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Actin-mediated feedback loops in B-cell receptor signaling

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Summary

Upon recognizing cognate antigen, B cells mobilize multiple cellular apparatuses to propagate an optimal response. Antigen binding is transduced into cytoplasmic signaling events through B-cell antigen receptor (BCR)-based signalosomes at the B-cell surface. BCR signalosomes are dynamic and transient and are subsequently endocytosed for antigen processing. The function of BCR signalosomes is one of the determining factors for the fate of B cells: clonal expansion, anergy, or apoptosis. Accumulating evidence underscores the importance of the actin cytoskeleton in B-cell activation. We have begun to appreciate the role of actin dynamics in regulating BCR-mediated tonic signaling and the formation of BCR signalosomes. Our recent studies reveal an additional function of the actin cytoskeleton in the downregulation of BCR signaling, consequently contributing to the generation and maintenance of B-cell self-tolerance. In this review, we discuss how actin remodels its organization and dynamics in close coordination with BCR signaling and how actin remodeling in turn amplifies the activation and subsequent downregulation process of BCR signaling, providing vital feedback for optimal BCR activation.

Keywords

B cells; B-cell antigen receptor; actin cytoskeleton; signal transduction; endocytosis

Introduction

B lymphocytes function to generate and maintain humoral immunity. Antibody resulting from B-cell activation neutralizes and flags pathogens and pathogen-secreted toxins for immune clearance. Resting B cells in circulation are activated in a clonally specific fashion through a two-stage signal. The initial signal is generated by the binding of cognate antigen to the B-cell antigen receptor (BCR), which leads to signaling cascades in the cytoplasm (1). Following antigen-induced BCR signaling, the receptor endocytoses antigen into the endocytic system, where the antigen is processed into a form recognizable by T cells. Through antigen presentation, B cells activate T cells and gain T-cell help (2, 3). The generation of both activation signals depends on the BCR: the ability of the receptor to transduce antigen binding into cytoplasmic signaling and to capture and transport antigen for processing. The two functions of the BCR regulate one another: BCR signaling enhances antigen processing while receptor endocytosis reduces BCR signaling at the cell surface (4). While the activation of BCR signaling has been extensively studied (1, 5–7), the molecular

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mechanisms underlying the regulation of signal transduction remain elusive. In particular, the negative regulatory mechanisms, which determine activation threshold, level, and duration, are not completely understood. Such negative regulation is essential for controlling the balance between effective activation of antibody responses against pathogens and strict control of autoreactive B-cell activation and autoantibody production (8). Recent studies have shown that the dynamic organization of BCRs at the cell surface is essential for their functions (9, 10). The cortical actin network immediately underneath the plasma membrane is known to impose physical barriers that control the organization of surface receptors (11, 12). In B cells, the dynamics of the cortical actin network have been demonstrated to be a regulatory factor for tonic signaling and antigen-induced signaling of the BCR (9, 13). In this review, we discuss recent advances in understanding how the signaling induced reorganization of the actin cytoskeleton generates positive or negative feedback to the signal transduction of the BCR.

Signal transduction of the BCR

Like most receptors expressed in immune cells, the BCR does not have any intrinsic enzymatic activity; instead, its cytoplasmic domains contain phosphorylation and docking sites for kinases and adapter proteins, which are called immunoreceptor tyrosine-based activation motifs (ITAMs) (14). The Ig α / β heterodimer of the BCR has one ITAM in each of the two cytoplasmic tails. This disulfide bond-linked heterodimer non-covalently associates with membrane immunoglobulin (mIg) that is responsible for recognizing specific antigen. This association involves their trans-membrane domains and is required for the expression of the BCR at the surface of B cells (15, 16). An additional tyrosine-based activation motif has been recently identified in the cytoplasmic domain of IgG (17). The biochemical make up of the BCR dictates its signaling mechanism: recruitment and organization of signaling molecules into supramolecular activation complexes (SMACs) or signalosomes.

The initiation of BCR activation has been shown to require receptor cross-linking by antigen (18, 19), which oligomerizes or organizes surface BCRs into membrane-bound clusters of different sizes depending on the valency and physical configuration of the antigen. Clustered receptors are preferentially associated with cholesterol- and glycolipid-rich lipid rafts, where ITAMs are phosphorylated by lipid raft-resident Src kinases, such as Lyn (20–22). Phosphorylated ITAMs recruit downstream kinases and adapter proteins. The key upstream signaling molecule Syk kinase binds to fully phosphorylated ITAMs, which activates its tyrosine kinase activity. Active Syk in turn phosphorylates and activates signaling molecules that are recruited to the BCR and lipid rafts, including phospholipase C β 2 (PLC β 2), phosphoinositol-3 kinase (PI3K), Bruton's tyrosine kinase (Btk), and adapter proteins, such as BLNK and Grb2, as well as costimulatory coreceptor CD19 (1, 23). These events lead to the formation of a group of BCR signaling microclusters as signalosomes, which induce biochemical cascades in the cytoplasm.

BCR signaling is relatively transient and tightly controlled by inhibitory phosphatases, like SH2-containing tyrosine (SHP) and phosphatidylinositol-5 phosphatase (SHIP) (8, 24, 25). A well known example of negative regulation is by Fc γ RIIB-mediated inhibition of BCR signaling. This occurs *in vivo* when most antigens are bound by antibody, forming immune complexes. The immune complexes colligate the BCR and Fc γ RIIB, which leads to the activation of SHIP (26). SHIP converts phosphatidylinositol-3,4,5-triphosphate [PtdIn(3,4,5)P₃] to PtdIn(3,4)P₂, which eliminates lipid raft-docking sites for PLC β 2, Akt, and Btk, consequently inhibiting their activation (27). BCR activation by antigen binding also induces SHIP activation. SHIP has been shown to bind BCR ITAMs with only one of the two tyrosines phosphorylated in anergic B cells, which is critical for keeping B cells in the anergic state (28). The downregulation of BCR signaling mediated by these inhibitory

phosphatases is critical for maintaining B-cell self-tolerance and controlling B-cell-mediated autoimmunity (8, 29).

Dynamic organization of surface BCRs

Recent advances in high resolution live cell imaging techniques have enabled us to reveal molecular details of receptor activation at the cell surface in real time. Upon interacting with antigen, particularly membrane-associated antigen, BCRs at the B-cell surface briefly increase their lateral mobility (30, 31). This is followed by immobilization of surface BCRs and concurrent formation of BCR microclusters (32). While the microclusters interact with lipid rafts and lipid raft-associated Lyn, tyrosine phosphorylation in the microclusters increases and Syk is recruited to the microclusters (18, 22, 33). Many additional signaling molecules are subsequently recruited to BCR microclusters, such as CD19, PLC 2, and Btk (34, 35), indicating that these microclusters function as signalosomes. Over a timescale of a few minutes, BCR microclusters grow by recruiting more receptors into the clusters while simultaneously moving towards one pole of the cells. In B cells interacting with membrane-associated antigen, BCR microclusters move towards the center of the contact surface between the B cell and the antigen-presenting membrane (B-cell contact zone). While moving centripetally, BCR microclusters merge into one another, forming a central cluster, a molecular complex similar to the immunological synapse formed between T cells and antigen-presenting cells (9, 36, 37). While most of these results were obtained by studies of B cells activated by membrane-associated or immobilized antigen, our recent studies show that multi-valent soluble antigen is capable of inducing similar receptor cluster dynamics and formation of a central cluster. However, the BCR central cluster induced by soluble antigen is more dynamic and less stable compared to that induced by membrane-associated antigen (38). It has also been shown that dynamic clusters of surface BCRs are targets for disruption by inhibitory signaling molecules. Colligation of the BCR with Fc RIIB by immune complexes inhibits the interaction of the BCR with lipid rafts and the formation of BCR microclusters and central clusters (39, 40). These findings further support the view that molecular dynamics and reorganization of BCRs at the B-cell surface are key events as well as regulatory targets during BCR-mediated B-cell activation.

While it has been clearly demonstrated that antigen-induced receptor clustering is required for BCR signaling activation (9, 36, 41), recent studies have shown that surface BCRs exist as clusters at the nanoscale in the absence of antigen binding. This was demonstrated by single molecule imaging using direct stochastic optical reconstruction microscopy (dSTORM) (42) and molecular interaction measurements using Forster resonance energy transfer (43). These BCR clusters are smaller than those induced by antigen, as they are not detectable using traditional confocal fluorescence microscopy. In addition to their size, BCR conformation and BCR-BCR interactions within these nano-clusters are likely different from those within antigen-induced clusters. The lateral mobility of these BCR nano-clusters has been implicated in regulating tonic signaling in resting B cells (42). BCRs within these nano-clusters have been postulated to be in an inhibitory conformation (43, 44). The physical constraints associated with antigen binding by BCRs have been shown to cause conformational changes in the receptor (10, 18, 32). BCR conformational changes may alter the ways in which BCRs in clusters interact with each other and allow BCRs to recruit additional receptors, and reorganize into signaling competent microclusters.

While BCR- and T-cell receptor-based SMACs share many common properties, the BCR central cluster is different from the T-cell immunological synapse in several aspects. First, the formation of BCR central clusters does not require the interaction of adhesion molecules between B cells and antigen-presenting cells, even though the interaction enhances BCR signaling at low antigen concentrations or density (45). Adhesion molecules can stabilize the

interaction between B cells and antigen presenting cells and thus facilitate antigen engagement by BCRs and BCR clusters. This suggests that antigen presented by professional antigen-presenting cells that express adhesion molecules, such as follicular dendritic cells in B-cell follicles, is more effective in activating B cells. Second, unlike T cells, multivalent soluble antigen can also induce the formation of BCR central clusters indicating that membrane-associated and immobilized antigen are not absolutely essential (38, 46). These antigen-binding properties of BCR, distinct from those of TCR, enable B cells to collect and respond to antigen in a wide variety of forms. Third, BCR central clusters are more dynamic and transient than the T-cell synapse, which is likely due to the rapid internalization of BCR-antigen complexes required for the initiation of antigen processing and presentation (47, 48). BCR endocytosis can destabilize the central cluster. This dynamic and versatile nature of BCR SMACs enables B cells to respond to as well as process and present antigen with diverse chemical and physical properties. Conversely, antigen properties can regulate the stability and life time of BCR SMACs as well as the kinetics and efficiency of antigen processing and presentation, consequently modulating both B-cell- and T-cell-mediated immune responses.

Actin remodeling during BCR signaling

Early studies have long noted that B-cell activation by antigen or mitogen stimulation induces actin remodeling (49, 50). The cortical actin network is the primary actin structure in B cells due to limited cytoplasmic space within resting lymphocytes. Cortical actin generally provides structural support for the plasma membrane, and its dynamic reorganization generates cell morphological changes. The cortical actin network is organized by actin cross-linking proteins into higher order structures such as actin bundles and branched meshworks, which generate filopodia and lamellipodia (51). The actin network is tethered to the plasma membrane by ezrin/radixin/moesin (ERM) family proteins that are capable of binding both F-actin and transmembrane proteins (52, 53). The actin cytoskeleton is highly dynamic, constantly undergoing polymerization and depolymerization, as well as association and disassociation with actin-binding proteins. In addition to driving changes in cell morphology, recent biophysical and cell biological studies reveal critical functions for cortical actin in controlling molecular dynamics and organization at the cell surface (11, 12). Because of its interactions with membrane anchor proteins, actin dynamics can physically influence the lateral movement of membrane proteins that have cytoplasmic tails extended into the cortical actin network by creating temporary mobility barriers, which leads to transient compartmentalization of membrane proteins. In B cells, the presence of such lateral mobility barriers has been demonstrated. Surface BCRs in actin- and ezrin-poor regions have a higher lateral mobility than those BCRs in actin- and ezrin-rich regions. Further, deletion of the cytoplasmic tails of the BCR increases its lateral mobility (54). These observations underscore the involvement of the actin cytoskeleton in regulating BCR lateral mobility and in the organization of surface BCRs.

Binding of antigen to the BCR first induces a transient disassembly of the cortical actin network, which is concurrent with a brief increase in BCR lateral mobility as discussed earlier (30, 31) (Fig. 1A). The actin disassembly is dependent on cofilin-mediated actin severing and the disassociation of ezrin from the plasma membrane (30, 31, 55). In B cells, ezrin has been shown to link the actin cytoskeleton to the plasma membrane by binding to a lipid raft-anchored protein, Csk-binding protein (55). Ezrin dephosphorylation induced by BCR signaling leads to the disassociation of ezrin from Csk-binding protein, consequently detaching cortical actin from the B-cell membrane. Following this brief disassembly, actin undergoes a rapid and dramatic reassembly. The reassembly does not restore actin back to the pre-activation structure but rather into dynamic, polarized, and BCR-centric structures. Early in this reassembly process, *de novo* actin polymerization is detected at locations of

BCR microcluster formation (38) (Fig. 1B), suggesting that signaling originated from the microclusters induces actin polymerization. In B cells activated by membrane-associated antigen, filopodia supported by actin bundles appear to contact the membrane first before BCR microclusters form. After establishing the initial contact, F-actin rapidly accumulates at the B-cell contact zone, particularly at the outer edge of this region, and generates dynamic filopodia and lamellipodia, which undergo rapid cycles of extension and contraction (38, 56). These actin-rich protrusions lead to B-cell spreading on antigen-presenting membranes. New BCR microclusters often form at the tip of extended filopodia and lamellipodia, and the contraction of these structures brings these microclusters inward (38, 56) (Fig. 1C). While polymerizing at BCR microclusters, actin undergoes retrograde flow at the cell periphery, concurrent with the centripetal movement of BCR clusters (57). The area of the B-cell contact zone continues to increase as actin accumulates over a period of several minutes post antigen stimulation, with the timescales depending on the nature of the stimulating antigen. Following this period of actin accumulation, the level of F-actin in the B-cell contact zone starts decreasing. While the actin cytoskeleton at the outer edge of the B-cell contact zone is maintained, actin disappears from the vicinity of merging BCR clusters and the BCR central cluster. Consistent with this spatial organization of actin, *de novo* actin polymerization occurs at the outer edge but not in the middle of the BCR central cluster. Concomitantly, B-cell membrane dynamics reduce and filopodia and lamellipodia contract rather than extend, which result in the contraction of the B-cell contact zone (38, 56) (Fig. 1D). These observations suggest that actin switches from a polymerization to a depolymerization phase, which mediates the transition from B-cell spreading to B-cell contraction. However, the mechanisms that mediate the contraction phase of B cells and the associated actin remodeling remain elusive.

Induction of actin remodeling by BCR signaling

Antigen-induced actin remodeling is dependent on BCR signaling. A number of proximal signaling molecules, including CD19, PLC 2, Vav, Btk, and Rap, have been shown to be involved in actin-mediated B-cell spreading and BCR clustering (34, 35, 58), but their exact roles in actin remodeling have not been fully examined. Using mouse models, we have demonstrated that the stimulatory kinase Btk and the inhibitory phosphatase SHIP-1 are essential for controlling actin remodeling in response to antigenic stimulation. Btk deficiency, which results from a point mutation in the Btk PH (pleckstrin homology) domain that blocks Btk activation (59), causes a dramatic reduction of *de novo* actin polymerization. Btk-deficient B cells not only fail to spread but also are unable to establish stable interactions with antigen-presenting membranes, sustain BCR microclusters, and form central clusters (56). The involvement of Btk in the formation of BCR signalosomes is not surprising, since other members of the Tec family kinases, such as Itk, are well known to be important for the formation of T-cell synapses (60, 61). The role of Btk in promoting actin polymerization demonstrates that activating actin assembly is one of the mechanisms by which Btk regulates BCR signaling besides directly phosphorylating its downstream signaling molecules. We also found that the inhibitory effects of the Btk PH domain mutation on actin-mediated events were much stronger than those resulting from gene knockout of individual actin regulators, such as Wiskott-Aldrich syndrome protein (WASP) (56, 62), which suggests that Btk may be involved in the activation of multiple actin regulators. Btk activates several signaling pathways upstream of the actin cytoskeleton, including the small GTPases Cdc42 and Rac, by activating the guanine nucleotide exchange factor Vav (63, 64) and the production of PtdIns by activating phosphatidylinositol-4-phosphate 5-kinase (PIP5K) (65). As a major downstream signaling molecule of CD19, the function of Btk in the activation of actin remodeling provides a mechanistic explanation for how the stimulatory co-receptor of the BCR, CD19, enhances antigen-induced B-cell spreading and BCR clustering (66, 67).

In contrast to Btk, SHIP-1 inhibits antigen-induced actin polymerization and F-actin accumulation at BCR activation sites. In B cells from B-cell-specific SHIP-1 knockout mice, F-actin accumulation levels are much higher than that in wildtype B cells, and F-actin accumulation persists around BCR clusters unlike in wildtype cells. Consequently, BCR microclusters fail to move centripetally, grow, and merge into a central cluster. Furthermore, SHIP knockout B cells spread to a greater extent than wildtype B cells and undergo a delayed contraction (56). SHIP can inhibit the activation of multiple signaling molecules downstream of the BCR, including Btk, PLC 2, and Akt, by converting their docking sites at the plasma membrane, PtdIn(3,4,5)P₃, into PtdIn(3,4)P₂ (27, 68). However, inhibiting Btk appears to be a primary mechanism for SHIP-mediated inhibition of actin polymerization and B-cell spreading, since treatment with the Btk inhibitor LFM A-13 inhibits the actin phenotypes observed in SHIP1-knockout B cells in a concentration-dependent manner (56). Upon BCR cross-linking by antigen, Btk activation induces actin polymerization and B-cell spreading. This is followed by the activation of SHIP (1, 26), which inhibits actin polymerization and B-cell spreading. When B cells encounter immune complexes, the colligation of the BCR with Fc RIIB triggers the activation of SHIP, which inhibits actin reorganization. This provides a mechanistic explanation for the inhibitory effect of BCR-Fc RIIB colligation on B-cell spreading and BCR clustering (40). Therefore, the balance of Btk and SHIP activation controls the dynamics and nature of actin remodeling, enabling a tightly controlled coordination of reorganization of actin and BCRs at the B-cell surface during signal transduction.

Actin-mediated positive feedback to BCR signaling

Actin remodeling has been demonstrated to be critical for BCR signaling activation. The primary target of the actin-mediated regulation is the BCR signalosome, including the formation, growth, and coalescence of BCR microclusters, and the recruitment of signaling molecules to BCR clusters. It was thought that antigens with multiple identical epitopes should be able to overcome the actin-mediated mobility barrier and physically bring multiple receptors together. However, this does not appear to be the case, since neither multivalent soluble nor membrane-associated antigen induces significant BCR clustering and signaling when the actin network is stabilized by jasplakinolide (38). On the contrary, upon dissolution of the actin mobility barrier by application of the actin depolymerization reagent latrunculin, BCRs are able to induce signaling and form microclusters in the absence of antigen (38, 54). Therefore, the transient disassembly and disassociation of cortical actin is essential for the formation of BCR microclusters. While BCR clusters formed by antigen-receptor interactions likely generate signals for actin disassembly, actin disassembly can release both antigen-bound and unbound BCRs from actin imposed mobility barriers, increasing their probability of interacting with one another. Our recent data show that B cells interacting with antigen immobilized on glass form mobile signaling active BCR clusters (Ketchum *et al.*, unpublished data), which supports the notion that antigen-induced actin reorganization also promotes the clustering of unbound BCRs.

While disassembly of cortical actin facilitates the formation of the initial BCR microclusters, polarized and organized actin reassembly is crucial for amplifying BCR clustering and signaling processes. This is demonstrated by much slower kinetics and lower levels of signaling and clustering of BCRs induced by the actin depolymerization reagent latrunculin as compared to those induced by antigen (38, 42). One mechanism by which actin remodeling amplifies BCR signaling is by mediating B-cell spreading, which maximizes the interaction of B cells with antigen-presenting surfaces (41). Actin dynamics drive filopodia and lamellipodia protrusions outwards and enable surface BCRs to bind antigen in the vicinity of the cell. The dynamic protrusions and retractions of filopodia and lamellipodia enable B cells to gather more antigen and antigen-bound BCRs. Membrane-associated

antigen can be continuously replenished under extending filopodia and lamellipodia by antigen lateral diffusion from areas external to the B-cell edge, further increasing the number of BCRs that engage antigen and get incorporated into clusters. The second mechanism by which actin remodeling amplifies BCR clustering is by mediating directional movement of BCRs and BCR microclusters (57). Actin polymerization at BCR microclusters and retrograde flow of actin at the cell periphery may drive BCRs and BCR microclusters into a center region, enhancing the physical interaction between BCR microclusters and allowing them to coalesce into larger clusters. This is supported by our recent observations that treating B cells with latrunculin after the formation of initial BCR microclusters stops the movement of the clusters and inhibits their growth (Ketchum *et al.*, unpublished data). While the directional flow of the actin cytoskeleton has mostly been studied in cells stimulated by membrane-associated antigen, our studies suggest that such actin flow may also occur in B cells stimulated by soluble antigen (38). In fact, polarized actin reassembly and directional actin flow may be more critical for optimal BCR activation in B cells interacting with soluble rather than membrane-associated or immobilized antigen, as in the absence of cell spreading, is the only actin-dependent mechanism that can facilitate the formation of BCR clusters. How B cells stimulated by soluble antigen break symmetry to identify a pole for directional actin flow and BCR cluster formation without a polarized stimulatory cue remains an open question.

Similar to the roles of actin dynamics in facilitating BCR clustering, actin disassembly and polarized reassembly potentially facilitate the interaction of BCRs and BCR clusters with signaling molecules anchored to or associated with the plasma membrane and lipid rafts. A recent study by Mattila *et al.* (42) shows that latrunculin-induced BCR signaling is dependent on the interaction of the BCR with CD19 in a complex with CD21 and CD81. In the absence of latrunculin, CD81 prevents CD19 from associating with BCRs probably by linking the CD19/CD21/CD81 complex to the actin cytoskeleton. These results predict that the actin-mediated diffusion barrier along with associated membrane proteins compartmentalizes these two proteins into separate membrane domains and that transient disassembly and disassociation of the cortical actin from the plasma membrane free both the BCR and CD19 complexes from their respective membrane compartments. In addition, directional movement of actin potentially helps to concentrate actin-associated, transmembrane, and/or lipid raft-associated signaling molecules, like CD19/CD23/CD81 complexes, to BCR clusters. While these hypotheses remain to be tested, they are supported by data accumulated in the last few decades. For examples, lipid rafts, labeled by cholera toxin, have been shown to co-cluster with surface BCRs (69, 70). The cortical actin network can compartmentalize lipid rafts by associating with lipid raft-resident membrane anchor proteins through ezrin (31, 55). Upon colligated with the BCR by complement-opsonized antigen, CD81 has been shown to enhance signaling by stabilizing the BCR and the CD19/CD21/CD81 complex in lipid rafts (71, 72). Furthermore, cytoplasmic signaling molecules can be recruited to BCR clusters by indirectly interacting with the actin cytoskeleton through actin-binding adapter proteins, such as the interaction of the signaling adapter protein Grb2 with actin through WASP (73).

The coupling of actin with lipid rafts, membrane proteins, and actin adapter proteins potentially enables antigen-induced actin remodeling to amplify BCR signaling by the dissolution of barriers that interfere with BCR-BCR and BCR-signaling molecule interactions and by actively enhancing the self clustering and co-clustering of BCRs with signaling molecules. All of these mechanisms likely work cooperatively to provide a positive feedback loop for BCR signaling.

Actin-mediated negative regulation of BCR signaling

While the cellular and molecular events for initiating and amplifying BCR signaling have been extensively studied, the mechanisms that negatively regulate the threshold for BCR signaling initiation and that downregulate BCR signaling after initiation are not well understood. However, it is evident that negative regulation mechanisms are crucial for preventing autoreactive B cells from activation, expansion, and differentiation, thereby generating and maintaining B-cell self-tolerance. A role for actin remodeling in the negative regulation of B-cell activation is suggested by reports that a significant percentage of Wiskott-Aldrich syndrome (WAS) patients have autoimmune diseases. WAS is the result of a genetic deficiency in the actin nucleation-promoting factor WASP (74–78). The significance of B cells in autoimmunity in WAS patients has been demonstrated by mouse models with B-cell-specific deletion of the *wasp* gene. These mice have increased numbers of germinal center B cells in the spleen and increased levels of autoantibodies in their serum (62, 79). Additional support for actin-mediated negative regulation comes from the finding that the well known inhibitory co-receptor of the BCR, Fc γ RIIB, which activates SHIP-1 upon colligated with BCRs by immune complexes (26), inhibits BCR clustering and B-cell spreading (40), two actin-mediated events necessary for initiation and amplification of BCR signaling. These data suggest that actin remodeling can contribute to negative regulation of BCR signaling by modulating B-cell morphology and BCR organization at the cell surface, similar to the mechanisms by which actin facilitates signaling activation.

Regulation of signaling threshold by actin mobility barrier

During signaling activation, actin disassembly is required for releasing BCR nano-clusters and signaling molecules, such as CD19/CD21/CD81 complexes, from actin-mediated compartmentalization, enabling them to interact with each other (31, 38, 42). These results suggest that the rigidity and organization of the cortical actin network not only control tonic signaling but also potentially affect the threshold for initiation of signaling. A rigid and stable actin network may impose higher energy barriers for antigen-induced interactions between BCRs and BCR nano-clusters to initiate the earliest signaling, as compared to a flexible and labile network, thereby negatively regulating BCR activation. The flexibility and stability of the cortical actin cytoskeleton in unstimulated B cells are probably controlled by tonic signaling. The level of tonic signaling may determine the activity levels of actin anchor proteins and actin regulators that control actin dynamics. For example, a higher basal level of phosphorylated ezrin will strengthen the association of cortical actin with the plasma membrane and actin-generated mobility barriers against BCR cluster formation, thereby suppressing receptor activation.

Negative feedback loop by actin reorganization

In addition to regulating the threshold for signaling initiation, our recent studies demonstrate that actin remodeling is important for the down regulation of BCR signaling subsequent to initiation. A decrease in actin polymerization and accumulation restrains the outward movement of the cell membrane, which may facilitate the contraction of the B-cell peripheral membrane to decrease the contact zone. B-cell contraction facilitates the merger of BCR microclusters into a central cluster. Our studies have demonstrated two different phases of signaling during the maturation of BCR clusters. Nascent BCR microclusters interact with lipid rafts (22, 33), recruit the key early signaling molecule Syk (18), and increase tyrosine, Btk, and Akt phosphorylation activities (56) as they grow. However, upon reaching a certain size, the interaction of BCR clusters with lipid rafts, Syk recruitment, and phosphorylation activities decrease as these microclusters merge into a central cluster (18, 22, 33, 56). This suggests that nascent BCR microclusters are signaling active, while the process of central cluster formation causes BCR clusters to transition from a signaling

activation to attenuation phase. The timing of the transition between these two signaling phases is concomitant with the timing of the transition of B-cell morphology from a spreading to contraction phase on antigen-presenting membrane, and the transition of F-actin levels from increasing to decreasing at the activation surface (38, 56). Inhibition or delays in the transitions of B-cell morphology and actin dynamics appear to result in persistent BCR microclusters, a failure in the formation of BCR central clusters, and prolonged high levels of signaling. Our recent studies show that the transitions of actin dynamics, B-cell morphology, BCR clustering, and signaling can be inhibited by a gene deletion of the negative regulatory signaling molecule SHIP-1 (56), the actin regulator N-WASP, or the actin adapter protein Abp1 (Liu *et al.* and Fallen *et al.*, unpublished data). Moreover, autoantibody levels are significantly elevated in mouse models where the gene of SHIP-1, N-WASP, or Abp1 is either specifically deleted in B cells or in all cells (80, Liu *et al.* and Fallen *et al.*, unpublished data). These results confirm the essential role for actin in the downregulation of BCR signaling. In the second phase of BCR clustering, the retrograde flow of actin potentially directs the movement of BCR microclusters, enabling them to merge with each other, and actin-mediated B-cell contraction provides an additional force for the merger of BCR microclusters into the central cluster. Thus, the transition in the function of actin remodeling from driving B-cell spreading to contraction and from facilitating BCR microcluster formation to coalescence leads to signaling downregulation of the BCR.

How the merger of BCR microclusters into the central cluster inhibits the signaling activity of BCRs is still unknown. Physical forces exerted by mobile actin filaments, contracting membrane and neighboring BCRs may again cause conformational changes of the BCR and/or alter the interactions of BCRs with other BCRs and with signaling molecules within a cluster. Thus, it is possible that the biochemical nature of BCR-BCR interactions and BCR-signaling molecule interactions are not the same for BCRs in clusters of different sizes. Logically, BCR-BCR interactions in the central cluster probably share some of the molecular interaction properties of BCRs in nano-clusters of unstimulated B cells (43, 44). While the differences in the biochemical nature of BCR-BCR interactions in different stages of BCR clustering remain to be elucidated, actin remodeling dynamics are likely to influence the interactions. Besides modulating BCR-BCR and BCR-signaling molecule interactions, another possible mechanism by which actin promotes signaling attenuation is by facilitating the recruitment of inhibitory signaling molecules to BCR clusters via actin regulators/adapters. We recently found that the actin nucleation promoting factor N-WASP and the actin adapter protein Abp1 are both recruited to BCR activation sites during signaling attenuation (Liu *et al.* and Fallen *et al.*, unpublished data). Abp1 has been shown to interact with the negative regulatory signaling molecule HPK1 and recruit HPK1 to T-cell synapses (81, 82). In B cells, HPK1 negatively regulates BCR signaling by inducing the phosphorylation and subsequent ubiquitination of BLNK (83). Additionally, N-WASP has been suggested to be responsible for localizing SHIP to actin tails of intracellular poxviruses (84). These findings support the idea that actin regulators that are involved in B-cell contraction and actin remodeling at the signaling attenuation stage may also facilitate the recruitment of inhibitory signaling molecules to BCR clusters, which amplifies signaling downregulation.

Binding of antigen to the BCR also induces receptor endocytosis, which enables antigen uptake into the endosomal system where antigen is processed for presentation (47, 85). Endocytosis removes BCRs and BCR clusters from the cell surfaces at the contact zone. Some of the associated signaling molecules may be endocytosed with the BCR, and BCRs in endosomes have been shown to be associated with cytoplasmic signaling molecules, such as JNK (86). However, signaling through cell surface BCRs is terminated upon receptor endocytosis, serving to downregulate BCR signaling. We have previously demonstrated that

BCR endocytosis requires actin. In particular, BCR endocytosis induced by antigen cross-linking but not constitutive endocytosis of the BCR is dependent on actin remodeling (87). BCRs undergo endocytosis at a low rate in unstimulated cells, but cross-linking by antigen, which induces actin remodeling, dramatically increases receptor endocytosis (47, 85). The primary role of actin was found to be at the membrane fission step, specifically detaching BCR-containing budding vesicles from the plasma membrane (87) but not at the step of recruiting proteins involved in endocytosis (87). Natkanski *et al.* (88) recently have reported that the actin motor non-muscle myosin II is required for pulling antigen from presenting membrane for endocytosis. Further support for the role of actin-dependent BCR endocytosis in down regulation of signaling comes from our finding that inhibiting endocytosis by latrunculin treatment enhances or prolongs BCR signaling (38, 87).

The data discussed thus far have provided strong evidence for an essential role of actin in the negative regulation of BCR signaling. Actin-dependent mechanisms can raise the BCR signaling threshold by increasing the rigidity and stability of the cortical actin network. Actin can facilitate the downregulation of BCR signaling by promoting B-cell contraction, the coalescence of BCR microclusters into a central cluster, the recruitment of negative regulatory signaling molecules to BCR clusters, and receptor endocytosis.

Actin regulators in positive and negative feedback to BCR signaling

The coordination between the actin cytoskeleton and the BCR involves a large number of actin regulators and actin adapter proteins. While the functions of these proteins are diverse, they share some common properties. Most bind to actin, G- or F-actin, and their activities are regulated by BCR signaling, commonly by phosphorylation and interactions with PtdIns and/or calcium. Here we discuss a few specific regulators that we and others have recently examined for their functions in actin-mediated positive and negative feedback to BCR signaling, including cofilin, WASP/N-WASP, and Abp1.

Cofilin in signaling initiation

Cofilin has recently been suggested to be critical for the early and transient actin disassembly during BCR activation, since neutralization of cofilin by antibodies inhibits antigen-induced actin severing and depolymerization (30). Cofilin belongs to a family of actin-depolymerizing factors and severs and depolymerizes actin filaments by distorting the helical twist of filaments and by promoting the disassociation of ADP-bound G-actin from the minus end of filaments (89, 90). F-actin binding of cofilin can be turned off and on by phosphorylation and dephosphorylation at its serine 3, respectively (89, 90). Based on studies in other cell types, the LIM domain kinase is found to be responsible for cofilin phosphorylation and slingshot phosphatase (SSH) for cofilin dephosphorylation (91, 92). In unstimulated cells, SSH is sequestered away from cofilin by the protein 14-3-3 (92). In B cells, stimulation by either soluble or membrane-associated antigen induces dephosphorylation of cofilin, allowing it to bind to F-actin and activating its severing and depolymerization functions (30, 38). SSH-mediated dephosphorylation of cofilin is dependent on the activation of Rap1 (30). We find that dephosphorylated cofilin is preferentially recruited to the vicinity of BCR microclusters and central clusters; however, it is not detectable by total internal reflection fluorescence microscopy (38), which visualizes 100~200 nm into the plasma membrane. This indicates that the recruited cofilin is not localized at the plasma membrane proximal region of the actin cytoskeleton but rather severs and depolymerizes F-actin from the cytoplasmic side of the cortical actin network. Cofilin-mediated actin severing and depolymerization may provide G-actin for actin polymerization at the leading edge of the spreading membrane and near moving BCR clusters. Together these activities create actin flows for B-cell spreading and the movement of BCR clusters. Indeed, blocking cofilin dephosphorylation by overexpression of a catalytically inactive

SSH or a dominant negative form of Rap1 or RapGAPII reduces B-cell spreading and BCR clustering (30), indicating cofilin functions in multiple steps of BCR clustering and signaling. Thus, cofilin, upon dephosphorylation by BCR signaling, contributes to actin disassembly and reassembly by severing and depolymerizing F-actin, providing positive feedback to BCR signaling.

WASP and N-WASP in signaling amplification and attenuation

WASP is one of the most well-studied actin regulators in immune cells, as mutations of the *wasp* gene cause complex immune disorders. WAS patients exhibit immune deficiency as well as high incidences of autoimmune diseases and lymphoid cancers (74, 77, 78). Its significance in B-cell functions is demonstrated by increased levels of autoantibody in mice with a B-cell-specific *wasp* gene deletion (62, 79). These data clearly point to critical functions for WASP-mediated actin remodeling in both positive and negative regulation of BCR signaling. WASP belongs to a family of actin nucleation promoting factors that includes N-WASP and WAVE (93, 94). While expressed in hematopoietic cells exclusively, WASP shares many properties with the other members of the family, inducing their function in the activation of actin polymerization at existing actin filaments, their multiple protein-protein interacting domain structures, and their activation mechanisms (95, 96). The activation mechanism of WASP family proteins has been well defined (75, 97). WASP exists in a closed autoinhibitory conformation which is opened and activated by binding to GTP-Cdc42 or Rac and PtdIn(4,5)P₂ via its GTPase-binding and PH domains, respectively (98). The open conformation is further stabilized by phosphorylation at the conserved tyrosine and serine sites (99, 100).

In B cells, we have demonstrated that WASP activation is induced by the Tec family kinase Btk. Btk activates WASP by inducing the phosphorylation of Vav that is the guanine nucleotide exchange factor for Cdc42 and Rac, the phosphorylation of PIP5K that produces PtdIn(4,5)P₂, and the phosphorylation of WASP (64). Conversely, the inhibitory phosphatase SHIP-1 suppresses WASP activation by inhibiting Btk activation (56). Active WASP preferentially localizes at BCR clusters and the leading edge of the spreading B cell membrane (38, 56, 64). WASP knockout causes significant decreases in antigen-induced actin polymerization as well as actin accumulation at BCR activation sites (56). These results demonstrate that WASP is a major contributor to actin reassembly during BCR activation. Consistent with the reduced actin reassembly, B-cell spreading, BCR clustering, as well as BCR internalization are reduced in WASP-deficient mouse B cells (56, 62), suggesting that WASP can contribute to both positive and negative feedback in BCR signaling. Reduction in BCR endocytosis can delay BCR signaling attenuation, providing a partial explanation for increases in calcium flux and enhanced proliferation response of WASP-deficient B cells (62, 79). However, the reductions caused by WASP deficiency in B-cell spreading, BCR clustering, and BCR internalization are surprisingly modest (56, 62), suggesting compensatory roles for other members of the WASP family.

We have recently pursued studies on the role of N-WASP, a close homologue of WASP in the family, in BCR signaling, using mice with B-cell-specific *n-wasp* gene knockout and *wasp/n-wasp* double gene knockout generated by Westerberg *et al.* (101). Our results show that B cells from double knockout mice exhibit much more dramatic decreases in actin accumulation at BCR activation sites, B-cell spreading, and BCR clustering than those in B cells from WASP knockout mice, as well as a strong inhibition of BCR signaling (Liu *et al.*, unpublished data). This demonstrates that both WASP and N-WASP are required for optimal BCR signaling activation. The finding that the double knockout leads to a nearly complete inhibition of these cellular events suggests that the other member of the WASP family, WAVE, may not have a significant role in these processes. Furthermore, neither WASP nor double knockout has any significant impact on B cell maturation in the bone

marrow (101), suggesting that the activation mechanisms used by the pre-BCR or the BCR in immature B cells may be different from those used by the BCR in mature B cells.

In contrast to what was observed in WASP knockout B cells, B-cell spreading is enhanced while B-cell contraction is delayed and the formation of BCR central clusters and BCR internalization are blocked in B cells from B cell-specific N-WASP knockout mice. Consequently, BCR microclusters persist, which is concurrent with enhanced and prolonged signaling (Liu *et al.*, unpublished data). This indicates that in addition to compensating for WASP, N-WASP has a unique role in downregulation of BCR signaling by promoting B-cell contraction, BCR central cluster formation, and BCR endocytosis, three actin-dependent negative regulatory mechanisms. These data together suggest that both WASP and N-WASP are required for optimal activation and attenuation of BCR signaling and that they have both compensatory and unique functions in the signaling process.

Abp1 in signaling attenuation

Abp1 is an actin adapter molecule that has been implicated in both BCR signaling and internalization. This protein has multiple protein-protein interaction domains, including an SH3 domains in its C-terminus, F-actin binding domains in its N-terminus, and a proline-rich domain (PRD) in the middle (102, 103). While the yeast homologue of Abp1 is capable of activating actin polymerization like WASP, the mammalian Abp1 loses this function (102, 104). Instead directly activating actin polymerization, Abp1 regulates actin dynamics by forming a complex with N-WASP or WASP family proteins (105, 106). We and others have revealed that BCR activation induces the phosphorylation of Abp1 at two tyrosines in its PRD domain and the recruitment of Abp1 to the B-cell surface. Abp1 recruitment depends on both its phosphorylation state and actin reassembly at the B-cell surface (107). Its ability to simultaneously bind F-actin and PRD or SH3 domain-containing proteins enables Abp1 to bring signaling molecules to BCR clusters and/or to connect F-actin to signaling molecule-associated BCR clusters. For example, Abp1 has been shown to be responsible for recruiting the negative signaling regulator HPK1 to the immunological synapse in T cells (81, 82). We have recently confirmed a similar function for Abp1 in B cells (Seeley-Fallen *et al.*, unpublished data). HPK-1, when recruited to BCR signalosomes, can inhibit the activation of the key signaling adaptor BLNK by phosphorylating its threonine residues and inducing its ubiquitination (83). Furthermore, we find that Abp1 is required for efficient BCR endocytosis. Its function in BCR endocytosis is dependent on the interaction of its SH3 domain with the PRD domain of dynamin, a GTPase that is responsible for constricting the necks of budding vesicles (107). This indicates that Abp1 can bring F-actin to dynamin-constricted necks of BCR-containing budding vesicles, where actin may generate a force to facilitate the scission and detachment of budding vesicles from the plasma membrane. The recruitment of Abp1 in response to BCR signaling and the interaction of Abp1 with dynamin provide a mechanistic explanation for why actin reorganization is specifically required for the membrane fission step in BCR internalization of antigen. Therefore, Abp1 is responsible for linking BCR signaling and actin remodeling with BCR endocytosis. The abilities of Abp1 to recruit HPK1 to BCR signalosomes and to facilitate BCR endocytosis predict its role in the downregulation of BCR signaling.

Concluding remarks

BCR signaling induced actin remodeling provides vital feedback to both BCR signaling activation and downregulation. Actin remodeling both upregulates and downregulates BCR signaling via several actin regulators and adaptors that are activated at different stages of BCR signal transduction. Activation of different actin regulators generates distinct stages of actin remodeling and reorganizations. The early stage of actin remodeling amplifies signaling activation by driving B-cell spreading, promoting BCR clustering, and recruiting

stimulatory signaling molecules to BCR clusters. The later stage of actin reorganization enhances signaling downregulation by mediating B-cell contraction, promoting the coalescence of BCR microclusters into a central cluster, facilitating endocytosis, and recruiting inhibitory signaling molecules to BCR clusters. From recent studies, we have learned a great deal about the identity of actin regulators and adaptors that are involved in actin remodeling during signaling initiation and activation and how these actin associated proteins translate BCR signaling into dynamic reorganization of actin and surface BCRs. However, the molecular mechanisms by which actin makes reorganizational transition to drive B-cell morphological changes from spreading to contraction and BCR signaling from activation to down regulation remain elusive. Our recent unpublished studies suggest that the actin contractile motor non-muscle myosin II, the ubiquitous homolog of WASP N-WASP, and the actin adapter protein Abp1 are specifically involved in B-cell contraction, the formation of BCR central clusters, BCR signaling downregulation, and the maintenance of B-cell tolerance. Based on what have been discussed in this review, we hypothesize that these three actin regulators (myosin II, N-WASP, and Abp1) coordinate with each other to inhibit actin dynamics at the B-cell surface, to enable B cells to contract their membrane, and to facilitate BCR central cluster formation and endocytosis. Furthermore, these actin regulators have two-way regulatory relationships with inhibitory signaling pathways of B cells. Delineation of the molecular details of actin-mediated signaling downregulation will help us to understand the mechanisms underlying B-cell peripheral tolerance and to discover new targets for suppressing B-cell-mediated autoimmunity.

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Fig. 1. Coordination of actin remodeling with dynamic reorganization of BCRs at the B-cell surface

(A) Upon antigen binding to the BCR, the cortical actin network undergoes rapid and transient depolymerization and detachment from the plasma membrane. This releases BCRs and BCR nano-clusters from mobility barriers and enables them to interact with each other. (B) Following actin disassembly, actin is reassembled at the activation surface. *De novo* actin polymerization is activated at BCR clusters and the membrane at the outer edge of the contact surface. (C) Actin reassembly enables B cells to spread and expand the contact area with antigen presenting surface. The extension of filopodia and lamellipodia enables the cell to gather more antigen for BCRs to bind, and their contraction brings BCR-antigen inwards to form larger microclusters. BCRs in microclusters are signaling active. (D) Actin-mediated B-cell contraction facilitates the merger of BCR microclusters into a central cluster. The BCR central cluster is surrounded by *de novo* actin polymerization sites and F-actin, and exhibits reduced signaling activity. Shown are images generated by Song's laboratory using interference reflection microscopy (IRM) and total internal reflection fluorescence microscopy (TIRFM). Scale bar, 2.5 μm .