Cytoskeletal control of B cell responses to antigens

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Preface

The actin cytoskeleton is essential for cell mechanics and has increasingly been implicated in the regulation of cell signalling. In B cells, the actin cytoskeleton couples extensively to B cell receptor (BCR) signalling pathways and its defects can either enhance or suppress B cell activation. Recent insights from single-cell imaging and biophysical techniques suggest that actin orchestrates BCR signalling at the plasma membrane through effects on protein diffusion, and that it regulates antigen discrimination through the biomechanics of immune synapses. These mechanical functions also play a role in the adaptation of B cell subsets to specialized tasks during antibody responses.

Introduction

The importance of the actin cytoskeleton for immunity was first recognized with the identification of immunodeficiencies caused by defects in actin polymerization[1]. Although a complete loss of actin polymerization is not compatible with life, immune cells express a set of unique actin regulators, which, when mutated, lead to altered dynamics of the actin cytoskeleton and cause disease. The best known immunodeficiency of this type is the **Wiskott-Aldrich syndrome**, caused by mutations in *WAS*, a gene encoding a key regulator of actin in hematopoietic cells[2,3]. Loss of WAS in both humans and in mouse models reduces the numbers and function of all hematopoietic cells, including platelets, myeloid cells and lymphocytes, and causes bleeding disorders as well as susceptibility to infections and cancer[4]. The fact that even a partial loss of cytoskeletal integrity causes broad and severe defects highlights the importance of actin for essential immune cell functions, such as cell migration, cell adhesion, proliferation and the engulfment of pathogens.

The cellular defects underlying Wiskott-Aldrich syndrome also revealed subtle, yet important effects of the actin cytoskeleton on signalling of immune cells, and in particular, B cells. For instance, the majority of patients with Wiskott-Aldrich syndrome suffer from antibody-mediated autoimmunity, which is largely caused by B cell hyperactivity[5-8]. These symptoms suggest that cytoskeleton dynamics are closely interwoven with both positive and negative regulation of B cell activation.

Recent advances in cellular imaging (Box 1) and biophysical techniques (Box 2) have made it possible to observe the detailed organization of actin filaments in B cells and begin to unravel the mechanisms by which cytoskeletal defects affect individual signalling pathways[9]. The emerging picture is that the cytoskeleton provides structural and energy-dependent regulation of biochemical reactions in the cell. On the sub-second timescale, which is shorter than the lifetime of individual actin filaments, the cytoskeleton modulates enzymatic reactions by altering the diffusion of membrane proteins and their availability for binding with their partners. This modulation impacts both the basal, resting state of the B cell, as well as antigen-induced signalling reactions at the plasma membrane. On a slightly longer time scale, over several seconds, rearrangement of the cytoskeleton clusters proteins, separates them or induces changes in their conformation and activity. This rearrangement has particular relevance for plasma membrane organization, cell

movement and transduction of signals and material in antigen-induced contacts of B cell with other immune cells. On even longer timescales, over several minutes, the cytoskeleton transports molecules and organelles into different regions or compartments of the cell. This segregation isolates biochemical reactions, which impacts on B cell polarity and decisions points that determine whether the cell proliferates or differentiates into effector cells.

In this review, I provide a current overview of the architecture of the cortical actin cytoskeleton and describe how its dynamics are regulated by B cell receptor (BCR) signaling pathways. I then discuss the biochemical mechanisms by which actin cytoskeleton modulates BCR signaling at the plasma membrane as well as the role of actin in the biomechanics of B cell immune synapses and antigen endocytosis. Finally, I highlight the relevance of the cytoskeleton for specialized functions of B cell subsets with a particular focus on germinal center (GC) B cells.

[H1] Structure of the cortical cytoskeleton

The mechanical functions of the actin cytoskeleton require reversible polymerization of actin monomers into thin, helical actin filaments. The polymerization is tightly controlled by cellular factors, the activity of which determine the density, orientation, structure, length and eventually function of the filaments. Two types of nucleation factors are responsible for the generation of new actin filaments in all cells, the ARP2/3 complex and formins (Fig. 1)[10]. The ARP2/3 complex binds to the side of an existing actin filament and nucleates a daughter filament branching at an oblique angle. In contrast, formins create filaments directly from actin monomers. The nucleated filaments can elongate at their barbed, but not pointed end, giving the filaments directionality. The length of the filaments depends on the activity of elongation factors, which processively add actin monomers to the growing filament, and capping proteins, which bind to the barbed end and prevent further growth. Filaments are then joined into networks by actin crosslinkers of various lengths. Actin is thus a highly versatile polymer that can create a range of structures adapted for different functions.

The area underneath the plasma membrane, the cortex, is a particularly prominent site of actin dynamics, supporting essential mechanical functions of the cell (Fig 1)[11].

The cortical cytoskeleton is generated by both ARP2/3 and formin activity at the plasma membrane, creating meshwork of short filaments[12-14]. The meshwork is approximately 100 nm thick, has a pore size of about 50 nm and attaches to the plasma membrane via ezrin, moesin and class I myosins[15-17]. Actin polymerization at the plasma membrane is constantly balanced by actin destruction at the cytosolic side of the cortex by actin severing proteins, **cofilin and destrin**[14,18,19], and by the debranching activity of **coronins**[20]. The cortical cytoskeleton is thus highly dynamic, turning over in under a minute[18]. These dynamics provide structural rigidity of the plasma membrane at short time scales, yet can also push or pull on the membrane to rapidly change cell shape and generate movement.

Pushing the membrane outward is mediated directly by bursts of actin polymerization against the plasma membrane[21]. Polymerization of thin, long bundles of filaments created jointly by ARP2/3, formins and crosslinker activity creates **filopodia**, while generation of short, branched filaments by ARP2/3 and capping proteins over extended areas creates **lamellipodia**[22]. Pushing by small foci of branched actin can also work together with membrane-bending proteins to deform the membrane inwards for endocytosis[23].

In addition to pushing on the membrane, the cortex also generates tension[11]. The tension is produced by class II myosins, represented in lymphocytes by myosin IIa. Myosin IIa is a motor protein that assembles into 300 nm-long minifilaments containing several mechanically active heads on each side[24,25]. The myosin heads bind to actin and exert power strokes in the direction of the barbed end, sliding actin filaments towards the center of the myosin minifilament, thereby contracting the network. Although linear actin filaments are the best substrates for myosin contractility, myosin also acts on the random meshwork of actin in the cortex. If myosin slides actin filaments that are structurally incompatible with contraction, the filaments buckle and break[26]. Therefore, myosin not only contracts, but also remodels the cortex[27,28]. In addition, myosin can induce flows of free actin filaments into aster-like formations[29,30], and potentially transport proteins via co-assembly with the cargo-binding protein MYO18A[31].

[H1] BCR signalling to the cytoskeleton

While the core elements of the actin cytoskeleton are present in all cells, their regulation by upstream pathways is cell type specific and is responsible for specialised functions, including the functions of immune cells. In B cells, the cytoskeleton is a major target of BCR signalling pathways. Even in resting B cells, the structure of the cortical cytoskeleton depends on the presence of BCR signalling components, suggesting that there is a constant influence of tonic BCR signalling on actin dynamics[32]. Antigen binding to the BCR triggers dramatic remodelling of the cortical cytoskeleton that induces cell spreading, formation of the immune synapse, and gathering of antigen for endocytosis. Biochemical and genetic studies show that this remodelling is mediated through all the major pathways of actin dynamics, including nucleation of branched and linear actin filaments, filament severing, and myosin contractility (Fig. 2).

[H2] The WAS family proteins. The first major pathway connecting the BCR with actin polymerization involves activation of the ARP2/3 complex by WAS proteins[3]. WAS proteins are regulators of actin polymerization in lamellipodia, filopodia and in endocytic pits[33,34]. B cells express both members of the family, the ubiquitous WASL and the hematopoietic WAS. Loss of WAS in mouse B cells leads to reduction of filamentous actin in resting B cells and impairs the development, adhesion and migration of B cells in the periphery (Table 1)[8,34]. In response to antigen, WAS-deficient B cells have reduced BCR internalization and increased signalling and antibody production[7,35], resembling the B cell phenotype in patients with Wiskott-Aldrich syndrome. Loss of WASL also increases B cell responsiveness to antigens[35]. However, simultaneous deletion of both WAS and WASL abrogates antigen-induced B cell responses, suggesting that while these proteins have non-redundant negative functions, they also act redundantly to promote B cell activation[36,37]. Surprisingly, while the absence of WAS reduces the amount of cortical actin and impairs the structure of immune synapses[7,34,35], the absence of WASL increases the amount of actin in B cell synapses[35], suggesting that their role in actin polymerization is also distinct.

BCR signalling to WAS family proteins occurs through multiple mechanisms. Biochemical studies indicate that activation of WAS requires binding by either the GTPase CDC42[38] or the NCK adaptors, NCK1 and NCK2 (Fig. 2)[39]. WASL is

activated either by NCK proteins or by GTPases of the RAC family, RAC1 and RAC2[40]. In addition, the activation of both WAS proteins is strongly promoted by interaction with phosphatidyl inositol (4, 5)-bisphosphate (PIP_2) in the plasma membrane[40,41] and by tyrosine phosphorylation by BTK and SRC-family kinases[42-44].

The pathway that leads to the activation of CDC42 and RAC proteins in B cells involves guanine-nucleotide exchange factors (GEFs) of the VAV family, of which VAV1 and VAV3 preferentially activate RAC, while VAV2 activates both RAC and CDC42[45,46]. VAV proteins are recruited to BCR signalling complexes via the phosphorylated cytoplasmic adaptor BLNK[47], and the transmembrane adaptors CD19[48,49] and LAT2[50]. CDC42 and RAC proteins are required for actin dynamics during immune synapse formation, but also for a number of other functions during B cell development, migration, adhesion and responses to antigens[51-54].

NCK proteins are recruited to the CD79A subunit of the BCR[55] and also to BLNK[47]. Deletion of both NCK proteins disrupts synaptic actin, although this is detectable only in some models[55]. Interestingly, biochemical measurements show that NCK clustering is required to activate WAS[56] and that two WAS proteins are needed to activate one ARP2/3 complex[57]. Activation of WAS proteins by the BCR may thus depend on the formation of multimeric complexes, where the mechanisms described above cooperate.

Additional insights into WAS family function come from studies of their binding partners. WAS is constitutively associated with WIPF1, another target of mutations causing immunodeficiency with B cell malfunction. In the absence of WIPF1, antigen-induced B cell synapses show loss of actin foci, reduction in phosphatidylinositol-3-OH kinase (PI3K) signalling, but enhanced proliferation[58-60]. Studies from non-B cells show that WIPF1 protects WAS proteins from degradation, but it also stabilizes actin filaments independently of WAS[61] and mediates the binding of WASL to NCK proteins[62], and the binding of WAS to DOCK8[63]. DOCK8 is a GEF for CDC42 that associates with the plasma membrane through binding to phosphatidyl inositol (3, 4, 5)-trisphosphate (PIP_3)[64]. Mutations in *DOCK8* cause immunodeficiency with impaired B cell synapse formation and activation[65], suggesting that the WAS-WIPF1-DOCK8-CDC42 complex underlies many of the positive functions of WAS in B cells.

[H2] The WAVE complex. The second major ubiquitous activator of ARP2/3 is the WAVE complex[10]. In B cells, the WAVE complex is composed of WASF2 and four other proteins (CYFIP2, NCKAP1L, ABI1, BRK1) [66]. The unique property of the WAVE complex is that it produces spreading arcs of branched actin polymerization, which promote the formation of lamellipodia[67,68]. The WAVE complex is activated by RAC GTPases via VAV proteins and by binding to PIP_3 at the plasma membrane[10]. In the absence of VAV or RAC proteins, or components of the WAVE complex, B cell development is severely impaired, however a direct role of the WAVE complex in antigen-induced B cell responses has not yet been demonstrated[52-54,69].

[H2] Cortactins. Branched actin networks in lymphocytes are also regulated by the cortactin-like protein HCLS1. HCLS1 is a weak activator of ARP2/3; however, it stabilizes ARP2/3 against debranching[10]. HCLS1 is phosphorylated after BCR stimulation and is required for B cell responses to antigens[70], although its precise connection to BCR pathways and role in actin dynamics in B cells are not known. Of note, HAX1, an interaction partner of HCLS1, binds to the cytoplasmic region of the BCR heavy chain where it enhances BCR internalization and antigen-induced apoptosis[71].

[H2] Formins and myosin. In addition to activating CDC42 and RAC proteins, the BCR also stimulates the third major GTPase involved in actin reorganization, RHOA[72]. The pathway responsible for RHOA activation has not been delineated, but it involves PI3K[72] and likely also VAV proteins[45]. RHOA is important for B cell development and survival[73] and stimulates a key formin, DIAP1. The importance of formin-mediated actin polymerization in B cells is largely unexplored, but studies in T cells suggest that DIAP1 and FMNL1, a formin activated by RAC and CDC42[74], are important for multiple aspects of immune synapse formation including adhesion, formation of contractile rings and polarization of microtubules[75-77].

RHOA also plays a major role in activating myosin IIa in B cells[78]. This is mediated by the kinase ROCK1, which phosphorylates activating sites on the myosin regulatory light chain and inhibitory sites on PPP1R12A, a myosin-targeting subunit of the phosphatase PP1[78-80]. Interfering with myosin activation reduces antigen extraction from immune synapses[80,81]. Notably, the BCR may also inhibit myosin by phosphorylation of the heavy chain, mediated by the protein kinase C family[79,82,83].

[H2] Cofilin. In addition to stimulating actin polymerization, BCR signalling leads to actin severing through the activation of cofilin[84,85]. Cofilin activity is controlled by inhibitory phosphorylation by LIMK1 kinase, downstream of RHOA via ROCK1[86], and downstream of RAC and CDC42 via PAK1 kinase[87]. After BCR stimulation, cofilin is dephosphorylated and activated by the phosphatase SSH1. The pathway that leads to cofilin dephosphorylation has not been fully characterized, but it is dependent on the GTPase RAP1A[84]. Interfering with cofilin dephosphorylation impairs B cell synapse formation[84], which is consistent with the need of actin severing for rapid remodelling of the existing cortical cytoskeleton.

[H1] Cytoskeletal regulation of BCR signalling

In addition to the extensive effects of BCR signalling on the cytoskeleton, actin filaments exert a reciprocal regulation of BCR signalling. Both in the absence and presence of antigen, BCR signalling operates in a continuous balance between kinase and phosphatase reactions. Pharmacological and genetic manipulations show that this balance is maintained by the cortical cytoskeleton[9], suggesting that actin regulates the access of membrane kinases and phosphatases to their substrates. These effects can be explained by several distinct mechanisms involving the influence of cortical actin on the diffusion of membrane proteins[88]. The different scenarios contribute to either positive or negative regulation and depend on the structure and dynamics of the actin filaments and their interaction with BCR signalling proteins (Fig. 3).

[H2] Effects on diffusion and sequestration. In resting B cells, the cortical cytoskeleton effectively partitions the plasma membrane into compartments of ca 50nm in diameter, corresponding to the pores of its meshwork. Notably, since B cells express on their surfaces on the order of 100,000 BCR molecules, and a similar number of other signalling proteins, such as CD19 [49], there is on average only about one of these proteins in a compartment. Thus, to meet their interaction partners, these proteins need to cross the actin barriers (Fig. 3a). Single molecule observations (Box 1) show that most membrane proteins diffuse fast enough to bump into the actin barriers of this density in less than 10 milliseconds[32]. However, crossing over the barriers is a rare event, which hinders the diffusion over longer times and distances. The barrier effect is increased dramatically if proteins interact

with the cytoskeleton. Proteins that bind strongly to actin become immobile and sequestered. In all of these situations, increased actin density reduces reaction rates, particularly with a substrate that also diffuses slowly.

This scenario applies to BCR signalling that leads to the phosphorylation of CD19, which involves transient interactions between the BCR-associated SRC family and SYK kinases and CD19, leading to CD19 phosphorylation and the activation of PI3K and ERK pathways [89]. Single molecule tracking shows that the IgM BCR in resting B cells diffuses approximately at 0.05 $\mu m^2/s$, a modest speed for a membrane protein[32,90]. In addition, up to twenty percent of the IgM molecules are completely immobile[90]. The IgD BCR is even slower, with the majority of the molecules immobile[32]. The diffusion of the BCR is particularly slow in areas of the membrane that are rich in actin and conversely, actin depolymerization increases BCR diffusion[32,60]. The exact molecular mechanisms by which IgM and IgD BCRs interact with actin filaments are not known, although it is likely that these mechanisms involve the cytoplasmic tails of the BCR signalling subunits CD79A and CD79B[32]. CD19 also diffuses slowly, although not due to hindrance from actin. Instead, CD19 diffusion is limited because it is included in large protein clusters organized by the tetraspanin **CD81**[49]. Acute depolymerization of actin triggers calcium flux and other downstream pathways that involve the BCR, CD19 and CD81, indicating that by hindering BCR diffusion, the cortical actin limits the access of BCR-associated kinases to the CD19-CD81 complex[32,49]. These findings suggest that the actin cytoskeleton controls the resting state of B cells and that actin reorganization is necessary to activate CD19-dependent signalling in response to antigens. Further evidence for the involvement of actin in the regulation of CD19 signalling is suggested by a defect in CD19 phosphorylation in WIPF1-deficient B cells, although in this case the defect seems to be caused by disruption of the CD19-CD81 complex itself[60]. B cell activation is sensitive to even a small increase in CD19 signalling *in vivo*[91], raising the possibility that increased BCR diffusion and CD19 phosphorylation underlie some of the B cell hyperactivity associated with genetic disruption of actin polymerization pathways.

[H2] Clustering of proteins. Cortical actin can also promote interactions between membrane proteins through the regulation of their clustering. Recent superresolution imaging studies (Box 1) suggests that the BCR is present in the plasma membrane

of resting B cells as a mixture of monomers and clusters of variable sizes[49,92,93]. The IgM BCR clusters are small, resembling previously described protein islands[94]. In contrast, the IgD BCR forms separate, larger clusters that co-localize with markers of lipid rafts[95]. The latter also colocalize with CD19, suggesting that the IgD BCR may communicate with CD19 independently of diffusion. Notably, the ratio of BCR monomers to clusters is stable over a 50-fold range of expression levels[93], which suggests that the clustering is not driven by a binding equilibrium, but by a separate, energy-dependent mechanism, compatible with a role of the cytoskeleton.

Actin has an important role in the organization of protein islands[94] and lipid rafts[96], although the effect of actin depolymerization on BCR clustering varies between studies[49,92]. One possible explanation is that BCR clustering is not dependent simply on the presence of actin, but on actin flow driven by myosin motor activity[30,97], which is actually enhanced by incomplete actin depolymerization[98]. This myosin-induced convective motion drags short actin filaments together into asters, thereby promoting transient clustering of actin-binding proteins[29] (Fig. 3b). Alternatively, because the BCR stimulates actin polymerization itself, transient BCR clustering may produce actin foci that absorb and stabilize the clusters, as has been observed with antigen-induced BCR microclusters described below. The exact role for ligand-independent BCR clustering is not completely understood, but it has been proposed as a means of keeping the BCR in an inhibited state[49,99].

[H2] Corralling and trapping of proteins. When the actin meshwork encircles two interaction partners, it increases their chances of rebinding, which can produce local bursts of signalling activity[100] (Fig. 3c). This mechanism is particularly effective at promoting processive kinase reactions that require the phosphorylation of several sites on one substrate to trigger a downstream signalling step, a common feature in antigen receptor signalling. Signalling complexes that activate the ARP2/3 complex can also embed themselves in actin filaments to protect them from dissociation. In this case, actin provides a positive feedback on antigen-induced signalling, particularly when these complexes assemble by otherwise weak interactions[101,102]. The cortical cytoskeleton is indeed important for proper function of antigen-induced BCR microclusters. Antigen-induced BCR signalling leads to the transient dephosphorylation of ezrin[103], which releases the plasma membrane from cortical actin and temporarily increases BCR diffusion, promoting BCR clustering[104]. After

BCR microclusters form, ezrin is rephosphorylated around them, resulting in corralling by the surrounding actin and stabilization of their signalling (Fig. 3c)[105].

[H2] Exclusion of proteins. In contrast to the BCR, membrane phosphatases diffuse rapidly and do not bind to actin (Fig. 3d). This type of diffusion was initially described for the abundant membrane phosphatase PTPRC[89,106]. Several new lines of evidence suggest that this high mobility extends to inhibitory co-receptors as well, and is in fact important for their inhibitory activity during antigen-induced responses.

CD22 is a transmembrane lectin that recruits the phosphatase PTPN6 to the membrane to dephosphorylate CD19 and VAV proteins[107]. Single molecule imaging of CD22 indicates that it bounces off actin barriers without being trapped[108]. Such behavior suggests that actin effectively increases CD22 concentration. The diffusion of CD22 is primarily regulated by interactions of its lectin domain with cell surface glycoproteins, including PTPRC. Mutation of the lectin binding domain increases CD22 diffusion and improves the negative regulation of BCR signaling[108-110].

A similar dependence on diffusion was found for the inhibitory capacity of FCGRB2[111]. This co-receptor binds to IgG in antigen-containing immune complexes and inhibits BCR signalling by recruiting the lipid phosphatase INPP5D. It diffuses rapidly at 0.3 $\mu m^2/s$ and is independent of the cytoskeleton. A lupus-associated mutation in the transmembrane domain of FCGRB2 causes slanting of the transmembrane helix, slowing its mobility. This mutation impairs FCGRB2's ability to inhibit BCR signaling upon stimulation with immune complexes[112], but can be rescued by allowing FCGRB2 to bind immune complexes before the BCR does[111]. Thus, the speed of diffusion of FCGRB2 is important to ensure rapid inhibition of activated BCRs before they propagate signalling onto downstream components.

Together, these mechanisms provide a passive framework for the regulation of B cell signalling reactions on the molecular level. After antigen binding, BCR signalling remodels the cytoskeleton on the cellular scale that provides a dramatically different regulation of B cell function, as discussed below.

[H1] The cytoskeleton in immune synapses

B cell activation *in vivo* depends on the recognition of antigens that are captured and retained by various accessory immune cells[113]. BCR binding to antigen on the

surfaces of these antigen-presenting cells (APCs) leads to cytoskeletal rearrangements that result in the formation of immune synapses. The synaptic cytoskeletal rearrangements first promote B cell spreading and adhesion to the APC, then mediate the extraction and endocytosis of the antigen and finally induce polarity of the B cell. Succesful completion of these steps is required for B cell antigen processing and presentation to helper T cells, driving antibody responses.

[H2] Immune synapse formation. Immediately upon recognition of antigen on an APC, the BCR triggers local depolymerisation of actin[84] and deactivation of ezrin[105], followed by the explosive generation of new actin filaments that propagate symmetrically outward from the initial point of contact[114]. The protrusions of the membrane drive the spreading of the B cells; this is dependent on BLNK- and CD19-mediated recruitment of VAV proteins and the activation of RAC2, implying that it is driven by activation of the ARP2/3 complex by WAS and WAVE proteins in a mechanism that is similar to generation of lamellipodia[52,89,115].

B cell spreading promotes antigen binding and BCR clustering, and therefore enhances B cell activation[114 116]. During spreading, BCR signalling also activates the integrins LFA1 and VLA4, which engage their ligands on the APCs and enhance adhesion of the B cell[114,117,118].

Actin polymerization in the periphery of the synapse generates a centripetal flow of actin that gathers antigen towards the center of the synapse for eventual endocytosis. Recent high resolution imaging of T cells revealed that, in addition to the ARP2/3 complex, formins also polymerize actin at the periphery of the spreading synapse, which contributes to centripetal transport of the T cell receptor (TCR)[76,119]. The linear actin fibers generated by formins grow perpendicular to the membrane and extend centripetally beyond the branched actin network, where they are bent and linked by myosin IIa into concentric rings. Myosin-dependent contraction of these rings carries antigen clusters towards the synapse center[76]. A role of formins in B cell synapse formation has not been reported, but myosin contributes to centripetal movement of BCR microclusters[81], suggesting that a similar mechanism may exist in B cell synapses.

In addition, BCR microclusters interact with microtubules via the cytoskeletal motor protein dynein, which contributes to the compaction of antigen clusters in the center

of the synapse and to the reorientation of the microtubule-organizing center (MTOC) towards the synapse[120,121].

[H2] Antigen extraction and endocytosis. Two to five minutes after B cell spreading, actin polymerization in the periphery ceases and the B cell synapse contracts. The actin pattern changes to a highly dynamic mixture of actin foci and short filaments interspersed with myosin IIa minifilaments. The actin foci transiently colocalize with BCR clusters and in conjunction with myosin contractility apply pulling forces on the BCR to extract the antigen from the APC[80] (Fig. 4a, c). Measurements with DNA-based tension sensors show that naive B cells generate extraction forces of about 10 pN per antigen molecule. Through a combination of BCR clustering and repetitive pulling, B cells can extract antigens that are anchored to the APC by bonds that are stronger than those with the BCR[81,122,123]. Interestingly, if the antigen is impossible to retrieve mechanically, B cells resort to secretion of lysozomal content into the synapse to liberate the antigen enzymatically[121,123].

Once the antigen is extracted, it is endocytosed by a mechanism that requires clathrin coated pits (CCPs) [80]. This is similar to the endocytosis of soluble antigen, which also requires CCPs[124]. The endocytosis of antigen depends on SRC-family kinases[125], BTK[126], activation of the ARP2/3 complex through WAS, WASL, and WIPF1[7,35,60] and linkage of CCPs to actin by DBNL[127]. However, the endocytic mechanism may not be identical to that involving canonical CCPs as it requires RAC1 and RAC2, but not the typical regulator of actin polymerization in CCPs, CDC42[50]. Endocytosis and BCR trafficking to degradative compartments lead to eventual termination of BCR signalling[124]. Thus, a reduction in BCR internalization caused by defects in actin polymerization is likely to contribute to B cell hyperactivity.

[H2] Induction of polarity. The recruitment of cytoskeletal components and the MTOC to the synapse establishes B cell asymmetry and a new axis of polarity. Although this initial polarization towards the APC is transient[123], a more persistent polarity axis follows the endocytosed antigen to the antigen-processing compartment, where antigenic peptides are loaded onto MHC II proteins over the next few days[128]. In the antigen processing compartment, late endosomes converge with lysosomes in the perinuclear space, a process that is dependent on CDC42 and other polarity components[121,129]. The polarity is reinforced by presentation of the peptide-MHC to helper T cells, which involves prolonged adhesion via ICAM1 and

signalling through CD40[130]. During subsequent B cell proliferation, the polarity axis is maintained, resulting in asymmetric inheritance of the antigen processing compartment and other components into the daughter cells[128,130]. The function of the asymmetric division remains to be fully explored, but it is possible that it contributes to the diversity of B cell fates after activation, for example by the unequal capacity of the daughter cells to present antigen[128], continue PI3K signalling[131] or regulate transcription by BCL6[130]. Through changes in polarity, the cytoskeleton may therefore regulate B cell differentiation into GC, plasma or memory cells.

The cytoskeleton thus emerges as an important regulator of multiple steps in antigen-induced B cell activation. Recently, attention also turned to the mechanical work of the cytoskeleton and its role in regulation of lymphocyte activation through cell mechanics.

[H1] Cytoskeletal regulation of biomechanics

As the previous section illustrates, the immune synapse is as much a conduit for biochemical signals as it is for intercellular mechanical forces. Sensing of the mechanical stress in the synapse provides feedback for signalling and cytoskeletal rearrangements, and has also been implicated in antigen discrimination[132]. Molecularly, the mechanical tension between cells either stretches sensitive proteins to activate them, or affects the dissociation of receptor-ligand interactions.

[H2] Mechanosensitivity of BCR signalling. The importance of mechanical tension in BCR activation was revealed in experiments showing that B cell responsiveness varies with the stiffness of the antigenic substrates[133]. Antigens presented on stiff substrates, which allow the generation of higher forces on the BCR, promote BCR signalling more efficiently than antigens on softer substrates, which limit tension. Precise measurements using DNA tension sensors (Box 2) revealed that the IgM BCR is poorly responsive when the tension on the BCR is below 20 pN, but responds better to tension between 20-40 pN, and reaches a maximum responsiveness to tension above 40 pN[134]. The low forces are likely generated passively, whereas the high forces require myosin contractility. Interestingly, signalling by the IgG BCR does not require detectable tension, possibly contributing

to its high sensitivity to antigen and to its ability to enhance differentiation of B cells into plasma cells.

One explanation is that mechanical forces directly induce the activation of the BCR or its signalling components. Mechanosensitivity has been extensively explored in the case of the TCR and several types of experiments suggest that TCR triggering involves mechanical stimulation[135-139]. The mechanosensitive component of BCR signalling has not been identified, but B cell activation involves phosphorylation of the adaptor protein NEDD9, which is known as a mechanosensor[140,141]. NEDD9 binds to SRC family kinases and to the cytoskeleton via the kinase PTK2. Mechanical stretching of NEDD9 in this complex promotes its phosphorylation and reveals binding sites for the adaptor protein CRKL[142]. CRKL intersects with a number of pathways, including the activation of RAC proteins through DOCK2, and activation of RAP1A through RAPGEF1. RAP1A in turn regulates actin filament elongation through VASP and also activates integrins[143]. NEDD9 thus promotes cell adhesion and could be involved in the mechanosensitive enhancement of synaptic signalling.

[H2] Biomechanics of affinity discrimination. Even in the absence of mechanosensitive activation of signalling, mechanical forces have strong effects on membrane receptor-ligand complexes by changing the rate of their dissociation. Most protein-protein bonds are **slip bonds**, which respond to applied forces by accelerated dissociation. In special cases, pulling can induce conformational changes within the proteins and paradoxically stabilize the bond. This type of bond, termed a catch bond, has been observed in various adhesion receptors, such as integrins, but also in the TCR[137]. Catch bonds formed by the TCR can contribute to mechanosensitivity because mechanical forces prolong the ligand-bound, active state of the TCR[137].

The BCR, however, forms slip bonds with antigens[80,144]. Nevertheless, pulling on the antigen-bound BCR against resistance from the APC contributes to affinity discrimination by B cells, because bonds with low affinity antigens, but not high affinity antigens, rupture before they can induce a biologically significant response. This is particularly important for affinity-dependent extraction of the antigen, where the bonds need to sustain extraction forces before the antigen is separated from the APC and endocytosed by the B cell[80]. Since the amount of antigen extracted by B cells and presented to T cells is the primary determinant of affinity-based B cell

clonal selection[145,146], mechanical extraction of antigen provides an advantage for high-affinity B cells during antibody responses[144].

Efficient mechanical testing of antigens requires strong resistance from the APC. The mechanical resistance to pulling is defined by the strength of antigen attachment to the presenting membrane and by the viscoelastic deformation of the presenting membrane during pulling[147,148]. Indeed, B cells achieve best affinity discrimination on stiff, non-deformable membrane substrates containing strongly tethered antigens[122,123]. Notably, APCs differ substantially in their mechanical properties[149]. For example, dendritic cells (DCs) have low cortical and membrane tension, therefore their membranes pull out easily into long, thin membrane nanotubes. The nanotubes behave as viscous, rather than elastic tethers, which limits tension on the BCR[123,150]. To endocytose the antigen, B cells pinch off the membrane nanotube by a trogocytic mechanism, without the need for separating the antigen from the membrane[80] (Fig. 4a). Consequently, B cells acquire antigens from DCs efficiently even through low affinity BCRs[123]. In contrast, follicular dendritic cells (FDCs) resist pulling out membrane tethers because of their high membrane and cytoskeletal tension. To endocytose the presented antigens, B cells have to separate them from the anchoring receptors by high forces (Fig. 4b). This high mechanical resistance of FDCs limits extraction of antigen by low affinity BCRs, thereby promoting affinity discrimination[123].

These studies open the possibility that mechanics of APCs regulate B cell responses. Inflammation enhances the stiffness and contractility of APCs through changes in their actomyosin cytoskeleton[149,151] and the active recycling of antigens by APCs suggests that they may also mechanically respond to B cell contacts[152,153]. Given that signalling can convert physical inputs into transcriptional and proliferative differences over time, it is possible that B cell mechanosensing in repeated contacts with APCs in lymphoid tissues has consequences for B cell responses that extend beyond the local adaptation of the B cell to the antigenic material[154].

[H1] Cytoskeletal regulation of B cell subsets

Because the actin cytoskeleton building blocks are common and modular, they often convey information between signalling pathways. An example is the synergy

between BCR and Toll-like receptor (TLR) signalling. TLR engagement increases BCR sensitivity to antigen and this depends on increased BCR diffusion and clustering induced by elevated severing activity of cofilin[155]. Interestingly, enhanced responsiveness to both TLR ligands and antigen in WAS-deficient B cells promotes the selection of autoreactive specificities in transitional B cells[6], and leads to loss of marginal zone B cells[156]. TLR-induced activation of cofilin may also be involved in the enhanced antigen reactivity of normal marginal zone B cells[155]. Thus, changes in cytoskeletal activity can have different effects on the responsiveness of specific B cell subsets.

[H2] GC B cells. The GC reaction combines the mutagenesis of immunoglobulin genes and the selection for antigen binding to improve the affinity of antibodies[157]. The selection is driven by GC B cell presentation of antigen peptides to T follicular helper cells[146]. Therefore, GC B cells need to be sensitive to antigen affinity during antigen extraction from FDCs. Recent studies indicate that the ability of GC B cells to discriminate antigen affinities is enhanced by the distinct architecture and mechanics of their immune synapses[81].

GC B cells are highly motile cells that contact FDCs through large dynamic lamellipodia[158-160]. High-throughput imaging of immune synapses of GC B cells *in vitro* revealed that unlike naive B cells, GC B cells do not gather antigen into the center of the synapse, but rather push the antigen outwards before ripping it from the substrate (Fig. 4d) [81]. The movement of the antigen is consistent with an outward flow of actin and with the perpendicular orientation of microtubules to the synapse in the cell periphery. Similarly to naive B cells, antigen extraction depends on tyrosine kinase signalling from the BCR, but does not require PI3K activation. Another distinguishing feature of GC B cell synapses is that antigen extraction in GC cells is uncoupled from endocytosis, as the extracted antigen is first transported along the sides of the cells away from the synapse before internalization (Fig. 4b).

The architecture of the immune synapses in GC B cells appears well suited for the application of tensile forces. Indeed, measurements with DNA tension sensors show that GC B cells pull on the antigen with stronger forces than naive B cells do. Stronger pulling by GC B cells is accompanied by higher activity of myosin IIa, which

is important not only for extraction, but also for reducing the size of the antigen clusters. These observations suggest that GC B cells use high pulling forces to regulate binding to the antigen. Accordingly, GC B cells are less efficient at extracting low affinity, but not high affinity, antigen compared with naive cells, resulting in more stringent affinity discrimination[81].

The high strength of GC B cells likely works in concert with the high stiffness of FDCs[123]. Extraction of antigen from FDCs requires the separation of the antigen from their surfaces, to which it is attached through immune complexes bound to the complement receptor CR2[152,161]. It is therefore possible that the affinities of antibodies in the immune complexes contribute to the strength of tethering of the antigen to FDCs and thus to the thresholds imposed on the selected BCR[162]. Thus, the mechanics of both B cells and FDCs likely contribute to affinity-based selection in GCs.

[H1] Conclusion and outlook

The actin cytoskeleton is a complex, self-assembling mechanical system with both physical and signalling functions that have important roles in the activation and differentiation of immune cells. Despite recent progress towards understanding both the regulation of the actin cytoskeleton and the signalling pathways activated by immune receptors, insights into the mechanisms that link these two processes remain fragmentary. Similarly, exactly how the cytoskeleton governs different mechanical responses in distinct B cell subsets, including GC B cells, remains poorly understood. These shortcomings are due to a number of complicating factors, such as the pleiotropic compensatory effects of actin regulatory networks in response to crude genetic and pharmacological manipulations, insufficient resolution of imaging methods and the lack of biophysical tools adapted for the work with lymphocytes. To understand how signalling pathways interact with cytoskeletal structures to achieve specific mechanical and regulatory functions, future studies will likely benefit from improvements in these areas. Advances in optical microscopy have been making long strides towards three-dimensional high-resolution imaging and it will be exciting to see this new technology employed for studies of immune cells[76,163-165]. Genetic manipulation is more accessible and precise than ever before, and new optogenetic and nanomechanical methods offer unprecedented temporal and spatial control of

manipulation of live cells[166,167]. Mathematical modelling of the cytoskeleton, for example using an active gel theory **[G]**[168], has also been informative and may allow to understand counterintuitive mechanisms by which actin regulates cellular functions[169].

Ultimately, these technological advances may improve our understanding of how defects in a specific pathway result in a complex phenotype, such as that seen in Wiskott-Aldrich syndrome. Based on current data, B cell defects associated with WAS deficiency arise from essential inhibitory functions of WAS in B cells, such as the regulation of BCR diffusion and endocytosis, as well as increased crosstalk with other signalling pathways, such as the TLRs. Many of the phenotypes result from malfunction of specific, less studied, B cell subsets, for example transitional, marginal zone and GC B cells. More detailed investigation of cytoskeletal regulation in these subsets will require new large-scale single cell approaches[81].

As immune cells probe their local environment, their function and malfunction in tissues is likely to be constantly influenced by cellular mechanics. With the emergence of new biophysical tools and concepts, the understanding of immune cell mechanics should facilitate new approaches to manipulate immune responses. This is illustrated by the recent discoveries of new phenomena, such as the mechanical potentiation of tumor cell killing by CD8 T cells[170], or by creation of synthetic immune receptors which are designed based on universal principles of mechanically-operated systems[171]. For B cells , insights into the biophysical aspects of their activation are likely to find applications in the design of new materials for vaccines and in strategies for the modulation of pathologically activated B cells in their microenvironment, such as in autoimmune reactions and in B cell malignancies.

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CFI

The authors declare no competing interests.

Box 1 Imaging techniques for studies of the cytoskeleton

Intravital microscopy. Two photon laser scanning confocal microscopy can image cells directly inside living organisms. The two-photon excitation penetrates deep into tissues and allows the imaging of the movement and interactions of fluorescently labelled cells. Light scattering, however, limits the resolution of subcellular structures.

Total-internal reflection fluorescence (TIRF) microscopy. This *in vitro* imaging technique excels in the imaging of processes near the plasma membrane[172]. The illuminating laser beam does not enter the sample, but reflects at an acute angle off the coverslip-sample interface, creating a thin (100-200 nm) evanescent

electromagnetic field that excites fluorophores in close proximity to the coverslip. The thin excitation volume eliminates fluorescent background from the rest of the cell, which greatly improves the detection of weak signals, for example in single molecule imaging.

Structured illumination microscopy (SIM). This technique improves resolution by projecting a series of patterns onto the sample and then using the patterned images to reconstruct a final image. SIM improves resolution modestly, to about 150 nm, however, it works in 3D and a number of recent improvements have combined it with TIRF or with photoswitchable fluorophores to improve resolution even further[163].

Super-resolution microscopy. Currently the highest resolution in light microscopy is achieved by two techniques. Stimulated emission depletion (STED) uses laser scanning microscopy with the excitation volume reduced by an emission-depleting beam. In contrast, localization microscopy (and its two major implementations, photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM)) uses the sequential localization of single molecules to build a super-resolution image. While initially designed for 2D imaging, 3D implementations are possible. Localization microscopy using TIRF illumination has been used to investigate the organization of the plasma membrane and the cortical cytoskeleton[9]. Its drawback is that undercounting or overcounting of the fluorescent labels can alter the details of the reconstructed structures[173].

Box 2. Biophysical techniques to study cell mechanics

Traction force microscopy. This technique measures forces that cells apply on substrates during adhesion or migration. The forces are measured as displacement of beads embedded in a gel-like substrate of known stiffness. A newer variation of the technique uses flexible micropillars[174].

Atomic force microscopy (AFM). AFM is based on the mechanical probing of cells or molecules by a nanoscopic tip attached to a miniature cantilever. The tip moves in three dimensions with nanometer accuracy, while bending of the cantilever measures the strength of the force to determine cell stiffness, or mechanical resistance of proteins and protein complexes to pulling[175].

Magnetic and optical tweezers. Tweezer techniques use a magnetic field or a focused laser beam, respectively, to manipulate microscopic beads[176,177]. These two techniques excel at applying low forces (less than 5 pN) in any direction, making them suitable for single molecule pulling assays.

Micropipette assays. In this assay, micropipettes are used to bring cells into contact with a force probe, which is typically a red blood cell containing ligands for cell surface receptors. As the cells are pushed together or pulled apart, the red blood cell deforms, allowing the calculation of the intercellular forces[178].

Nanoscopic mechanosensors. The sensors are composed from a stretchable linker flanked by fluorophores, whose proximity reports on the strength of the stretching force. These sensors have been designed using synthetic polymers, peptides, or DNA and are small enough to incorporate into cell surface ligands or receptors. The newest designs of nanosensors use DNA hairpins or duplexes, whose unfolding by mechanical forces can be tuned by the DNA sequence to achieve a digital response with threshold ranging from 5 to 56 pN[179].

Figure legends

Figure 1. The cortical cytoskeleton in B cells. The cortical cytoskeleton of resting B cells is composed of a dynamic meshwork of actin filaments attached to the inner leaflet of the plasma membrane. Actin polymerization is regulated by monomeric actin binding proteins (profilin, thymosin) and actin filament nucleators (ARP2/3 complex, formins). The dynamics and structure of the meshwork are controlled by elongation factors, capping proteins, crosslinkers, severing and debranching

proteins, and by motor activity of myosin II. The filaments are attached to the plasma membrane by ERM proteins and class I myosins. Boxes show genes encoding the actin regulators expressed in B cells based on www.imgen.org and reference[180].

Figure 2. BCR signalling to the cytoskeleton. The schematic shows antigen-induced BCR signalling pathways that lead to actin remodelling. Activation of SYK, LYN and BTK tyrosine kinases by the BCR triggers actin severing by activation of cofilin via RAP1. Phosphorylation of the BCR and of the adaptors BLNK, CD19 and LAT2 leads to activation of the WAS and WAVE complexes through NCK proteins, and RAC and CDC42 GTPAses. WAS and WAVE proteins induce actin polymerization by activating the ARP2/3 complex at the plasma membrane, generating lamellipodial protrusions and endocytic pits. RHOA activates actin polymerization by the formin DIAP1 and also stimulates myosin contractility through ROCK1.

Figure 3. Effects of the actin cytoskeleton on signalling reactions in the plasma membrane. (a) Left panel; actin filaments hinder diffusion and sequester mobile proteins (shown in blue), reducing their collisions with stationary partners (red). The right panel illustrates how a reduction in actin density increases diffusion and reaction rates **[Au: should this be more clearly illustrated by including more blue/red doublets, and maybe making the difference in actin density more obvious?]**. (b) Myosin II (green) induces the convective motion of free actin filaments (left panel) into asters (right panel), promoting the clustering of actin-associated proteins. (c) Actin corrals promote the repeated collisions of proteins (left panel) and stability of signalling complexes (right panel). (d). The left panel illustrates how actin filaments channel the diffusion of inhibitory proteins (yellow) and promote the rate of their encounters with targets (blue). After the formation of active signalling complexes (blue, shown in the right panel), the rate of collisions with the inhibitory receptor (yellow) is critical for shutting down the response.

Figure 4. Immune synapses of naive and germinal center B cells. (a, b) Sideview of immune synapses formed between B cells and APCs illustrating the

mechanism of antigen extraction. (a) Naive B cells deform soft APCs to pinch off antigen with membrane tethers in the center of the synapse. (b) GC B cells rip antigen from stiff APCs at the periphery of the synapse. Endosomes containing antigen fuse with lysozomes (green), where antigen processing is initiated. (c, d) Top view of the synapses of naive (a) and germinal center (b) B cells showing localization of antigen clusters (red) and actin filaments (blue) .

Table 1. B cell phenotypes in mouse knockouts of cytoskeletal regulators.

Gene Name	Protein name(s)	Phenotype in vivo	Phenotype in vitro	References
WAS	Wiskot-Aldrich syndrome protein (WASP)	Disturbed development of B1a, transitional and marginal zone B cells, enhanced GC formation, production of autoantibodies	Enhaced B cell activation, reduced BCR endocytosis, disturbed immune synapse formation, reduced migration	5, 6, 7, 8, 34, 35, 156
WASL	Neural- Wiskot-Aldrich syndrome protein (N-WASP)	Reduced numbers of marginal zone B cells, production of autoantibodies	Enhanced B cell activation, reduced BCR endocytosis, disturbed immune synapse formation	35, 37
WIPF1	WAS/WASL-interacting protein family member 1 (WIP)	Reduced B cell responses	Enhanced B cell proliferation, reduced PI3K signaling, reduced plasma cell differentiation, reduced migration	59, 60
DOCK8	Dedicator of cytokinesis protein 8 (DOCK8)	Disturbed development of B1a and marginal zone B cells, reduced GC formation, reduced antibody production	Disturbed immune synapse formation	65
NCKAP1L	Nck-associated protein 1-like (NCKAP1L), Hematopoietic protein 1 (HEM-1)	Impaired B cell development	Not known	69
EZR	Ezrin	Enhanced B cell responses	Increased BCR clustering, altered BCR signaling,	104

			enhanced proliferation, enhanced plasma cell differentiation	
HCLS1	Hematopoietic lineage cell-specific protein (HS1)	Reduced antibody production	Reduced proliferation	70

Glossary

Wiskott-Aldrich syndrome. X-linked primary immunodeficiency characterized by recurrent infections, bleeding disorders, eczema and autoimmune reactions.

Cofilin and destrin. Proteins with actin-severing activity.

Coronins. Proteins that disassemble branched actin filaments; Coronin 1A is the target of mutations causing primary immunodeficiencies.

Filopodia. Thin, long cellular extensions that contain linear actin filaments and are used in cellular sensing and migration.

Lamellipodia. Large, flat cellular protrusions containing branched actin filaments that drive cell motility.

CD81. A palmitoylated, cholesterol-binding tetraspanin membrane protein that is essential for CD19 expression and function.

Slip bonds. Bonds whose stability decreases with applied pulling forces.

Active gel theory. An area of soft matter physics describing the mesoscopic behavior of gels, such as those composed of actin filaments, maintained in a non-equilibrium state by constant consumption of energy.

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Biography

Pavel Tolar received his PhD from Charles University, Prague, Czech Republic and trained as a postdoctoral fellow at the National Institutes of Health. He then established a research group at the National Institute for Medical Research and later at the Francis Crick Institute, London, UK. His research is focused on the molecular and biophysical mechanisms of B cell activation.

Online summary

- The cortical actin cytoskeleton regulates cellular mechanics and signalling from plasma membrane receptors.
- In B cells, the cortical cytoskeleton is controlled through multiple connections to the signalling pathways downstream of the B cell antigen receptor.
- Cortical actin regulates B cell signalling by controlling the diffusion and reaction rates of plasma membrane proteins.
- Remodelling of the cortical actin in response to antigen regulates B cell immune synapses, antigen internalization, and cell polarization.
- Mechanical work produced by the cytoskeleton promotes affinity discrimination during B cell antigen extraction from antigen presenting cells.
- Differences in actin dynamics in B cell subsets contribute to subset-specific regulation of B cell activation, for example by improving affinity discrimination in germinal center B cells.

Subject categories **[Au: Editor to insert]**

ToC blurb **[Au: editor to insert]**