Intrinsic Differences in the Initiation of B Cell Receptor Signaling Favor Responses of Human IgG+ Memory B Cells over IgM+ Naive B Cells

Angel M. Davey and Susan K. Pierce

*J Immunol* 2012; 188:3332-3341; Prepublished online 29 February 2012;
doi: 10.4049/jimmunol.1102322

http://www.jimmunol.org/content/188/7/3332

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/02/29/jimmunol.1102322.DC1

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article cites **50 articles**, 25 of which you can access for free at:
http://www.jimmunol.org/content/188/7/3332.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Intrinsic Differences in the Initiation of B Cell Receptor Signaling Favor Responses of Human IgG+ Memory B Cells over IgM+ Naive B Cells

Angel M. Davey and Susan K. Pierce

The acquisition of long-lived memory B cells (MBCs) is critical for the defense against many infectious diseases. Despite their importance, little is known about how Ags trigger human MBCs, even though our understanding of the molecular basis of Ag activation of B cells in model systems has advanced considerably. In this study, we use quantitative, high-resolution, live-cell imaging at the single-cell and single-molecule levels to describe the earliest Ag-driven events in human isotype-switched, IgG-expressing MBCs and compare them with those in IgM-expressing naive B cells. We show that human MBCs are more robust than naive B cells at each step in the initiation of BCR signaling, including interrogation of Ag-containing membranes, formation of submicroscopic BCR oligomers, and recruitment and activation of signaling-associated kinases. Despite their robust response to Ag, MBCs remain highly sensitive to FcγRIIB-mediated inhibition. We also demonstrate that in the absence of Ag, a portion of MBC receptors spontaneously oligomerized, and phosphorylated kinases accumulated at the membrane and speculate that heightened constitutive signaling may play a role in maintaining MBC longevity. Using high-resolution imaging, we have provided a description of the earliest events in the Ag activation of MBCs and evidence for acquired cell-intrinsic differences in the initiation of BCR signaling in human naive and MBCs. *The Journal of Immunology*, 2012, 188: 3332–3341.

The acquisition of Ab memory is critical for protection from many human infectious diseases and is the basis for most current human vaccines (1, 2). Re-exposure to Ags induces rapid, high-tiered, high-affinity Ab responses that are mediated by an expanded memory B cell (MBC) population expressing somatically mutated, isotype-switched IgG BCRs (3). In contrast, primary Ab responses involve naive B cells that express low-affinity, unswitched IgM and IgD BCRs. It is likely that the efforts to develop vaccines for infectious diseases for which we have none, including malaria and AIDS, would benefit from a thorough understanding of the biology of MBCs.

A variety of studies comparing gene expression profiles, cellular phenotypes, and responses to diverse stimuli have provided evidence of intrinsic differences in human naive and MBCs that may allow MBCs to respond more efficiently than naive B cells in secondary Ab responses (4). As compared with naive B cells, MBCs were shown to have increased expression of genes involved in activation, costimulation, and survival (5–7), to have a greater proliferative capacity and to produce more Ab-secreting plasma cells in response to a variety of stimuli including IL-2, IL-10, CD40L, and TLR ligands and anti-Ig (8–12). Taken together, these results define MBCs as long-lived, quiescent B cells that express high-affinity, somatically mutated, isotype-switched BCRs and are differentiated to respond rapidly to external stimuli, giving them an intrinsic advantage over naive B cells in immune responses.

Although our understanding of the biology of MBCs has advanced recently, what remains largely unknown is the molecular basis of Ag activation of MBCs even though our knowledge of BCR signaling in model systems has progressed considerably. B cell responses to Ags begin when Ags bind to the BCRs, resulting in BCR clustering and recruitment of the first kinases in the BCR signaling cascade, including Lyn and spleen tyrosine kinase (Syk) that trigger at least four different signaling cascades (13–16). Our understanding of where B cells engage Ag and initiate signaling has also advanced (17). Although B cells can respond to Ag in solution, B cells were shown to respond robustly to Ag presented on APCs in vitro (18). Moreover, the use of two photon laser scanning microscopy has provided strong evidence that B cells recognize Ag on APCs in vivo (19–22). Through the application of live-cell imaging techniques, it has recently become possible to view the earliest events in Ag-driven B cell activation that trigger the downstream signaling cascades (23, 24). High-resolution imaging has provided a detailed picture of how B cells engage Ag presented on membrane surfaces and respond to it. Fleire et al. (25) first described the remarkably dynamic nature of B cell Ag engagement. They observed for mouse primary B cells that during the first 2 min after contact with Ag-containing bilayers, the BCRs form microclusters that trigger B cells to spread over the bilayer and to accumulate BCRs at the interface of the cell and the bilayer leading to B cell activation. We characterized the BCR-intrinsic events leading up to microcluster formation using single-molecule tracking, live-cell imaging and provided evidence in mouse cells that Ag binding to BCRs leads to the formation of submicroscopic, immobile signaling-active BCR oligomers by a novel mechanism that involves interactions between the membrane-proximal ectodomains of the BCR’s Ig (26). Following oligomer-
ization, BCR microclusters grew with time in both the number of BCRs in the cluster and the area of the cluster, and the size of the cluster correlated with strength of signal (27). We demonstrated that IgG BCRs, as compared with IgM BCRs, more efficiently formed clusters that grew more rapidly and signaled more robustly (27). These studies established that intrinsic properties of IgG BCRs accounted for their enhanced ability to cluster and signal. Our understanding of the initiation of BCR signaling has come from the study of mouse model systems, and to date, these events have not been investigated in human B cells or in MBCs of either mice or humans.

Evidence is also accumulating that the early events in B cell activation are regulated by B cell coreceptors. We showed in mouse B cells that FcyRIIB, a potent inhibitory B cell coreceptor (28), when ligated to the BCR through immune complexes, blocked the earliest events in BCR signaling in primary mouse B cells, including BCR oligomerization, microcluster growth, and cell spreading (29). Whether FcyRIIB regulation of BCR signaling is similar in naive and MBCs in either mice or humans has not been investigated in detail. Diamond and colleagues (30) reported that the expression of FcyRIIB was upregulated on human MBCs as compared with naive human B cells but failed to be upregulated on MBCs in patients with systemic lupus erythematosus (SLE). The failure to increase expression of FcyRIIB correlated with the decreased ability of FcyRIIB to regulate BCR signaling in SLE, suggesting a critical role for FcyRIIB in the regulation of human MBCs.

In this study, we use quantitative total internal reflection fluorescence (TIRF) microscopy at the single-cell and single-molecule levels to investigate the early Ag-driven events in IgM+ and isotype-switched IgG+ human peripheral blood (PB) B cells as representative naive and MBCs, respectively. We provide evidence that the quality of the earliest events in B cell activation distinguish naive versus MBCs. We show that these early events are highly sensitive to inhibition by FcyRIIB. We also describe cell-intrinsic differences in constitutive BCR signaling in naive and MBCs that could contribute to the longevity of MBCs.

### Materials and Methods

**Cells and Abs**

B cells were purified from PBMCs from healthy, anonymous, adult blood bank donors via negative selection magnetic cell separation using a human B cell isolation kit II from Miltenyi Biotec (Bergisch Gladbach, Germany). Human B cells were frozen in FBS (Life Technologies, Grand Island, NY) containing 7.5% DMSO (Sigma-Aldrich, St. Louis, MO), kept at −20°C for at least 24 h, and then stored at −196°C in liquid nitrogen before use. B cells were thawed and washed in media (RPMI 1640 medium containing 10% FBS, and 50 μM 2-ME) immediately prior to each experiment as described by Crompton et al. (31).

Cy3 and DyLight 649-conjugated Fab goat Abs specific for human IgM, or F(ab′)2 goat anti-IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). DyLight 649 Fab anti-IgG was either obtained from Jackson ImmunoResearch Laboratories or prepared by conjugating intact Fab-specific Ab to DyLight 649 using an Abs labeling kit (Pierce, Rockford, IL) and subsequently preparing Fab using a Fab micro preparation kit with protein A/G purification (Pierce). A "nonblocking" Fab mouse mAb specific for human FcγRIIB (clone number 3H7) (Fab anti-FcγRIIB) was provided by MacroGenics (Rockville, MD) and conjugated to Alexa Fluor 568 using an Ab labeling kit (Molecular Probes, Eugene, OR). Cy3-labeled Fab mouse Ab pan specific for human MHC class I (Cy3-Fab anti-MHC-I) was prepared from intact Fab Ab purchased from AbD Serotec (Raleigh, NC) by conjugating the Ab to Cy3 using an Ab labeling kit (Amersham Biosciences, Piscataway, NJ) and preparing Fab using a Fab micro preparation kit with protein A purification (Pierce). Mouse mAb specific for human CD27 (anti-CD27) (Exbio, Vestec, Czech Republic) was conjugated to Alexa Fluor 568 using an Ab labeling kit (Molecular Probes) and Fabs were prepared using a Fab micro preparation kit with protein A purification (Pierce). Rabbit Abs specific for phospho-ZAP70 (Tyrosine 319) (Syk (Tyrosine 352) (anti-phosphorylated Syk [pSyk]) and for phospho-PI3K p85 (Tyrosine 458/p55 (Tyrosine 499) (anti-phosphorylated PI3K [pPI3K]) were from Cell Signaling Technology (Danvers, MA). Rabbit Abs specific for phospho-ERK1 (Thr202/Tyr204) (ERK2 (Thr185/Tyr187) (anti-phosphorylated ERK [pERK]) and for phospho-p38 MAPK (Thr180/Tyr182) (anti-phosphorylated p38 [p38]) were from R&D Systems. Primary Abs were detected using Alexa Fluor 488-conjugated F(ab′)2 goat Ab specific for rabbit IgG (H+L). Biotin-conjugated Fab specific for human IgG + IgM (H+L) [biotin-F(ab′)2] anti-Ig was purchased from Jackson ImmunoResearch Laboratories and used unlabeled or was conjugated to Alexa Fluor 568 using an Ab labeling kit ( Molecular Probes). F(ab′)2 goat Ab specific for human λ chain [Fab′(α)] anti-κ was purchased from MyBioSource (San Diego, CA), biotin-conjugated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce), and used unlabeled or conjugated to Alexa Fluor 488. Biotin-conjugated mouse mAb specific for human FcγRIIB (clone number 4A5.1) (biotin-anti-FcγRIIB) was purchased from AbD Serotec.

**Preparation of anti-Ig−containing planar lipid bilayers**

Planar fluid lipid bilayers were prepared as described previously (32–34) on Nanostrip (Cytect, Freeport, CA) cleaned glass coverslips attached to the bottom of LabTek chambers (Thermo Fisher Scientific, Rochester, NY). As described by Sohn et al. (35), biotin-containing lipid bilayers were prepared by fusing unilamellar vesicles of 99% 1,2-dioleoyl-sn-glycerol-3-phospho-cytidylylcholine and 1% 1,2-dioleoyl-sn-glycerol-3-phospho-ethanolamine-cap-biotin (Avanti Polar Lipids, Alabaster, AL) to the coverslips. Biotin-containing lipid bilayers were then incubated with 50 nM streptavidin (Jackson Immunoresearch Laboratories) for 10 min, washed, and incubated with 10 nM biotin–F(ab′)2 anti-Ig either alone or in combination with 20 nM biotin-anti-FcγRIIB for 20 min.

**TIRF microscopy and image analysis**

TIRF images were acquired for live and fixed cells using an Olympus IX-81 microscope (Melville, NY) equipped with a TIRF port, Cascade II 512 × 512 electron-multiplying charge-coupled device camera (Roper Scientific, Tucson, AZ), Olympus 100 × 1.45 N.A. objective lens, and heated stage maintained at 37°C. A 488/514-nm argon gas laser and a 568/647-nm red krypton/argon laser were used as indicated. Alexa Fluor 488, Cy3, Alexa Fluor 568, and DyLight 649 were excited at 488-, 514-, 568-, and 647-nm and detected after 525/550 BP; 550/560 ET BP; 605/600 BP; and 665 LP emission filters, respectively, after a 488/568/647 or 514/647 dichroic filter wheel cube, as appropriate. Image acquisition was controlled by Metamorph software (Molecular Devices, Sunnyvale, CA), with a 100-ms exposure time for a 512 × 512 image, unless otherwise noted. For live-cell, time-lapse imaging, cells were incubated with 200 nM fluorescently labeled Fab anti-IgM and/or Fab anti-IgG, washed twice, and added to chambers containing planar lipid bilayers. When indicated, cells were added to bilayers simultaneously incubated with 200 nM fluorescently labeled Fab anti-FcγRIIB, Fab anti–MHC-I or 200 nM Fab anti-CD27. To visualize the underlying anti-Ig (or anti-κ), 7% of the unlabeled biotin-F(ab′)2 anti-Ig [or biotin-F(ab′)2 anti-κ] was replaced with Alexa Fluor 568-labeled biotin-F(ab′)2 anti-Ig [or biotin-F(ab′)2 anti-κ]. TIRF images were acquired at ≤2-s intervals after loading the chamber with cells. For fixed-cell imaging, cells were prepared as described for live-cell imaging and incubated on the bilayers for the indicated times. As described by Depoil et al. (36), the chambers were washed, and the cells were fixed in 4% paraformaldehyde for 10 min at 37°C. Cells were permeabilized with 0.1% Triton X-100 for 5 min at 20°C and blocked with 1% BSA, 1% FCS, 1% goat serum, and 0.05% Tween 20 in PBS. Cells were incubated with anti-FcγRIIB, anti-κ, or pp38 primary rabbit Abs and labeled with fluorescent F(ab′)2 goat-anti-rabbit IgG.

The contact area of B cells with anti-Ig−containing lipid bilayers, the mean fluorescence intensity (Fl) of BCR, anti-Ig, pSyk, pp313, pERK, or pp38 within the contact area (i.e., per unit area), and the number of pSyk microclusters per unit area were measured and counted using ImageJ software (National Institutes of Health, available at http://rsbweb.nih.gov/ij). Unpaired two-tailed t tests were performed for statistical comparison (95% confidence interval). To monitor changes in the relative FI over the contact area during live-cell, time-lapse imaging, the autotracking function of Image Pro Plus software (Media Cybernetics, Silver Spring, MD) was used (35). A 3 × 3 pixel autotracking mode was used, and the background FI outside the contact area was used as the lower-end threshold for segmentation. Individual BCR normalized FI curves were normalized to one beat-norm plot with fluorescence at 0 s on a single graph. For these time-lapse measurements, statistical significance (95% confidence interval) was assessed using the "compareGrowthCurves" function from the statmod statistical modeling package (available at http://bioconductor.org/).
software (compareCurves) available from the R Project for Statistical Computing.

Linear regression analyses were conducted to assess the relationship between pSyk cluster number or mean FI and B cell contact area or BCR mean FI from fixed-cell images using Prism software (GraphPad, LaJolla, CA). Colocalization between the BCR and the underlying anti-Ig or FcγRIIB was quantified from background subtracted images via intensity correlation analysis as described by Li et al. (37) using the Wright Cell Imaging Facility plugin of ImageJ to obtain the Pearson’s correlation index (29), and unpaired two-tailed t tests were again performed for statistical comparisons.

Single-particle tracking and analysis

For single-molecule TIRF imaging, human B cells were labeled with 0.025 or 0.25 nM DyLight 649-Fab anti-IgM or anti-IgG, respectively, such that single BCR molecules could be visualized without the need for photo-bleaching (26). Cells were washed three times after labeling, incubated with Ag-presenting lipid bilayers for 5–10 min, and imaged with a 633-nm laser at 5 mW (at the objective lens in epifluorescence mode). To achieve an exposure time of 35 ms, a 100 × 100 pixel region of interest was used for imaging, and streamliner acquisition mode was used to image single BCR molecules for 300 frames over ~10 s. Data from at least 20 cells was acquired for each condition in each experiment.

The processing of single molecule TIRF videos was detailed previously (26), with tracking and analysis performed using Matlab (Mathworks, Natick, MA) code, based on available tracking algorithms (38, 39).

RESULTS

Early events in the response to membrane-bound anti-Ig distinguish human naive B cells and MBCs

We used live-cell TIRF microscopy to examine the early events that follow the encounter of IgM+ and IgG+ human PB B cells with anti-Ig, as a surrogate Ag, incorporated into lipid bilayers. We studied the B cell response to anti-Ig incorporated into lipid bilayers as a variety of studies have provided evidence that B cells are readily activated by Ag presented by APCs or incorporated into lipid bilayers as APC mimics in vitro. Two-photon laser

FIGURE 1. Interactions with anti-Ig− and anti-κ−-containing lipid bilayers distinguish human MBCs and naive B cells. (A) Representative time-lapse TIRF images over 180 s (Supplemental Video 1) of IgM+ B cells (top panels) and IgG+ B cells (bottom panels) placed on fluid lipid bilayers that did not contain F(ab′)2 anti-Ig (–anti-Ig) or did contain anti-Ig (+anti-Ig). The images shown are of Cy3-Fab anti-IgM (green); and the overlay of IgM and anti-Ig (red). Scale bar, 1

3334 INITIATION OF SIGNALING IN HUMAN NAIVE AND MEMORY B CELLS

(available at http://physics.georgetown.edu/). Trajectories were visually inspected and occasional tracking errors were corrected manually. A two-dimensional Gaussian fit was used to refine the positions of the diffraction-limited spots in the trajectories. Mean square displacement and instant diffusion coefficients for each of a BCR molecule’s trajectories were calculated from positional coordinates as described by Douglass and Vale (38, 39). Short-range single-molecule diffusion coefficients (μm²/s) were calculated from linear fits to mean square displacement data of individual molecules for the time intervals 35–140 ms and plotted as cumulative probability distribution graphs. The proportion of diffusion slower than 0.01 μm²/s is classified as immobile (26), so the greater the immobile fraction, the slower the rate of diffusion.

Statistical comparison of BCR mean FI accumulated within the B cell contact area per unit area as quantified from images of IgM+ and IgG+ human B cells labeled as in (A) and placed on −anti-Ig or +anti-Ig fluid lipid bilayers (B) or on anti-κ− or anti-κ+ fluid lipid bilayers (C). (D) Statistical comparison of BCR mean FI accumulated within the B cell contact area per unit area as quantified from images of IgM+ and IgG+ human B cells labeled as in (A) and fixed after 10 min of anti-Ig stimulation. (E) Normalized MHC-I FI quantified from several time-lapse image series over a period of 180 s for cells labeled with DyLight 649-Fab anti-IgM or -IgG and Cy3-Fab anti–MHC-I (Supplemental Fig. 2) and placed either on –anti-Ig or +anti-Ig fluid lipid bilayers. Normalized BCRs and anti-Ig FIs quantified from several time series over a period of 180 s for cells labeled with DyLight 649-Fab anti-IgM or anti-IgG and placed on anti-Ig [Alexa Fluor 568-F(ab′)2 anti-Ig]-containing bilayers (F) or on anti-κ [Alexa Fluor 568-F(ab′)2 anti-κ]−containing bilayers (G). The data in (B), (E), and (F) and in (C), (D), and (G) were averaged over two and three independent experiments, respectively, and the bars indicate the mean ± SD.
scanning fluorescence microscopy has provided evidence that recognition of Ag on APCs may be highly relevant to B cell activation in vivo. CD19+ B cells were purified from PBMCs by negative selection using Ab-coated magnetic beads and incubated with a mixture of Cy3-conjugated Fab goat Abs specific for human IgM (Cy3-Fab anti-IgM) and DyLight 649-conjugated Fab goat Abs specific for human IgG (DyLight 649-Fab anti-IgG) to label IgM+ and IgG+ B cells, respectively. The cells were washed and placed on fluid lipid bilayers that either contained or did not contain fluorescently labeled biotinylated F(ab')2 goat Ab specific for human IgG + IgM (H+L) (F(ab')2, anti-Ig) attached to streptavidin lipid incorporated into the bilayers. As measured by flow cytometry, ~1.3-fold more F(ab')2 anti-Ig bound to IgG- versus IgM-expressing B cells (Supplemental Fig. 1A); however, IgG+ B cells were slightly larger (~1.2-fold) than IgM+ B cells (Supplemental Fig. 1B), indicating that the density of BCRs was similar on the two cell types.

Time-lapse images showed that when IgM+ or IgG+ B cells were placed on bilayers that did not contain anti-Ig, the BCRs formed initial contact points with the bilayer but did not spread substantially and did not cluster (Fig. 1A, Supplemental Video 1). When placed on anti-Ig–containing bilayers the BCRs of both IgM+ and IgG+ cells clustered upon contact with the bilayers and the cells then rapidly spread over the bilayer (Fig. 1A, Supplemental Video 1). After maximal spreading, the cells showed a subtle contraction, with only a small inward movement of BCRs from the cell periphery. However, in contrast to the observations in mouse B cells (18, 25, 34), neither IgM+ nor IgG+ B cells formed well-organized immune synapses even after 10 min on the bilayers (data not shown). We also imaged the fluorescently labeled anti-Ig in the bilayer (Fig. 1A). Before B cells contacted the bilayer, the anti-Ig appeared evenly distributed, producing dim, uniform FI. Upon B cell encounters, the anti-Ig accumulated underneath the B cell contact areas, resulting in what appears to be corresponding areas of anti-Ig FI that overlap with the areas of BCR FI to varying degrees.

To quantify the accumulation of BCRs at the interface of the B cell with the bilayer, we tracked several individual cells for 3 min, measuring the BCR FI within the contact area of B cells with the bilayers (Fig. 1B). When placed on anti-Ig–containing bilayers, IgG+ B cells accumulated BCRs in the contact area more rapidly than IgM+ B cells, resulting in ~2-fold more BCRs after 3 min on the bilayer. The accumulation of the BCRs in the contact area was highly anti-Ig–dependent as neither IgG+ nor IgM+ B cells accumulated BCRs in the contact area in the absence of anti-Ig (Fig. 1B). To ensure that the IgG and IgM BCRs did not behave differently due to differences in binding to anti-Ig, we also quantified BCR accumulation upon stimulation with biotinylated F(ab')2 goat Abs specific for human κ L chain (anti-κ) incorporated into bilayers (Fig. 1C). Although the overall levels of BCR accumulation were slightly lower with anti-κ stimulation as compared with anti-Ig stimulation, the differences in the rate and level of accumulation between IgG+ and IgM+ B cells were similar, confirming the generality of the observation made with anti-Ig. BCR accumulation was also quantified per unit area from images of IgM+ and IgG+ human B cells labeled as described for Fig. 1A and fixed after 10 min of anti-Ig stimulation (Fig. 1D). After 10 min on anti-Ig–containing bilayers, the IgG+ B cells accumulated more BCR mean FI per unit area at the interface of the B cell with the bilayer than did IgM+ B cells.

The accumulation of BCR was selective for both IgM+ and IgG+ B cells as we observed no increase in MHC-I FI over the contact area in the absence of anti-Ig and only a slight, but not statistically significant, increase in the presence of anti-Ig (Fig. 1E). Furthermore, time-lapse TIRF images demonstrated that the MHC-I molecules did not colocalize with the BCR upon anti-Ig stimulation (Supplemental Fig. 2). This result indicates that MHC-I molecules neither accumulated in nor were excluded from the interface of the B cell with the bilayers.

We also imaged fluorescently labeled anti-Ig in the contact area of the B cell with the bilayer. For IgM+ B cells, we observed that the anti-Ig FI increased at a rate similar to that of the BCR (Fig. 1F), indicating that the BCRs that accumulated at the interface were bound to anti-Ig. In contrast, for IgG+ B cells, the BCR FI increased more rapidly than the anti-Ig FI between 30 and 150 s after contact with the bilayer, indicating that a portion of IgG BCRs that were not bound by anti-Ig accumulated in the contact area in an anti-Ig ligation-independent fashion. Consistent with this observation, although both IgM and IgG BCRs appeared to colocalize with anti-Ig to a relatively high degree (Fig. 1A), when colocalization was quantified and given as a Pearson’s correlation coefficient, it is clear that fewer IgG BCRs colocalized with anti-Ig than did IgM BCRs (Supplemental Fig. 3). We observed a similar phenomenon when B cells were stimulated with anti-κ incorporated into the bilayer. For IgM+ B cells, the BCR and anti-κ FI increased at similar rates in the contact area (Fig. 1G). However, for IgG+ B cells, a portion of BCRs accumulated in an anti-Ig ligation-independent manner for the first 150 s on the bilayer. This Ag ligation-independent accumulation of IgG BCRs may be a mechanism that allows MBCs to rapidly amplify BCR signaling at the initiation of the response.

**BCRs on MBCs readily oligomerize in response to anti-Ig**

Our previous studies in mouse B cells demonstrated that one of the earliest events following the B cell’s encounter with membrane-associated Ag is the formation of signaling-active immobile BCR oligomers (26). To compare the formation of immobile BCR oligomers on human naive versus MBCs, we used single-molecule TIRF microscopy to monitor the diffusion of the BCRs on cells after placing them on bilayers that either contained anti-Ig or not. Time-lapse imaging showed that the mobility of single BCR molecules was inhibited upon anti-Ig binding (Fig. 2A, Supplemental Video 2). To quantify the extent of formation of immobile BCR oligomers, cumulative probability distributions were compared...

---

**FIGURE 2.** Immobile BCR oligomers form in response to anti-Ig-containing fluid lipid bilayers. (A) Cumulative probability plots of the diffusion coefficients of single BCR molecules were obtained from time-lapse TIRF videos (Supplemental Video 2) of human B cells labeled with DyLight 649-Fab anti-IgM or -IgG and placed on −anti-Ig or +anti-Ig bilayers. The numbers (n) of trajectories collected from two independent experiments and used to construct each probability curve are indicated on the graph (*p < 0.0001). (B) The percentages are given of BCR molecules in the mobile and immobile fractions, defined by a cutoff diffusion coefficient of 0.01 μm²/s.
piled from >1000 single molecule trajectories (Fig. 2A). For both IgM- and IgG-expressing B cells placed on bilayers that do not contain anti-Ig, a fraction of BCRs were immobile (diffusion coefficient \( \leq 0.01 \mu m^2/s \)). This observation is consistent with previous studies of mouse primary B cells (26, 27, 40), and we have speculated that these spontaneous immobile BCR oligomers may play a role in tonic signaling (23). The IgG+ cells showed a statistically significantly higher fraction of immobile oligomers as compared with IgM+ cells (23 versus 17\%) (Fig. 2B), and the overall diffusion of the BCRs on IgG+ cells was significantly slower as compared with IgM+ cells (0.109 versus 0.131 \( \mu m^2/s \)). When placed on anti-Ig–containing bilayers, the BCRs on IgG+ cells more readily formed immobile BCR oligomers (46\% immobile fraction and an average overall diffusion coefficient of 0.043 \( \mu m^2/s \)) as compared with those on IgM+ cells (42\% immobile fraction and an average overall diffusion coefficient of

FIGURE 3. Phosphorylated kinases accumulate at the B cell membrane in response to anti-Ig–containing lipid bilayers. (A) Representative TIRF images show the BCR (red; labeled with DyLight 649-Fab anti-IgM or -IgG) and pSyk (green; rabbit anti-pSyk detected using Alexa Fluor 488-F(ab’)_2 rabbit Ig-specific Abs) distribution for IgM+ and IgG+ human B cells placed on anti-Ig–containing fluid lipid bilayers for 10 min, fixed, and labeled as described in Materials and Methods. Scale bar, 1 \( \mu m \). Also shown are the pSyk mean FI, pSyk cluster number, and ratio of pSyk mean FI to BCR mean FI quantified from several TIRF images of B cells placed on +anti-Ig or –anti-Ig bilayers. Each data point represents one cell analyzed in one of three independent experiments, and the bars indicate the mean ± SD. (B) The pPI3K mean FI and the ratio of pPI3K mean FI to BCR mean FI, (C) the pERK mean FI and the ratio of pp38 mean FI to BCR mean FI, and (D) the pp38 mean FI and the ratio of pp38 mean FI to BCR mean FI, all quantified as described for (A), using rabbit anti-pPI3K, -pERK, or -pp38, detected with Alexa Fluor 488-F(ab’)_2 rabbit Ig-specific Abs. (B–D) Each data point represents one cell analyzed in one of two independent experiments, and the bars indicate the mean ± SD.
0.062 μm²/s) (Fig. 2B), suggesting that MBCs are inherently better able to respond to anti-Ig.

Enhanced anti-Ig-driven and constitutive kinase phosphorylation in MBCs as compared with naive B cells

To determine whether the differences observed between IgG⁺ and IgM⁺ human B cells in the early events of B cell activation resulted in enhanced signaling, we quantified the amount of pSyk, pPI3K, pERK, and pp38 recruited to the B cell membrane by TIRF microscopy. B cells were labeled with Cy3-Fab anti-IgM and DyLight 649-Fab anti-IgG, placed on bilayers that either contained anti-Ig or did not for 10 min, fixed, permeabilized, stained with fluorescently labeled Abs specific for pSyk, pPI3K, pERK, or pp38, and imaged. Levels of phosphorylated signaling molecules were quantified as either pSyk, pPI3K, pERK, or pp38 FI over the B cell contact area or as the number of pSyk clusters. A representative image is given showing the BCR and pSyk distribution for IgM⁺ and IgG⁺ B cells (Fig. 3A). The overall staining patterns for pPI3K, pERK and pp38 were similar to that of pSyk. The amount of pSyk recruited and the number of pSyk clusters was significantly higher for IgG⁺ B cells as compared with IgM⁺ cells (p = 0.0001 in both cases) (Fig. 3A). In both cases the pSyk recruited to the membrane colocalized with the BCR. However, the pSyk to BCR FI ratios were similar for anti-Ig–stimulated IgM⁺ and IgG⁺ B cells (p = 0.072), indicating that the individual IgG BCR microclusters were not more signaling active than IgM BCR microclusters. Because the contact area of IgG⁺ B cells with the anti-Ig–containing lipid bilayer is greater than the area of IgM⁺ B cells, to confirm that the higher pSyk levels observed for IgG⁺ B cells were not simply a function of B cell size, we carried out correlation analyses. The results showed a positive correlation between BCR FI and pSyk cluster number for both IgM⁺ and IgG⁺ cells that was even more pronounced for IgG⁺ cells (Supplemental Fig. 4A). In contrast, pSyk cluster number did not correlate with the size of the contact area for either B cell type (Supplemental Fig. 4B).

The amount of pPI3K (Fig. 3B), pERK (Fig. 3C), and pp38 (Fig. 3D) recruited to the membranes of IgG⁺ cells placed on anti-Ig–containing bilayers was also significantly greater than the amount recruited to membranes of anti-Ig–stimulated IgM⁺ cells (p < 0.0001 in all cases). As was the case for pSyk, the ratio of pPI3K, pERK, and pp38 FIs to BCR FIs were similar for naive and MBCs. If cells were stimulated on anti-Ig–containing bilayers for only 2 min prior to fixation, phosphorylated kinase levels followed the same trend as that observed after 10 min of stimulation (data not shown).

For B cells placed on bilayers that did not contain anti-Ig, we observed little difference in pSyk FIs or cluster numbers between

FIGURE 4. The effect of BCR-FcγRIIB coligation on the response of B cells to anti-Ig–containing fluid lipid bilayers. (A) Representative time-lapse TIRF images over a period of 60 s of IgM⁺ B cells (top panels) and IgG⁺ B cells (lower panels) placed on –anti-Ig or +anti-Ig fluid lipid bilayers or bilayers that contained both anti-Ig and anti-FcγRIIB (anti-FcR). The images shown are of B cells labeled with DyLight 649-Fab anti-IgM (red, top) or –IgG (red, bottom) and Alexa Fluor 568-Fab anti-FcγRIIB (green). Scale bar, 1 μm. (B) Colocalization of BCR and FcγRIIB after 60 s on –anti-Ig, +anti-Ig, or +anti-Ig +anti-FcR bilayers quantified by Pearson’s correlation index. The data represent the mean ± SD of IgM –anti-Ig (n = 4); IgM +anti-Ig (n = 7); IgM +anti-Ig +anti-FcR (n = 7); IgG –anti-Ig (n = 5); IgG +anti-Ig (n = 3); and IgG +anti-Ig +anti-FcR (n = 7) in one of three independent experiments. (C) Normalized BCR FI quantified from several time-lapse image series, as in (A), over a period of 180 s from two independent experiments with the bars indicating the mean ± SD.

Downloaded from http://www.jimmunol.org/ by guest on November 30, 2017
IgM+ and IgG+ cells (Fig. 3A). In contrast, the amounts of pPI3K, pERK, and pp38 at the membrane in resting IgG+ B cells were significantly greater than those in resting IgM+ B cells ($p < 0.0001$ in all cases) (Fig. 3B–D), translating to a highly significant increase in the ratio of phosphorylated kinase FIs to BCR FIs. Given that the amount of pSyk at the membrane was not increased in MBCs in the absence of anti-Ig, the increase in pPI3K, pERK, and pp38 may be through a Syk-independent pathway, possibly through CD19. For PI3K, we determined that the IgG BCR and pPI3K were not highly colocalized (data not shown), consistent with this possibility.

**Naive and MBCs are sensitive to FcγRIIB-mediated inhibition**

FcγRIIB is a key negative regulator of BCR signaling (28) that we recently showed blocks the early events in mouse naive B cell signaling (29). To determine whether early events in the activation of human naive and MBCs are similarly affected by coengagement of the BCR and FcγRIIB, B cells were labeled with DyLight 649-Fab anti-IgM or -IgG and with a nonblocking Alexa Fluor 568-conjugated Fab mouse mAb specific for human FcγRIIB (Alexa Fluor 568-Fab anti-FcγRIIB). By flow cytometry, IgG+ cells expressed $\sim 1.25$-fold more FcγRIIB as compared with IgM+ B cells (Supplemental Fig. 1C), as previously reported (30, 41), but because MBCs also express $\sim 1.3$-fold more BCRs, the ratio of FcγRIIB to BCRs is similar for the two cell types. The cells were washed and placed on bilayers containing biotinylated F(ab’)$_2$ anti-Ig bound to streptavidin–lipid, either alone or in combination with biotinylated mouse mAb specific for human FcγRIIB (anti-FcγRIIB) to coligate the BCR and FcγRIIB. Time-lapse images (Fig. 4A) and the corresponding colocalization analysis at 60 s after stimulation (Fig. 4B) showed that anti-Ig alone, in the absence of coligation to FcγRIIB, induced a significant increase in colocalization of the BCR and FcγRIIB in IgM+ cells ($p = 0.012$) but not in IgG+ cells ($p = 0.2$), suggesting that the FcγRIIB may play a role in BCR signaling in the absence of coligation in naive B cells. Coligation of the BCR and FcγRIIB increased the colocalization of the BCR and FcγRIIB in both IgM+ ($p = 0.0013$) and IgG+ B cells ($p = 0.0011$) (Fig. 4B) and reduced the accumulation of BCRs in the contact area with the anti-Ig–presenting membrane (Fig. 4C) ($p < 0.0001$ in both cases).

The effect of BCR-FcγRIIB coligation on the accumulation of BCRs anti-Ig and pSyk in the interface between the B cell and the bilayer was determined at later time points. IgM+ and IgG+ cells were placed on bilayers containing F(ab’)$_2$ anti-Ig alone or in combination with anti-FcγRIIB for 2 or 10 min and then fixed. With BCR cross-linking alone, for both IgM+ and IgG+ B cells, the amount of BCRs and anti-Ig that accumulated in the interface increased from 2 min (Fig. 5A) to 10 min (Fig. 5B), and BCR-FcγRIIB coligation blocked this accumulation as well as the accumulation of pSyk.

**FIGURE 5.** BCR and anti-Ig accumulation at the B cell–anti-Ig–containing bilayer interface are inhibited upon BCR-FcγRIIB coligation. BCR mean FI (top panels), anti-Ig mean FI (middle panels), or pSyk mean FI (bottom panels) 2 min (A) or 10 min (B) after B cells were placed on −anti-Ig, +anti-Ig, or +anti-Ig +anti-FcγRIIB (anti-FcR) bilayers quantified from images of fixed B cells labeled with DyLight 649-Fab anti-IgM or -IgG (top panels), of Alexa Fluor 568-F(ab’)$_2$ anti-Ig (middle panels), or of Alexa Fluor 488-F(ab’)$_2$ Ig specific to anti-pSyk (bottom panels). Each data point represents one cell analyzed in one of two independent experiments for each time point, and the bars indicate the mean $\pm$ SD.
CD27 expression correlates with enhanced efficiency of early BCR signaling events

Substantial heterogeneity among human MBCs has been documented by a variety of studies (4, 42, 43). At present, the relationship between the subsets and their relative contribution to long-lived B cell memory has not been established. For example, even though CD27 has been considered to be a marker for human MBCs, CD27−IgG+MBC populations have been reported (44), and conversely, a proportion of human CD27+ B cells has not undergone isotype switching (45). We determined that ∼70% of IgG+ cells in PB were CD27+, whereas only ∼10% of IgM+ cells were CD27+ (data not shown). The B cells in the subpopulations defined by IgG, IgM, and CD27 expression differed in size with IgM+CD27− < IgG+CD27− < IgM+CD27+ = IgG+CD27+ (Supplemental Fig. 1D). However, the origin and function of human IgM+CD27+ MBCs remains controversial (4, 45). We investigated the early events in B cell activation and their sensitivity to FcγRIIB inhibition in IgM+ and IgG+ B cells that were either CD27− or CD27+. B cells were labeled with either DyLight 649-Fab anti-IgM or -IgG and Alexa Fluor 568-conjugated Fab mouse Ab specific for human CD27 (Alexa Fluor 568-anti-CD27), placed on bilayers containing anti-Ig alone or in combination with anti-FcγRIIB for 10 min, fixed, permeabilized, stained with rabbit anti-pSyk detected using Alexa Fluor 488 F(ab′)2 secondary Ab to rabbit Ig, and imaged. In the absence of anti-Ig stimulation, there were no significant differences in BCR accumulation or pSyk recruitment between IgG+CD27− and IgG+CD27+ B cells or between IgM+CD27− and IgM+CD27+ B cells, indicating that BCRs on CD27+ B cells of either isotype are not in a more constitutively activated state as compared with those on CD27− B cells. Upon anti-Ig stimulation, as compared with IgM+CD27− B cells, IgM+CD27+ B cells accumulated more BCRs and pSyk (p < 0.0001 in both cases) in the interface between the B cell and the bilayer (Fig. 6A). The anti-Ig–induced accumulation of BCRs and recruitment of pSyk were sensitive to FcγRIIB inhibition for both CD27+ and CD27− IgM B cells. CD27+IgG+ B cells also accumulated more BCRs and pSyk in the interface between the B cell and the bilayers as compared with IgG+ B cells that did not express CD27 (p < 0.0001 in both cases) (Fig. 6B). For both CD27+ and CD27− IgG-expressing B cells, both BCR accumulation and pSyk recruitment were sensitive to FcγRIIB inhibition. However, a comparison of IgM+CD27− cells and IgG+CD27+ cells showed that CD27 expression did not result in IgM+ cells accumulating as much BCR and pSyk in the interface upon anti-Ig stimulation as IgG+ cells (p < 0.0001 in both cases). Collectively, these results indicate that B cells that express CD27 are more responsive to anti-Ig than CD27− B cells. However, IgG BCRs are more efficient than IgM BCRs, independent of CD27 expression.

Discussion

Despite the key role that Ab memory plays in protection from a variety of infectious diseases (1, 2), little is known about the responses of human MBCs and naive B cells to Ag. High-resolution live-cell imaging is providing the tools necessary to define and order the early events in BCR signaling that ultimately lead to B cell proliferation and differentiation. In this study, we used these tools to compare the responses of human PB IgM+ B cells and IgG+ B cells as representative naive B cells and MBCs, respectively. Our results show that human MBCs are more robust at each step in the initiation of BCR signaling, from interrogation of the lipid bilayer to the formation of submicroscopic BCR oligomers to the recruitment and activation of kinases in the BCR signaling cascade. We recently provided evidence in mouse primary B cells that these early events in the initiation of BCR signaling were highly sensitive to the affinity of the BCR for Ag (40). As human MBCs have undergone somatic hypermutation and express high-affinity BCRs, we predict that in response to specific Ags, the differences in the response of human MBC and naive
B cells would be even more dramatic than those shown in this paper using anti-Ig as a surrogate Ag. The robust responses of MBCs to anti-Ig and anti-κ that we observed in vitro could provide an advantage in responding to Ag in the competitive environments of the lymphoid tissues in vivo, explaining in part the rapid, high-titered Ab responses characteristic of B cell memory. In addition, a number of studies have provided evidence for cell-intrinsic differences between human naive and MBCs in gene expression and responses to a variety of stimuli, including T cell help, cytokines, TLR ligands, and anti-Ig, that are consistent with the ability of MBCs to respond quickly and robustly during an immune response, giving MBCs an intrinsic advantage over naive B cells (4). Such differences would serve to further amplify the intrinsic advantage conferred on MBCs through their efficient Ag-driven initiation of BCR signaling.

We observed in human B cells a new phenomenon that we first described in mouse IgG-expressing B cells (27), namely that IgG BCRs, unlike IgM BCRs, show an Ag ligation-independent phase in the recruitment of BCRs to BCR microclusters. From 30 to 150 s following anti-Ig encounter, the FI of IgG BCRs in the interface between the B cells and the bilayer increased more quickly than did the FI of the underlying anti-Ig, indicating an accumulation of IgG BCRs that were not bound to anti-Ig. In contrast, the FIs of IgM BCRs and underlying anti-Ig accumulated at the same rate. We speculate that this may be a novel mechanism to rapidly amplify early IgG BCR signaling in MBCs.

We also found differences between naive B cells and MBCs that were independent of BCR ligation. We observed that in the absence of anti-Ig, MBCs were more signaling active, having more spontaneously oligomerized BCRs that recruit more pP3K, pERK, and pp38 to the membranes. Rajewsky and colleagues (46) recently demonstrated a key role of P3K in BCR tonic signaling. These authors suggested that modulation of P3K signal may be the key element in the control of mature B cell survival mediated by the BCR. Although studies have suggested that Ag binding is not required for the maintenance of IgG MBCs (47), the possibility that a constitutive or tonic BCR signal is needed has not been ruled out. In fact, Hikida et al. (48) proposed that the BCR uses phospholipase C-γ2 for MBC generation and maintenance, supporting the involvement of BCR signaling in maintaining MBC longevity. Other recent work suggested that tonic BCR signals are propagated via ERK, showing that treatment with a MEK inhibitor blocked immature B cell differentiation and that immature B cells with low BCR expression were rescued by rescue of expression of a constitutively active N-Ras (49). It is possible that the higher constitutive level of pP3K and pERK that we observed in MBCs plays a role in the survival of MBCs, allowing them to achieve their remarkable characteristic longevity. Adachi and Davis (50) recently provided evidence that the phosphorylation of ERK and p38 are dramatically different in naive and Ag-experienced T cells following TCR stimulation and suggested that these differences could account for differences in responses to Ag. It is possible that the difference in naive and MBC recruitment of pERK and pp38 in resting cells may subsequently impact Ag-driven signaling in an analogous fashion.

We also show that the robust early responses of MBCs to anti-Ig were completely inhibited by coligating the BCR and FcγRIIB. Thus, differentiation to MBCs does not alter the B cell’s sensitivity to FcγRIIB-mediated inhibition. We also observed a difference in the spatial relationship between the BCR and FcγRIIB following BCR cross-linking alone in naive and MBCs. Only in naive B cells did FcγRIIB show significant microscopic colocalization with the BCR. It is possible that the spatial proximity of FcγRIIB and the BCR serve to limit BCR signaling in naive B cells. It is becoming increasingly clear that MBCs are not a homogeneous subpopulation of cells but rather a phenotypically and functionally heterogeneous population of Ag-experienced B cells (42, 43). CD27 expression was considered to be a reliable marker for human MBCs, but it has more recently become apparent that up to 30% of IgG B cells are CD27−. IgGCD27− and IgGCD27+ subpopulations may have distinct origins and functions in memory responses. IgMCD27+ and IgM−IgG−CD27+ human B cells have also been described previously, but their origins and functions remain less well understood. We found that B cells that expressed CD27 were more robust in the early events in B cell activation but that IgGCD27− B cells demonstrated stronger responses to anti-Ig than did IgMCD27+ cells.

Collectively our results suggest that differences in the early events following anti-Ig binding between IgM naive B cells and IgG MBCs may explain in part the heightened recall Ab responses observed in vivo. A better understanding of the molecular mechanisms that determine the responses to Ag at the early stages of B cell activation in normal human naive and MBCs may provide new strategies to attenuate this process in systemic autoimmune diseases and to design more effective vaccines.

Acknowledgments
We thank Dr. Wanli Liu for helpful suggestions and guidance and Dr. Joseph Brzostowski for expert advice on imaging in the National Institutes of Health/National Institute of Allergy and Infectious Diseases, Laboratory of Immunogenetics Imaging Facility.

Disclosures
The authors have no financial conflicts of interest.

References