

Receptor Dissociation and B-Cell Activation

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Abstract The B-cell antigen receptor (BCR) is one of the most abundant receptors on the surface of B cells with roughly 100,000–200,000 copies per cell. Signaling through the BCR is crucial for the activation and differentiation of B cells. Unlike other receptors, the BCR can be activated by a large set of structurally different ligands, but the molecular mechanism of BCR activation is still a matter of controversy. Although dominant for a long time, the cross-link model (CLM) of BCR activation is not supported by recent studies of the nanoscale organization of the BCR on the surface of resting B cells. In contrast to the prediction of CLM, the numerous BCR complexes on these cells are not randomly distributed monomers but rather form oligomers which reside within membrane confinements. This finding is more in line with the dissociation activation model (DAM), wherein B-cell activation is accompanied by an opening of the auto-inhibited BCR oligomers instead of a cross-linking of the BCR monomers. In this review, we discuss in detail the new findings and their implications for BCR signaling.

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Current Topics in Microbiology and Immunology (2016) 393: 27–43
DOI 10.1007/82_2015_482

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Published Online: 2 October 2015

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1 Introduction

The humoral immune response depends on the activation and clonal expansion of B cells and their differentiation into antibody-producing plasma cells. A B-cell recognizes a specific antigen via its B-cell antigen receptors (BCR), comprising the membrane-bound form of the immunoglobulin (mIg) and the Ig α /Ig β heterodimer, the former acting as the antigen binding subunit and the latter as the signaling subunit. Signals from the BCR control the proliferation and differentiation of B cells. These signals are initiated by the binding of the BCR to its cognate ligand (referred to as antigen). Antigen binding is accompanied by a spatial reorganization of the BCR and its interaction with two different protein tyrosine kinases, namely the Src-family kinase Lyn and the spleen tyrosine kinase (Syk). As a consequence, the two tyrosines of the immune receptor tyrosine-based activation motif (ITAM) located in the cytosolic tails of Ig α and Ig β are phosphorylated. This results in the formation of a BCR/Syk complex and the activation of several different signaling pathways controlled by the BCR.

B cells express a large amount of BCR complexes on their cell surface. How these abundant receptors remain silent on resting B cells and how they are activated by a diverse set of structurally different antigens are core questions of B-cell biology. Studies from the past decades have falsified several features of the cross-linking model (CLM) of BCR activation. For example, contrary to the predictions of CLM, the numerous BCR complexes are not monomers on the surface of resting B cells but rather are organized as auto-inhibited oligomers. The binding of an antigen to the BCR also does not primarily result in a cross-linking but to a disruption of the oligomeric BCR organization, as discussed in detail below.

2 The Structure of the BCR Complex

In its monomeric form, the BCR comprises the mIg molecule and the Ig α /Ig β heterodimer (Fig. 1a) (Hombach et al. 1990a). The mIg is nearly identical to an antibody. The two molecules differ only in the C-terminus of the heavy chain

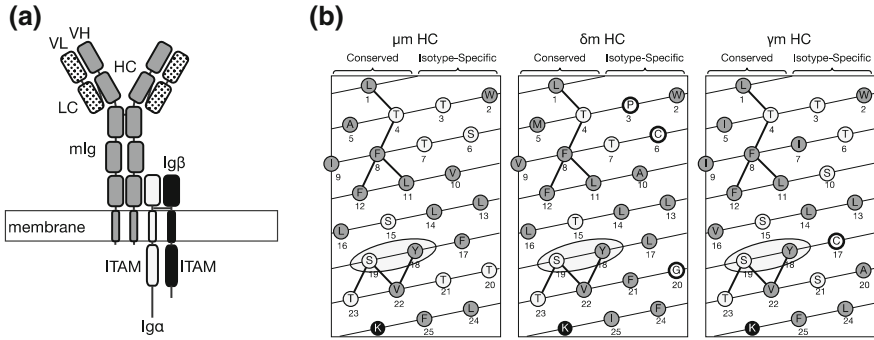


Fig. 1 Schematic drawing of IgM-BCR and comparison of the TM region of HCs of different isotypes. **a** Schematic drawing showing the components of BCR. **b** Scheme of the TM α -helix of membrane-bound μ , δ , and γ HC. Amino acids are indicated by single-letter code in *circles* with different *shades* indicating their properties (*Light*, polar/hydrophilic; *Dark*, nonpolar/hydrophobic; *Black* charged; *White* with *thicker* outline, C, P, G). Conserved amino acids in the conserved side of the TM region were indicated by the *thick lines* connecting them. And the tyrosine and serine residues involved in the binding of Ig α /Ig β were highlighted by an *ellipse*

(HC) where the antibody HC ends with a short hydrophilic sequence, while the mIg HC (mHC) ends with a longer sequence, part of which is the hydrophobic trans-membrane (TM) region that anchors the mIg molecule in the membrane (Alt et al. 1980; Rogers et al. 1980). Each B cell expresses mIgs with a unique antigen binding specificity, which is determined by the combination of the variable domains (VH and VL) of the mHC and the light chain (LC).

While the mIg molecule binds to antigen, the Ig α /Ig β heterodimer functions as the signaling subunit of the BCR (Reth 1989). Ig α and Ig β share similar structural features. They both carry an extracellular Ig domain, an evolutionarily highly conserved TM region, and a cytoplasmic tail with an ITAM motif. Ig α is covalently associated with Ig β through an extracellular disulfide bond between cysteine residues situated at the linker regions between the extracellular Ig domains and the TM regions (Siegers et al. 2006).

All five mIg isotypes (IgA, IgD, IgE, IgG, and IgM) are non-covalently associated with the common Ig α /Ig β heterodimer (Venkitaraman et al. 1991). The proper assembly of mIg with Ig α and Ig β in the endoplasmic reticulum is required for export of the BCR onto the B-cell surface (Hombach et al. 1990b). An exception to this rule is a special, GPI-linked form of the mIgD molecule that comes on the surface without Ig α /Ig β (Wienands and Reth 1992). Important contact sites between the mIg molecule and the Ig α /Ig β heterodimer are formed both by the membrane-proximal Ig domains of mIg and the Ig domains of Ig α and/or Ig β , as well as the TM regions of the respective molecules with each other (Hombach et al. 1990a; Radaev et al. 2010). A comparison of the TM region of all mIg isotypes (Fig. 1b), which probably traverse the membrane as a single α -helix, revealed a conserved side, which is implicated in Ig α /Ig β binding (Reth 1992). Mutation of a

tyrosine and a serine residue on this side of the μ m HC prevents proper BCR assembly (Sanchez et al. 1993; Shaw et al. 1990).

Formerly, it was thought that, similar to the T-cell antigen receptor (TCR), the BCR is a symmetric complex with each of the two mHCs of the mIg molecule bound to one Ig α /Ig β heterodimer (Reth 1992). However, this 1:2 stoichiometry was not confirmed by blue native polyacrylamide gel electrophoresis (BN-PAGE), which, together with other biochemical experiments, clearly showed a 1:1 stoichiometry of the mIg:Ig α /Ig β complex (Schamel and Reth 2000). A 1:1 stoichiometry was also found by comparing the fluorescence ratio of labeled mIg to labeled Ig α or Ig β components across a range of expression levels (Tolar et al. 2005). Although we still lack a confirmatory crystal structure, it is generally accepted that in its basic structure, the BCR is composed of one mIg molecule binding to a single Ig α /Ig β heterodimer.

3 The Resting BCR and Models of Its Activation

3.1 *The Monomeric BCR and the CLM*

In 1972, the fluid mosaic model for the organization and behavior of membrane lipids and proteins was proposed (Singer and Nicolson 1972). Although the possibility of lateral protein–protein, protein–lipid, and lipid–lipid interactions was discussed in the original publication, one widely accepted aspect of this model was that membrane proteins are randomly distributed and freely diffusing in the lipid bilayer of the plasma membrane. The fluid mosaic model was also the basis for the CLM, which suggested that the numerous BCR complexes exist as freely diffusing signaling-inert monomers on the resting B-cell surface and that it is the cross-linking of two BCR monomers by multivalent antigen or anti-HC antibodies which initiates the BCR activation process. The CLM was supported by the finding that only dimeric F(ab)₂ but not monomeric Fab fragments of anti-BCR antibodies could stimulate BCR, using the lymphocyte agglutination test and uridine/thymidine uptake as a readout for B-cell stimulation (Woodruff et al. 1967). However, alternative explanations for these observations (see below) were not considered in this publication. Further support for CLM came from *in vivo* experiments showing that monovalent antigens are often poor vaccines in that they do not generate high antibody titers (Dintzis et al. 1976; Puffer et al. 2007; Schlossman et al. 1966). Some monovalent antigens, however, can stimulate B cells very well (Avalos et al. 2014; Kim et al. 2006). At the time the CLM was proposed, it was not possible to directly monitor the nanoscale organization of the BCR on the B-cell surface. Thus, the notion that the many BCR complexes randomly diffuse as monomers on the surface of resting B cells was not experimentally proven at that time.

One attempt to experimentally verify the assumptions of CML was undertaken by Tolar et al. (2005). They studied the behavior of BCR complexes on living cells employing the Förster resonance energy transfer (FRET) method (Tolar et al. 2005).

Since the energy transfer from the FRET donor to the FRET acceptor is based on dipole–dipole coupling, FRET can only occur if molecules are within close proximity to each other, typically less than 100 Å (Förster 1946). This feature makes FRET a powerful tool for studying protein–protein interaction in living cells. In their study, Tolar et al. used monomeric versions of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) as FRET a donor and acceptor, respectively. They fused the donor and acceptor to Ig α and reconstructed IgM-BCR and IgG-BCR in an Ig α -deficient B-cell line with these constructs. They found for both BCR isotypes, a low FRET efficiency between the CFP- and YFP-tagged Ig α . This result was taken as a proof that BCRs are monomers on the B-cell surface. Unfortunately, FRET efficiency is influenced by many factors, such as the abundance ratio between the donor and acceptor or the orientation of the FRET pairs, which were not well controlled in this study. Therefore, while a high FRET value can indicate a protein–protein interaction, a low FRET value does not prove the absence of a protein–protein interaction (Vogel et al. 2006). For example, in the same publication, a rather low FRET value was found for the bona fide Ig α /Ig β heterodimer (Tolar et al. 2005). In summary, FRET is clearly not the appropriate method to prove the absence of BCR homo-oligomers on the surface of B lymphocytes.

3.2 The Monomeric BCR and the Conformation-Induced Oligomerization Model

With the development of total internal reflection microscopy (TIRFM), single particle tracking (SPT) methods have been used to follow movements of BCR clusters on the surface of living cells (Tolar et al. 2009). After staining surface BCRs with very low amounts of fluorescent anti-Ig Fab fragments, Tolar et al. studied the lateral diffusion of the labeled BCRs on B cells with TIRFM. They found that during the spreading of B cells on antigen-coated bilayers, the mobility of the BCRs is reduced. BCR complexes become immobile and converge to microclusters. This result was taken as evidence for the existence of freely diffusing BCR monomers that oligomerize during B-cell activation. In addition, they proposed that the immobilization and clustering of BCR were mediated by the constant region of mHC. Based on these results, they proposed a conformation-induced oligomerization model in which the constant region (Fc) of HC contains a homo-typic clustering interface that is not accessible in the resting fast-diffusing BCR monomers. According to this model, antigen binding induces a conformational unmasking of the Fc interface, resulting in BCR clustering and signaling (Pierce and Liu 2010).

The problem with these SPT studies using a light microscope with a diffraction limit of 250 nm is that one does not directly “see” single BCR complexes of about 10 nm dimensions. Since only a very small portion of the BCR is labeled in these

experiments, it is impossible to determine whether the single Fab labels an isolated BCR monomer or a BCR as part of an oligomer. One thus tries to obtain insight into the conformation and behavior of individual BCR molecules from the study of BCRs with unknown molecular composition. This can lead to quite misleading conclusions. For example, by overestimating the resolution of SPT, the concatenation of large membrane structures that occurs on the scale of hundreds of nanometers can be misinterpreted as the cross-linking of the BCR which takes place in the 10–30 nm range.

3.3 The Higher Organization of the Lymphocyte Membrane

It is rare that a scientific model is not adapted from its original form to fit with new findings as they accumulate over time. Indeed, the fluid mosaic model has been challenged from early on when limited diffusion of both lipids and membrane proteins were discovered (Chang et al. 1981; Fowler and Branton 1977; Schindler et al. 1980). More recently, the development of the high-speed SPT technique and the tracking of the lateral movement of a single labeled target with a temporal resolution of 25 μ s have challenged the notion of freely diffusing proteins in the lipid bilayer (Fujiwara et al. 2002). These studies on fibroblasts showed a partitioning of membrane proteins into submicron compartments which limits their free diffusion and only rarely allows “hopping” between compartments (Kusumi et al. 2005). It was proposed that the compartment boundaries are generated by the actin cytoskeleton, functioning as a “fence” with the cytoskeleton-anchored membrane proteins as “pickets.” This view is supported by experiments showing that small drug inhibitors of the cytoskeleton alter the diffusion behavior of membrane proteins (Andrews et al. 2008; Charrier et al. 2006; Treanor et al. 2010).

A compartmentalization of membrane proteins was not only found on fibroblasts but also on lymphocytes. Early studies of membrane sheets from mast cells with the transmission electron microscope (TEM) showed clear evidence for a clustering of receptors at nanodistances (Wilson et al. 2001). These studies were extended by Lillemeier et al. and indicate that not only the TCR, but most proteins on the T lymphocyte membrane reside in nanosized compartments called protein islands (Lillemeier et al. 2006). The TEM analysis of the T-cell plasma membrane was complemented by superresolution microscopic studies using photoactivated localization microscopy (PALM) or direct stochastic optical reconstruction microscopy (dSTORM) (Lillemeier et al. 2010; Owen et al. 2010).

3.4 The Oligomeric BCR and the DAM Hypothesis

Accompanying the changing view of membrane protein and lipid organization, newer studies have revealed that many receptors on the cell surface are actually not

monomers but rather form oligomers already before ligand binding. Typical examples are the insulin receptor (IR) (de Meyts 2008), the epidermal growth factor receptor (EGFR) (Tao and Maruyama 2008) and, under certain conditions, the T-cell receptor (TCR) (Fernández-Miguel et al. 1999). This seems also to be the case for the BCR. The search for a higher ordered BCR structure started with the finding that the mIg and Ig α /Ig β heterodimer form a 1:1 complex. In such a complex, the hydrophilic amino acids in mHC TM region would be partially exposed to the hydrophobic lipid environment but could be sheathed if it would form BCR homo-oligomers (Reth et al. 2000; Schamel and Reth 2000). The first hint for an oligomeric organization of the BCR was found by a BN-PAGE analysis. Both the IgM-BCR and the IgD-BCR, when solubilized with mild detergent, run as large macro-molecular complexes in such gels. In addition, it was found that a mutation of all hydrophilic and aromatic amino acids on the isotype-specific side of the TM region of the δ m HC reduced the size of the BCR oligomer. Because the detection of BCR oligomers by BN-PAGE is dependent on the detergent used for lysis, it is possible that the complexes are artifacts of the cellular lysis.

To test whether the BCR also forms oligomers in living cells, we next employed the bifluorescence complementation (BiFC) method (Yang and Reth 2010a). This method is based on the reconstruction of a complete fluorescent protein from two non-fluorescent fragments. This reconstruction is facilitated when the non-fluorescent fragments are coupled to proteins that interact with each other (Kerppola 2006). In this way, one can monitor a protein–protein interaction in living cells by the amount of fluorescence generated. We fused the half-domains of YFP and CFP (YN and CC) to Ig α and expressed these proteins together with Ig β and the mIg molecule in *Drosophila* S2- or Ig α -deficient B cells. The fluorescence signal from the reconstructed YFP then allowed us to monitor the oligomerization of the BCR on living cells. This study showed that the formation of oligomers is an intrinsic feature of the BCR.

Having in hand, an assay for BCR oligomerization allowed us to search for BCR mutants that are defective in this process. We thus found a double-mutant form of the IgD-BCR that failed to form oligomers. The BCR mutant had several amino acid alterations at the isotype-specific side of the TM region of the δ m HC and lacks the Ig α cysteine that mediates the disulfide bridge between Ig α and Ig β . The involvement of the isotype-specific side of the mHC TM region in BCR oligomerization is in line with our previous BN-PAGE results (see above). Importantly, the monomeric BCR mutant was found to be more active and less stably expressed on the B-cell surface. In contrast, a BiFC-stabilized BCR dimer is less active in signaling and is internalized more slowly. These findings contradict the assumption of the CLM that BCR monomers do not signal and suggest that BCR oligomerization keeps the receptor silent and more stably expressed on the B-cell surface. Based on these findings, we proposed that most BCR complexes form auto-inhibited oligomers in resting B cells. Inside the BCR oligomer, the ITAMs of the Ig α /Ig β may be orientated in a way that they are not directly available for the phosphorylation mediated by kinases such as Lyn and Syk. Upon antigen binding, BCR oligomers could open, exposing the ITAM of Ig α /Ig β tail and

initiating intracellular signaling. Thus, B-cell activation seems to involve the dissociation of BCR oligomers rather than the cross-linking of BCR monomers as suggested by CLM. We thus named this model as dissociation activation model or DAM (Yang and Reth 2010b).

This new model seems contradictory to the observation that BCR is activated by dimeric $F(ab)_2$ but not monomeric Fab fragments of anti-BCR antibodies (see above). However, in the frame of DAM, an alternative explanation for this experiment is that only the dimeric $F(ab)_2$ is able to disrupt the BCR oligomer by keeping two BCRs apart. In fact, previous findings explained by CLM could all be explained by DAM as well. It is also notable that the DAM may not only apply to BCR, but rather represent a common type of mechanisms for surface receptors. For example, it was found recently that pigment epithelium-derived factor (PEDF) receptors form homo-oligomers under basal conditions, and PEDF dissociates the homo-oligomer to activate the receptors (Cheng et al. 2014).

3.4.1 Dissociation Activation of the BCR

Due to the irreversibility of the BiFC assay, it is only possible to monitor the formation but not the dissociation of BCR oligomers. The receptor dissociation, as predicted by the DAM, could not be directly proven by such an assay. Other studies also challenged the notion that oligomers are the dominant form of the BCR on resting B cells (Pierce and Liu 2010). For example, in the already mentioned SPT studies of the diffusion behavior of the BCR, it was found that 20 % of these receptors are immobile (Tolar et al. 2009). The authors later suggested that these 20 % of immobile receptors are oligomers and that they are a minority rather than the dominant form of the BCR on the surface of resting B cell (Pierce and Liu 2010). However, as already discussed, it is rather difficult from studies of the diffusion behavior of receptors with a light microscope to come to valid conclusion about the nanoscale organization of receptors.

With the emergence of superresolution microscopy, it is now possible to reach such optical resolution, beyond the diffraction barrier with fluorescence labeled proteins (Huang et al. 2010). Mattila et al. (2013) have used this method to study the BCR organization in primary cells. In line with our finding, both IgD-BCR and IgM-BCR were found to be mostly organized as preformed nanoclusters in resting B cells. Surprisingly, the authors found that the size and distribution of BCR nanoclusters are not altered after attaching the Fab-labeled cells to a stimulatory antigen-coated surface. This is in conflict with other studies finding major alterations in the organization or movement of the BCR upon B-cell activation (Harwood and Batista 2008; Tolar et al. 2009). The authors suggest that in their study, only a small portion of the BCR gets activated and thus the majority of the BCRs are still in the resting state. However, it has been shown that the majority of the BCR complexes form microclusters when the B cells are placed on similar or identical antigen-coupled lipid bilayers (Fleire et al. 2006; Tolar et al. 2009). It is notable that dSTORM does not really achieve single molecular resolution directly.

dSTORM images are actually results of complicated calculations. Thus, the interpretation of a dSTORM image is strongly affected by parameters such as the labeling efficiency, over-counting problems, and the data-processing procedure. This may be the source of the discrepancy between this superresolution study and other studies. We therefore developed the Fab-based proximity ligation assay (Fab-PLA), which permits, for the first time, nanoscale studies without these difficulties (Kläsener et al. 2014).

The *in situ* proximity ligation assay (PLA) detects the close proximity of two target proteins using a polymerase-mediated amplification step after ligating oligos coupled to secondary antibodies (2-PLA) or primary antibodies (1-PLA) (Söderberg et al. 2008). The use of antibodies in this assay allows the detection of two targets in close proximity without the need of genetically engineered proteins. This offers a unique tool to study protein–protein interactions in their native form, such as *ex vivo* samples. However, considering the size of an antibody (Harris et al. 1997), it is worth noting that the maximum detection range can reach 80 and 40 nm for 2-PLA and 1-PLA, respectively. Thus, a positive PLA signal does not necessarily represent direct protein–protein interaction in a standard antibody-based PLA experiment. To deal with this issue, we prepared Fab fragments and conjugated them to oligos directly, thereby reducing the theoretical limit of PLA detection to the 10–20 nm range. The effectiveness of this improved Fab-PLA in detecting BCR oligomerization level was confirmed using both BCR-expressing S2 cells and with IgM-coupled beads. Positive Fab-PLA results for BCR:BCR proximity were only obtained from S2 cells expressing wild type, but not a mutant, monomeric form of IgD-BCR. Furthermore, we showed that this assay could reliably distinguish pentameric from monomeric IgM. Employing this method, we were able to show that BCR activation, for both IgM-BCR and IgD-BCR, results in a clear reduction of Fab-PLA signals corresponding to two closely situated BCRs, indicating that activated BCRs move apart from each other. These results thus provided direct evidence for the opening/dissociation of BCR oligomers during BCR activation, supporting DAM but not CLM.

Interestingly, employing the same method, we also found that inhibiting the function of the ITAM-interacting kinase Syk, but not Lyn, with pharmacological inhibitor prevents the opening of BCR. Correspondingly, splenic cells isolated from Syk-deficient mice, but not Lyn-deficient mice, failed to open BCR. These results thus revealed a novel role of Syk in opening BCR oligomers. Syk can phosphorylate ITAM tyrosines of Ig α /Ig β and bind to the phosphorylated ITAM. To determine whether the opening of the BCR by Syk is due to the phosphorylation of the ITAM tyrosines or to the binding of its tandem SH2 domains to the phosphorylated ITAM, we constructed expression vectors for truncated forms of Syk containing either the kinase domain or the tandem SH2 domain and expressed them separately, or together, in IgM-BCR-expressing S2 cells. Strong Fab-PLA signals representing the proximity between IgM-BCRs were detected in S2 cells expressing IgM-BCR alone, and these signals were lost only upon the coexpression of both the kinase domain and the tandem SH2 domains of Syk, suggesting that it is the binding of Syk to the phosphorylated ITAM that opens the BCR. These results do

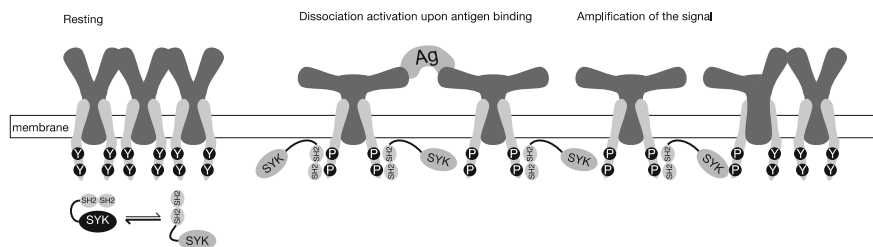


Fig. 2 Schematic drawing explanation of the dissociation activation of BCR

not exclude the possibility that the BCR can begin to open following the phosphorylation of its ITAM tyrosines and this opened structure is then stabilized by Syk binding.

We found that Syk is an allosteric enzyme, regulated by a positive product feedback. Its activity is blocked by interactions between its tandem SH2 domains and the kinase domain, while binding of its tandem SH2 domain to the phosphorylated ITAM stabilizes the open, active form (Rolli et al. 2002). Our Fab-PLA study now supports the idea that the binding of the Syk SH2 domain to the phosphorylated ITAM could also stabilize the BCR in an open, active form. Thus, even a low amount of antigen would be able to activate a large amount of the BCR through these feedbacks. Upon binding of a limited amount of antigen, only a small amount of the BCR will be opened and their ITAMs phosphorylated by either Lyn or Syk. Syk will then be recruited to the phosphorylated ITAMs. The formation of this kind of small BCR/Syk seeding complex would stabilize both Syk and the opened BCR monomers, allowing the phosphorylation of neighboring ITAMs. Subsequent recruitment of more Syk and opening of more BCRs cause an amplification of the signal (Fig. 2) (Kläsener et al. 2014).

3.4.2 Unsolved Questions

These recent Fab-PLA studies have provided valuable insights into DAM. However, to achieve a more complete understanding of the mechanism of BCR activation, there are still several questions that need to be addressed.

The Assembly of the BCR Monomer and the Formation of BCR Oligomers

It is well accepted now that BCR monomer is a 1:1 complex of mIg molecule and Ig α /Ig β heterodimer. What is less clear is how exactly it is assembled, especially the relative orientation of Ig α or Ig β toward the mIg molecule. On the one hand, a study of the assembly and intracellular transport of the BCR suggested that the Ig β is closer to the mHC than Ig α (Brouns et al. 1995). On the other hand, the conservation of polar amino acids in the Ig α TM region, and the finding that only the

glycosylation of Ig α but not that of Ig β varies upon binding to different mIg isotypes, favored the notion that Ig α is closer to the mHC than Ig β (Campbell et al. 1991; Reth 1992). However, these structural considerations become obsolete if one considers that the single Ig α /Ig β heterodimer may not bind to only one side of the mIg. When we compare the TM region of Ig α and Ig β , which most likely also cross the membrane as one α -helix, it appears that they also contain conserved amino acids lying on the same sides of the TM helices (Fig. 3a). It is thus possible that in the plasma membrane, both Ig α and Ig β interact with one chain of the mHC by this conserved side. Interestingly, a mutagenesis study exploring the interaction between refolded human Ig α /Ig β Ig domains with human Fc μ carrying the C μ 2–C μ 4 domains of μ HC has suggested that both Ig α and Ig β Ig domains are involved in binding to the C μ 4 region (Radaev et al. 2010). Due to the symmetry of mIg, the TM interaction between mIgs and Ig α /Ig β would place Ig α and Ig β separately on the outside of mIg homo-dimer. However, the interaction with the C μ 4 region and the formation of the disulfide bond between Ig α and Ig β would require them to be close in the extracellular part. Thus, we suggest that Ig α and Ig β are twisted upon their binding to mIg to fulfill both structural requirements simultaneously. Correspondingly, it may cause a rotating effect of the mHC TM, leading to a partial exposure of the originally sheathed isotype-specific side of the mIg in the assembled BCR (Fig. 3b); the formation of the disulfide between Ig α and Ig β would fix the conformation of Ig α , Ig β , and the mHC, allowing the exposed isotype-specific side to interact with another BCR to form BCR oligomers, thus explaining the role of the isotype-specific side of mHC TM and the disulfide bond of Ig α /Ig β in BCR oligomerization.

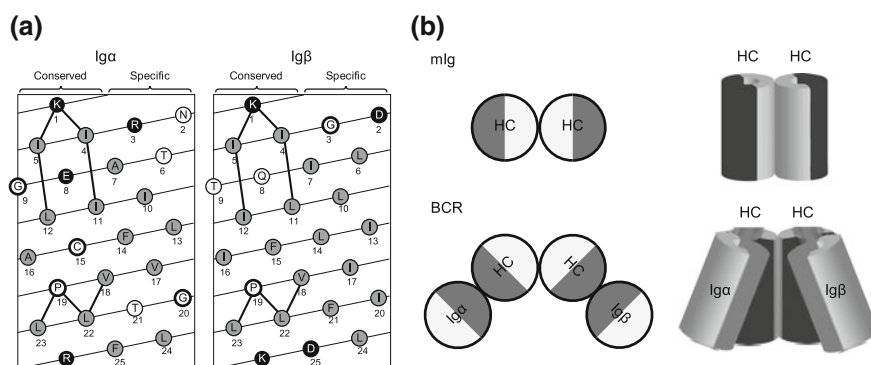


Fig. 3 Comparison of the TM region of Ig α and Ig β and schematic drawing of the arrangement of BCR components in the form of mIg and BCR. **a** Scheme of the TM α -helix of Ig α and Ig β . Amino acids are indicated by single-letter code in circles with different shades indicating their properties (Light, polar/hydrophilic; Dark, nonpolar/hydrophobia; Black, charged; White with thick outline, C, P, G). Conserved amino acids in the conserved side of the TM region were highlighted by the thick lines connecting them. **b** Comparison of the relative arrangements of TM region of BCR components. Dark-shaded part, conserved side of TM. Light-shaded part, specific side of TM, Upper, mIg; lower BCR. Left, view from the cytosolic side; right, lateral view

Mature B cells express IgM-BCR and IgD-BCR on their surface, both with the same antigen specificity (Kerr et al. 1991). We have shown that both IgM-BCR and IgD-BCR are able to form oligomers and both are opened upon activation (Kläsener et al. 2014; Yang and Reth 2010a). However, it is not clear whether they form oligomers separately or together. Earlier studies with B cells expressing both isotypes of BCR showed that stimulation with one type of anti-HC antibodies induces phosphorylation of only the Ig α associated with the corresponding BCR (Gold et al. 1991; Schamel and Reth 2000). This suggests that IgM-BCR and IgD-BCR may be physically separated from each other. Recent Fab-PLA studies have shown that during BCR activation, IgD-BCR loses its proximity to the coreceptor molecules CD19/CD20 and the GM-1 ganglioside, while IgM-BCR gains proximity toward these molecules. These results support a scenario in which IgM-BCR and IgD-BCR forms isotype-specific oligomers. However, this notion still needs to be vigorously tested by a more direct readout, since Fab-PLA results using different pairs of Fab are not directly comparable.

The Dissociation of Oligomeric BCR and Signal Initiation

When we suggested the DAM hypothesis in 2010 (Yang and Reth 2010b), we only considered the activation of the BCR by polyvalent antigen, and we proposed “A polyvalent antigen binding to mIg can interfere with the formation or promote the dissociation of the BCR oligomer.” However, since we still do not precisely know how the oligomeric BCR dissociates upon antigen stimulation, it is not appropriate to exclude the possibility that the binding of monomeric antigen to BCR could also disrupt the BCR oligomers and initiate BCR signaling. An examination of BCR organization by Fab-PLA upon the addition of monomeric antigen would clarify this issue.

Based on the new fence-picket model of the membrane (Kusumi et al. 2005), and recent studies on the role of cytoskeleton in BCR activation (Song et al. 2014), it is very possible that BCRs directly or indirectly interact with the cytoskeleton. Alteration in the structure of the cytoskeleton could affect the organization of oligomeric BCR. In fact, it was shown that simply disrupting the cytoskeleton with drugs such as Latrunculin A (Lat-A) can induce strong signaling, depending on the BCR signaling components (Mattila et al. 2013; Treanor et al. 2010). In our recent Fab-PLA study, treating B cells with Lat-A also resulted in the loss of Fab-PLA signals for BCR:BCR proximity. However, whether the dissociation of the oligomeric BCR following antigen binding is caused by the disruption of actin cytoskeleton remains to be determined.

Even if the BCR dissociation is achieved through the disruption of the cytoskeleton, a more important question still remains to be answered. How does the binding of antigen to the BCR lead to these events? Interestingly, by studying the syntaxin clustering dynamics in membranes by combining superresolution microscopy, biochemistry, fluorescence recovery after photobleaching (FRAP) analysis, and *in silico* simulations, Sieber et al. (2007) have suggested that oligomerization of

membrane proteins depends on a balance between weak homo-philic protein–protein interactions and crowding-induced steric repulsions and that this is sufficient to explain both the size and the dynamics of syntaxin clusters. For the BCR, the involvement of the isotype-specific side of ITAM in the formation of the BCR oligomer, and the possible isotype-specific oligomerization of BCR, has underlined the role of such kinds of homo-philic interactions between the mIg part of the BCR. It is feasible that the stability of BCR oligomers is also governed by the balance between these homo-philic interactions and the crowding-induced repulsions between BCR monomers. The repulsion power may come from the tight packing of mIg in the oligomer. Due to the flexible hinge region, the angle between the two arms of mIg is also flexible. For IgD, it varies between 65° and 144° (Løset et al. 2004). It is reasonable to expect that the mIg in a monomeric BCR would be T-shaped (144°), while a Y-shaped (65°) conformation is more suitable for BCRs forming the oligomer (Fig. 4). Antigen binding to the Fab region of mIg thus may release them from the Y-shaped conformation, resulting in the enhancement of repulsion power and subsequently the opening of the BCR oligomer.

Upon antigen binding, the BCR would have to transmit this binding event through the plasma membrane and convert it to downstream signaling, such as the phosphorylation of Ig α /Ig β ITAM tyrosines. In the framework of DAM, we would suggest that this could be achieved by simply exposing the originally inaccessible Ig α /Ig β ITAMs upon opening of the BCR oligomer. The cytoplasmic tail of ITAM-containing proteins, including Ig α and Ig β , is able to form homo-oligomers, depending on the concentration of the protein (Sigalov et al. 2004). Thus, disruption of BCR oligomers will lead to a reduction in local Ig α and Ig β concentrations, thus freeing the tail from homo-oligomerization. This is in agreement with the finding that BCRs carrying a truncated version of Ig α , without the ITAM part, are more sensitive to antigen stimulation (Kraus et al. 1999).

It is also possible that the initiation of BCR signaling is due to the translocation of the dissociated BCR to a different microenvironment. This notion was supported by a recent Fab-PLA study showing that the proximity between BCR and

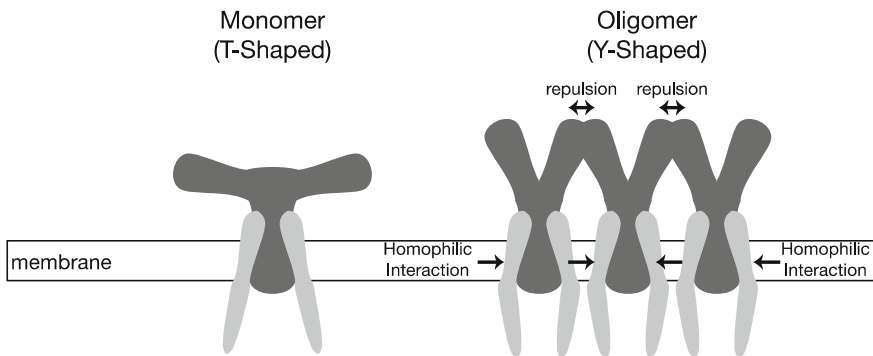


Fig. 4 Schematic drawing of mIg in the form of monomeric and oligomeric BCR

coreceptors such as CD19 is changed during BCR activation. Interestingly, studies on TCR activation have suggested that the activation of the TCR involves the convergence of pre-organized TCR “protein islands” with the protein island of linker for activation of T-cells (Lat), a key adaptor for TCR signaling (Lillemeier et al. 2006). Although the BCR may not necessarily interact with its coreceptors and signaling adaptors following the TCR model, the dissociation of BCR oligomers would enhance its chance to meet with those molecules and might be an important step in BCR signal initiation.

Another non-exclusive possibility is that the opening of BCR oligomers is also accompanied by a conformational change in the Ig α /Ig β tail. It has been reported that Ig α /Ig β tails undergo conformational changes during BCR activation since the FRET signal between CFP- and YFP-tail-labeled Ig α and Ig β changes upon BCR activation (Tolar et al. 2005). However, since most resting BCR exists as oligomers, FRET between labeled Ig α /Ig β tails does not necessarily happen only between the Ig α /Ig β tails inside of one BCR monomer (intra-BCR). It may also happen between the Ig α and Ig β belonging to two different BCR monomers (inter-BCR). The observed FRET change can be the result of Ig α /Ig β tail conformational change, but it also can be explained by changes in the BCR oligomerization level. To answer this question properly, it is thus necessary to develop tools which are able to distinguish between the intra-BCR and inter-BCR interactions of Ig α /Ig β .

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2016, X, 231 p., Hardcover

ISBN: 978-3-319-26131-7