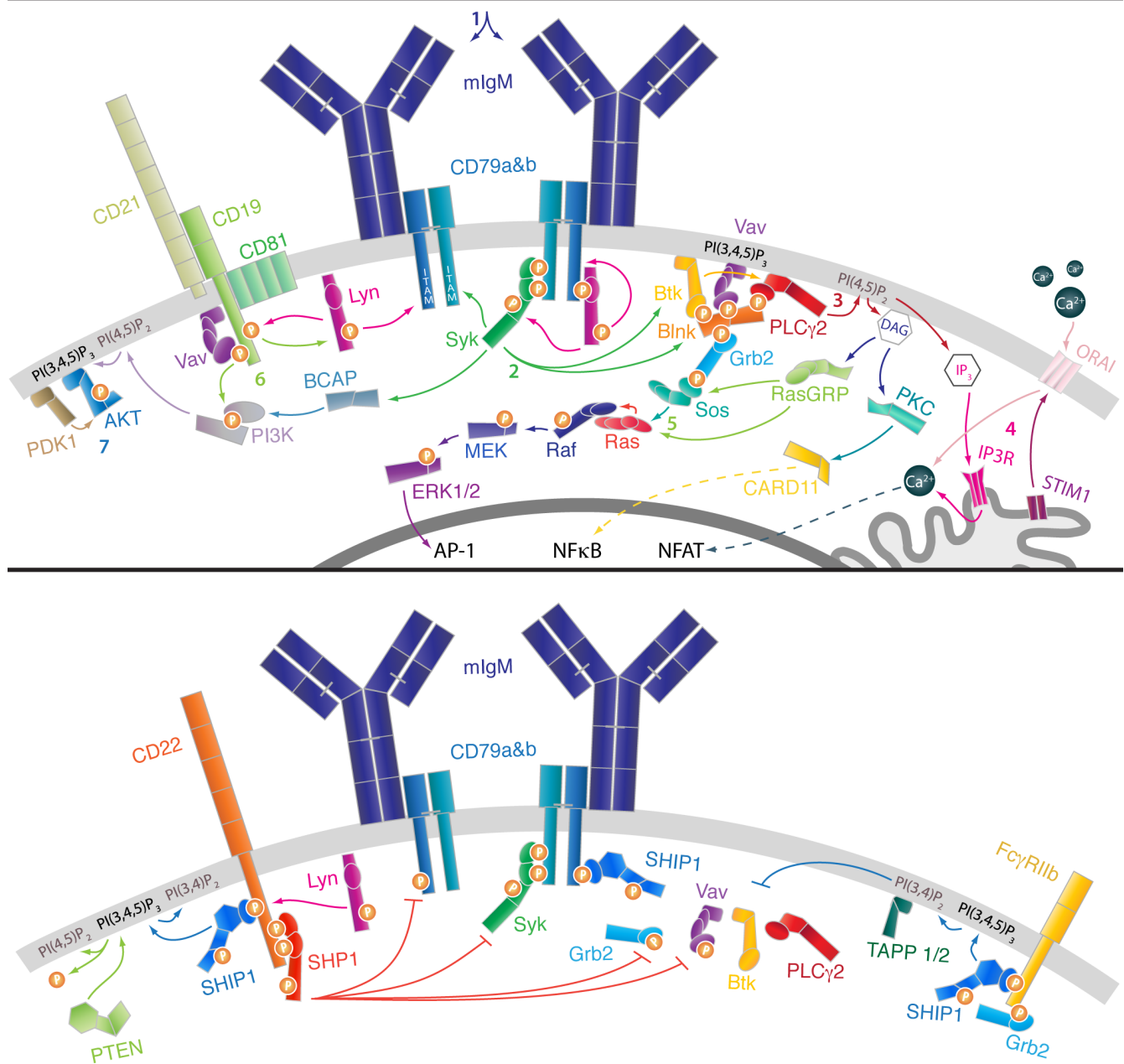
An important and controversial topic in the B cell field is the initiation of BCR signaling. In most cases, cell-surface receptors signal via ligand-induced subunit oligomerization, conformational changes, or a combination of these. These receptor/ligand interactions have conformational limitations, likely preserving specificity through a process of coevolution. In contrast, BCR ligands are incredibly diverse and it is imperative to accommodate and discriminate a much broader range of ligand affinity and presentation than other receptors. Put another way, B cell effector function—including secretion of antibody critical to immunity—relies on the ability of the repertoire

of BCRs to respond to every possible epitope, in diverse physical contexts, derived from an array of pathogens that are limited only by evolution. As the immune response continues, the pathogen may attempt to evade by modifying antigens, and B cells must keep up with these minor modifications, while also increasing the receptor affinity of the responding B cells. This diversity of ligand/receptor affinity, orientation, and presentation makes it difficult to resolve a clear mechanism of BCR signal initiation. Thus, many groups have proposed alternative mechanisms of signal initiation and amplification to complement, or dispute, the long established model that simple aggregation of receptors is sufficient to initiate signaling. Here we discuss recent progress and points of contention related to BCR initiation and function.

Overview of BCR signaling

The BCR is composed of membrane-bound immunoglobulin (mIg) non-covalently associated with a disulfide-linked heterodimer of CD79a (Ig α) and CD79b (Ig β) [1-4] (see Figure 1). Following ligation of the mIg, signals are transduced across the plasma membrane, leading to phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) contained in the cytoplasmic tails of the CD79a and b [1,2,5-7]. The kinase primarily

Figure 1. B cell receptor signaling and regulation. Upper panel B cell activation



Upon ligation of the BCR (1), ITAMs become phosphorylated via activity of SFKs (such as Lyn) and Syk. Syk and SFKs then phosphorylate signalosome components (2). The signalosome is associated with CD79a non-ITAM phosphotyrosine residues via binding of the adaptor protein Blnk (not illustrated). Activated Btk phosphorylates PLCγ2, which in turn cleaves the phosphoinositide PI(4,5)P₂, releasing IP₃ into the cytosol and forming DAG (3). IP₃ binds IP3R in the endoplasmic reticulum, releasing Ca²⁺ into the cytoplasm. The decrease in endoplasmic reticulum [Ca²⁺] activates STIM1, which binds ORAI in the plasma membrane, forming the CRAC channel and allowing for the influx of extracellular Ca²⁺ ions (4). RasGRP and protein kinase C (PKC) are activated by binding DAG, and feed into the MEK/MAP kinase (5) and NFκB activation pathways, respectively. CD19 plays an important role in amplifying the BCR signal via processive activation of Lyn, and activation of PI3K (6). Along with the recruitment of PH domain-containing signalosome components, the accumulation of PI(3,4,5)P₃ drives activation of Akt (7). **Lower panel B cell deactivation:** Lyn phosphorylates immunoreceptor tyrosine-based inhibition motifs (ITIMs) in CD22 and FcγRIIb. These ITIMs activate SHP1 and SHIP1, which function to inhibit BCR signaling. The protein phosphatase SHP1 has many substrates, including CD79, Syk, Grb2, and Vav, as well as others not shown. Additionally, ITIMs and mono-phosphorylated ITAMs can activate the lipid phosphatase SHIP1. SHIP1 hydrolyzes the phosphate at position 5 of PI(3,4,5)P₃, while PTEN removes that at position 3. This decrease in PI(3,4,5)P₃ concentration results in the disassociation of many PH domain-containing molecules, inhibiting signalosome assembly and downstream signaling. Illustrated structures not to scale, references in text.

responsible for this phosphorylation is thought to be a Src-family kinase (SFK): particularly, Lyn, which is the predominant SFK expressed in B cells [8]. Phosphorylation of ITAMs leads to SFK binding by Src-homology 2 (SH2) domains and the upregulation of kinase activity [7]. Dual phosphorylation of ITAM tyrosines leads to Syk binding via its tandem SH2 domains [9]. This binding results in the activation and phosphorylation of Syk [10,11].

Once Syk is activated, the BCR signal is propagated via a group of proteins associated with the adaptor protein B-cell linker (Blnk, SLP-65), which has been referred to as a signalosome [12]. Blnk binds CD79a via non-ITAM tyrosines and is phosphorylated by Syk [13]. Phospho-Blnk serves as a scaffold for the assembly of the other components, including Bruton's tyrosine kinase (Btk), Vav 1, and phospholipase C-gamma 2 (PLC γ 2) [14]. Btk, Vav 1, and PLC γ 2 contain pleckstrin-homology (PH) domains that bind lipid phosphoinositides. These phosphoinositides are enriched in the inner leaflet of the plasma membrane and are important for the propagation of multiple signaling pathways. Phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P $_3$) is the product of the phosphorylation of PI(4,5)P $_2$ at position three by phosphoinositide-3-kinase (PI3K). PI(3,4,5)P $_3$ is a critical second messenger in BCR signaling and is the specific target of lipid phosphatases that regulate signaling.

Following their recruitment by Blnk, Btk phosphorylates PLC γ 2, which in turn cleaves the phosphoinositide PI(4,5)P $_2$, generating IP $_3$ and DAG [15,16]. IP $_3$ binds the IP $_3$ receptor (IP $_3$ R) in the membrane of the endoplasmic reticulum, resulting in the release of the intracellular stores of Ca $^{2+}$ ions. This reduction in calcium stores activates calcium-release-activated channels, which allow the influx of extracellular calcium ions [17,18]. DAG binds to Ras guanyl nucleotide-releasing protein (RasGRP) as well as the classical members of the protein kinase C family, such as PKC β in B cells promoting their activity [19-21].

Distal from these events, BCR signaling becomes quite branched, activating multiple kinase cascades (e.g. Erk/MAP kinases) and guanine nucleotide exchange factors (e.g. Ras/Raf). The consequences of these signals include the translocation of transcription factors (e.g. NF κ B, AP-1, and NFAT); the net result of these processes drives activation of the B cell, antigen presentation, cytokine production, cell proliferation and differentiation.

These activating signals are necessary to initiate responses against antigens with pathogenic potential. Regulating these responses, as well as silencing

autoreactive B cells, are multiple receptors and coupled pathways mediated by phosphatases. Key regulators of proximal BCR signaling include the SH2 domain-containing phosphatase 1 (SHP1), a protein tyrosine phosphatase, as well as SH2 domain-containing inositol 5'-phosphatase 1 (SHIP1) and phosphatase and tensin homolog (PTEN), lipid phosphatases. Multiple studies have shown that disruption of negative regulatory circuitry can result in subversion of tolerance mechanisms and autoimmunity [22-25].

Together, BCR signaling and its regulation represent complex and dynamic fields of study. For the sake of brevity, we will focus this review on a few of the major recent advances in the contentious area of BCR signal initiation.

Initiation of signaling

The definitive requirements for BCR engagement, minimally sufficient to initiate signaling, remain controversial. Additionally, these requirements may differ depending on the physical context of the antigenic epitope. B cells can recognize an antigen in soluble and immobilized forms, such as adsorbed onto or embedded in the plasma membrane of another cell, bacterium or virus. In the case of free soluble antigen, crosslinking of two or more BCRs seems to satisfy the minimal requirements for induction of a detectable downstream signal, whereas monovalent soluble ligands do not [26,27]. In contrast, monovalent membrane-bound antigens can reportedly induce conformational alterations in the BCR that initiate association with neighboring BCR, and this can lead to signaling [28]. Both of these signal initiation models coalesce in the idea that two or more BCRs become closely associated following ligand binding, allowing for transactivation via SFK and Syk.

Still others propose that it is not aggregation but rather disassociation of preformed oligomeric BCR complexes that releases the receptors from an inhibited state and triggers signaling [29-33]. Reth and colleagues propose a disassociation activation model in which resting B cells have preaggregated BCR that are inhibited via methyltransferase activity and Syk serine kinase activity [31,32]. This model proposes that following ligand binding, BCRs move from "closed"-monomeric or oligomeric states to an "open" cluster conformation that initiates positive signaling. The proponents of this model argue that the flexibility of the hinge in mIg and variability of antigen binding would result in inefficient activation via traditional crosslinking due to steric requirements for signaling, describing this incongruity of antigen/BCR as an "unsolved problem" of clonal selection [30,33]. However, there are two key problems with this

argument: firstly, no orientational requirements have been described for the induction of BCR signaling, thus they may not exist, and secondly, the hypothesis fails to account for the diversity of the BCR repertoire. The first counterpoint is that flexible mIg, and non-covalently associated CD79—in *the round*—likely have a diverse assortment of signaling competent orientations. As evidence against steric restriction of the BCR signal consider signaling by intact monoclonal antibodies; it was demonstrated decades ago that monoclonal antibodies recognizing spatially distinct IgM epitopes can induce signaling [34]. If there existed a strict orientational requirement for BCR signal, some or most of these antibodies should be incapable of replicating this orientation and inducing signaling. The second argument of clonal selection and repertoire is simple: the clonal selection theory predicts that the clones capable of responding to an antigen do respond. Thus, clonal selection is a tautology that is self-evident as responding B cells express a BCR capable of signaling regardless of the mechanism of antigen engagement employed.

Other arguments against initiation via aggregation include observations that BCRs exist in preformed oligomers, as shown by native gel electrophoresis [29]. It is likely that BCRs are clustered near each other in highly ordered areas of the plasma membrane, which would explain their apparent association in low-detergent conditions. However, if they were truly oligomers, this interaction should persist in more harsh detergents, such as the observed association of CD79 with mIg. Additionally, Förster resonance energy transfer (FRET) experiments failed to show evidence for oligomerization prior to stimulation [35]. Perhaps BCRs are simply in organized, quiescent clusters poised to signal upon encountering an aggregating ligand. In any case, pre-organization of receptors is not *a priori* indicative of inhibition, as shown by the pre-ligand-binding assembly domains of tumor necrosis factor receptors [36]. Along with the pre-organization of BCRs, there exists evidence that Syk can be activated by a single dual-phosphorylated ITAM [37-39]. To this end, we completely agree. However, this argument is downstream of signal initiation, as either the ITAM must first be phosphorylated, or SFKs and Syk must be activated, subsequently phosphorylating ITAMs. Thus, we discover a “catch-22” in which at least one ITAM must first be dually phosphorylated before Syk can be activated. As SFKs are the likely candidate for this activity, we think the aggregation model allows for the most efficient recruitment of a nearby SFK to drive this activation. Together, most researchers in the field of antigen receptor signaling are in agreement that BCR crosslinking is sufficient to induce signaling, and is the model that best fits current evidence.

Responses induced by different physiochemical forms of antigen may vary in their requirement for proximal signaling effectors. Recent work by Mukherjee et al. suggested that monovalent antigen induction of BCR signaling is dependent on SFKs, whereas SFKs play a less critical role in responses to multivalent antigens [40]. This is in keeping with previous findings using SFK knockout models, suggesting that the Syk tyrosine kinase may be sufficient to initiate signaling in response to multivalent antigens, mediating phosphorylation of receptor ITAMs [8,41]. The authors employed *in silico* modeling to predict the differential roles of Syk and SFKs in responses to multivalent antigens that induce clustering or to homogenous, “monovalent” antigens. These models predict that Syk is critical for BCR signaling via positive feed-forward functions and is able to compensate for SFK deficiency when the stimulating antigen is sufficiently avid. The model's predictions were interrogated by *in vitro* experiments measuring responses in the presence of Syk and SFK pharmacologic inhibitors. The results of these experiments support the model, though interpretations are compromised somewhat by use of the MD4 system: MD4 B cells are capable of signaling in response to soluble “monovalent” hen egg lysozyme (HEL) in the absence of inhibitors [40,42]. This appears to be a unique property of HEL, as others have demonstrated that monovalent soluble antigens are incapable of inducing signaling [27]. Indeed, with the exception of HEL, previous studies demonstrate that monovalent antigen-induced signaling depends on antigen adsorption to lipid membranes [28]. An explanation of the ability of “monovalent” HEL to induce signaling probably lies in the highly charged nature of HEL, and, likely, rapid spontaneous multimerization, or adsorption on cell surface carbohydrates. Decades of studies indicate that truly monovalent soluble antigens (e.g. monovalent Fab fragments of anti-BCR antibodies) do not induce detectable signaling. Nonetheless, the findings of Mukherjee et al. support the concept that responses to paucivalent antigens are more dependent on SFKs than responses to high avidity antigens, confirming the authors' general predictions.

Signal amplification by CD19

CD19, a B cell-restricted membrane glycoprotein, functions both as an accessory in BCR signaling and, when associated with CD21, as a BCR co-receptor for complement C3dg fragments. Recent findings by Depoil et al. indicate that CD19 is uniquely required for responses to membrane-adsorbed antigens [43]. The requirement for CD19 in this response may be related mechanistically to the apparent requirement for SFK in BCR signaling induced by low-avidity antigens [40]. It seems likely that the forward-feeding mechanism of CD19 as both a

substrate and processive activator of SFKs may provide amplification of SFK activity critical to sustain cell activation when BCR stimulation is suboptimal [44]. Thus, experimental approaches towards understanding minimal thresholds of BCR stimulation should consider requirements for SFKs and CD19, due to their complementarity.

Interestingly, Mattila et al. recently demonstrated that spontaneous BCR signaling as a result of cytoskeletal disruption is dependent on CD19 and CD81 [45]. The authors use superresolution microscopy (dSTORM) to demonstrate the existence of BCR in preformed "nanoclusters" that associate with Syk upon antigen stimulation, and higher level clustering of BCR induced by anti-Ig antibodies. Despite decades of knowledge that the cytoskeleton affects BCR signaling, the advent of advanced imaging techniques has allowed for a more refined understanding of these mechanisms. The cytoskeleton acts both in organizing and immobilizing BCRs in the resting cell, as well as amplifying signaling by allowing for B cell spreading and synapse formation. These advanced techniques and concepts are reviewed in excellent detail by Harwood & Batista [46].

Further, CD19 and CD81 are shown to be dispensable for the initiation of signaling in response to soluble, multivalent antigens. These latter results seem inconsistent with previous reports that suggested a critical role for CD19 in the activation of PH domain-containing signaling intermediaries. However, this discrepancy may be explained by the redundant action of B-cell adaptor for phosphoinositide-3-kinase (BCAP) in activation of PI3K, allowing for the propagation of the BCR signal in the absence of CD19 [47,48]. Thus, CD19 may be critical for signaling in response to low-avidity antigens because it enhances SFK and PI3K activation, though in response to high-avidity antigens, it may be redundant with the activity of Syk and BCAP.

Signal modulation

As well as having differing antigen avidity requirements for the initiation of signaling, Syk and SFKs play different roles in signal propagation. Syk appears completely positive, both in its forward-feeding amplification of ITAM and auto-phosphorylation, as well as activation of downstream components. By contrast, the SFK Lyn activates both positive and negative regulators of BCR signaling, such as CD22, Fc γ RIIb, SHP1 and SHIP1 [49]. Recent studies suggest that by toggling between mono- and dual-phosphorylated states, ITAMs drive Syk-mediated activation versus Lyn-mediated inhibitory signaling. Indeed, in chronically antigen-stimulated anergic B cells, CD79a is monophosphorylated, driving

chronic activation of inhibitory SHIP1 signaling [24,50]. This plays a role in preventing activation of anergic B cells.

SHIP1 and SHP1 have recently been shown to play an additional role in B cells, modulating BCR signaling in the germinal center where B cells engage in the somatic mutation of their BCR, and those with resultant increased affinity for antigen are selected for survival and class switching. These phosphatases are upregulated in germinal center B cells and oppose positive BCR signaling at differing levels throughout the cycle of these highly proliferative cells. As a net result of this signal dampening, cells bearing higher affinity receptors are more competitive, leading to affinity maturation [51,52].

The delicacy of the balance of positive and negative signaling is also demonstrated by the effects of altering Lyn levels, as both under and over-expression results in autoimmune phenotypes [53,54]. Other SFKs expressed by B cells, e.g. Fyn, have been demonstrated to have only activating signaling qualities, while Blk is localized on endoplasmic reticulum structures where it appears to regulate IP3 receptor function [55,56]. These observations illustrate an important role for Lyn in tolerance, while Fyn and Syk are more important as signaling intermediaries in cell activation.

Underscoring the importance of regulating signals transduced by the BCR is the fact that a high proportion of circulating B cells are autoreactive [57]. Many of these self-specific cells are probably silenced by virtue of ignorance because their autoantigens are monovalent or in low abundance and therefore do not induce signaling. Multivalent autoantigens may, by inducing chronic signaling, render specific B cells anergic. Two recent studies bear on the status of these potentially autoreactive cells. The first, by Zikherman, Parameswaran & Weiss, shows that in specific-pathogen-free mice, a significant proportion of mature naïve B cells show evidence of ongoing BCR signaling [58]. Signaling was reported in these studies by GFP expression in B cells from transgenic mice expressing GFP under control of the Nur77 promoter, a transcript driven by early antigen-receptor signaling events. Interestingly, they found that transitional-3 cells, a compartment previously shown to contain anergic cells, were highly enriched for GFP⁺ cells [59]. Additionally, they found that some GFP⁺ B cells were present in virtually every compartment, characterized by decreased surface IgM. These findings suggest that a significant proportion of peripheral B cells are stimulated by autoantigens but are spared from activation by regulatory mechanisms. A second study involved analysis of the autoantigen-specific B cells in the normal repertoire of animals expressing an ectopic autoantigen

(soluble ovalbumin) as well as the natural autoantigen glucose-6-phosphate isomerase (GPI) [60]. The authors demonstrate that in the case of these soluble, presumably monovalent, self antigens, the autoreactive cells appear to be ignorant of antigen in their environment. This is not completely unexpected, and echoes the previously discussed studies: namely, soluble, monovalent antigens fail to trigger BCR signaling. Interestingly, they did find B cell intrinsic tolerance, similar to anergy, when the autoantigen ovalbumin was membrane-bound, and therefore sufficient to induce a BCR signal.

Summary and conclusion

To summarize, BCR signaling is initiated via ligation of mIg under conditions that induce phosphorylation of the ITAMs in CD79. This results in the activation of Syk, SFKs, and other signaling intermediaries (i.e. Blnk, Btk, Vav1, PLC γ 2). These are recruited via association with BCR or PH-domains binding to PI(3,4,5)P₃. While SFKs play roles throughout the process, they appear to be especially necessary in the initiation of signaling by low-avidity antigens. The glycoprotein CD19 is dually important in its amplification of activation of the SFK Lyn, and in activation of PI3K. It is our opinion that this role for CD19 in the amplification of Lyn and activation of PI3K is most critical in examples of low-avidity reactions, whereas, in the case of high-avidity stimuli, Syk and BCAP exhibit compensatory capabilities.

There exist multiple negative regulators of BCR signaling, critical for B cell-intrinsic tolerance. However, recent studies have shown that many potentially autoreactive cells appear functionally naïve, and those that have encountered sufficient antigen to signal can be found in all B cell compartments, and are characterized by lower surface IgM expression.

It is likely that these cells are maintained in the periphery with decreased levels of BCR stimulation and do not become activated due to the “conditional” dominance of inhibitory signaling (i.e. in the steady-state the B cell is not activated by autoantigen). Though this anergy may seem to flirt with autoimmunity, one could imagine that this is necessary to avoid extensive exploitation of molecular mimicry by pathogens. Further, an increase from normal physiological levels of certain self-antigens is associated with some tumors and other pathophysiology. Thus, the maintenance of these autoreactive B cells, poised to react to homeostatic disruption, may be important for tumor surveillance and pathogen defense.

We hope we have conveyed the evolving status of principles that govern B cell antigen receptor signal initiation and its outcome. Signaling is determined by

many currently ponderable and imponderable factors. These factors include antigen valency, affinity, concentration, physical context, and chronicity of stimulation; additionally, intrinsic properties of the responding cell, including the relative levels of receptors and signaling intermediaries that function to propagate and regulate signal transmission. Finally, given the complexity of the universe of antigens, the net effect of these factors must be to both respond to pathogenic stimuli and limit these responses, while preventing responses to autoantigens.

Abbreviations

BCAP, B-cell adaptor for phosphoinositide-3-kinase; BCR, B cell antigen receptor; Blnk, B-cell linker; Btk, Bruton’s tyrosine kinase; DAG, diacylglycerol; Fc γ R1b, low-affinity immunoglobulin gamma Fc region receptor II-b; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; GPI, glucose-6-phosphate isomerase; HEL, hen egg lysozyme; IP₃, inositol triphosphate; IP3R, inositol triphosphate receptor; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; mIg, membrane-bound immunoglobulin; PI3K, phosphoinositide-3-kinase; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-triphosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PH, pleckstrin-homology; PKC, protein kinase C; PLC γ 2, phospholipase C-gamma 2; PTEN, phosphatase and tensin homolog; RasGRP, Ras guanyl nucleotide-releasing protein; SFK, Src-family kinase; SH2, Src-homology 2; SHIP1, SH2 domain-containing inositol 5’-phosphatase 1; SHP1, SH2 domain-containing phosphatase 1.

Disclosures

The authors declare that they have no disclosures.

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