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Viewing the antigen-induced initiation of B-cell activation in living cells

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Summary: The binding of antigen to the B-cell receptor (BCR) induces BCR clustering and signaling cascades that lead to the activation of a variety of genes associated with B-cell activation. Over the last several years, our understanding of the molecular details of the BCR signaling pathways have been considerably advanced; what remains only poorly understood are the molecular events that initiate BCR clustering and how clustering leads to activation. Here, we review our progress using live cell imaging technologies to view the earliest events that follow the B cell's binding of antigen. We provide a model for BCR clustering and B-cell activation that involves an intrinsic tendency of the BCR to cluster and does not require direct crosslinking of the BCR by multivalent antigens. We suggest that local membrane topology and lipid composition play key roles in BCR clustering and initiation of signaling. We believe that our model for B-cell activation, in which receptor interactions with monovalent antigens on membrane surfaces lead to receptor clustering, may be highly relevant to the mechanisms by which other immune receptors cluster including the T-cell receptor in response to monovalent peptide–major histocompatibility complex complexes.

Keywords: *B cells, live cell imaging, signal transduction, membrane microdomains*

What we do not know about the initiation of B-cell activation

B cells are activated by the binding of antigen to the B-cell receptor (BCR). The B cell's binding of multivalent, soluble antigens results in clustering of the BCRs and ultimately to the movement of the clusters to one pole of the cell to form a cap (1). These events trigger signaling cascades leading to the activation of a variety of genes associated with B-cell activation (2). Under the influence of the induced signaling, the BCR also internalizes antigen into an intracellular compartment, where the antigen is processed and ultimately presented on major histocompatibility complex (MHC) class II molecules to helper T cells (3). Recent studies using intravital two photon imaging suggest that B cells contact antigen not in solution but rather on the surfaces of antigen-presenting cells (APCs) in lymphoid organs (4). Studies *in vitro* showed that B cells encountering antigen on the surface of an APC or on a planar lipid bilayer,

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approximating an APC surface, form an immune synapse (5–7), a bull's eye-like structure composed of the BCR and antigen in the center surrounded by a ring of the adhesion molecule leukocyte function-associated antigen-1 (LFA-1). The immune synapse formation has been associated with B-cell activation. What we do not know is how antigen binding to the BCR triggers these events.

The BCR, like all members of the family of multichain immune recognition receptors (MIRRs), is composed of a ligand-binding chain, for the BCR a membrane form of the antigen-specific immunoglobulin (Ig). The membrane ligand-binding chains have short cytoplasmic tails and no means of connecting directly with the cell's signaling apparatus. To do so, the members of the MIRR family associate with additional membrane proteins that contain in their cytoplasmic domains immunoregulatory tyrosine-based activation motifs (ITAMS) that interact directly with the cell's signaling machinery. For the B cell, these proteins are Igα and Igβ (8). One of the earliest events observed following antigen binding to B cells is the phosphorylation of the ITAM tyrosines in the Igα and Igβ chains by the membrane-tethered Src family kinase Lyn. Phosphorylation of the BCR leads to the recruitment of the src homology 2 domain (SH2)-containing kinase Syk and the initiation of a variety of downstream signaling pathways (2). What we do not know is what changes in the antigen-bound BCRs allow Lyn to discriminate between the monomeric BCRs and the antigen-bound BCRs and to phosphorylate only the antigen-bound BCRs.

The crystal structure of antibody Fab bound to specific antigens provides no evidence for an allosteric conformational change that could propagate the information that the BCR has bound antigen along the Ig chains to the cytoplasmic domains (9). However, as there is as of yet no structure of a complete BCR containing Ig, Igα, and Igβ, it is possible that the conformation of the BCR does change with antigen binding and that such conformational changes are important to the initiation of signaling. Nonetheless, without evidence for an antigen-induced conformational change in the BCR, one is left with clustering as the indicator that the BCRs have bound antigen. What we do not know is by what mechanism BCRs cluster and what aspect of the clustered BCRs is essential to the initiation of B-cell activation.

Why live cell imaging?

The critical events that initiate B-cell activation are likely to occur within seconds of antigen binding to the BCR and may be highly dynamic and ephemeral in nature, involving many weak protein–protein and protein–lipid interactions. In general, the biochemical approaches that have been used so effectively to describe the BCR signaling pathways are inadequate to capture events that occur as rapidly and as transiently as those predicted to initiate BCR signaling. In addition, antigen binding to the B cell involves a dramatic spatial change in the BCRs, resulting in patching and capping of the BCRs and the formation of an immune synapse. All this potentially important spatial information is lost with the addition of detergents to cells for biochemical analyses. The use of detergents is particularly problematic in the study of the early membrane changes that accompany antigen binding by BCRs. Indeed, the addition of detergent can destroy or artifactually create lipid structures within the membrane. Live cell imaging technologies allow analyses of the BCR and components of the BCR signaling pathway with the temporal and spatial resolution necessary to view the earliest events in B-cell activation without the complications that the addition of detergents introduce.

What new have we learned from live cell imaging about the nature of the BCR on resting B cells?

To better understand the molecular basis of BCR clustering and initiation of signaling, it will be necessary ultimately to understand the structure of the BCR. As discussed above, although the structures of several antibodies bound to antigens were solved some time ago, there is as yet no structure of the Ig, Igα, and Igβ ectodomain complex of the BCR. Moreover, we have no information on the overall conformation of the cytoplasmic domains. We focused our efforts on understanding the conformation of the cytoplasmic domains, given their importance in the initiation of signaling. To investigate the conformation of the cytoplasmic domains of the BCR in living cells, we determined the interactions between the component chains of the BCR, Ig, Igα, and Igβ, engineered to contain fluorescent proteins, using confocal microscopy in combination with fluorescent resonance energy transfer (FRET) (10). Because of the extreme sensitivity of FRET efficiencies to the distance between FRET donor and acceptor fluorescent proteins, FRET imaging has proven to be a highly sensitive molecular ruler to measure the interactions between proteins. We generated cell lines that expressed BCRs that contained all possible combinations of FRET donor and acceptor fluorescent proteins engineered into Igγ, Igμ, Igα, and Igβ (*Fig. 1*). These combinations allowed us to determine the proximity of the chains of the BCRs one to another within an individual BCR. In addition, to report on the interactions between two individual BCRs, cell lines were generated in which the BCRs contained an Igα

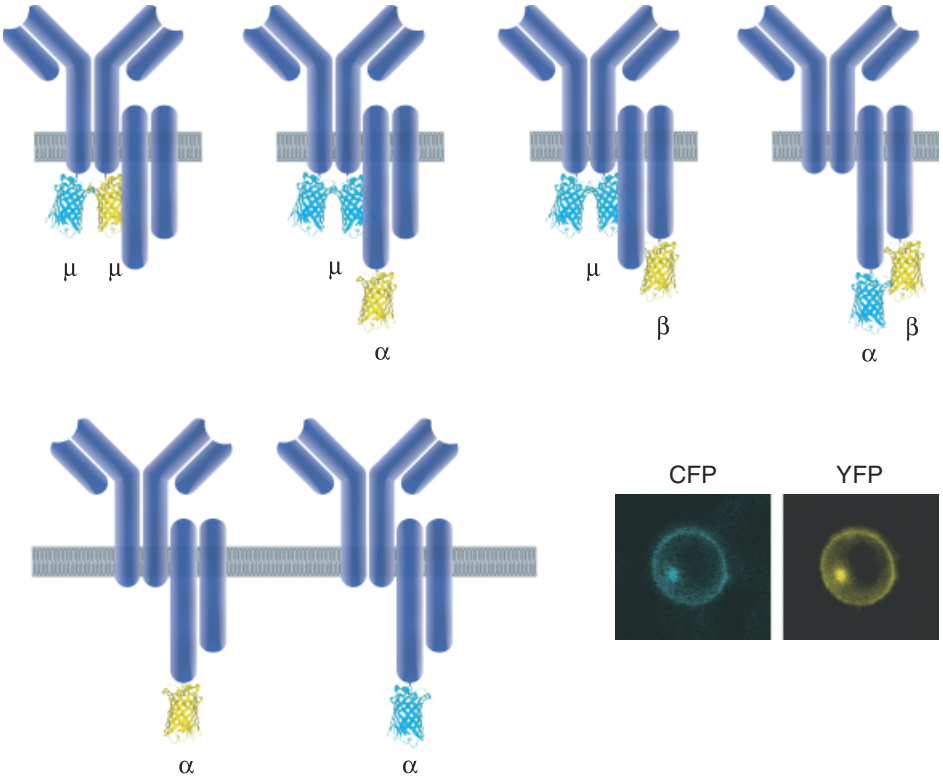

***Fig. 1.* Fluorescent BCR constructs used to probe the interactions of the BCR chains by FRET imaging.** B-cell lines were generated that expressed all possible combinations of the BCR's Ig, Igα, and Igβ chains engineered to contain FRET donor CFP and FRET acceptor YFP fluorescent proteins to report on interchain distances within a BCR (Top). Depicted are constructs containing Igμ. Corresponding cell lines were generated using Igγ. A cell line was also generated that expressed both Igα-CFP and Igα-YFP in approximately equal amounts to report on interactions between BCRs (bottom). Images are of a cell expressing Igμ-CFP and Igα-YFP.

engineered to contain either a FRET donor or acceptor fluorescent protein (*Fig.* 1). An initial analysis of the cell line expressing Igμ cyan fluorescent protein (CFP) and Igα yellow fluorescent protein (YFP) in combination with fluorescent antibodies specific for the ectodomain of the BCR allowed us to determine that the stoichiometry of the BCR was 1 Ig:1 Igα:1 Igβ (10). This observation confirmed the stoichiometry determined by biochemical analyses (11).

Information about the spatial relationship of the BCR chains one to another was provided by FRET analyses. We quantified FRET efficiencies by calibrated sensitized acceptor emissions (12). The experimental design to measure FRET is shown in *Fig.* 2. The cells were illuminated at 458 nm to excite the FRET donor CFP, and the emissions in the range of 469–555 nm were collected in the CFP channel. If the CFP donor is in close proximity, within 100 Å, of the FRET acceptor YFP, the CFP transfers energy to YFP, and its emissions in the range of 523–608 nm are collected in what is referred to as a FRET channel. Lastly, the B cells are scanned a second time, illuminating the cells at 514 nm to excite YFP, and the emissions in a range of 523–608 nm are collected in a YFP channel. The scans are repeated every 10–20 s to provide a real time image of the cells. After correcting for cross talk between channels, FRET efficiencies were calculated and used to estimate the distances between the BCR chains. The results of the FRET analysis provided two new important pieces of information about the BCR expressed by resting cells, namely, that the receptor was a monomer showing no inter-BCR FRET and that the cytoplasmic domains within an individual BCR were ordered, showing discrete FRET values and thus discrete distances between the chains. In addition, the results of FRET analyses for BCRs that contained Igγ versus Igμ chains were nearly identical, indicating that the biological differences in the outcome of signaling through Igγ versus Igμ BCRs cannot be accounted for by their stoichiometry, oligomerization, or the order of their cytoplasmic domains.

The first observation that the receptors are monomeric in live B cells is in contrast to the results of biochemical analyses that indicated that BCRs purified from B cells were oligomers (13). It was suggested from the biochemical observations that antigen activates B cells not by clustering the BCRs but rather by disrupting the preexisting BCR oligomers (14). This idea was

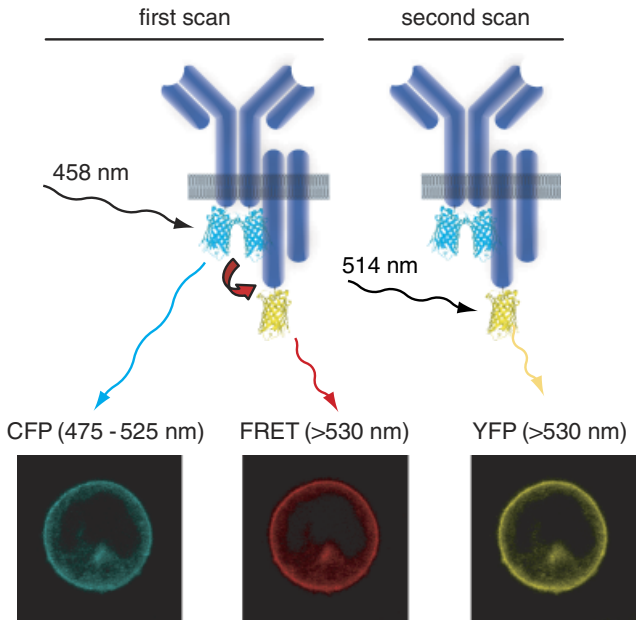

***Fig. 2.* FRET confocal microscopy in living B cells.** Depicted is the experimental design for the measurement of FRET by calibrated sensitized acceptor emission. Fluorescence was collected from three channels, namely CFP (CFP excitation and CFP emissions), FRET (CFP excitation and YFP emissions), and YFP (YFP excitation and YFP emissions). Data from these three channels along with controls correcting for bleed through were sufficient to calculate FRET efficiencies and to estimate the distance between the chains of the B-cell receptor .

attractive, as it would account, at least in part, for the ability of B cells to respond to the range of sizes and shapes of antigens in nature that are predicted to rarely present B-cell antigenic epitopes in a spatial array that would facilitate the ordered clustering of monomeric BCRs. Binding of such antigens could, however, serve to disrupt a pre-existing, well-defined BCR oligomers. As we discuss below, our analysis by total internal reflection fluorescence microscopy (TIRFM) of B cells responding to antigens on planar bilayers suggests an alternative mechanism by which B cells expressing BCR monomers are able to recognize and respond to the topological diversity of the natural antigenic world. The observation that BCRs tend to form oligomeric structures in detergent may be important in indicating an intrinsic tendency of the BCR to cluster. This tendency may be an important feature of the BCR's ability to form signaling active clusters following antigen binding.

The significance of the observed order of the cytoplasmic domains of the BCR is in the possibility that the order could be altered by receptor clustering, providing a signal of antigen binding from the cell's exterior to interior. FRET confocal microscopy provided the tool to test this prediction in live B cells as they bound antigen.

Live cell imaging reveals a conformational change in the cytoplasmic domains of the microclustered BCR that accompanies signaling

Using FRET confocal microscopy and the B-cell lines described above (*Fig. 1*), we investigated the relationships between the cytoplasmic domains of BCRs as they bound antigen from solution and initiated signaling (10). To do so, B cells were placed in warmed chambers and imaged as antigen was added to the chamber. Labeling BCR extracellular domains with Ig-specific Fab conjugated with the FRET donor and acceptor pairs, Cy3 and Cy5, we observed that antigen binding was followed by an increase of FRET between the BCR bound Cy3- and Cy5-coupled Fab fragments that we interpreted as clustering of the antigen-bound BCRs (*Fig. 3*). The FRET between the BCR-bound antigens persisted over several minutes, indicating stable clustering of the BCR ectodomains. When FRET between the fluorescent proteins in the cytoplasmic domains of the BCRs was analyzed, the pattern was very different (*Fig. 3*). Immediately upon addition of antigen, there was a rapid gain in FRET that we interpreted as indicating the clustering of the cytoplasmic domains of the antigen bound BCRs. The FRET, however, did not persist. Rather, several seconds after the peak in FRET, the FRET dropped and remained low. Importantly, the FRET did not drop below the levels of FRET in the unligated BCR monomer. Such that for the example shown, a BCR expressing Igγ-CFP and Igα-YFP showed FRET between CFP and YFP in the monomeric receptor before antigen-induced clustering, due to the close molecular proximity of Igγ and Igα. The FRET increased upon antigen binding and then decreased and remained at a level above that of the BCR monomer. Thus, the loss of FRET was not due to a dissociation of the receptor chains themselves, for example, a dissociation of Ig from Igα and Igβ, as had been suggested by earlier biochemical analyses (15, 16). Because the same pattern of FRET gain and loss was observed in all cell lines shown in *Fig. 1*, including cells that expressed a mixture of Igα-CFP and Igα-YFP that reported on the interactions between individual BCRs, we interpreted the loss of FRET as an opening up of the cytoplasmic domains in the clustered BCR, perhaps much like the opening of an umbrella (*Fig. 3*).

The conformational change in the cytoplasmic domains of the antigen-clustered BCR correlated with the initiation of signaling and did not occur in the presence of a Lyn inhibitor or in BCRs in which the ITAM tyrosines were mutated to phenylalanines. The opening was reversible with the addition of PP2 to inhibit Lyn's activity, suggesting that continued phosphorylation of the ITAMs was necessary to maintain the open conformation, and in the absence of Lyn's activity,

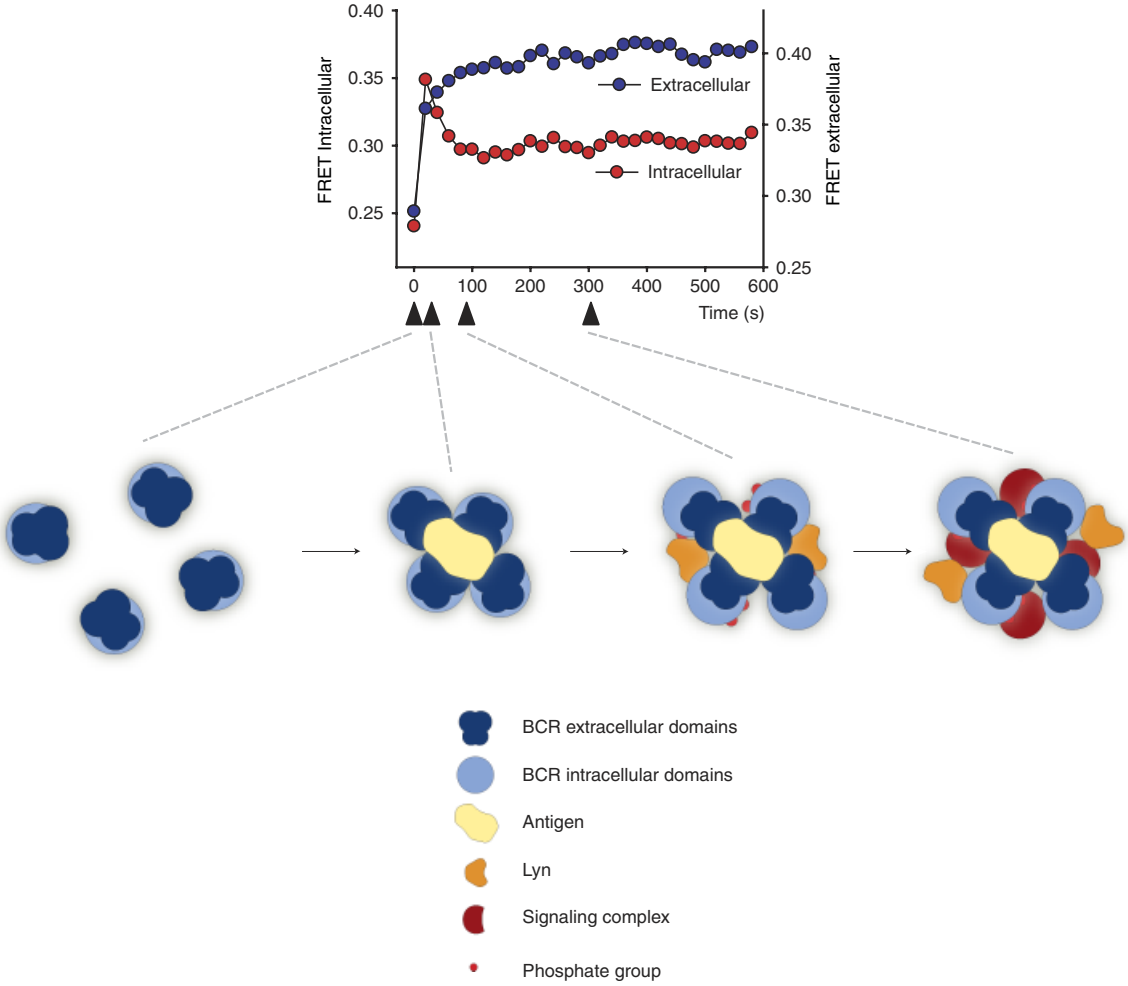

***Fig. 3.* FRET imaging of live B cells revealed a conformational change in the cytoplasmic domains that accompanied BCR clustering and signaling.** Shown are the results of an analysis of FRET from Ig-specific Fab probes containing FRET donor and acceptor fluorescent tags after the addition of the antigens to B cells. Also shown is an example of the FRET efficiencies for the cytoplasmic domains of a BCR containing Igγ-CFP and Igα-YFP. The interpretation of these data is depicted below. The FRET between the ectodomain of the BCR increases with the time, reaches a maximum, and is maintained at that level, indicating that the BCRs are brought into close molecular proximity in a stable cluster. The pattern of FRET between the cytoplasmic domains is strikingly different. FRET increases with the addition of antigen, peaks, and then decreases. Because this pattern was similar for all of the combinations of BCR CFP- and YFP-containing chains shown in *Fig. 1*, including the Igα-CFP and Igα-YFP expressing cell line that reports on the interactions between individual BCRs, we interpret this result to reflect an initial antigen-induced clustering of the BCR cytoplasmic domains and then an opening of the domains. The opening correlates with the phosphorylation of the BCR by Lyn and precedes the recruitment of Syk.

opposing phosphatases dephosphorylate the cytoplasmic domains of the clustered BCR, resulting in a closed state. The open conformation was not dependent on an intact cytoskeleton and occurred in BCR clusters in cells treated to block internalization. Syk was recruited to the BCR microclusters several seconds after they achieved an open conformation (*Fig.* 3), but the open conformation was not dependent on Syk binding or on Syk's activity nor on the activity of B-cell linker protein (BLNK), which was also recruited to BCRs in the open conformation (P. Tolar, unpublished data). Thus, we concluded from these results that the antigen-clustered BCRs undergo a change in the conformation of their cytoplasmic domains that occurs simultaneously with the phosphorylation of the receptor by Lyn and triggers subsequent recruitment of key signaling components including Syk and BLNK to the BCR.

We do not yet know the mechanism underlying the change in conformation of the cytoplasmic domains of the antigen-clustered BCR. In particular, we do not know the relationship between Lyn's phosphorylation of the BCRs and the change in conformation. Does Lyn's phosphorylation simply stabilize an open conformation, or does phosphorylation induce the open conformation? As described below, we favor a model in which the BCR undergoes a conformational change

from a closed signaling inactive form to a signaling active open conformation that is stabilized and kept open by phosphorylation by Lyn.

Imaging B-cell activation at the surface of APCs provides a new view of the spatial organization BCR signaling

In the studies described above, B cells were provided multivalent antigen in solution, as is conventional for analyses of B-cell responses *in vitro*. However, several recent studies have provided evidence that the relevant mode of antigen recognition by B cells *in vivo* may be of antigen on the surfaces of APCs. By two-photon intravital imaging, naive B cells were shown to engage antigen on the surfaces of dendritic cells, leading to signaling outside the lymph node follicle (4). Results using similar imaging technology suggested that B cells scan antigen trapped on the surfaces of follicular dendritic cells in germinal centers during affinity maturation (17, 18). In addition, studies *in vitro* show that B cells actively form immune synapses when interacting with antigen either on the surfaces of APCs or on planar lipid bilayers, simulating APCs (7, 19).

To visualize the earliest events in the initiation of B-cell activation following binding of antigen on an APC, we turned to TIRFM to image B cells encountering antigen on a planar lipid bilayer. The advantage of TIRFM is that only a small region of the cell is illuminated, approximately the thickness of the B cell's membrane, as it rests on a lipid bilayer. Because only the plasma membrane is viewed and the fluorescence within the cell is excluded, TIRF provides a contrast that is sufficient to resolve individual BCRs. Planar lipid bilayers were prepared on glass slides to which the antigen and the intercellular adhesion molecule-1 (ICAM-1) were attached by His tags to nickel-containing lipids (*Fig.* 4). Both the antigens and ICAM-1 were completely mobile in the planar bilayers. As predicted by the studies of Batista et al. (6, 7), we observed that when placed on an ICAM-1- and antigen-containing lipid bilayer, splenic B cells spread and formed an immune synapse (Tolar *et al.*, unpublished observations) (*Fig.* 4). Engagement of the antigen – and ICAM-1-containing bilayers led to rises in intracellular calcium and later to upregulation of CD86, a marker of B-cell activation. In the absence of antigen, the interactions of the B cell with the bilayer were unstable and no activation resulted. In the absence of ICAM-1, the interaction of the B cell with the antigen-containing bilayer was less stable as compared with that with antigen – and ICAM-1-containing bilayers, and the resulting responses were reduced (20).

Imaging the B cell by TIRFM during the first several seconds of interaction with the bilayer, we observed that the B cells first contacted the bilayers in one or more discrete points that likely represented protrusions of the B cell's membrane extending outward to touch the bilayer (*Fig.* 5). Labeling the BCR with fluorescent monovalent Fab anti-Ig and the B-cell membrane with the lipophilic dye DIC16, we showed that the BCR first formed microclusters in these initial contact points where the B cell's plasma membrane and the BCRs colocalized. The microclusters grew, and after a few seconds, a spreading response was triggered that was visible as a broadening of the contact areas, eventually resulting in a homogenous distribution of the B cell's membrane over the growing contact area. As the B cell spreads on the bilayer, the BCR microclusters that formed in the first contact points remained at these points as additional microclusters formed at the leading edge of the cell. After the cells had maximally spread, microclusters continued to form in the ruffling membranes at the periphery of the cells and later moved directionally toward the center, fusing with one another on their way. After 5 min on the bilayer, the membrane ruffling and the formation of BCR microclusters subsided and the B-cell lamellipodia contracted. These TIRF images suggested that BCR microclusters were formed in membrane protrusions, first in the initial points of contact of the B cells with the bilayer, then in the spreading edge of the B cells, and lastly in the membrane ruffles of the maximally spread cells. These membrane protrusions are of interest, as they may provide a specialized

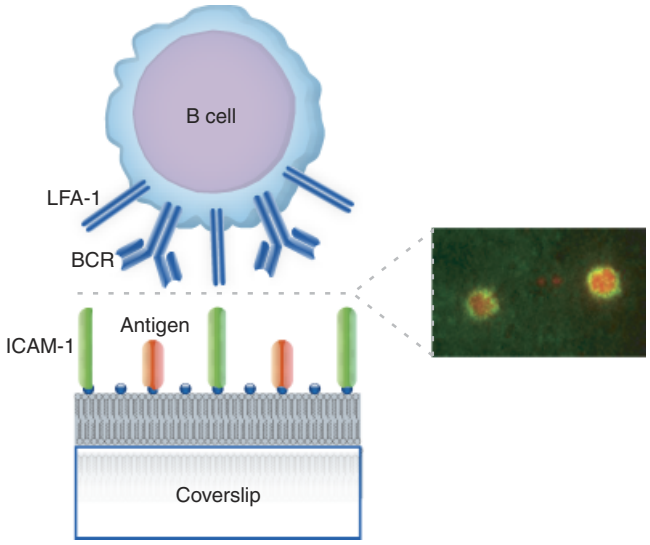

***Fig. 4.* TIRFM imaging of antigen-specific B cells as they encounter antigen- and ICAM-1-containing planar lipid bilayers.** NIP-specific splenic B cells incubated on red-fluorescent NIP-antigen– and green fluorescent ICAM-1-containing lipid bilayers. Images obtained by TIRFM show that the B cells have formed immune synapses with the NIP-antigen clustered in the center surrounded by ICAM-1.

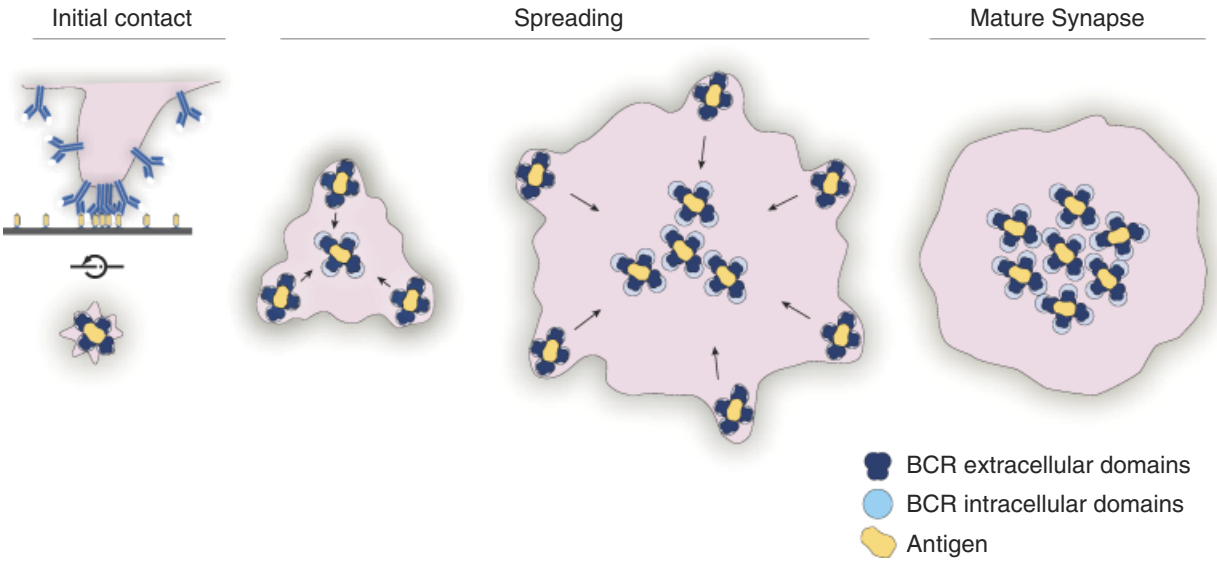

***Fig. 5.* A depiction of the results obtained by TIRFM and FRET of B cells encountering antigen on a planar lipid bilayer.** Beginning from the left, the B cell first encounters the antigen-containing bilayer by small membrane protrusions that reach towards the bilayer. Depicted are both a TIRFM view and a side view of the B-cell protrusion touching the bilayer. The BCRs in these protrusions cluster following antigen binding and undergo a conformational change to a signaling open form. The formation of the clusters was similar for monovalent and multivalent antigens. The BCR signaling triggers a spreading response, and new BCR clusters are formed and open in the protrusions at the leading edge of the cell. These open clusters are actively transported toward the center of the contact area forming the immune synapse. The clusters continue to form and open in the ruffling membranes in the periphery of the spread cell and move to the center of the synapse.

topology that concentrates the BCR and promotes or facilitates clustering.

By several criteria, the BCR microclusters that first form are signaling active. Simultaneous imaging of intracellular calcium levels and the BCR-bound antigen as the B cell first touched the bilayer showed that a calcium response was initiated at the point of microcluster formation prior to cell spreading. Using FRET in conjunction with TIRFM, we determined that the FRET in the individual microclusters showed the same pattern as that described above for the BCR clusters that formed following antigen binding from solution. The FRET between the cytoplasmic domains of the BCRs forming microclusters first increased and then decreased, indicating that the BCRs were in an open active conformation. The BCR microclusters remained in an open conformation as they accumulated in the synapse. Lastly, Syk was recruited from the cytoplasm to the microclusters as they formed. Interestingly, Syk did not appear to remain associated with the BCR microclusters as they accumulated in the synapse.

Taken together, the TIRF images provide a view of the antigen-triggered activation of B cells in which antigen binding induces BCR microclusters to form in topologically restricted protruding membranes. These microclusters are signaling active as they first form, obtaining an open conformation, recruiting Syk, and signaling for calcium fluxes. As the microclusters accumulate in the synapse, they remain in the 'open' conformation, but the signaling from the clusters appeared to have progressed to a point where Syk was no longer associated. An important question raised by these observations in what signaling functions are associated with BCR clusters as they first form to the point where they accumulate in the synapse. This issue is highly controversial for T cells, where the signaling function of the synapse continues to be debated. For B cells, the synapse is the site from which the clustered BCRs are internalized by a clathrin-dependent mechanism (P. Tolar, unpublished observations). We have also observed that BCR signaling continues as the BCR is internalized and trafficked to the multivesicular bodies (MVBs) (Chaturvedi *et al.*, unpublished observations). Indeed, looking downstream to mitogen-activated protein kinase signaling, it is clear that phosphorylated forms of p38 and c-Jun N-terminal kinase (JNK) are not associated with BCR clusters until they are internalized into the MVBs. These observations suggest that the spatial organization of the BCRs may be critical to the outcome of signaling.

TIRFM has provided a view of BCR signaling with new spatial dimensions. The TIRFM images suggest a process of B-cell activation in which BCR microclusters form, obtain an open conformation, and initiate signaling by the recruitment of the first components of the pathway in the membrane fingers of the B-cell advancing over an antigen-containing surface. The microclusters are actively moved to the synapse.

The signaling from the synapse is qualitatively different than that from the initial microclusters and may be involved in the active internalization from the plasma membrane. The quality of the signaling from the BCR continues to change as the BCR moves to the MVBs. Through this process, the various phases of BCR signaling are spatially segregated, perhaps for the purpose of regulating signaling or providing an opportunity for the BCR to interact with coreceptors in well-defined environments. An important goal of future studies will be to provide spatial dimensions to the well-characterized biochemical events in BCR signaling. Hopefully, the additional spatial and temporal information about the BCR signaling cascades will provide insights into how these cascades are regulated and integrated with signals emanating from coreceptors.

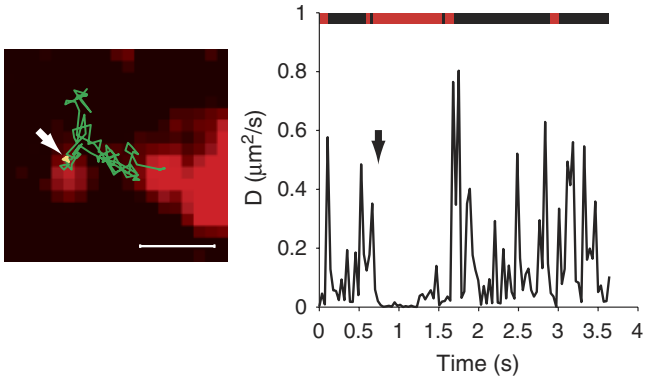

***Fig. 6.* Single molecule tracking of BCRs as they first encounter antigen in a planar lipid bilayer.** The BCRs were labeled with fluorescent monovalent Fab anti-Ig under conditions that allowed tracking of individual BCR (green) within one minute of their initial contact with an antigen-containing bilayer together with simultaneous dual-color imaging of the fluorescent antigen microclusters (red). The BCRs were highly mobile outside the antigen clusters but showed a high probability of stopping once they entered an antigen cluster. Shown is one example in which the BCR moves rapidly until it enters the antigen cluster, where it stops (yellow) and in this case exits the cluster, resuming its rapid movement.

What TIRFM tells us about how BCR microclusters form

The clustering of immune receptors is fundamental to the mechanisms by which immune receptors signal, and yet we understand very little about this process at a molecular level. For the BCR, the observation that activation of B cells by antigen provided in solution required that the antigen be multivalent led to the conclusion that BCR crosslinking by antigen was a requisite for clustering and signaling. Because the BCR is bivalent, clustering by multivalent antigen was assumed to proceed by physically crosslinking BCRs into lattice-like clusters. However, TIRFM of B cells encountering antigen in a lipid bilayer provided a very different view of the mechanism by which BCRs cluster.

The first most unexpected finding was that BCR microclusters formed in nearly an identical fashion, resulting in identical signaling when BCRs engaged monovalent versus multivalent antigens in planar lipid bilayers (Tolar *et al.*, manuscript submitted). Thus, BCR microclusters are not the result of physical crosslinking of bivalent BCR by multivalent antigen. How then do microclusters form? Our results of single molecule TIRFM showed that both monovalent and multivalent antigen binding dramatically decreased the mean diffusion of the BCRs and increased the fraction of immobile BCRs. Tracking single BCRs within the first seconds of their contact with ICAM-1 and antigen-containing bilayer, with simultaneous dual color imaging of antigen microclusters that had already formed, showed that most immobile BCRs were inside clusters (*Fig. 6*). The mobile BCR often bounced off the boundaries of the antigen clusters and on some occasions entered the clusters, followed by an abrupt stopping of the BCR. This antigen cluster-induced arrest in BCR mobility was independent of the initiation of BCR signaling, as evidenced by the observation that blocking the Src family kinase activity had no effect on this process. Taken together, the results from TIRFM suggest that BCR microclusters are formed in B-cell membrane protrusions where BCRs accumulate by a mechanism involving the intrinsic tendency of the BCR to cluster at these points. The microclusters grow by trapping mobile BCRs by mechanisms we do not yet understand but could involve local membrane perturbations, as described below.

These findings may provide a general framework for understanding microclustering of other immune receptors, including the clustering of the monovalent T cell receptor by MHC–peptide complexes. In addition, the observation that BCR clustering is induced by monovalent interactions between the BCR and the antigen provides an explanation for how B cells are able to recognize and respond to the topologically diverse world of pathogen antigens in which the BCR epitopes are unlikely to be spatially arrayed so as to facilitate BCR crosslinking. Lastly, the monovalent recognition of antigen by the BCR suggests a mechanism by which B cells are able to be highly discriminating of their affinities for antigen. If BCR clustering was dependent on the crosslinking of the divalent BCR to multivalent antigen, it is difficult to understand how small differences in BCR affinities could be discerned in the face of the overwhelming affect of the avidity provided by the multivalent BCR–antigen interactions. If B cells do encounter antigens on surfaces of APCs, monovalent interactions of BCRs, even with multivalent antigens, would provide a mechanism by which B cells could read the affinity of the antigen.

Live cell imaging provides evidence for dynamic membrane changes during BCR antigen-induced clustering

Earlier biochemical analyses of the detergent solubility of immune receptors including the BCR, T-cell receptor, and IgE receptor provided evidence that the local lipid microenvironment of the immune receptors and of the Src family kinases in the plasma membrane may play important roles in the earliest events in the initiation of immune cell signaling (21–24). Membrane microdomains were proposed to segregate the antigen receptors from the Src family kinases in resting cells and facilitate their association after antigen binding, thereby triggering signaling cascades. Indeed, lateral heterogeneities in the membranes of living cells, enriched in sphingolipids and cholesterol, coined 'lipid rafts,' have been hypothesized to function in receptor signaling and trafficking in a variety of cells types (23, 25, 26). Lipid rafts have been operationally defined by their relative detergent insolubility, due to the tight packing of the saturated chains of the raft lipids and by their dependence on cholesterol. By these criteria, we showed that in resting B cells the BCR was excluded from lipid rafts that contained Lyn and that following antigen binding the BCR associated with lipid rafts and was phosphorylated by Lyn (27).

The 'lipid raft hypothesis' is particularly attractive in the case of B-cell signaling, as it provided a mechanism by which the BCR's interaction with Lyn can be controlled in resting and activated cells. Is the lipid hypothesis correct? At present, the nature and function of lipid rafts and indeed their existence are highly controversial (28, 29). The critics of the raft hypothesis point to the paucity of data directly supporting the existence and function of lipid rafts in living cells. To a large degree, the controversy stems from the limitations of the widely used experimental tools, namely detergent solubility and cholesterol depletion, to investigate lipid rafts in cells. Indeed, current evidence indicates that lipid rafts are highly dynamic, submicroscopic assemblages of saturated lipids, and cholesterol that are not amenable to study by conventional microscopy or biochemical analyses (30, 31). However, high-resolution FRET confocal microscopy offered the opportunity to quantify the interactions of the BCR with raft lipids in live cells over the time and length scale necessary to capture the earliest events in antigen-initiated B-cell activation. Using live cell FRET confocal imaging, we recently provided direct evidence for dynamic membrane changes in the initiation of B-cell signaling (32).

To study the interactions of raft lipids with BCRs, we created cell lines that expressed a BCR containing the FRET donor fluorescent protein CFP and the FRET acceptor protein YFP, tethered to the membrane by either 'raft lipids' or by 'non-raft lipids' (*Fig.* 7). To tether YFP to the lipid rafts, we generated chimeric proteins that contained the first 16 residues of Lyn (Lyn16-YFP) that resulted in the myristoylation and palmitoylation of YFP and its association with lipid rafts. To target YFP to non-raft regions of the membrane, chimeric proteins were generated that contained the targeting sequence of two membrane-associated proteins that are not in lipid rafts, namely the C-terminus of Rho resulting in the geranylgeranylation of YFP (Ger-YFP) or the first 15 residues of Src (Src-YFP) containing only a myristoylation sequence. As predicted, Lyn16-YFP associated with detergent insoluble, lipid raft membranes, whereas both Src-YFP and Ger-YFP were in the soluble, non-raft membranes of B cells.

By confocal microscopy, the raft marker Lyn16-CFP appeared uniformly distributed over the B-cell surface and completely colocalized with the BCR's Igα-CFP both in resting cells and in cells 30 s after the addition of antigen. Thus, at the level of confocal microscopy, there is no apparent organization of the raft marker. However, FRET confocal microscopy of living B cells revealed that within seconds of antigen binding, the BCR selectively and transiently associated with the Lyn constructs and that this association preceded the triggering of calcium fluxes by several seconds. The association of the antigen-bound BCR with the raft lipid probe was prolonged by coengagement of the BCR, and the CD19/CD21 coreceptor complex that serves to enhance BCR signaling. Conversely, the association of the BCR with the raft lipid probe was blocked by the coengagement of the BCR with the potent inhibitory receptor, the FcγRIIB (Sohn *et al.*, unpublished observations). Thus, the FRET measurements provided the first direct evidence for the antigen-induced association of the BCR with raft lipids in living cells. The association was more dynamic than predicted by the earlier detergent solubility studies, suggesting that detergent may stabilize lipid structures that are far more dynamic in living cells. Taken together, the biochemical analyses of detergent solubility and the live cell imaging studies support a role for raft lipids in the initiation of BCR signaling in showing that the antigen-clustered BCR becomes associated with raft lipids.

TIRFM reveals the interactions of BCR microclusters with membrane raft lipids are transient but lead to stable association with Lyn

The results described above suggest that the antigen-clustered BCRs condensed raft lipids around them, forming a highly dynamic, transient assemblage of BCRs and raft lipids.

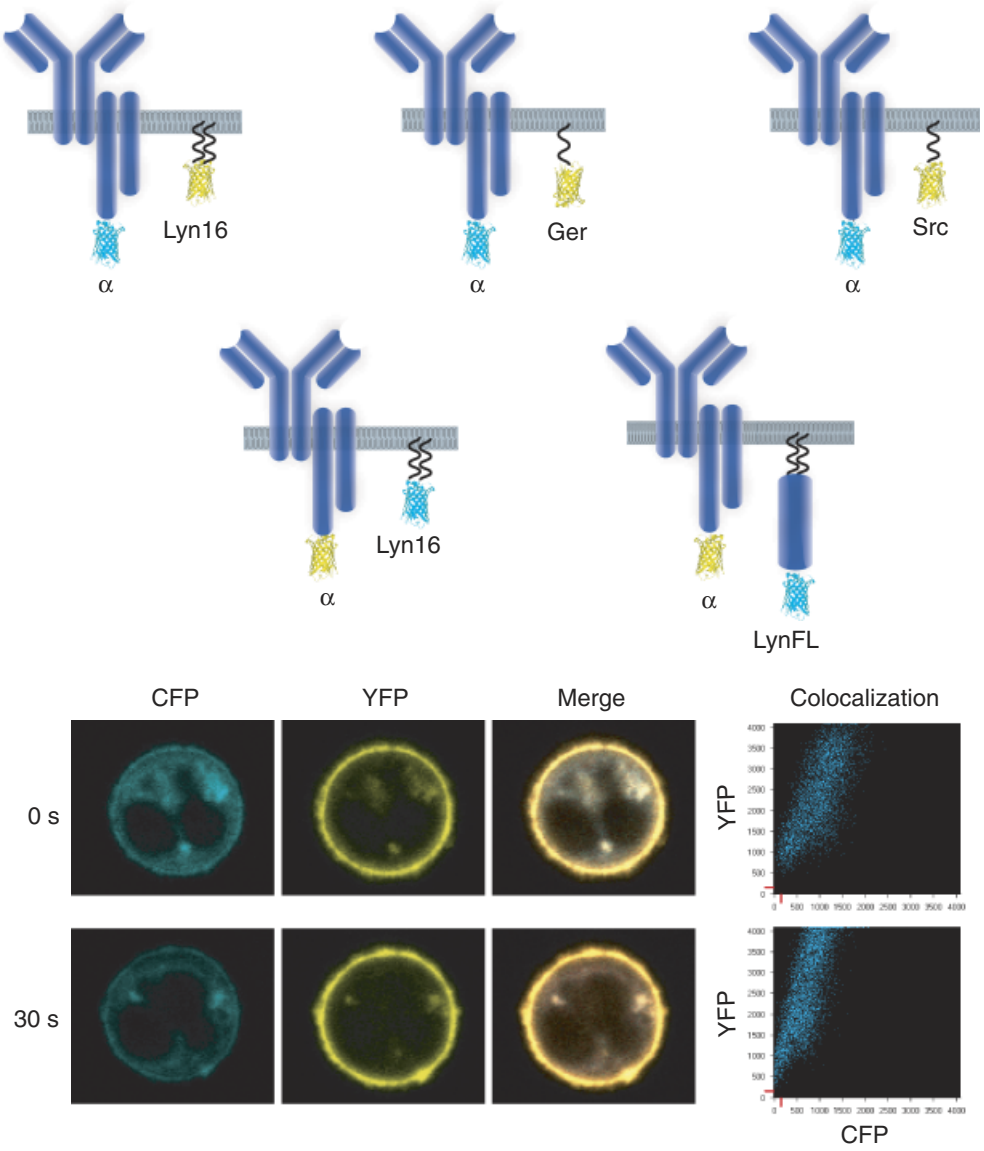

Fig. 7. **Fluorescent protein-containing constructs used to probe the interaction of the BCR with membrane lipids and with Lyn.** Cell lines were generated to investigate the interactions of the BCR with membrane lipids that expressed combinations of Igα-CFP and YFP tethered to the membrane by either the first 16 amino acids of Lyn, containing a myristoylation and palmitoylation site (targeted to detergent–insoluble raft membranes), a C-terminal geranylgeranyl sequence (targeted to detergent–soluble, non-raft membranes), or the first 15 residues of Src containing only a myristoylation site (targeted to non-raft membranes). To investigate the interactions between the BCR and Lyn, cell lines were generated that expressed Igα-YFP and either Lyn16-CFP or the full length (FL) Lyn kinase-CFP. Below is a confocal image of a cell line expressing Igα-CFP and Lyn16-YFP before (0 s) or after (30 s) the addition of antigen and the results of a colocalization analysis of CFP and YFP showing complete colocalization at both the 0 and 30 s time points.

Presumably, the association of the Lyn kinase with the BCR would initially follow the same kinetics as that of the Lyn16-YFP probe relying simply on BCR–lipid interactions. We hypothesized that the initial interaction of Lyn with the BCR through lipid–protein interactions facilitates the initiation of a more stable interaction of Lyn with the BCR through protein–protein interactions. To test this hypothesis and to better resolve the spatial and temporal order of BCR clustering and raft association, we turned to TIRFM. Using TIRFM in conjunction with FRET, we analyzed the cell lines described above (*Fig.* 7), including one expressing the full-length Lyn kinase as they interacted with a planar lipid bilayer containing ICAM-1 and antigen. We determined that the FRET between Lyn and the BCR was indeed greater and more prolonged as compared with FRET between the raft probe and the BCR (*Fig.* 8). Analyzing individual B-cell clusters in more detail, we determined that several seconds after BCRs formed microclusters in the periphery of the contact area of the B cell with the bilayer, the

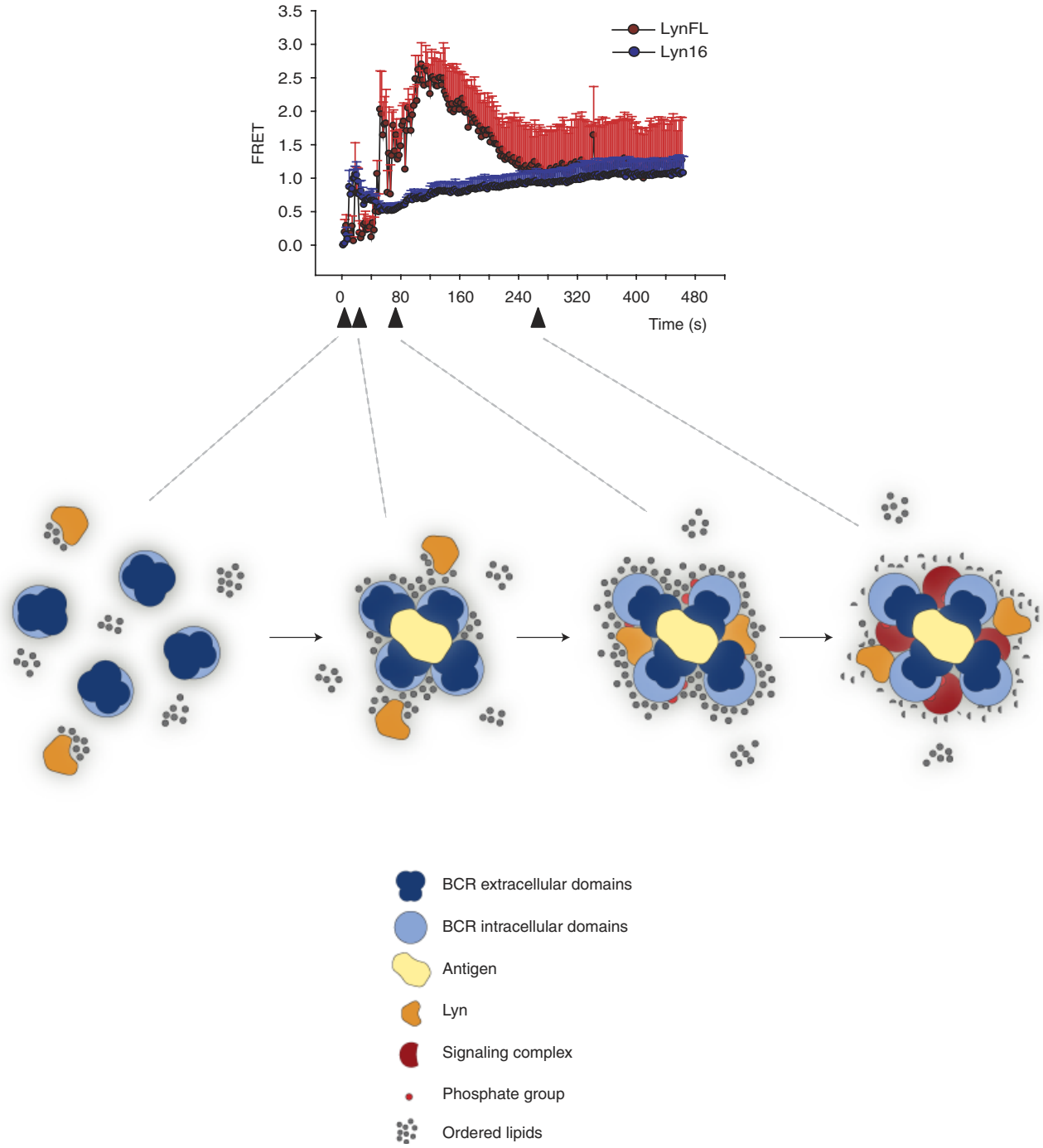

***Fig. 8.* FRET TIRFM reveals that the transient association of raft lipids with the BCR is followed by stable interactions of the BCR with Lyn.** The FRET efficiencies for cell lines expressing Igα and either the raft lipid (Lyn16) or the full-length Lyn (LynFL) as described in *Fig. 7* are shown with time after the contact with antigen. Below is a depiction of the interpretation of these data in terms of the BCR clustering described in *Fig. 3*. Before antigen binding, the BCR shows no FRET with either the raft-lipid probe or Lyn. After antigen binding, there is a rapid transient FRET between the BCR and raft-lipid probe followed by a higher more stable FRET between the BCR and Lyn. We propose that the BCR clusters condense raft lipids around them, facilitating the conformational change in the BCR that leads to Lyn's association, phosphorylation of the receptor, and finally Syk recruitment.

microclusters associated with the lipid raft probe. Thus, by TIRFM and FRET, it was possible to establish an order to the BCR microcluster formation and the association with raft lipid, clearly showing that the raft lipids condense around the formed BCR microclusters. Tracking individual BCR clusters revealed that the association of the raft probe with the BCR microclusters was transient and did not persist as the BCR clusters moved toward and accumulated in the synapse. The association of Lyn with the BCR microclusters also first occurred in the periphery of the cells, but in contrast to the raft probe, the association of

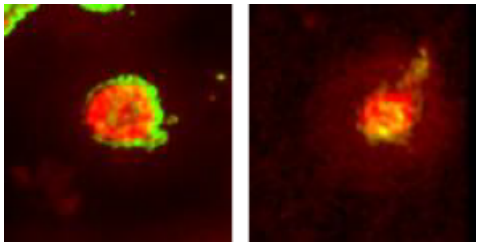

***Fig. 9.* The raft lipid probe associates transiently with newly formed BCR clusters in the periphery in contrast to Lyn that remain associated with the BCR microclusters in the synapse.** Shown are TIRFM FRET merged images of the BCR (red) and FRET (green) between the BCR and the raft probe (Lyn16) or the full-length Lyn (LynFL). For the raft probe, FRET occurs in the periphery of the cell and not with the BCR accumulating in the synapse. In contrast for Lyn, the BCR and FRET overlap in the synapse.

Lyn with the clustered BCR was more stable and persisted as the BCR clusters moved to the synapse. Indeed, the association of a BCR cluster with Lyn in the periphery was predictive of its ability to move to the synapse, suggesting a causal relationship between Lyn binding and movement of the BCR clusters to the synapse. Thus, as the BCR accumulates in the synapse, FRET between the BCR and Lyn persists (*Fig.* 9). In contrast, FRET between the BCR and the raft marker is restricted to the new BCR clusters forming in the periphery of the contact area (*Fig.* 9). Thus, the results of FRET and TIRFM indicate that raft lipid-BCR interactions predominate within the first several seconds following BCR clustering. These interactions appear to serve to facilitate the more stable protein–protein interactions between the BCR and Lyn.

And what might lipid rafts be good for?

Having provided evidence that raft lipids associated with BCR microclusters in living cells, a key question remains: what functions do lipid rafts provide in the initiation of BCR signaling? As originally proposed, the lipid rafts may serve to create microheterogeneity in the membrane that serves to facilitate the association of the BCR with Lyn. Our data support this function, showing that the dynamic condensing of raft lipids around the clustered BCR brings the raft lipid-associated Lyn in close physical proximity to the BCR cluster, promoting its stable association with the BCR.

The observation that the cytoplasmic domains of the clustered BCR open at the same time and in the same location in the B cell as the raft lipids associate with the clustered BCR (*Fig.* 8) suggests another fundamental role for the raft lipids, namely to induce the conformational change in the cytoplasmic domains, allowing Lyn to bind and phosphorylate the BCR. Two well-documented features of lipid rafts could account for the ability of raft lipids to exert a conformational change in the clustered BCR. The lipid raft membranes are thicker than more fluid membranes, due to the packing of the rigid saturated lipid tails (33). In response to a thicker membrane, the transmembrane domain may be stretched, exerting force on the cytoplasmic domains to open (34). The composition of lipid rafts also causes the membranes to curve. To accommodate a curvature in the membrane, the cytoplasmic domains may open. It may be possible to test this prediction in reconstitution studies *in vitro*, similar to studies demonstrating the dramatic affect of raft lipids on the conformation of the mechanosensitive ion channel (34).

Putting it all together: a model for the antigen-induced initiation of B-cell activation

The live cell imaging results reviewed here provide several new clues as to how antigen binding to the BCR results in BCR clustering and B-cell activation. Chief among these are the following observations: BCRs form microclusters in response to monovalent antigens, microclusters grow by trapping and immobilizing BCRs, clustering precedes the association of BCRs with raft lipids leading to the association of Lyn with the BCR, and the clustered BCR cytoplasmic domains undergo a conformational change that correlates with Lyn's phosphorylation of the BCR and precedes recruitment of Syk. Putting these findings together in the current context of B-cell activation suggests the following model.

The B cell initially interrogates APC surfaces by cellular membrane protrusions. If antigen is engaged, BCRs are immobilized and, due to their intrinsic tendency to cluster, start building up into signaling active microclusters. The newly formed microclusters trigger a spreading response. In the spreading cell, BCR microclusters continue to form as the cell encounters antigen in the protrusions in the leading edge and in the ruffles at the edge of a fully spread cell. We suggest that the mechanism of microcluster formation is the same at each site and involves an inherent tendency of the BCR to cluster that may be facilitated by the topological restriction of the membrane protrusions at the initial contact sites, the leading edge and the ruffles.

The BCRs in the microclusters undergo a conformation change to an open form, to which Lyn and Syk are recruited. The microclusters grow by trapping mobile BCRs, and the larger clusters are actively trafficked to the synapse. The

microcluster condenses raft lipids around it by mechanisms that are still unclear but may simply reflect the preference of the transmembrane domains of the microclustered BCR for the ordered environment provided by the saturated raft lipids. We propose that the condensing of the raft lipids has two repercussions. The first is to facilitate the association of Lyn with the BCR by concentrating the raft lipid-modified kinase around the BCR microclusters. The second is more fundamental, which is to alter the membrane topology so as to induce a conformational change in the BCR microclusters to a signaling active form that is stabilized by the phosphorylation by Lyn. The condensing of the raft lipids around the microclusters may also serve to trap mobile BCRs around the microcluster, facilitating the growth of the BCR cluster. This model, in which BCR antigen interactions are monovalent, provides a mechanism by which B cells are able to recognize pathogens in a highly discriminating fashion independent of the antigens' topology.

We predict that as live cell imaging is more widely used, new details about the earliest events in B-cell activation will be forthcoming. The new information will likely both add to what we know already and lead us to revise and refine our interpretation of existing data. In either case, these are indeed exciting times in B-cell biology.

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