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Understanding B cell activation: from single molecule tracking, through Tolls, to stalking memory in malaria

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Abstract

B lymphocyte activation is initiated by the binding of antigens to the clonally expressed B cell receptors (BCRs) triggering signaling cascades that lead to the transcription of a variety of genes associated with B cell activation. Provided with the appropriate T cell help and the microenvironment of germinal centers antigen drives B cells to proliferate and differentiate into long-lived plasma cells and memory B cells that together constitute immunological memory. Here I describe efforts in my laboratory to gain an understanding of the cellular and molecular mechanisms that underlie three processes central to B cell biology namely, the initiation of BCR signaling, the interactions of the BCR with the innate immune system Toll-like receptors and the generation and maintenance of B cell memory. Such knowledge is likely to aid research efforts in two areas of high public health priority, namely, the development of new therapeutics to control B cell responses in autoimmune disease and the design of effective vaccines to control infectious diseases.

Keywords

B cells; B cell receptors; signaling; malaria; immunological memory

Using live cell imaging and single molecule tracking to uncover the molecular basis of the initiation of BCR signaling

B cell activation is initiated by the binding of antigen to the B cell receptor (BCR) that triggers a number of signaling cascades that ultimately lead to B cell activation. Over the last several years our knowledge of the biochemical nature of the signaling cascades that follow BCR antigen binding has advanced considerably (1). However, what remains relatively poorly understood are the molecular events that trigger the initiation of signaling. We understand that the BCR, like other members of the multichain immune recognition receptor family, is composed of a ligand binding chain, for the BCR a membrane form of Ig, mIg, that has a short cytoplasmic domain with no capacity to interact directly with the components of the signaling cascades. To do so the mIg associates with two additional chains, Igα and Igβ, that contain within their cytoplasmic domains immunoregulatory tyrosine activation motifs (ITAMs) (2). The BCR has no inherent kinase activity but following antigen binding one of the first events observed is the phosphorylation of the BCR ITAMs by the Src-family kinase, Lyn. At present there is little understanding of the changes that occur in the BCR following antigen binding that allow Lyn to discriminate the antigen-bound BCR from the unbound BCR. Based on the crystal structure of antibodies bound to their antigens there is at present no evidence for an

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antigen-induced structural change in the BCR that could propagate the information that the BCR has bound antigen from the BCR ectodomains, across the membrane, to the cytoplasmic domains (3). In the absence of such an allosteric effect of antigen binding one is left with antigen-induced clustering of the BCR as the trigger for the initiation of signaling. However, we have little understanding of how antigens induce BCRs to cluster and how BCR clustering leads to the initiation of signaling.

The critical events that trigger BCR signaling are likely to occur within seconds of antigen binding to the BCR and to be highly dynamic, 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 evokes a dramatic spatial change in the BCRs resulting in their patching and capping and the formation of an immune synapse (4). 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. Consequently we have taken advantage of live cell imaging technologies that 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.

The stoichiometry, oligomerization state and conformation of the BCR on resting cells

Most of our understanding of the critical parameters of the BCR such as the stoichiometry of the BCR's mIg, Igα and Igβ chains, the oligomerization state of the BCR and the conformation of the cytoplasmic signaling domains is derived from the results of biochemical analyses. To study these parameters in living B cells we used confocal microscopy in combination with fluorescent resonance energy transfer (FRET) to analyze B cells that expressed BCRs engineered to contain FRET donor and acceptor fluorescent proteins in their cytoplasmic domains. 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 (5,6). We generated cell lines that expressed BCRs that contained all possible combinations of FRET donor, cyan fluorescent protein (CFP) and acceptor, yellow fluorescent protein (YFP) engineered into Igγ, Igμ, Igα, and Igβ cytoplasmic domains (7). These combinations allowed us to determine the molecular 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 Igα of the BCRs was engineered to contain either a CFP or YFP in their cytoplasmic domains. To determine the proximity of the extracellular domains Fab anti-Ig conjugated to FRET donor and acceptor fluorophores were used. An initial analysis of the cell line expressing Igμ-CFP and Igα-YFP in combination with fluorescent antibodies specific for the ectodomain of the BCR allowed us to determine that the stoichiometry of the BCR in living cells was 1 Ig: 1 Igα: 1 Igβ (7) confirming the stoichiometry determined by biochemical analyses (8).

We quantified FRET efficiencies by calibrated sensitized acceptor emissions to determine the spatial relationship of the BCR chains one to another (7). The results of the FRET analysis provided three new important pieces of information about the BCR expressed by resting cells. First, the receptor on the cell surface was a monomer showing no inter-BCR FRET. This observation in live B cells is in contrast to the results of biochemical analyses that indicated that BCRs purified from B cells may be oligomers (8). 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 (9). This idea was attractive as it could 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. As discussed 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 monomeric BCRs are able to recognize and respond to the topological diversity of the natural antigenic world. The second observation was that the cytoplasmic domains within an individual BCR were ordered, showing discrete FRET values and thus discrete distances between the chains. The observed order of the cytoplasmic domains of the BCR is significant, raising the possibility that the order could be altered by receptor clustering providing a signal of antigen binding from the cell's exterior to its interior. As described below, FRET confocal microscopy provided the tool to test this prediction in live B cells as they bound antigen. Lastly, 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 state or the order of their cytoplasmic domains.

Live cell imaging revealed a conformational change in the cytoplasmic domains of the BCR microclusters that accompanies signaling

Using FRET confocal microscopy and the B cell lines described above, we investigated the distances between the cytoplasmic domains of BCRs as they bound antigen from solution and initiated signaling (7). 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 Fabs that 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 strikingly different. Immediately upon addition of antigen, we observed a rapid gain in FRET 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 indicating that the loss of FRET was not due to a dissociation of the receptor chains themselves, for example, a dissociation of Igμ from Igα and Igβ, as had been suggested by earlier biochemical analyses (10,11). Because the same pattern of FRET gain and loss was observed in all cell lines, 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 BCRs. Using a variety of inhibitors, mutant BCRs and signaling components containing fluorescent proteins we provided evidence 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.

Imaging B cell activation at the surface of antigen presenting cells provides a new view of the spatial organization BCR signaling

In the studies described above, B cells were stimulated with multivalent antigens in solution as is conventional for analyses of B cell responses in vitro. However, several recent studies provided evidence that the relevant mode of antigen recognition by B cells in vivo may be of antigen on the surfaces of APCs. During infection, many pathogen-associated antigens are captured and retained on the surfaces of antigen-presenting cells (APCs) (12), such as dendritic cells (13,14) and subcapsular macrophages (15–17). In addition, antigens ultimately reach B-cell follicles where they are retained on follicular dendritic cells to serve as a reservoir of antigen for germinal center formation and affinity maturation (18). B cells have been shown

to interact with membrane-tethered antigens both in vitro and in vivo (4,13,15,19). In vitro, the initial contact with the antigen-containing membrane triggers B cells to spread, a response that is dependent on Lyn and Syk, the first two kinases in the B cell signaling cascade and accompanied by an intracellular calcium response (20). The B cells then contract to form an immunological synapse (21,22). We used TIRFM to image B cells encountering antigen on a planar lipid bilayer that also contained ICAM (unpublished data). We imaged splenic B cells from B1.8 transgenic mice that express an NP-specific BCR by TIRFM during the first several seconds of interaction of B cells with a fluid planar lipid bilayer that contained the antigen NIP_{14}-BSA and the intercellular adhesion molecule, ICAM-1, tethered to the bilayer via engineered histidine tags bound to nickel-containing lipids. TIRFM provided high resolution images that allowed us to observe the formation of individual BCR microclusters. 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. Labeling the BCR with fluorescent monovalent Fab anti-Ig and the B cell membrane with the lipophilic dye, DIC16, we observed that the BCRs 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, visible as a broadening of the contact areas, eventually resulting in a homogenous distribution of the B cell's membrane over the contact area. As the B cells spread on the bilayer 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 to form a synapse fusing with one another on their way. After five minutes on the bilayer the membrane ruffling and the formation of BCR microclusters subsided and the B cell lamellipodia contracted. This behavior of the B cell was similar to that recently described by Batista and colleagues (23).

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 individual microclusters underwent a conformational change to an 'open' active conformation immediately following antigen binding and 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.

The mechanisms underlying BCR clustering

The clustering of immune receptors is fundamental to the mechanisms by which signaling is initiated and yet at present we understand 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 lead to the conclusion that physically crosslinking BCRs into lattice-like clusters was required for signaling. However, observing TIRFM of B cells encountering antigen in a lipid bilayer we discovered that BCR microclusters formed in nearly an identical fashion resulting in identical signaling when BCRs engaged monovalent antigen in a fluid planar lipid bilayer that could not physically crosslink the BCRs versus multivalent antigens (unpublished data). 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 binding of either monovalent or multivalent antigens 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 BCR were inside clusters. 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 blocking the Src-family kinase activity had no effect on this process. Analyzing B cells interacting with bilayers that contained very few molecules of monovalent antigen that limited the ability of BCRs to cluster we learned that binding of the antigen alone did not induce the individual BCRs to stop. Analyzing the behavior of several mutant BCRs we learned that stopping and clustering required the Cμ4 membrane proximal domain of the Fc region of the mIg. Thus, mIgμ with a well studies transmembrane mutation (YS→VV) that allowed the mIgμ to be expressed at the cell surface stopped and clustered upon antigen binding but a deletion mutant missing Cμ4 did not stop upon antigen binding and failed to signal. These results suggested that monovalent antigen induced a change in the BCR ectodomain that reveals an interaction face that allows oligomers to form between antigen-engaged BCRs. Conversely, the Cμ4 domain expressed alone on the B cell surface clustered spontaneously and activated B cells. These results support a novel mechanism for the initiation of BCR signaling in which antigen binding induces a conformational change in the Fc portion of the BCR revealing an interface that promotes BCR clustering.

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. In addition, monovalent interactions of BCRs with antigens would provide a mechanism by which B cells could read the affinity of the antigen by avoiding the overwhelming affect of the avidity provided by the multivalent BCR-antigen interactions. In addition, these findings may provide a general framework for understanding microclustering of other immune receptors, including the clustering of the monovalent TCR by monovalent MHC-peptide complexes.

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

Our observations from single molecule tracking of the BCR provided evidence for a novel mechanism by which antigen engaged receptors oliogmerized. However, these studies left unanswered the question: how does the oligomerized BCR but not the monomer trigger signaling by recruiting the first kinase in the pathway, the Src family kinase, Lyn. Earlier biochemical analyses of the detergent solubility of immune receptors including the BCR, TCR and IgE receptor provided evidence that the local lipid microenvironments 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 (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 (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 earlier 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). However, the use of detergents and cholesterol-depleting drugs are fraught with potential artifacts including creating the lipid heterogeneities we set out to study. 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 the selective association of the BCR with raft lipids following antigen binding (28).

To study the interactions of raft lipids with BCRs we generated cell lines that expressed a BCR containing the FRET donor fluorescent protein CFP and the FRET acceptor fluorescent protein, YFP, tethered to the membrane by either 'raft lipids' or by 'non-raft lipids' (28). FRET confocal microscopy of living B cells revealed that within seconds of antigen binding from solution the BCR selectively and transiently associated with the 'lipid raft' probe and that this association preceded the triggering of Ca^{++} fluxes by several seconds. The association of the antigen bound BCR with the lipid-raft 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 lipid-raft probe was blocked by the coengagement of the BCR with the potent inhibitory receptor, the FcγRIIB (29). Collectively, these FRET measurements provided the first direct evidence for the antigen-induced association of the BCR with lipid rafts in living cells that was regulated by B cell coreceptors.

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

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 expressing the 'raft' and 'nonraft' fluorescent probes as well as a cell line expressing the full length Lyn kinase. We determined that several seconds after BCRs formed microclusters, the microclusters associated selectively with the lipid raft probe (30). Association of the lipid raft probe with the clustered BCR preceded signaling and was not dependent on a signaling competent BCR. Tracking individual BCR microclusters revealed that the association of the raft probe with the BCR microclusters in the cell's periphery 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 followed by seconds the association of the raft probe but in contrast to the raft probe the association of 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. These results provided a new view of the dynamic process of antigen-induced BCR microclustering and the effect of microclustering on the local lipid microenvironment and recruitment of Lyn.

A model for the initiation of B cell activation based on results from live cell imaging

The results of our live cell imaging studies suggest the following model for B cell activation [reviewed in (31)]. B cells initially interrogate APC surfaces by cellular membrane protrusions. If antigen is engaged, BCRs form signaling active microclusters that 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 fully spread cell. The mechanism of microcluster formation involves binding to monovalent antigen and an inherent tendency of the BCR to cluster through the Cμ4 domain that may be facilitated by the topological restriction of the membrane protrusions at the initial contact sites. Once formed, the BCR microclusters condense raft lipids around them by mechanisms that are still unclear but may simply reflect an inherent property of transmembrane domains of the microclustered BCR to associate with saturated raft lipids. 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. 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. 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.

The synergistic interactions of the BCR receptor with innate immune system receptors

Our interest in the mechanisms underlying the antigen-induced initiation of BCR signaling led us to investigate the regulation of these events by B cell coreceptors of the innate immune system. BCR signaling is regulated and fine tuned by an array of innate immune system coreceptors many of which are present on the cell surface where they interact with the BCR. These include both the B cell inhibitory receptor, FcγRIIb, that recognizes immune complexes and the stimulatory coreceptor complex, CD19/CD21, that recognizes complement coupled antigens, as described above. However, members of one family of receptors, the Toll-like receptors (TLRs), that have been shown to influence BCR signaling are located in intracellular endosomes and not on the plasma membrane. Thus, it is not clear how synergy between the two receptors is achieved. One endosomal TLR, TLR9, that recognizes unmethylated CpG-DNA motifs has been shown to enhance BCR signaling resulting in hyperactivation of B cells to proliferate and differentiate into antibody secreting plasma cells in response to DNA-containing antigens (32). Recent evidence indicates that synergistic signaling between the BCR and TLR9 underlies the production of self-reactive antibodies in systemic autoimmune diseases and partly explains the preponderance of auto-antibodies that react with DNA-containing antigens (33,34).

Both the TLR9- and BCR-signaling pathways ultimately result in phosphorylation of p38, JNK and activation of NFKB. Although the BCR and TLR9 initiate signaling from two spatially distinct sites, the BCR from the plasma membrane, and TLR9 from endosomes, recent unpublished observations from my lab that the BCR-induced phosphorylation of the MAP kinases, p38 and JNK, occurs only after the BCR has internalized and trafficked to intracellular compartments suggests that these may be sites of synergistic signaling between the BCR and TLR9 in response to DNA-containing antigens. Using confocal microscopy and antibodies specific for the BCR, TLR9, phospho-p38 and phospho-JNK we recently showed that following the binding and internalization of DNA-containing antigens, the BCR signals for the recruitment of TLR9 from multiple small endosomes to a large autophagosome-like structure into which the BCR trafficks antigen and where synergistic signaling through to p38 and JNK occurs (35). The recruitment of TLR9 to the autophagosome-like compartment was by a dynein-mediated, microtubule-network dependent process and was necessary for B cell hyper-responses to DNA-containing antigens. This unique mechanism for BCR-induced TLR9 recruitment resulting in B cell hyper-responses may provide new targets for therapeutics for autoimmune disease.

The generation and maintenance of B cell memory in response to vaccination and natural malaria infection

My laboratory's work until recently has focused almost exclusively on the cellular and molecular mechanisms that initiate and regulate early BCR signaling events. Over the last few years, in collaboration with our NIAID colleagues, we have expanded our focus to investigate a much further downstream antigen-driven event, namely, the generation and maintenance of memory B cells. The hallmark of adaptive immunity is antigen-specific immunological memory. Immunological memory is a phenomenon that having been exposed to a pathogen and survived the infection the experience is 'remembered' by the immune system such that

upon re-exposure to the same pathogen an individual's immune response is more rapid and stronger such that the individual may experience no clinical systems of the infection. Indeed, all vaccines are predicated on the phenomenon of immunological memory. However, despite its importance we still have an incomplete understanding of the cellular and molecular mechanisms that underlie the generation, maintenance and activation of immunological memory. For most vaccines, neutralizing Ab plays a critical role in protective immune responses (36), and thus the mechanisms that underlie the generation and maintenance of B cell memory are of considerable interest. A vaccine to combat malaria is a highly desirable public health tool to reduce morbidity and mortality in African children. Our colleagues in the NIAID in the Malaria Vaccine Development Branch (MVDB) under the leadership of Dr. Louis Miller are working to develop such a vaccine. In malaria, parasite-specific antibodies have been shown to play a crucial role in controlling disease. Indeed, the transfer of antibodies from immune adults living in endemic areas but not from nonimmune adults to children with severe clinical malaria and high parasitemia resulted in significant reduction in both disease symptoms and parasite levels (37,38). Thus a detailed understanding of the generation and maintenance of memory B cells both in response to vaccination and to natural infection should be of value in informing the vaccine development effort in the MVDB.

In humans, B cell memory is encoded both in long-lived memory B cells and in plasma cells that reside in the bone marrow (39). B cell memory is long lived in that memory B cells generated in response to small pox and polio vaccination, for example, were detected in the peripheral blood of individuals 60 years later in the absence of any exposure to the viruses that cause these diseases (39–41). The mechanisms by which memory B cells or plasma cells are generated and maintained over a life time are not known although recent studies have implicated the TLRs in this process (42). Current evidence, primarily from serological epidemiological studies, indicates that immunological memory to malaria is slow to be acquired, incomplete and short lived (43). Thus, despite nearly constant exposure to *P. falciparum* from birth from infectious mosquito bites, children in endemic areas do not acquire immunity that protects them from severe disease until the age of five. Consequently, children under five years of age are susceptible to severe disease that accounts for over two million deaths each year in Africa alone. Immunity that protects against severe disease but not against mild disease is acquired during adolescence and an immunity sufficient to prevent disease but not to eliminate parasites is acquired only in early adulthood. Our current hypothesis is that *P. falciparum* infection disrupts the normal mechanisms by which B cell memory is generated, maintained or activated.

The memory B cell response to vaccination in malaria naive individuals

The interpretation of results from an analysis of the memory B cells generation and maintenance in endemic areas would be greatly facilitated by an understanding of the normal acquisition and maintenance of memory B cells in individuals in response to vaccination in nonendemic areas. At present there is no information on the kinetics of the acquisition of memory B cells or the frequency of memory B cells generated in naive individuals after exposure to antigen. Phase 1 malaria vaccine trials carried out by the MVDB provide unique opportunities to describe the acquisition of B cell memory to malaria antigens in the absence of infection. In collaboration with our MVDB colleagues, we described the acquisition of antigen-specific memory B cells in the peripheral blood of volunteers enrolled in two clinical trials of the malaria vaccine composed of *P. falciparum* apical membrane antigen 1 (AMA1) or merozoite surface protein 1_{42} ($MSP1_{42}$) on alum either alone or in combination with the TLR9 agonist, CpG. Memory B cells were identified by the method of Ahmed and colleagues (44) that relies on the unique ability of memory B cells, as compared to naive B cells, to respond to a mixture of CpG, pokeweed mitogen and SAC by proliferating and differentiating into clones of antigen-specific antibody secreting as measured by ELISPOT assays. We confirmed the validity of this

assay showing that the memory B cells ($CD19^+$, $CD27^+$, $CD38^-$) sorted from peripheral blood responded in this assay while the naive B cells ($CD19^+CD27^-CD38^-$) did not. We found that the acquisition of memory B cells is a dynamic process in which the antigen-specific memory B cell pool rapidly expands and then contracts following vaccination (unpublished data). In individuals who received CpG-containing vaccine antigen, specific memory B cells appeared more rapidly, in greater numbers, and persisted for longer. The percentage of vaccine-specific memory B cells present at the time of re-immunization predicted antigen-specific antibody levels 14 days later; and at steady state, there was a positive correlation between antigen-specific memory B cells and antibody levels. We also observed an antigen-independent decrease in the total IgG^+ memory pool in circulation 3 days after each vaccination, possibly the result of adjuvant-induced trafficking of memory B cells into tissues. Consistent with this possibility we observed a large increase in the total number of plasma cells in circulation, suggesting that memory B cells induced to leave the circulation gave rise to plasma cells. Such results are consistent with those of Bernasconi *et al.* (42) showing that revaccination of immune individuals with the current tetanus vaccine resulted in a nonspecific activation of all memory B cells to differentiate to plasma cells that the authors attributed to an adjuvant effect working through TLRs expressed by memory but not naive B cells. These are the first data on the naive human memory B cell response to vaccination and will serve as a baseline for similar analyses in endemic areas. We are in the process of analyzing peripheral blood samples that have been collected in a similarly designed MVDB vaccine trial testing AMA1 on alum with or without CpG in adults living in a malaria endemic area in Mali, Africa.

The memory B cell response to natural malaria infection

To determine the kinetics and magnitude of the acquisition of memory B cells with age and the impact of malaria infection on the acquisition and maintenance of memory B cells we are conducting a longitudinal study on a cohort of 225 volunteers, 2–25 years of age, in a village outside of Bamako, the capital city of Mali in collaboration with colleagues in the Malaria Research and Training Center (MRTC) at the University of Mali (Crompton *et al.* **JID** 2008, in press). The study was initiated in June 2006 prior to the malaria transmission season which runs July through December. This transmission season with six months of malaria exposure and six months free of malaria offers a near ideal condition to evaluate the impact of malaria infection on B cell memory generation and maintenance. Peripheral blood samples were collected every two months and 14 days after the first case of malaria for each individual. Our field data shows 298 cases of uncomplicated malaria with a predicted gaussian distribution of malaria cases over the transmission season peaking in October. The proportion of individuals who were malaria free decreased in a clear age-dependent fashion during the course of the season. A determination of the frequency of hemoglobin types in a multiple linear regression analysis including age and gender showed a surprising finding that an AS hemoglobin type was associated with significant delay to the first malaria infection. This observation will be important to take into account in vaccine trials that assess time to first malaria case as a measure of immunity. Taken together these results indicate that we have acquired quality clinical data on which to interpret the results of our B cell analyses.

We have just begun our analyses of the B cells in these samples and have already made a striking observation related to the differentiated state of the B cells. In other persistent or chronic infections, for example, in both HIV In both HIV (45–48) and hepatitis C virus (49) infections, T cells are driven into what has been termed an exhausted phenotype, presumably as a result of chronic stimulation through their antigen receptors. Exhausted T cells have increased expression of inhibitory receptors, mainly PLD1, and decreased expression of activating receptors. An exhausted B-cell phenotype has also been described recently for memory B cells in HIV-infected individuals (50). The HIV-specific B cells in individuals with high viremia were selectively driven into a premature poorly responsive state that might

contribute to the impaired B-cell responses in HIV disease. In normal individuals in non-endemic areas B cells with an exhausted phenotype represent only 1–2% of peripheral blood B cells. In the volunteers living in Mali, we observed that the percent of exhausted B cells reaches 20–60% even in children as young as two years of age (unpublished data). This observation provides the first evidence that malaria interferes with B cell development.

Summary

The research in the research in my laboratory over the last few years has focused on fundamental questions concerning the initiation of B cell activation. We have brought new live cell imaging technologies to bear on problems that could not be addressed using existing biochemical approaches. The results of these studies are providing a new view of the earliest events in antigen-induced B cell activation and how these events are regulated by potent co-receptors. We have also addressed questions concerning the role of intracellular signaling of the BCR particularly in response to DNA-containing antigens that play an important role in autoimmune disease. We provided evidence for a novel mechanism by which the BCR and TLRs interact namely by intracellular trafficking to a common signaling site. Lastly, the NIAID has provided a unique environment in which to begin to apply our newly gained understanding of basic B cell biology to the biology of infectious disease. With our collaborators in the MVDB and at the MRTC in Mali we are carrying out studies to determine the underlying cellular and molecular basis of the deficit in immunological memory to malaria infection.

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