



Published in final edited form as:

Nat Rev Immunol. 2010 November ; 10(11): 767–777. doi:10.1038/nri2853.

The tipping points in the initiation of B cell signalling: how small changes make big differences

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Abstract

B cells are selected by the binding of antigen to clonally distributed B cell receptors (BCRs), triggering signalling cascades that result in B cell activation. With the recent application of high-resolution live-cell imaging, we are gaining an understanding of the events that initiate BCR signalling within seconds of its engagement with antigen. These observations are providing a molecular explanation for fundamental aspects of B cell responses, including antigen affinity discrimination and the value of class switching, as well as insights into the underlying causes of B cell tumorigenesis. Advances in our understanding of the earliest molecular events that follow antigen binding to the BCR may provide a general framework for the initiation of signalling in the adaptive immune system.

With the cooperation of helper T cells and the involvement of cells of the innate immune system, antigen has the potential to drive naive B cells to proliferate and differentiate into memory B cells and antibody-secreting plasma cells through processes that involve somatic hypermutation and class switching¹. These events occur in specialized compartments in which B cells must compete for limited niches and resources in order to survive². B cell receptors (BCRs) are clonally distributed and the life or death process for B cells begins with the clonal selection of specific B cells by antigen binding to BCRs on their cell surfaces³.

The BCR comprises a membrane-bound immunoglobulin and a disulphide-linked heterodimer composed of Ig α and Ig β ^{4,5} (FIG. 1). Ig α and Ig β are transmembrane proteins with extracellular domains that, on the basis of their amino acid sequence, are predicted to have immunoglobulin-like folds, and intracellular domains that each contain an immunoreceptor tyrosine-based activation motif (ITAM). Upon antigen binding to mature B cells, the BCR is phosphorylated on its ITAM tyrosines by the first kinase in the BCR signalling pathway, primarily LYN, and then SYK (spleen tyrosine kinase) is recruited through its SH2 domain to the phosphorylated Ig α –Ig β heterodimer, resulting in the triggering of at least four different signalling cascades^{6,7}.

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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

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SUPPLEMENTARY INFORMATION

See online article: S1 (figure) | S2 (figure)

Over the past several years, biochemical and genetic approaches have elucidated a great deal about the nature of the complex signalling cascades that are triggered by antigen binding to the BCR^{6,7}. However, it was not until the advent of live-cell imaging, particularly at the single-molecule level, that the field acquired tools (BOX 1) that could provide the temporal and spatial resolution necessary to begin to understand how BCRs perceive that antigen has bound to their ectodomains and how this information is translated across the membrane to trigger signalling cascades.

Box 1

Application of imaging technologies to the study of B cell activation

Scanning electron microscopy

Scanning electron microscopy (SEM)⁷³ uses a high-energy electron beam to scan the surface of samples with nanometre-scale resolution, providing information on three-dimensional characteristics. However, because SEM cannot be used for live-cell imaging, it cannot provide kinetic data with high temporal resolution.

Confocal laser-scanning microscopy

Confocal laser-scanning microscopy (CLSM)⁷⁴ is one of the most widely used live-cell imaging techniques. CLSM uses a focused beam of laser light to excite fluorophores in a sample at a selected depth. Emitted light that is out of focus is prevented from reaching the detectors by the use of a pinhole device. CLSM generally produces spatial resolution of several hundred nanometres, approaching the limit of light diffraction (200 nm) and temporal resolution of several hundred milliseconds in living cells. However, CLSM does have limitations — it cannot produce quality images of samples thicker than 80 μm , and the depth of optical sections in CLSM is relatively thick, typically greater than 0.6 μm , making imaging thin specimen planes, such as the plasma membrane, difficult owing to unwanted signals reaching the detector.

Two-photon laser-scanning fluorescence microscopy

Two-photon laser-scanning fluorescence microscopy (2PM)⁷⁵ allows imaging of living tissues up to a depth of approximately 1 millimetre. Typically, 2PM uses a long-wave infrared laser to excite a fluorophore that requires the absorption of two infrared photons to emit a single photon. Thus, 2PM produces the desired optical sectioning without the assistance of a pinhole device. The long-wavelength infrared photons can deeply penetrate tissues more efficiently than short-wavelength lights owing to limited scattering.

Total internal reflection fluorescence microscopy

Total internal reflection fluorescence microscopy (TIRFM)⁷⁶ allows fluorophores to be visualized with a superior signal-to-noise ratio because the depth of optical section in TIRFM is limited to a 100 nm space above the cover slip on which a cell is placed. Typically, the glass cover slip has antigen absorbed onto it or supports a fluid lipid bilayer into which antigen is incorporated. A laser pointed at an angle to the cover slip generates an exponentially decaying evanescent light at the glass–cell interface. Single fluorophores can be individually resolved, as long as they are separated by a distance greater than the light diffraction limit (~200 nm). In addition, the application of particle-tracking methods can further improve the resolution to a sub-diffraction limit level. Typically, the temporal resolution of TIRFM can reach tens of milliseconds. These features make TIRFM an ideal tool for studying protein diffusion dynamics at or near the plasma membrane. TIRFM is especially useful when integrated with other fluorescence-

based imaging techniques, described below. TIRFM cannot be applied to the study of B cells interacting with antigen-presenting cells and is limited in that regard.

Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET)^{77,78} is the transfer of energy from a donor fluorophore to an acceptor fluorophore within a limited distance (10 nm). Because of the extreme sensitivity of FRET efficiencies to the distance between FRET donor and acceptor, FRET has proved to be a highly sensitive molecular ruler with which to measure the interactions between proteins or proteins and lipids. In combination with TIRFM, FRET imaging allows the detection of molecular interactions of membrane-associated proteins at the contact interface of cells with glass or glass-supported membranes.

Single particle tracking

Single particle tracking (SPT)⁷⁹ is an imaging technique that is widely used to examine the diffusion mobility of molecules. Multicolour imaging with SPT can provide information concerning the spatial and temporal relationship between receptors of interest or between receptors and important cellular structures. For single molecules to be resolved and tracked, they must be fluorescently labelled at a low level that is usually determined by the fluorescence intensity profile and bleaching characteristics. When labelling the target molecules, traditional organic dyes (such as Cy3) are widely used, however their application in long-term SPT (many tens of trajectory steps) is limited owing to photobleaching⁸⁰.

Quantum dots

Quantum dots (Qdots)⁸⁰ are semiconductor materials with excellent fluorescence and photostability. In combination with TIRFM, molecules of interest labelled with Qdots can be tracked at the single-molecule level for long periods of time^{71,72}. Qdots are especially useful in multicolour SPT TIRFM.

We think of these very early events that follow antigen binding to the cell surface BCR as ‘tipping points’: the crucial events that propel the BCRs towards triggering signalling cascades and all the sequelae that follow. In this Review, we describe the tipping points of antigen-induced initiation of BCR signalling that have been revealed by live-cell imaging. We suggest that very small differences in these early events result in big differences in the quality of the resulting humoral immune response.

We begin with a description of the BCR in resting cells and briefly review how and where B cells ‘see’ antigen. We describe the response to antigen at the whole-cell level, then zoom in on individual BCRs and a view of the molecular events that occur within seconds of antigen binding to the BCR, including the initial oligomerization of the receptor and the subsequent growth of BCR microclusters. We detail how these early BCR-intrinsic events can account for antigen affinity discrimination and explain the value of class switching. Mechanisms are proposed to explain how BCR oligomerization triggers signalling, and the regulation of early events in the initiation of BCR signalling and their implications for B cell tumorigenesis and autoimmunity are discussed. Finally, the BCR is a member of a family of multichain immune-recognition receptors (MIRRs) that includes the T cell receptor (TCR) and the high-affinity receptor for IgE (FcεR1). We attempt to put the advances in our understanding of molecular events that follow antigen binding to the BCR into a general framework for understanding the initiation of signalling by MIRRs in the adaptive immune system.

The BCR in resting cells

Although in the absence of antigen B cells are referred to as resting cells, they are not quiescent and are probably constantly hovering near a tipping point of activation. The expression of the BCR is essential for the survival of B cells at all developmental stages, leading to the hypothesis that the BCR must provide constitutive low-level ‘tonic’ signals that promote survival, either spontaneously or on interaction with ligands in the environment^{8,9}. Recent genetic and biochemical analyses have shed light on the nature of the tonic signalling pathway, suggesting that the initiation of tonic signalling may not be identical to that of antigen-induced signalling, and may bypass the requirement for the phosphorylation of BCR ITAMs. Evidence was provided that phosphoinositide 3-kinase (PI3K) signalling is crucial for the survival of mature B cells and that the PI3K pathway might be connected to the BCR in resting cells by the GTPase TC21 (also known as RRAS2), which directly recruits the catalytic subunit of PI3K to the unphosphorylated ITAM of the BCR^{8,10}.

However, regardless of the biochemical nature of the tonic signalling pathway, the mechanism by which the BCR initiates tonic signalling is not fully understood. Recent high-resolution imaging in live B cells has provided insights into the behaviour of BCRs in resting cells that have implications for how tonic signalling might be initiated in mature B cells. Live-cell total internal reflection fluorescence microscopy (TIRFM) in conjunction with analyses of fluorescence resonance energy transfer (FRET) (BOX 1) between BCRs containing FRET donor and acceptor fluorescent proteins indicated that in resting B cells most BCRs were not in close molecular proximity, but instead were monomeric⁵. In addition, tracking individual BCR molecules in resting B cells by single particle tracking (SPT) (BOX 1) in the absence of antigen showed the short-range movement of most BCRs to be consistent with free diffusion^{11,12}. In the context of the ‘picket fence’ model of plasma-membrane organization (BOX 2) — in which the actin cytoskeleton (the fence) and trans-membrane proteins (pickets) compartmentalize both proteins and lipids — we interpret these observations to mean that, in resting B cells, most BCRs are freely diffusing and highly mobile as monomers within, and hop relatively easily between, compartments created by the actin cytoskeleton. These highly mobile BCR monomers seem to exist on the B cell surfaces with less mobile dimers or more highly ordered BCR oligomers (FIG. 1). We observed that approximately 20% of BCRs were immobile in the membrane of resting B cells⁵, which suggests that these BCRs may have formed either spontaneous or ligand-induced oligomers, and undergone ‘oligomerization-induced trapping’ (BOX 2), which decreases the long-range diffusion of membrane molecules (FIG. 1; Supplementary information S1 (figure)). Because antigen-induced signalling-active BCR oligomers show similar behaviour (see below), an in-depth analysis to determine whether these immobile BCRs are the source of tonic signals and the relationship, in mature B cells, between these immobile microclusters and PI3K and TC21, the proposed mediators of tonic signals, should be of interest.

Box 2

A contemporary view of the plasma membrane

An appreciation of the interpretation of much of the data produced by live-cell imaging requires an understanding of the organization and function of the plasma membrane. Current evidence indicates that the plasma membrane is not simply a fluid mosaic structure of freely diffusing proteins in a phospholipid bilayer, as proposed by Singer and Nicholson nearly 40 years ago⁸¹. Current data better fit a model in which the plasma membrane is partitioned into highly dynamic compartments that range in size from tens to hundreds of nanometres, depending on the cell type^{31,32}. The current view of the

compartmentalized plasma membrane accounts for an important observation made using single particle tracking (SPT) techniques — namely that the mobility of proteins, as well as lipids, is considerably slower in the plasma membranes of cells than in artificial lipid bilayers^{31,82}. These data suggest that proteins and lipids are in some way confined in the plasma membrane and cannot freely diffuse.

Of great importance to understanding the early events in B cell receptor signalling is the observation that the formation of oligomers of membrane proteins formed by crosslinking resulted in dramatically reduced motility in the plasma membranes of cells, a behaviour not observed for protein oligomers in model membranes. The nature of the confinement became clear when Kusumi and colleagues applied extremely high-resolution microscopy at the microsecond scale to describe the movement of membrane proteins⁸². These remarkable images showed that, in the short term, proteins and lipids diffused freely within confined areas of the plasma membrane, and in the longer term hopped between these compartments. Electron tomography produced a three-dimensional structure of the membrane cytoskeleton showing that the entire cytoplasmic surface of the plasma membrane is covered by a meshwork of the actin cytoskeleton that forms boundaries of a size that correlated well with the size of the membrane compartments determined by SPT⁸³. Kusumi and colleagues proposed that membrane proteins and lipids are confined in their movement by actin cytoskeleton ‘fences’ and actin-anchored protein ‘pickets’; termed the picket fence model³¹. According to this model, monomeric proteins can readily hop between compartments, whereas larger oligomers are less able to hop and are consequently ‘trapped’ in the compartment in which the oligomers first formed.

As recently reviewed, there is considerable experimental support for Kusumi’s picket fence model³². The model implies that cells have evolved mechanisms to control the long-range diffusion of membrane molecules, allowing compartmentalization of receptors and ‘oligomerization-induced trapping’ of receptor complexes. The immobilization of receptor oligomers could have an important role in facilitating the assembly of signalling complexes on the cytoplasmic domain of the receptors. In addition, the trapping of receptors at the site of antigen encounter may be important in orienting cell polarization.

Recently, Yang and Reth¹³ provided evidence for the existence of antigen-independent BCR dimers on the surface of cells using bifluorescence complementation (BiFC) in conjunction with confocal microscopy. They observed surface fluorescence in S2 *Drosophila* Schneider cells expressing membrane-bound immunoglobulin heavy and light chains and Ig β , and two types of Ig α molecules that contained either the amino-terminal or carboxy-terminal half-domains of yellow fluorescent protein (YFP) such that each BCR had either an Ig α -NYFP or an Ig α -CYFP. Upon BCR dimerization a complete YFP forms, resulting in a fluorescent signal (the BiFC method). Confocal microscopy revealed YFP fluorescence on the cell surface, indicating the presence of dimers. However, the authors did not determine what percentage of BCRs on the surface of the living cells were in dimers, and only quantified the dimers in cell lysates. An important question is whether the percentage of dimerized BCRs in resting B cells that was detected by BiFC in this study was similar to the percentage detected by SPT (which was ~20%). The authors concluded that the observed dimers were signalling inactive because mutations that decreased dimerization increased signalling in response to antigen. The authors also speculated that the signalling-inactive dimers were in equilibrium with signalling-active monomers that provide tonic signals (see below).

Using two-colour SPT (BOX 1), Treanor *et al.*¹² recently directly visualized the behaviour of individual BCRs and the actin cytoskeleton in mature resting B cells. They observed two

distinct BCR populations: BCRs with high diffusion coefficients were present in actin-poor regions of the membrane, whereas BCRs with low diffusion coefficients that were essentially immobile were in actin- and ezrin-rich regions. Confinement in actin-rich regions seemed to be mediated through the cytoplasmic domain of Ig β . Alterations introduced into the actin network by drugs that either stabilized or disassembled the actin cytoskeleton had the dramatic effect of fully activating the B cell in the absence of antigen. These authors suggested that in resting B cells, tonic signalling is maintained by the dynamic organization of the actin cytoskeleton, either by concentrating activators of tonic signalling or by excluding inhibitors of tonic signalling, and that alterations in the actin cytoskeleton disrupt this steady state and result in full BCR signalling. It is also possible that, in addition to actin, other proteins that form extracellular matrixes and have the ability to organize membrane receptors, such as galectins, have a role in controlling BCR tonic signalling¹⁴. Although speculative, these SPT studies suggest that tonic signalling might result from the transient trapping of highly dynamic BCR oligomers, promoting the assembly of signalling complexes.

The BCR response to antigen

Where B cells encounter antigen

B cells can respond to multivalent antigens in solution by BCR clustering and capping, and, ultimately, provided with the appropriate help, by proliferating and differentiating into antibody-secreting cells. Indeed, much of what we know about the nature of the BCR signalling pathways has come from biochemical studies of B cells activated by multivalent antigens in solution.

However, results of recent two-photon laser-scanning fluorescence microscopy (BOX 1) indicate that B cells recognize antigen on the surfaces of antigen-presenting cells (APCs) and that such recognition may be highly relevant for B cell activation *in vivo*^{15–20}. Batista and colleagues²¹ first showed that the recognition by B cells of antigens on APCs *in vitro* was highly efficient and resulted in the formation of an immune synapse, a process similar to that described for T cells. Using antigen-containing fluid lipid bilayers as surrogate APCs, Fleire *et al.*²² provided evidence that the response of B cells to antigen encountered on membranes was much more active and dynamic than that predicted from the response of B cells to antigen in solution. They observed that B cells first engaged antigen through membrane protrusions, inducing the BCRs to form microclusters that resulted in B cell spreading over the lipid bilayer, forming additional microclusters in the peripheral lamellipodia of the cell. The microclusters then moved to the centre of the contact area through mechanisms that require the BCR to associate with the actin cytoskeleton and, following maximal spreading of the B cell, contracted to form an immune synapse. The B cell-spreading response to membrane-bound antigens was dependent on cooperation between phospholipase C γ 2 and the guanine-nucleotide exchange factor VAV, and on the small GTPase RAC2, and was regulated by Rap GTPases^{23–25}.

Thus, B cells encounter and respond to antigen both in solution and on the surfaces of APCs. An important unanswered question is: are the B cell responses to antigen in solution and on APCs qualitatively and quantitatively similar? Also, will the rules established by the study of B cell responses to antigen in solution apply to B cells that are encountering antigen on APCs?

The formation of BCR oligomers: a tipping point

That the encounter of B cells with antigen (whether in solution or on the surface of APCs) leads to the formation of microclusters suggests that microclusters may be the elementary signalling unit for B cells and raises the questions: how does antigen drive the formation of

microclusters, and are these mechanisms the same for antigen in solution and on APC surfaces? It is commonly observed that B cell activation by antigen in solution requires that the antigen be multivalent. Monovalent antigen does not induce the characteristic clustering and capping of BCRs that is observed after engagement of multivalent antigen. The striking difference between the effects of monovalent and multivalent antigen led to the conclusion that B cell activation required the physical cross-linking of the BCR by antigen²⁶. Physical crosslinking is a common mechanism for the activation of various growth factor and chemokine receptors^{27,28}. For these receptors, crosslinking by their growth factor or chemokine ligands brings the receptors into a spatially well-defined orientation in which kinases associated with the intracellular domains are in close molecular proximity, allowing for cross-phosphorylation of the receptor and the initiation of signalling^{29,30}. By contrast, BCRs respond to a universe of foreign antigens of all sizes and shapes, and of spatial arrays of their antigenic epitopes. It is difficult to imagine how these diverse antigens can each succeed in bringing the crosslinked BCRs into spatially well-defined oligomers that can recruit LYN.

We recently provided the unexpected evidence that physical crosslinking of BCRs is, in fact, not required for B cell responses to antigens on membrane surfaces and that monovalent engagement of BCRs by membrane-bound antigens triggers signalling¹¹. We followed individual BCRs by SPT TIRFM (BOX 1) as B cells first engaged fully mobile monovalent or multivalent antigens incorporated into a fluid lipid bilayer. Antigen binding greatly slowed the diffusion of the BCRs in the B cell plasma membrane, such that they were essentially immobile (Supplementary information S1 (figure)). This ‘stopping’ behaviour of the BCRs was indistinguishable between B cells responding to monovalent antigens and those responding to multivalent antigens, as long as the antigens were associated with planar lipid bilayers. The interpretation of the stopping behaviour in the context of the picket fence model of plasma-membrane organization (BOX 2) is that the receptors oligomerized after antigen binding and were trapped in membrane compartments formed by actin ‘fences’ and membrane protein ‘pickets’ and were unable to freely diffuse^{31,32}.

BCR immobilization was highly dependent on the concentration of the antigen in the fluid lipid bilayer¹¹, and antigen-bound BCRs did not stop when the concentration of antigen in the fluid lipid bilayer was low, decreasing the probability of random bumping between antigen-bound BCRs. We also observed that antigen-bound BCRs were much more likely to stop when encountering pre-existing BCR microclusters¹¹. Through extensive analyses of BCRs with mutations — which resulted in the elimination of the Ig α -Ig β heterodimer, alterations in the transmembrane domain of membrane-bound immunoglobulin or deletions of portions of the membrane-bound immunoglobulin ectodomains — it was determined that oligomerization and immobilization was a BCR-intrinsic event that did not require the signalling apparatus of the BCR, and that the membrane-proximal C μ 4 portion of the ectodomain of the membrane-bound IgM of the BCR was both necessary and sufficient for BCR oligomerization and signalling (FIG. 2). Similar results were obtained for the C γ 3 membrane-proximal domain of membrane-bound IgG.

Taken together, these results support a model for the initiation of BCR signalling that we termed the ‘conformation-induced oligomerization model’³³ (FIG. 2). In this model, it is proposed that in the absence of antigen, the C μ 4 portion of the BCR ectodomain is not accessible for oligomerization and that the binding of the BCR to a monovalent antigen in an opposing membrane exerts a force that brings the C μ 4 domain into an oligomerization-receptive orientation. The exertion of this force did not require that the BCR be attached to the actin cytoskeleton because C μ 4-dependent oligomerization of the BCR was observed for BCRs that lacked the Ig α -Ig β heterodimer and so had no means of associating with the actin cytoskeleton¹¹. The antigen-bound BCR is still mobile and it is not until the BCR

encounters another antigen-bound BCR that oligomerization occurs and the oligomerized BCRs are immobilized (FIGS 2,3). This model is attractive because it provides a mechanism by which the universe of foreign, structurally distinct antigens can bring the BCRs into a precise signalling-active oligomer. But we suspect that this model does not apply to B cells responding to multivalent antigens that physically crosslink BCRs. BCRs that lack the membrane-proximal domains of membrane-bound immunoglobulin are readily immobilized by multivalent antigen presented on membranes¹¹. We suggest below that physically crosslinked BCRs might mimic oligomerized BCRs at later points during the process of signal initiation.

As mentioned above, Yang and Reth recently proposed an alternative model for BCR clustering, termed the 'dissociation activation model', in which most BCRs exist on the B cell surface in signalling-inactive 'closed' dimers in equilibrium with a small number of signalling-active 'open' BCR monomers¹³. This model is based, in part, on the results from BiFC studies (see above) in which the number of BCR dimers on living B cells was not directly quantified. In the dissociation activation model, multivalent antigens disrupt the closed dimers, resulting in open monomers that are then clustered by multivalent antigen in a manner that prevents the formation of inactive closed dimers within the cluster. An advantage of this model is that it accounts for the ability of structurally different antigens to activate B cells, because in this model antigen keeps BCRs apart rather than bringing them together into well-ordered oligomers. However, this is not a unique advantage, because the data supporting the conformation-induced oligomerization model for BCR clustering, which show that monovalent antigens presented on membranes induce BCR oligomerization, also account for the ability of structurally distinct antigens to oligomerize BCRs. The dissociation activation model does not accommodate the data from SPT and FRET analyses, which indicate that only a small fraction of BCRs in resting cells exist in dimers and that antigen binding results in a marked increase in FRET between BCRs in clusters, indicating the formation of dimers or oligomers. Neither does this model accommodate the observation that physical crosslinking of BCRs by multivalent antigens is not required for BCR oligomerization and clustering when antigens are presented to the B cell on an opposing membrane.

The growth of BCR microclusters

During TIRF imaging of B cells on antigen-containing fluid lipid bilayers, BCR microclusters can be seen to grow with time, providing a mechanism to amplify signalling³⁴ (FIG. 3). Microclusters, although stationary, grew both in terms of the number of BCRs in the microcluster and of the size of the microcluster³⁴. The SPT results described above, in which individual antigen-bound BCRs were immobilized as they entered pre-existing microclusters, suggest that the growth of microclusters may be driven by oligomerization-induced trapping (BOX 2), as proposed by the picket fence model of plasma-membrane organization. By pre-treating B cells with inhibitors of Src family kinases and of SYK, it was shown that the early growth of the BCR microcluster, during the first 60 seconds, was a BCR-intrinsic event, similar to the initial oligomerization itself³⁴. However, growth of the microcluster beyond a certain size at later stages required LYN and SYK activity, and presumably BCR signalling components further downstream. Early microcluster growth may reflect a mechanism by which B cells count the number of antigens captured. The recruitment of BCRs to the microcluster is BCR intrinsic until a sufficient number of BCRs is trapped in the microcluster, which serves as a tipping point for the recruitment of LYN and the triggering of the signalling cascades. Subsequent signalling-dependent microcluster growth may allow the initial signal to be amplified in response to increased antigen. The mechanisms that underlie signalling-dependent microcluster growth are not known but are of interest.

Affinity maturation and class switching

Two hallmarks of humoral immune responses are affinity maturation and class switching³⁵. As antibody responses to immunization mature, and after secondary immunization, antigen-specific antibodies become of increasingly higher affinity and predominantly of the IgG isotypes through the linked molecular processes of somatic hypermutation and class switching. Because the humoral immune response functions through clonal selection, the affinity maturation and class switching of antibody responses presumably reflect an advantage of B cells expressing high-affinity, class-switched BCRs in the selection process. The question is: how early in this process are high-affinity, class-switched BCRs an advantage?

Several important studies have provided strong evidence that high-affinity B cell clones out-compete low-affinity B cell clones for survival in adoptive transfer experiments, presumably reflecting a competition at the clonal level for limiting quantities of antigen, T cell help or appropriate niches^{36–42}. At the level of individual B cells there is evidence that signalling through high-affinity, as opposed to low-affinity, BCRs is qualitatively different⁴³ and, most recently, the affinity of the BCR for antigen was shown to dictate the degree to which B cells spread over antigen-containing fluid lipid bilayers, allowing increased accumulation of antigen and enhanced responses²². Similarly, several seminal studies showed that class-switched B cells out-competed unswitched B cells for survival *in vivo* and that this survival advantage could be attributed to the cytoplasmic tail of membrane-bound IgG^{44,45}.

Both membrane-bound IgM and IgD expressed by unswitched B cells have short, three-amino-acid tails in contrast to all membrane-bound IgG isotypes, which have highly conserved cytoplasmic domains of 28 amino acids^{44,45}. The membrane-bound IgG tail has been demonstrated to enhance B cell proliferative responses to antigen and to trigger signalling cascades that are qualitatively different from those triggered by membrane-bound IgM^{46–49}. Engels *et al.*⁴⁹ recently showed that enhanced signalling through membrane-bound IgG was dependent on the phosphorylation of a tyrosine in the IgG tail that served to recruit growth-factor-receptor-bound protein 2 (GRB2), resulting in sustained kinase activation and generation of second messengers culminating in enhanced B cell proliferation.

Using high-speed, high-resolution SPT TIRFM imaging, we recently provided evidence that the earliest events that occur following antigen binding to the BCR are highly sensitive to both the affinity of the BCR for antigen and the isotype of the BCR^{34,50}. These results place the ability to discriminate antigen affinity and class switching at the tipping point in the initiation of BCR signalling, namely BCR-intrinsic oligomerization. Using an experimental system in which the affinity of BCRs for antigen differed by 50-fold, it was shown that high-affinity BCRs more readily formed immobile BCR microclusters that grew more rapidly, thereby resulting in larger microclusters that recruited more SYK and signalled for more robust calcium responses, than did low-affinity BCRs³⁴. The robust signalling that is triggered by high-affinity BCRs should allow high-affinity B cells to respond to the low levels of antigen that may be presented by APCs.

Using SPT, we also discovered that BCRs of the IgG isotype were dramatically enhanced in their ability to oligomerize and in the growth of the resulting BCR microclusters, ultimately resulting in the increased recruitment of SYK and in more robust calcium responses compared with BCRs of the same affinity for antigen but of the IgM isotype⁵⁰. Through an extensive domain swapping and mutagenesis analysis it was determined that the 12 membrane-proximal residues of the IgG tail conferred enhanced oligomerization⁵⁰. This region of the tail does not contain the tyrosine residue that is phosphorylated following IgG-

containing BCR crosslinking and that recruits GRB2 (REF. 49). Thus, these studies revealed a new function for the membrane-proximal region of the IgG tail, which was not previously appreciated to have a role in IgG-containing BCR signalling. We speculate that this region of the IgG tail binds to an adaptor protein of some sort or interacts with the inner leaflet of the plasma membrane in such a way as to promote the stabilization of the BCR oligomer and growth of the BCR microcluster.

These results place both affinity maturation and class switching at the earliest step in the initiation of BCR signalling: the point of BCR oligomerization. At this tipping point, relatively small differences in affinity and a small portion of the immunoglobulin tail make a big difference during B cell activation.

How BCR oligomerization triggers signalling

The studies reviewed thus far indicate that BCRs oligomerize and form microclusters in a BCR-intrinsic fashion, raising the important question: how do BCR oligomers recruit LYN and trigger the signalling cascade? One simple explanation is that the structure of the cytoplasmic domain of the BCR monomer is distinct from that of the oligomer and that LYN can only phosphorylate the oligomeric form (FIG. 4a). In this model it might be predicted that LYN is constitutively associated with the cytoplasmic domains of some monomeric BCRs but only phosphorylates BCRs in *trans* when the BCRs are clustered. If this model is correct, oligomerization should be sufficient to trigger signalling.

However, other studies suggest that the mechanisms for the recruitment of LYN to the BCR oligomers are more complicated. One model is based on the observation that the BCR oligomer perturbs the local lipid microenvironment, leading to the coalescing of lipid rafts⁵¹ around the BCR oligomers. Various other studies indicated that crosslinking of BCRs resulted in their association with detergent-insoluble lipid rafts⁵². More recently, we provided evidence that in living B cells, antigen-engaged BCR microclusters transiently associated with lipid rafts, and that this was followed by a more stable association of the BCR microcluster with lipid-raft-tethered LYN⁵³. These results suggest that the recruitment of LYN to the BCR oligomers is a repercussion of the association of the BCR oligomers with lipid rafts (FIG. 4b). It is not known how the BCR microclusters perturb the local lipid environment but, as reviewed by Viola and Gupta⁵⁴, it is possible that BCR microclusters signal for local dissolution of the membrane cytoskeleton, allowing the coalescence of lipid rafts with the BCR microclusters. Such perturbation of the local lipid microenvironment has been observed both for BCR oligomers formed in response to monovalent membrane-associated antigens and for BCRs physically crosslinked by multivalent antigens^{53,55}. This suggests that the ability of BCR microclusters to perturb the local lipid environment, resulting in the recruitment of LYN, may provide a common mechanism by which BCRs responding to multivalent or monovalent membrane-associated antigens initiate signalling.

Lipid perturbation may have additional roles in transducing signals. Saturated raft lipids form thicker membranes⁵⁶ and have the potential to induce a pronounced curvature in the membrane⁵⁷ that could cause alterations in the BCR cytoplasmic domains, providing a docking site for LYN. Consistent with these possibilities are results from an analysis of BCRs that contained FRET donor and acceptor fluorescent proteins in their cytoplasmic domains⁵. After antigen binding, BCRs clustered and FRET occurred between the cytoplasmic domains, indicating that they were in close molecular proximity. However, the FRET signal then rapidly decreased, although the oligomers stayed intact, indicating a change in the distance between the cytoplasmic domains. This decrease in FRET was referred to as an 'opening' of the BCR cytoplasmic domain and this opening occurred simultaneously with the association of the BCR microcluster with LYN^{5,53}. The finding

mirrored the results of Wucherpennig and colleagues⁵⁸ showing that the cytoplasmic tail of the CD3 ϵ chain of the TCR associated with the membrane through the deep insertion of two key tyrosines, resulting in their sequestration from Src kinases in the unligated receptor. In the ligated TCR these tyrosines were phosphorylated. Although the mechanism by which the CD3 ϵ tail became accessible is not known, it is possible that perturbations in the local lipid environment induced the dissociation of the CD3 ϵ tail from the membrane.

To fully understand the molecular basis of the antigen-induced initiation of BCR signalling it will be essential to determine the structure of the BCR and, ultimately, the entire structure as it exists in the membrane. An important step towards this goal was the recent solution of the structure of a disulphide-linked homodimer of Ig β , which allowed the modelling of an Ig α -Ig β heterodimer with the existing structure of the C α 4 domain⁵⁹ (Supplementary information S2 (figure)). Combined with solution- binding studies and microcluster mutagenesis analyses, these results predicted an extensive contact surface between membrane-bound immunoglobulin and both Ig α and Ig β through multiple charged residues. The extensive interactions between membrane-bound immunoglobulin and the Ig α -Ig β heterodimer suggest the possibility that changes in the membrane-proximal region of the membrane-bound immunoglobulin induced by binding to membrane-tethered antigens could alter the association of the membrane-bound immunoglobulin with Ig α -Ig β . In turn, this could result in changes in the cytoplasmic domains of Ig α -Ig β that recruit LYN (FIG. 4c). Although these are still early days, current evidence from live-cell imaging and from structural studies of the BCR suggest that the information that antigen has bound to the BCR ectodomain may be transduced across the membrane by perturbations of either the local lipid microenvironment or by the association of the ectodomain of the membrane-bound immunoglobulin with the Ig α -Ig β heterodimer.

Regulation of the initiation of BCR signalling

B cell responses are highly regulated by various co-receptors, raising the question: are the earliest events in the initiation of BCR signalling targets of regulation? Although, to date, few studies have addressed this question, the early indication is that two key regulators of BCR signalling, the Fc receptor IIB for IgG (Fc γ RIIB) and CD19, influence early events.

Fc γ RIIB expressed by B cells is a potent inhibitor of BCR signalling⁶⁰. Fc γ RIIB blocks BCR signalling at steps downstream of the early activation events by reversing the BCR signalling-induced activation of PI3K through the recruitment of the lipid phosphatase SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP) to the co-ligated complex. In addition, when co-ligated to the BCR, Fc γ RIIB inhibits the formation of immobile BCR oligomers by an unexpected mechanism⁶¹. FRET analyses showed that Fc γ RIIB-mediated blocking of BCR oligomerization did not require molecular proximity of the cytoplasmic tails of the BCR and Fc γ RIIB, but instead appeared to be the repercussion of the ability of Fc γ RIIB to tip the balance of the association of the BCR with raft lipids by associating itself with raft lipids in a highly stable way. Consistent with this interpretation, a loss-of-function mutation in the transmembrane domain of Fc γ RIIB that inhibits the ability of ligated Fc γ RIIB to associate with lipid rafts^{62,63} blocked the ability of Fc γ RIIB to inhibit BCR oligomerization. The ability of the Fc γ RIIB to target two different points in the BCR signalling pathway through two entirely different mechanisms may account for its potent inhibitory function.

CD19 has also recently been shown to have an integral role in early BCR signalling events⁶⁴. CD19 is an activating B cell co-receptor that is phosphorylated in response to BCR antigen binding and functions as a molecular adaptor that recruits VAV, PI3K and LYN to augment BCR signalling⁶⁵. CD19 is expressed on the surface of B cells, both alone and as

part of a complex containing CD21, and is the receptor for the Cd3 component of complement, allowing complement-coupled antigen to co-ligate the CD19–CD21 complex with the BCR. Batista and colleagues⁶⁴ provided evidence that CD19 alone functions to regulate early events in BCR signalling. They showed that in response to antigen presented on fluid lipid bilayers, BCR microclusters transiently associated with CD19. This association seemed to be essential for BCR activation, because CD19-deficient B cells failed to form signalling-active BCR microclusters or to trigger the spreading response, suggesting that the BCR–CD19 complex may be a basic signalling unit.

Collectively, these studies indicate that the earliest events in the initiation of BCR signalling are regulated by co-receptors. Understanding which B cell-surface receptors influence these early events and the mechanisms underlying their effects may reveal new tipping points in the initiation of BCR signalling.

Alterations in early signalling events in disease

Understanding that the early BCR-intrinsic events in the initiation of BCR signalling are regulated raises the possibility that naturally occurring mutations affecting BCR oligomerization could result in spontaneous BCR clustering and hyperactivation of B cells, as occurs in systemic autoimmune disease or even tumorigenesis. In collaboration with Staudt and colleagues, we recently analysed the mobility of BCRs on activated B cell-like diffuse large B-cell lymphomas (DLBCLs) that a small interfering RNA screen had demonstrated were dependent on the BCR for survival⁶⁶. Remarkably, we observed that the BCRs on these tumours were immobilized in microclusters. By contrast, BCRs on several B cell tumour types that were not dependent on the BCR for survival, including Burkitt's lymphoma, mantle cell lymphoma and germinal centre B-cell-like DLBCL, were highly mobile. These findings are exciting because they raise the possibility that by understanding the molecular basis of constitutive oligomerization, therapies could be developed to selectively disrupt the BCR oligomers. It is of interest that constitutive oligomerization of the Epstein Barr virus (EBV) latency membrane protein 2A (LMP2A), which contains ITAMs in its cytoplasmic tail and functions to mimic BCR signalling, is required for the transformation of B cells by EBV⁶⁷. It will be of interest to determine the activation state of BCRs in cells of individuals with systemic autoimmune disease, with a view towards targeting spontaneous oligomerization for therapy.

Similarities in immunoreceptor signalling

The BCR, TCR and FcεR1 are all members of the family of MIMRs, raising the question: do these receptors initiate signalling by similar mechanisms? The engagement of antigen by the BCR, TCR and FcεR1 induces receptor microclusters that are essential for productive signalling. Each receptor has also been shown to associate with detergent-insoluble lipid rafts after antigen engagement, suggesting similar mechanisms for signal transduction⁵². The TCRs engage antigenic peptides bound to MHC monomers on the surface of APCs, suggesting that physical crosslinking of the TCRs may not be required for activation. Two-colour SPT imaging in conjunction with confocal laser-scanning microscopy provided evidence that antigen engagement of the TCR resulted in the immobilization of the kinase LCK, CD2 (a TCR co-receptor) and linker for activation of T cells (LAT), which is an adaptor protein in the TCR microclusters⁶⁸. Similarly, our recent studies showed that antigen engagement by the BCR resulted in the immobilization of SYK with BCR microclusters¹¹. For FcεR1, McConnell and colleagues^{69,70} showed that monovalent antigen incorporated into a fluid lipid bilayer was sufficient to trigger the aggregation of IgE-loaded FcεR1 on mast cells, as well as their degranulation. These authors proposed that monovalent mobile antigen aggregated the FcεR1 by means of thermodynamic forces acting on a

topologically constrained system, although they did not rule out a role for antigen-induced conformational changes in the FcεR1. In recent experiments, Lidke and colleagues⁷¹ used SPT to follow FcεR1 molecules that were labelled with quantum dots (BOX 1) in cells expressing fluorescent actin. They showed that the highly mobile FcεR1 molecules became immobilized within seconds of antigen binding in an actin-dependent manner at high antigen concentration. However, at low antigen concentration, small FcεR1 oligomers remained mobile and signalling active, suggesting that in mast cells the organization of the actin cytoskeleton might serve not to trap oligomers efficiently but to support mast cell degranulation⁷².

Collectively, these data suggest that the BCR, TCR and FcεR1 may share common mechanisms for the initiation of receptor signalling that involve immobilization of receptors and membrane perturbations. Future SPT imaging of TCRs and mutational studies of the TCR and FcεR1 to determine what features of the receptor are required for immobilization in signalling complexes should reveal just how similar these processes might be.

Conclusions and perspectives

High-resolution live-cell imaging has provided the first view of the events that occur within seconds of the encounter of BCRs with antigen and that ultimately lead to B cell activation. So far, this technology has been used to explore only a small area of B cell biology. It will be of interest to understand how BCRs behave on B cells at different developmental stages and in diseases that involve B cell hyper- or hypoactivation. Further advances in multi-colour SPT should provide the tools necessary to meet the next challenge of understanding the dynamic behaviour of the BCR in relation to important signalling molecules and cell structures. Ultimately, the goal of such studies is to understand the tipping points in B cell activation, with the long-term goal of applying this knowledge to the development of therapies for autoimmunity and B cell cancers and to the engineering of effective vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Brzostowski for expert comments on live-cell imaging techniques. This work has been supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

Glossary

Somatic hypermutation	A unique mutation mechanism that is targeted to the variable regions of rearranged immunoglobulin gene segments. Combined with selection for B cells that produce high-affinity antibodies, somatic hypermutation leads to affinity maturation of B cells in germinal centres
Class switching	The somatic recombination process by which the class of immunoglobulin is switched from IgM to IgG, IgA or IgE
Microclusters	Microscopic assemblies of receptor oligomers in the plasma membrane that recruit signalling molecules. They first form in the contact areas between the B cell and antigen-presenting cell, and

	ultimately form the central supramolecular activation cluster of the immune synapse
BCR clustering and capping	The binding of multivalent ligands to B cell receptors (BCRs) induces the redistribution and aggregation of the bound receptors into clusters (clustering). Capping, which requires metabolic energy and cytoskeleton dynamics, represents the coalescence of clusters to form a single aggregate called a cap
Immune synapse	The specialized contact area between a T or B cell and one or more antigen-presenting cells. The synapse is dynamic and shows lipid and protein segregation, signalling compartmentalization and bidirectional information exchange through soluble and membrane-bound transmitters
Lamellipodia	Thin sheet-like processes that extend at the leading edge of moving cells. They are actin-rich zones formed in response to chemokine signals, and propel a migrating cell forward
Lipid rafts	Cholesterol- and sphingolipid-rich membrane microdomains that provide ordered structure to the lipid bilayer and have the ability to include or exclude specific signalling molecules and complexes
Small interfering RNA	Short double-stranded RNAs of 19–23 nucleotides that induce RNA interference, a post-transcriptional process that leads to gene silencing in a sequence-specific manner

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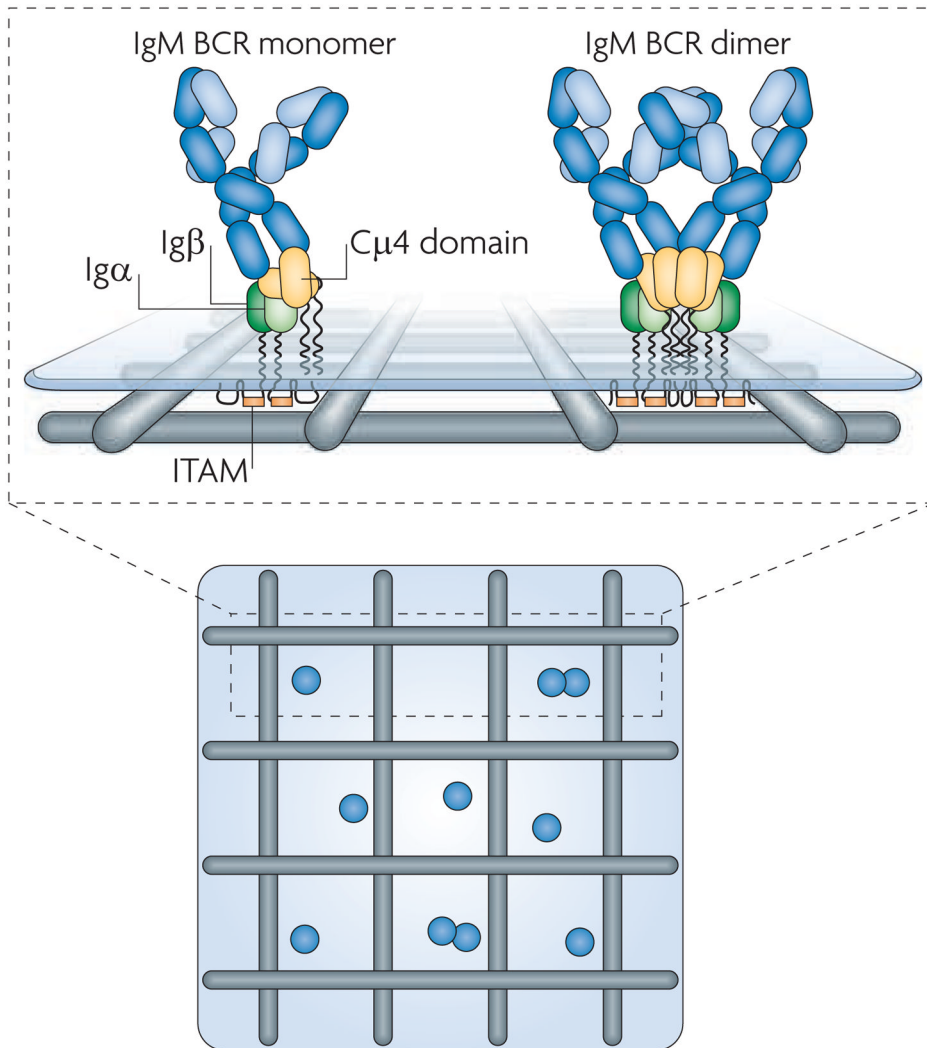


Figure 1. The structural organization of the B cell receptor

The four-chain structure of the IgM B cell receptor (BCR), composed of the membrane form of IgM associated with a covalently linked heterodimer of Iga and Igβ, is depicted. Cell surface BCRs are shown in a compartmentalized plasma membrane such as that proposed by Kusumi and colleagues (BOX 2). This schematic is based on observations from single particle tracking experiments from live-cell imaging¹¹ showing that the BCRs exist mainly as highly mobile monomers on resting B cells, with only ~20% comprising less-mobile dimers or more highly ordered oligomers. The membrane-proximal Cμ4 portion of the membrane-bound IgM (or the Cγ3 portion of membrane-bound IgG, not shown) was determined to be required and sufficient to induce BCR oligomerization and immobilization upon membrane-bound monomeric antigen binding¹¹. The immunoreceptor tyrosine-based activation motif (ITAM)-containing cytoplasmic tails of the BCR extend through the cytoskeleton into the cytoplasm.

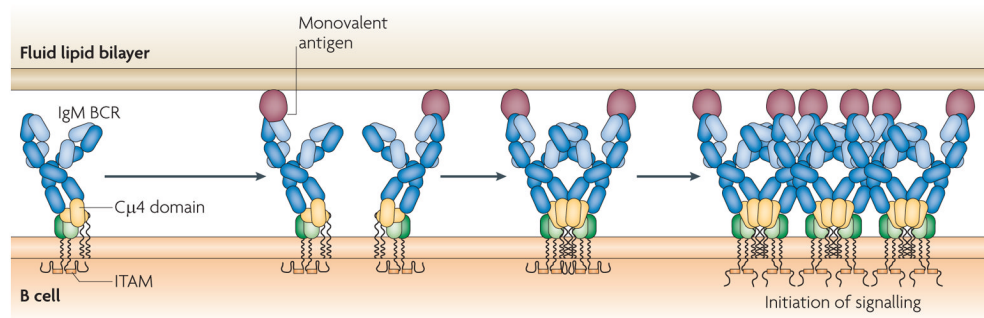


Figure 2. The 'conformation-induced oligomerization model' for B cell receptor microcluster formation

Depicted is an IgM B cell receptor (BCR) in the absence of antigen and in a conformation that is not receptive to oligomerization. The binding of monovalent antigen in a fluid lipid bilayer that cannot physically crosslink the BCR exerts a force that brings the BCR into an oligomerization-receptive form. The subsequent random bumping of antigen-bound BCRs results in their oligomerization through the membrane-proximal C μ 4 domain and in the initiation of signalling. ITAM, immunoreceptor tyrosine-based activation motif.

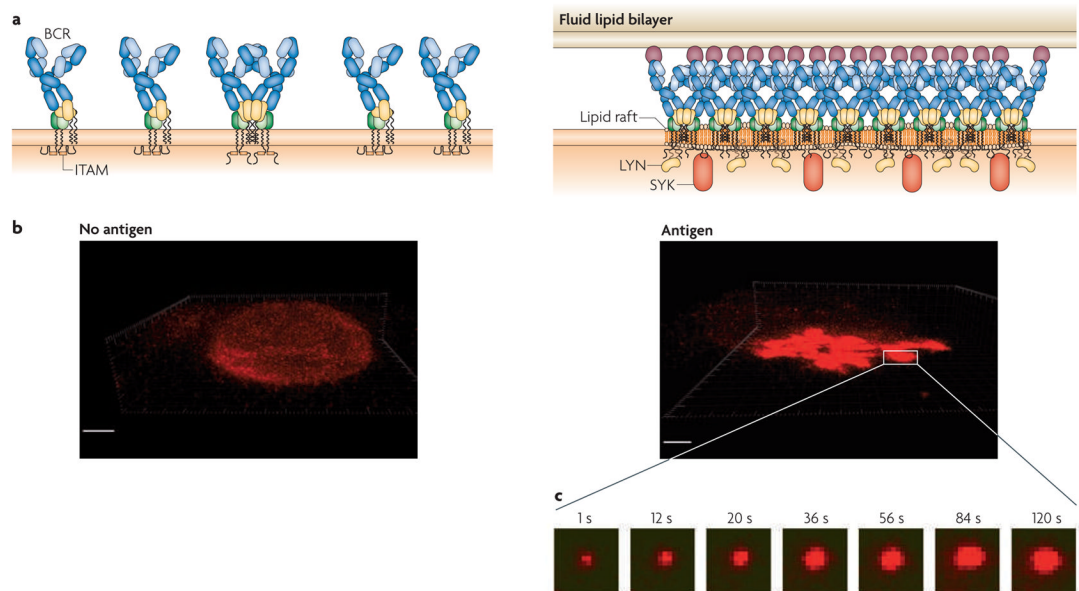


Figure 3. B cell microclusters grow with time after antigen binding

a | B cell receptors (BCRs) in the absence of antigen (left) and in the presence of antigen (right). **b** | Confocal images showing the spatial distribution of BCRs on the surface of a B cell placed on a fluid lipid bilayer in the absence of antigen (left), showing the BCRs covering the entire B cell surface, and (right) on an antigen-containing fluid lipid bilayer, showing the BCRs accumulating at the interface of the B cell with the bilayer. **c** | Total internal reflection fluorescence microscopy images demonstrating the growth, over time, of the microcluster within the area indicated by the white box. SYK, spleen tyrosine kinase.

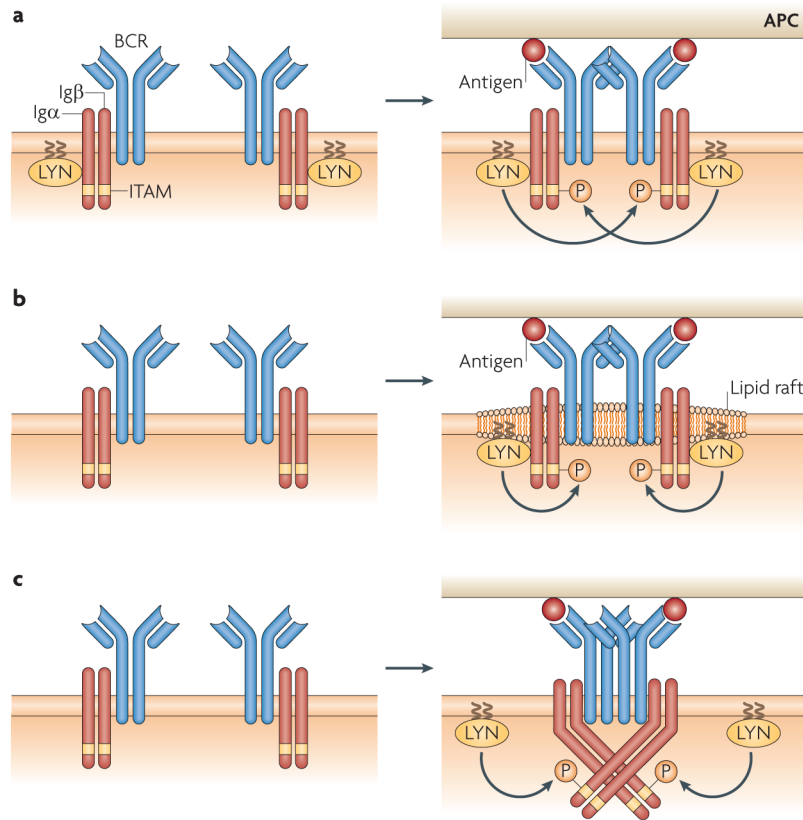


Figure 4. Models for how B cell receptor oligomers trigger signalling

a | B cell receptor (BCR) oligomers may simply bring the BCR-associated **tyrosine** kinase LYN into close proximity with the Igα–Igβ heterodimer for *trans*-phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs). **b** | Alternatively, they may perturb the local lipid environment, causing the coalescence of lipid rafts and bringing the lipid-raft-associated LYN into close proximity with ITAMs of the Igα–Igβ heterodimer for phosphorylation. **c** | Yet another possibility is that BCR oligomers alter the association of membrane-bound immunoglobulin and Igα–Igβ, inducing changes in the cytoplasmic tails of Igα–Igβ and allowing LYN to access the ITAMs for phosphorylation. APC, antigen-presenting cell.