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Through an ITIM-Independent Mechanism the FcγRIIB Blocks B Cell Activation by Disrupting the Colocalized Microclustering of the B Cell Receptor and CD19

Liling Xu,*† Gen Li,*† Jing Wang,* Yilin Fan,* Zhengpeng Wan,* Shaosen Zhang,* Samina Shaheen,* Jing Li,‡ Li Wang,‡ Cai Yue,‡ Yan Zhao,‡ Fei Wang,§ Joseph Brzostowski,‖ Ying-Hua Chen,* Wenjie Zheng,†∥ and Wanli Liu*,**

B cell activation is regulated through the interplay of the BCR with the inhibitory coreceptor FcγRIIB and the activating coreceptor CD19. Recent studies suggest that Ag-driven BCR microclusters are efficiently converted to a signaling active state on colocalization with CD19 microclusters. Using total internal reflection fluorescence microscopy–based, high-resolution, high-speed live-cell and molecule imaging approaches, we show that when co-ligated to the BCR, the FcγRIIB can inhibit B cell activation by blocking the colocalization of BCR and CD19 microclusters within the B cell immunological synapse. Remarkably, this inhibitory function of FcγRIIB is dependent not on its well-characterized ITIM-containing cytoplasmic domain, but its transmembrane domain. Indeed, human primary B cells from systemic lupus erythematosus patients homozygous for gene encoding the loss-of-function transmembrane domain mutant FcγRIIB-I232T fail to block the synaptic colocalization of the BCR with CD19, leading to dysregulated recruitment of downstream signaling molecule p-PI3K to membrane proximal signalingosome. This inhibitory function of FcγRIIB in impairing the spatial-temporal colocalization of BCR and CD19 microclusters in the B cell immunological synapse may help explain the hyper-reactive features of systemic lupus erythematosus patient B cells in reported studies. These observations may also provide new targets for therapies for systemic autoimmune disease. The Journal of Immunology, 2014, 192: 000–000.

The primary function of B lymphocytes is to establish and maintain Ab memory for foreign Ags. It is critical for B cells to distinguish self-Ag from non–self-Ags, which is accomplished through surface-expressed BCRs (1, 2). Once bound by Ag, the BCR initiates the activation of B cells (1–3). Aberrant B cell activation induced by self-Ags is associated with B cell tumors and autoimmune diseases such as systemic lupus erythematosus (SLE), although the underlying mechanisms are incompletely understood (4–6). To balance immunoprotection and immunopathology, B cell activation is under strict control by either the activating CD19 or the inhibitory FcγRIIB coreceptor (7–9). Early biochemical studies identified that the actions of CD19 and FcγRIIB on the BCR is dependent on the cytoplasmic domains of the respective coreceptor (8, 9). Mouse model studies demonstrated that Ab responses in CD19-deficient mice are severely impaired (10–12), whereas FcγRIIB-deficient mice show susceptibility to autoimmune diseases in some certain genetic backgrounds, consistent with their respective activating and inhibitory functions (13, 14). Mechanistically, biochemical studies showed that FcγRIIB exerts inhibition of BCR signaling through its ITIM in the cytoplasmic domain to recruit the SH2-domain containing inositol 1,4,5-triphosphate 5-phosphatase SHIP (15).

The recent application of advanced live-cell imaging technologies to B cell activation studies have provided an updated view of the dynamic, complicated, yet ordered molecular events that follow within seconds of Ag recognition by the BCR. These studies have revealed that BCR microclusters function as a central platform for the signaling cascades that mediate B cell activation (3, 16), which, in turn, are subject to the regulation imposed by CD19 and FcγRIIB. Batista and colleagues (17) were the first to report the key importance of BCR and CD19 microcluster colocalization in the initiation of BCR signaling, which has been advanced by their recent study using super-resolution microscopy to show the modestly concomitant concentration of BCR and CD19 molecules in Syk signaling microclusters upon BCR-Ag recognition (18).

Recently, we showed by live cell imaging technique that BCR microclusters change their integrated fluorescence intensity (FI)
over time shortly after BCR binding to Ags in a process we termed BCR microcluster growth (19), which is itself subject to regulation by FcγRIIB (20). At physiological conditions, B cell activation could be under competitive regulation by both FcγRIIB and CD19 coreceptors. In this study, we used total internal reflection fluorescence microscopy (TIRFM)–based, high-resolution, high-speed live-cell and molecule imaging techniques to show that FcγRIIB can efficiently disrupt the spatial-temporal colocalization of BCR and CD19 microclusters within B cell immunological synapse (IS), suggesting a new inhibitory function of FcγRIIB. Moreover, we showed that this inhibitory feature relies on the transmembrane (TM) domain of FcγRIIB and not the ITIM-containing cytoplasmic domain, suggesting that TM domain based protein–protein and protein–lipid interactions may play an important role in the regulation of receptor-mediated cross-membrane signaling transduction. We linked these findings to clinical diseases demonstrating that primary B cells from SLE patients harboring the single nucleotide polymorphism I232T within the TM of FcγRIIB, a mutation previously shown to be significantly associated with SLE in its homozygous format (21, 22), have lost the ability to block the synaptic colocalization of BCR with CD19 and downstream signaling molecular microclusters. This inhibitory function of FcγRIIB in impairing the spatial-temporal colocalization of BCR and CD19 microclusters in the B cell IS may help explain the reported hyper-reactive features of SLE patient B cells (4–6).

Materials and Methods

Mice, cells, Ags, and Abs

B1–8–specific B cells were isolated from spleens of IgH B1–8/BL1–8 Igκ−/− transgenic mice as described previously (19). A20II.6, Ramos, and ST486 B cell lines were gifts for laboratory scientific studies from Dr. Susan K. P. (National Institute of Allergy and Infectious Diseases, National Institutes of Health [NIH]), all of which were originally purchased from American Type Culture Collection. Biotin-conjugated goat F(ab′)2 anti-mouse IgG, biotin-conjugated goat F(ab′)2 anti-human IgA+IgG+IgM (H+L), and rabbit Fab anti-mouse IgG specific for FcRIIa were purchased from Jackson ImmunoResearch Laboratory. Pacific Blue–conjugated mouse mAb anti-human CD19 and FITC-conjugated mouse mAb anti-human CD19 (clone hIB19) were obtained from eBioscience. Biotin-conjugated anti-mouse CD32/CD16 (clone 2.4G2) was purchased from BD. Biotin-conjugated anti-human CD32 (clone AT10) was purchased from AbD Serotec. Alexa 647–conjugated goat Fab anti-human IgM specific for Fcμa and secondary Ab Alexa Fluor 568–conjugated F(ab′)2 goat Abs specific for Fab were obtained from Invitrogen (Carlsbad, CA). Alexa Fluor 633–phospho–Zap-70 (Tyrosine 633) and CD19 (Tyro 159) were purchased from Cell Signaling Technology. Goat Fab anti-mouse CD19 (clone 1D3) and mouse Fab anti-human CD19 (clone hIB19) were made by using Fab micropreparation kit following a published protocol in our published studies (19, 20, 23–25). Conjugations of Abs with Alexa Fluor 405, Alexa Fluor 488, 568, or 647 were carried out using Alexa Fluor mAb labeling kit (Molecular Probes) following manufacturer’s protocols, whereas biotinylating Abs were carried out following manufacturer’s protocols of EZ-labeling kits (Molecular Probes) following manufacturer’s protocols, whereas biotinylating Abs were carried out following manufacturer’s protocols of EZ-labeling kits (Molecular Probes) whereas published studies (19, 20, 23–25). Conjugations of Abs with Alexa Fluor 405, Alexa Fluor 488, 568, or 647 were carried out using Alexa Fluor mAb labeling kit (Molecular Probes) following manufacturer’s protocols, whereas biotinylating Abs were carried out following manufacturer’s protocols of EZ-labeling kits (Molecular Probes) whereas published studies (19, 20, 23–25). Conjugations of Abs with Alexa Fluor 405, Alexa Fluor 488, 568, or 647 were carried out using Alexa Fluor mAb labeling kit (Molecular Probes) following manufacturer’s protocols, whereas biotinylating Abs were carried out following manufacturer’s protocols of EZ-labeling kits (Molecular Probes) whereas published studies (19, 20, 23–25).

Preparation of PLBs

PLBs were prepared containing biotinylated Ags to which biotinylated BCR and FcγRIIB ligands were attached through streptavidin following our published protocol (19, 20, 24, 25) and were provided as online resources that describe any necessary reagents, materials, and methods. In brief, biotin liposomes were prepared by sonication of 1,2-dioleoyl-sn-glycerol–3-phosphocholine and 1,2-dioleoyl-sn-glycerol–3-phosphoethanolamine–cap-biotin (Avanti Polar Lipids) in a 25:1 molar ratio in PBS at a lipid concentration of 2.5 mM. The PLBs were formed in Lab-Tek chambers (Nalge Nunc) in which the cover glasses were coated with streptavidin. The PLBs membranes containing surrogate Ags were at 37°C, 5% CO2. The fixed B cells were permeabilized with 0.1% Triton X-100 and pretreated with blocking reagent containing 100 μg/ml goat nonspecific IgG (Jackson Immunoresearch Laboratory) and 2% BSA. Subsequently, cells were stained with phospho–Zap-70 (Tyrosine 633), and CD19 (Tyro 159) and CD19 (Tyro 159) were incubated with either 20 nM biotinylated goat F(ab′)2 anti-mouse IgG or goat F(ab′)2 anti-human IgM specific for FcRIIa. In addition, the BCR with streptavidin followed by the biotinylated Abs. The labeled cells were washed twice and then loaded to the lipid bilayer containing Ags that cross-link BCR alone or co-ligate BCR and FcγRIIB. Cells were fixed with 4% paraformaldehyde 15 min after incubation with the PLBs membranes containing surrogate Ags at 37°C, 5% CO2. The fixed B cells were permeabilized with 0.1% Triton X-100 and pretreated with blocking reagent containing 100 μg/ml goat nonspecific IgG (Jackson Immunoresearch Laboratory) and 2% BSA. Subsequently, cells were stained with phospho–Zap-70 (Tyrosine 633) and CD19 (Tyro 159) were incubated with either 20 nM biotinylated goat F(ab′)2 anti-mouse IgG or goat F(ab′)2 anti-human IgM specific for FcRIIa. In addition, the BCR with streptavidin followed by the biotinylated Abs. The labeled cells were washed twice and then loaded to the lipid bilayer containing Ags that cross-link BCR alone or co-ligate BCR and FcγRIIB. Cells were fixed with 4% paraformaldehyde 15 min after incubation with the PLBs membranes containing surrogate Ags at 37°C, 5% CO2. The fixed B cells were permeabilized with 0.1% Triton X-100 and pretreated with blocking reagent containing 100 μg/ml goat nonspecific IgG (Jackson Immunoresearch Laboratory) and 2% BSA. Subsequently, cells were stained with phospho–Zap-70 (Tyrosine 633) and CD19 (Tyro 159) were incubated with either 20 nM biotinylated goat F(ab′)2 anti-mouse IgG or goat F(ab′)2 anti-human IgM specific for FcRIIa. In addition, the BCR with streptavidin followed by the biotinylated Abs.
for 60 min at 37 °C and washed thoroughly for further use. Immune complexes (ICs) were made by mixing 10 nM biotin-conjugated NIP8-BSA with 20 nM of either rabbit IgG of BSA-specific (for IgG-IC) or F(ab′)2 rabbit BSA-specific (for F(ab′)2-IC). F(ab′)2-IC or IgG-IC was then incubated on biotin-containing lipid bilayers for 20 min for binding. After washing, the F(ab′)2-IC or IgG-IC containing lipid bilayers for 60 min at 37 °C and washed thoroughly for further use. Immune complexes (ICs) were made by mixing 10 nM biotin-conjugated NIP8-BSA with 20 nM of either rabbit IgG of BSA-specific (for IgG-IC) or F(ab′)2 rabbit BSA-specific (for F(ab′)2-IC). F(ab′)2-IC or IgG-IC was then incubated on biotin-containing lipid bilayers for 20 min for binding. After washing, the F(ab′)2-IC or IgG-IC containing lipid bilayers.
were ready to be used in total internal reflection fluorescence (TIRF) imaging.

Molecular imaging by confocal fluorescence microscopy
A20II.6 cells were stained with Alexa Fluor 568–conjugated goat Fab anti-mouse CD1 (clone 1D3) and Alexa Fluor 405–conjugated rabbit Fab antimouse IgG specific for Fcγ and washed twice, then placed on PLB membranes containing surrogate Ags to cross-link BCR alone or to coligate BCR and FcγRIIB for 15 min at 37 °C, 5% CO₂, and then fixed with 4% paraformaldehyde. Images of stained molecules were captured by confocal fluorescence microscope (LSM710-3channel; Zeiss) with a 100× oil objective lens. Images were analyzed using ImageJ (NIH) software as reported in our previous studies (19, 20, 25, 27). In brief, images were subtracted for background and then marked with regions of interest (ROIs). The mFI values obtained were the ratio of integrated FI of the ROIs to the total area of pixels in the same ROIs.

Mathematical quantification of BCR, pSyk, pCD19, and p-P13K microclusters for FI information using a Matlab-based two-dimensional Gaussian analysis algorithm
The mathematical quantification of BCR, pSyk, pCD19, and p-P13K microclusters for precise FI information was performed following our published protocol by a Matlab-based two-dimensional Gaussian analysis algorithm. Through this analysis, precise two-dimensional positions and integrated FIs of these microclusters can be acquired by means of least squares fitting of a two-dimensional Gaussian function. Such a two-dimensional Gaussian function is used to quantify each of the two-dimensional FI profiles. Because the profiles of some of these microclusters in TIRFM images were not perfectly circularly shaped, we thus allowed the Gaussian function to analyze an elliptical shape. The two-dimensional Gaussian function used in this study is provided (19):

\[ f(x, y) = \frac{1}{\sqrt{2\pi\sigma_x\sigma_y}} \exp\left(-\frac{(x-x_c)^2}{2\sigma_x^2} - \frac{(y-y_c)^2}{2\sigma_y^2}\right) \]

We define the ellipticity by

\[ \epsilon = \sqrt{\frac{\sigma_y}{\sigma_x}}. \]

We define the two-dimensional width of each microcluster as

\[ \sigma_2^2 = \sqrt{\sigma_x^2 + \sigma_y^2}. \]

Through the two-dimensional Gaussian mathematical fitting, we are able to get several key parameters for each microcluster including the position (x_c, y_c), integrated FI (I), background FI (z_0), and full width at half-maximum peak height of the intensity distribution.

Images processing, mathematical, and statistical analyses
Unless specifically indicated, the display range of a set of TIRFM images in each figure is the same to allow direct visonial comparison of intensity. The colocalization index of BCR and CD19 or BCR and signaling molecules microclusters within the IS were calculated based on the intensity correlation analysis as described previously (19, 20, 24, 25). In this report, the mathematical quantifications by means of Pearson’s correlation index (PCI) analyses for the synaptic distribution of two molecules of interest were analyzed by the WCIF plugin of ImageJ software. Student t tests were performed for statistical comparisons. A p value <0.05 was considered to indicate a significant difference.

Results
FcγRIIB TM DOMAIN DISRUPTS COCLUSTERING OF BCR AND CD19
First, we asked whether the colocalization of BCR and CD19 microclusters could be regulated by the BCR inhibitory coreceptor FcγRIIB. To do so, we imaged the spatial and temporal distribution of both BCR and CD19 microclusters within the IS by TIRFM on human Ramos B cells that were placed on PLBs. Using an improved experimental system that is similar to the ones in our published report (20), we simultaneously tethered goat anti-human IgM F(ab’)₂ fragment and mouse anti-FcγRIIB mAb to PLBs to co-ligate BCR and FcγRIIB. To cross-link BCR alone, we used goat anti-human IgM F(ab’)₂ fragment and the isotype matched mouse mAb. The colocalization of BCR and CD19 microclusters was quantified by the PCI following our published protocol (24, 25, 27). Co-ligation of the BCR with FcγRIIB did not affect the colocalization of the BCR and CD19 after 5 min of engagement with the PLBs (p = 0.4056; Fig. 1A and 1B). However, BCR and CD19 colocalization was significantly disrupted at 10 min (p = 0.0012; Fig. 1C and 1D) and continued to be so at 15 min (p < 0.0001; Fig. 1E and 1F). The accumulation of BCR and CD19 into the IS was also downregulated by FcγRIIB and is consistent with our and others’ previous reports (20, 28, 29).

We also assessed the colocalization of BCR and CD19 microclusters using primary B cells from IgH B1-8/B1-8 Igκ−/− transgenic mice ( termed B1-8 primary B cells (30, 31). B1-8 primary B cells expressing B1-8-IgM-BCRs specifically recognize the hapten 4-hydroxy-3-nitrophenyl (31). We placed B1-8 primary B cells on PLBs tethering ICs composed of biotin-conjugated NIP8-BSA and rabbit polyclonal anti-BSA Abs either in their whole IgG format ( termed IgG-IC to co-ligate B1-8 BCR and FcγRIIB) or F(ab’)₂ fragment format ( termed F(ab’)₂-IC to

FIGURE 3. A loss-of-function FcγRIIB I232T mutant in SLE patients cannot block the synaptic colocalization of BCR and CD19 microclusters. (A and D) Representative TIRFM images of BCR (red) and CD19 (green) within the IS of A20II.6 B cells (A) or ST486 B cells (D) expressing either human FcγRIIB-WT or the loss-of-function mutant FcγRIIB-I232T are shown. Cells were activated for 15 min by BCR cross-linking alone or BCR and FcγRIIB co-ligation. Relative FI along a white line is shown. Scale bars, 1.5 μm. (B, C, E, and F) Shown are mathematical quantifications by PCI analyses for the synaptic colocalization of BCR and CD19 microclusters under BCR cross-linking alone or co-ligating BCR and FcγRIIB in its FcγRIIB-WT (B and E) or FcγRIIB-I232T (C and F) format. Data are from at least 21 cells over 3 independent experiments, with each dot representing the value of 1 cell. Bars indicate mean ± SD. A Student t test was performed with the p value indicated, ***p < 0.001.
shown. Bars indicate mean ± SD from at least 11 cells over 2 independent experiments.

Next, we addressed the molecular mechanism of the inhibitory function of FcγRIIB by transfecting a series of FcγRIIB mutants into A20II1.6 mouse B cells lacking endogenous FcγRIIB (Fig. 2A). These mutants showed proper plasma membrane localization and equivalent level of cell-surface expression (Supplemental Fig. 1B), consistent with earlier studies (32). FcγRIIB-WT potently disrupted the synaptic colocalization of BCR and CD19 microclusters (Fig. 2B). Unexpectedly, we found that FcγRIIB-DL238, which lacks the entire cytoplasmic domain (Fig. 2A), also showed equally efficient inhibitory ability as the FcγRIIB-WT (Fig. 2C). Earlier biochemical studies reported that tyrosine 235 (Y235), which is predicted to be located proximal to the inner leaflet of plasma membrane, is phosphorylated upon co-ligation of BCR and FcγRIIB (32). Because Y235 is intact in FcγRIIB-DL238, we tested the function of this residue by examining BCR and CD19 colocalization in B cells expressing a Y235 to F235 point mutation in either WT FcγRIIB (FcγRIIB-Y235F) or the truncation mutant FcγRIIB-DL238 (FcγRIIB-DL238-Y235F; Fig. 2A). We found that FcγRIIB-Y235F still maintained its inhibitory function (\( p = 0.0021 \); Fig. 2D) and FcγRIIB-DL238-Y235F only mildly impaired the synaptic colocalization of BCR and CD19 (\( p = 0.0778 \); Fig. 2E). These results suggest that the TM domain, but not the cytoplasmic domain, of FcγRIIB might be responsible for its inhibitory function. To confirm this speculation, we swapped the type I TM domain of FcγRIIB-WT with a type I TM domain of similar length from the fruit fly neural-cadherin protein (FcγRIIB-FF-TM; Fig. 2A). In the transfected A20II1.6 B cells, FcγRIIB-FF-TM showed good plasma membrane location and comparable surface expression as FcγRIIB-WT (Supplemental Fig. 1B). PCI analyses on BCR and CD19 TIRFM images indicated that FcγRIIB-FF-TM completely lost the ability to block the synaptic colocalization of BCR and CD19 (\( p = 0.1585 \); Fig. 2F), endorsing this new inhibitory function of FcγRIIB depending on its TM domain, but not the cytoplasmic tail.

**FcγRIIB can inhibit BCR and CD19 colocalization under the same synaptic recruitment efficiency of BCR (or CD19) microclusters**

PCI is a reliable tool to quantify the codistribution of two molecules and has been widely used by us and others to quantify TIRFM images (17, 19, 24, 25, 27). However, it is in principle an intensity-based method that can produce reduced PCI values resulting from a reduction in the mean FI (mFI) of one of the molecules in the ROI being analyzed. Therefore, we compared the inhibitory function of FcγRIIB-WT and FcγRIIB-FF-TM under co-ligating conditions for the BCR and FcγRIIB when equivalent amounts of BCR or CD19 molecules were recruited to the IS (Supplemental Fig. 1C). Still, FcγRIIB-FF-TM lost the ability to block the synaptic colocalization of BCR and CD19 microclusters compared with FcγRIIB-WT (Supplemental Fig. 1D). To circumvent the inhibitory effects from the cytoplasmic tail of FcγRIIB, we made an additional mutant FcγRIIB-FF-TM-DL238, in which the TM domain of the truncation mutant FcγRIIB-DL238 (see Fig. 2A) was swapped with the TM domain of fruit fly neural-cadherin protein (Supplemental Fig. 2A). FcγRIIB-DL238 or FcγRIIB-FF-TM-DL238 was expressed at the surface of A20II1.6 mouse B cells to comparable levels (Supplemental Fig. 2B) and equivalent levels of BCR, or CD19 was recruited to the IS of both cell types (Supplemental Fig. 2D). As expected, FcγRIIB-DL238 maintained and FcγRIIB-FF-
TM-DL238 lost its ability to block the synaptic colocalization of BCR and CD19 microclusters (Supplemental Fig. 2E). These analyses confirmed that the TM domain of FcγRIIB has the ability to inhibit the synaptic colocalization of BCR and CD19 microclusters. However, we cannot exclude the possibility that the decreased colocalization of BCR and CD19 microclusters is also contributed by the impaired recruitment of BCR and CD19.

A loss-of-function FcγRIIB I232T mutant in SLE patients is incapable of blocking the synaptic colocalization of BCR and CD19 microclusters

Early epidemiological studies demonstrated a loss-of-function polymorphism in the TM domain of human FcγRIIB (FcγRIIB-I232T) in SLE patients where FcγRIIB-I232T cannot perturb and associate with sphingolipid- and cholesterol-rich lipid raft microdomains upon BCR and FcγRIIB co-ligation (20–22). Because this mutation lies within the TM domain, we next tested its function to regulate the synaptic colocalization of BCR and CD19 microclusters by expressing FcγRIIB-I232T in A20II1.6 B cells. As expected, FcγRIIB-WT potently inhibited the colocalization of BCR and CD19, whereas the FcγRIIB-I232T mutant did not (Fig. 3A–C). Similar results were obtained in human ST486 B cells lacking endogenous FcγRIIB (Fig. 3D–F). We confirmed that FcγRIIB-WT and FcγRIIB-I232T show good plasma membrane location and comparable surface expression on the cells that were imaged by TIRFM (Supplemental Fig. 3A).

The inability of FcγRIIB-I232T to block the synaptic colocalization of BCR and CD19 could be a result of the impaired recruitment of FcγRIIB-I232T to the B cell IS compared with FcγRIIB-WT. To check this point, we simultaneously examined the recruitment of BCR, FcγRIIB, and CD19 into the IS of human ST486 B cells using an advanced three-color-TIRFM time-lapse imaging approach (Fig. 4A, 4B, Supplemental Fig. 3B, 3C, and Supplemental Videos 1–7). The kinetics of FcγRIIB-WT or FcγRIIB-I232T accumulation into the IS was analyzed following our published protocol (20, 24, 25, 33). We found that both molecules were equally recruited to the B cell IS under co-ligating conditions compared with BCR cross-linking alone (Fig. 4A, 4B, Supplemental Fig. 3B, 3C, and Supplemental Video 5). We also performed the PCI analysis to quantify the colocalization of BCR with either FcγRIIB-WT or FcγRIIB-I232T. We found that BCRs similarly colocalized with either FcγRIIB-WT or FcγRIIB-I232T after co-ligation (Supplemental Fig. 3D and Supplemental Videos 2 and 4), suggesting a similar physical cross-linking effect in the process of BCR and FcγRIIB co-ligation. These two independent analyses demonstrated that the defect of FcγRIIB-I232T is not due to the impaired recruitment of FcγRIIB-I232T to the B cell IS compared with the case of FcγRIIB-WT upon its co-ligation to BCRs.

Lastly, we quantified the colocalization dynamics of the BCR and CD19 using PCI analysis over the time lapse and found an obvious increase in colocalization efficiency of BCR and CD19 microclusters.
shortly after the initiation of B cell activation regardless of FcγRIIB involvement (Fig. 4C, 4D, and Supplemental Videos 6 and 7). However, starting at 3–5 min, FcγRIIB-WT but not the FcγRIIB-I232T mutant impaired the synaptic colocalization of BCR and CD19 microclusters (Fig. 4C and 4D). These results are consistent with the end-point evaluations of human Ramos B cells (Fig. 1) and primary human B cells (Fig. 5 and Supplemental Fig. 4) discussed in further detail later in this report, demonstrating again that FcγRIIB relies on its TM domain to efficiently block the synaptic colocalization of BCR and CD19 microclusters.

Reduction in the colocalization of BCR and CD19 is mainly specific to the lipid raft regions

The experiments using the human loss-of-function mutant FcγRIIB-I232T suggested that the association of FcγRIIB with lipid rafts plays an important role in regulating the colocalization of BCR and CD19 microclusters. We were curious to know where BCR and CD19 show the colocalization, in the lipid-raft–rich or in the lipid-raft–poor regions. We used lipid raft marker Lyn-m16-CFP to probe the lipid rafts on the plasma membrane and checked the distribution of BCR (or CD19) microclusters in the condition of BCR cross-linking alone or BCR and FcγRIIB co-ligation. We observed much higher efficiency of BCR and CD19 colocalization in lipid-raft–rich regions (strong Lyn-m16-CFP FI microdomain) compared with lipid-raft–poor regions (weak Lyn-m16-CFP FI microdomain; Supplemental Fig. 3E), suggesting BCR and CD19 mainly colocalized in the lipid raft regions (Supplemental Fig. 3F and 3G). More importantly, a comparison of the synaptic colocalization of BCR and CD19 in the condition of BCR cross-linking alone or BCR and FcγRIIB co-ligation suggested that the observed reduction in the colocalization of BCR and CD19 is mainly specific to the lipid-raft–rich regions (Supplemental Fig. 3H and 3I). Then we asked whether the defect of FcγRIIB-I232T can be reversed by substituting its TM domain with the TM domain of a lipid-raft–resident protein, like LAT. To test this hypothesis, we made the construct of FcγRIIB-LAT-TM-DL250, in which the TM domain of human FcγRIIB is substituted with the TM domain of LAT and its colocalization with BCR is inhibited by FcγRIIB-WT (Fig. 8). Together, these results demonstrate that the impaired synaptic colocalization of BCR and CD19 microclusters contributes to the inhibition in the recruitment of signaling molecules to the membrane proximal signalosome. FcγRIIB-I232T does not inhibit the integrated FI change of pCD19, pSyk, and p-P13K microclusters when co-ligated to the BCR.

Recent high-resolution and high-speed live-cell imaging experiments have revealed the nature of the dynamic recruitment of intracellular signaling molecules to the IS and BCR microclusters, which, in turn, transduce BCR signals and ultimately activate B cells (3, 16). In this study, we examined how FcγRIIB will regulate the synaptic recruitment of two such early signaling molecules, Syk and P13K, into the BCR signalosome by TIRFM. Under co-ligating conditions, we found that FcγRIIB-WT efficiently impaired the synaptic colocalization of BCR and pSyk microclusters (Fig. 6A–C) and the recruitment of pSyk molecules into the IS (Fig. 6E). However, the FcγRIIB-I232T mutant failed to show such inhibitory function (Fig. 6A, 6B, 6D, and 6F). Because phosphorylation at Y482 and/or Y513 of CD19 is needed to recruit P13K to the membrane proximal BCR signalosome (9, 18, 34), we next examined the effect of FcγRIIB on the synaptic colocalization of phosphorylated CD19 to BCRs within the IS. FcγRIIB-WT potently blocks the synaptic accumulation of pCD19 in the IS and colocalization of p-P13K and BCR microclusters, whereas FcγRIIB-I232T loses these capabilities (Fig. 7). Finally, using an anti–p-P13K p85 (Tyr458)/p55 (Tyr199) Ab (35), we showed that the synaptic recruitment of the phosphorylated P13K p85 subunit (termed p-P13K in this report) and its colocalization with BCR is inhibited by FcγRIIB-WT, but not FcγRIIB-I232T (Fig. 8). Together, these results demonstrate that the impaired synaptic colocalization of BCR and CD19 microclusters contributes to the inhibition in the recruitment of signaling molecules to the membrane proximal signalosome.

**FIGURE 6.** Impaired synaptic colocalization of BCR and CD19 microclusters downregulate the recruitment of pSyk to BCR microclusters. (A and B) Representative TIRFM images of BCR (red) and pSyk (green) within the IS of human ST486 B cells expressing either FcγRIIB-WT or FcγRIIB-I232T. Relative FI profiles for BCR (red) and pSyk (green) are given (B). Scale bar, 1.5 μm. (C-F) Mathematical quantification for the synaptic colocalization (C and D) or accumulation (E and F) of BCR and pSyk microclusters for human ST486 B cells as introduced in (A) and (B). Data are from at least 26 cells over 3 independent experiments with each dot representing the value of 1 cell. Bars indicate mean ± SD. A Student t test was performed with the p value indicated, ***p < 0.001.
To understand how FcγRIIB might modulate downstream BCR effector molecules, we imaged thousands of BCR and BCR signaling molecule microclusters, including pSyk, pCD19, and p-PI3K, within the IS by TIRFM, and computed the changes of these microclusters in terms of integrated FI through a Matlab algorithm-driven two-dimensional Gaussian fitting protocol as reported in our published studies (19, 20, 25, 27). Accordingly, FcγRIIB-WT dampened the FI of BCR microclusters by 84.3% in B cells under co-ligation conditions compared with B cells activated by BCR cross-linking alone (Fig. 9A and 9B), consistent with our published studies quantifying the function of WT mouse FcγRIIB on BCR microclusters (20). In contrast, the FcγRIIB-I232T showed only a partial inhibitory effect with a 60.3% reduction in FI (Fig. 9A and 9B). Clearly, the growth features of BCR microclusters is subjected to downregulation by not only the TM, but also the cytoplasmic domains of FcγRIIB, consistent with the fluorescence resonance energy transfer data in our early report (20). When we quantified pCD19 (Fig. 9C and 9D), pSyk (Fig. 10A and 10B), and p-PI3K (Fig. 10C and 10D) microclusters, we found that FcγRIIB-I232T mutant completely lost the ability to inhibit the growth in integrated FI of these microclusters compared with FcγRIIB-WT. These results suggest that the conversion of BCR microclusters from signaling inactive to active state is under strict regulation by the TM domain of FcγRIIB.

Primary B cells from SLE patients bearing the FcγRIIB-I232T homozygous mutant show defects in blocking early B cell activation events

We next studied the inhibitory function of FcγRIIB in primary B cells from SLE patients and healthy control subjects. B cells from peripheral blood was obtained from 6 healthy control subjects and 37 diagnosed SLE patients, and genotyping of FcγRIIB...
was performed following reported protocol (22). Only three SLE patients were homozygous for the FcγRIIB-I232T mutation, consistent with a <10% incidence of homozygosity in epidemiological studies for Asian populations (5, 37–41). For live-cell TIRF imaging experiments, B cells were obtained from three donors with sex and age selected from the following categories: SLE patients heterozygous or homozygous for FcγRIIB-I232T, SLE patients WT for FcγRIIB, healthy donors heterozygous for FcγRIIB-I232T, and healthy donors WT for FcγRIIB. The nine SLE patients registered for the TIRF-based in vitro cell biology study presented with a similar severity of clinical lupus syndrome.

In our imaging analyses, we found that B cells from healthy individuals WT for FcγRIIB (Fig. 5A) or heterozygous for FcγRIIB-I232T (Fig. 5B) blocked the synaptic colocalization of IgM-BCR and CD19 microclusters. Intriguingly, B cells from SLE patients WT for FcγRIIB (Fig. 5C) or heterozygous for FcγRIIB-I232T (Fig. 5D) also responded similarly to Ag activation. However, strikingly, for B cells from SLE patients homozygous for FcγRIIB-I232T, a severe defect in blocking the synaptic colocalization of IgM-BCR and CD19 microclusters was observed (Fig. 5E). Because primary B cells can be positive for either IgM-BCR or IgG-BCR in human peripheral blood (35), we also analyzed in parallel the synaptic colocalization of IgG-BCRs with CD19 microcluster and found similar results (Supplemental Fig. 4A–C).

![FIGURE 9.](http://www.jimmunol.org/)

**FIGURE 9.** FcγRIIB-I232T cannot inhibit the integrated FI change of pCD19 microclusters when co-ligated to the BCR. (A and C) Shown for each indicated condition (BCR cross-linking alone or co-ligation with WT or mutant FcγRIIB) are TIRFM images for two representative BCR (A) or pCD19 (C) microclusters (top), and their fitted pseudocolored two-dimensional (middle) and 2.5-dimensional (bottom) Gaussian images (bottom). Scale bar, 1.5 μm. (B and D) Shown are statistical comparisons for the integrated FI of the indicated number of BCR (B) or pCD19 (D) microclusters within the IS of ST486 human B cells expressing either FcγRIIB-WT (top panel) or FcγRIIB-I232T (bottom panel). Bars indicate mean ± SEM collected from at least 20 cells over 2 independent experiments. A Student t test was performed with the p value indicated, ***p < 0.001.
B cells from SLE patients homozygous for FcγRIIB-I232T completely lost the ability to block BCR and p-PI3K colocalization, and thus the ability to impair the recruitment of p-PI3K to the IS (Fig. 11C, 11F, and 11I), suggesting hyper-activated PI3K signaling. We conclude that B cells of SLE patients homozygous for the TM domain FcγRIIB-I232T mutant show defects in blocking early activation events, re-emphasizing the importance of this domain in maintaining the balance of immunoprotection and immunopathology.

Discussion

B cell activation is under strict control by BCR activating and inhibitory receptors that are usually coexpressed on the cell surface. In this report, we identified a previously uncharacterized inhibitory function of FcγRIIB that is dependent on its TM domain to block the synaptic colocalization of BCR and CD19 microclusters. Unexpectedly, this inhibitory function does not require the well-characterized inhibitory ITIM motif present in the cytoplasmic domain (8, 15, 42–44). We propose that the TM domain provides an extra layer of inhibitory function for FcγRIIB to secure its unique position as the only inhibitory IgG FcR to downregulate immune responses (45). Indeed, the data in this report support the concept that FcγRIIB is a potent inhibitory coreceptor against B cell activation by two mutually integrated mechanisms: 1) impairing the synaptic recruitment of BCR and CD19 molecules, and 2) destabilizing the synaptic colocalization of BCR and CD19 microclusters. Cambier and colleagues (32, 46) were the first to...
report the importance of the TM domain of FcγRIIB in inhibiting B cell activation, demonstrating that FcγRIIB-mediated dephosphorylation of CD19 relies on the TM domain but not the cytoplasmic tail of FcγRIIB. Although the molecular mechanism remains unclear, we speculate that our results, showing that the TM domain of FcγRIIB can inhibit the recruitment of CD19 to the B cell IS, may help explain their observations. The importance of the FcγRIIB TM domain is also supported by a rich amount of epidemiological studies that have revealed a strong positive association of the FcγRIIB-I232T mutant with SLE disease (7). The I232T polymorphism and its conferred susceptibility to human SLE when homozygous were first identified in Japanese, Chinese, and Thai populations (5, 37–41), and then later in African (47) and white populations (6, 48).

How does the TM domain of FcγRIIB exert its inhibitory effect independent of the cytoplasmic ITIM motif? Although we do not understand the exact mechanism, we speculate that the TM domain modulates the association of FcγRIIB with lipid rafts. Such association has been reported by us and others (20–22). A number of cell biological studies have clearly shown the importance of the dynamic interaction of lipid rafts and TM domain of a protein in the activation of B cells (49). Indeed, the respective interaction patterns of lipid rafts with BCR, CD19, or FcγRIIB are quite different. The association of BCR with lipid rafts is transient and dynamic (50, 51), whereas FcγRIIB-WT with lipid rafts is rather in a rapid and sustained manner (20). More importantly, the interaction of BCR and lipid rafts is under the dual regulation by FcγRIIB and CD19. It was found that BCR cross-linking alone induces the translocation of BCRs into the lipid raft microdomain; however, upon co-ligation with FcγRIIB, FcγRIIB destabilized BCR and lipid rafts interactions (29). Several other studies have also revealed that CD19, when co-ligated with the BCR, increases the amount of BCRs that are translocated into lipid rafts and prolongs their residency, which likely accounts for the enhanced B cell activation (52, 53). Based on these data, we speculate that the sustained condensing of raft lipids around the TM domain of FcγRIIB molecules could be very effective in blocking the subsequent association of lipid raft microdomains with either BCR or CD19 microclusters. Our speculation is partially supported by early studies that showed the FcγRIIB-I232T mutant is unable to associate with the lipid raft microdomain upon co-ligation with the BCR (20–22).

Then, a following question is: can the TM domain of a lipid-raft–resident protein, like LAT, substitute the function of the TM domain of FcγRIIB? We tested this hypothesis and found that a chimeric construct of FcγRIIB-LAT-TM-DL250 carrying the TM of LAT did not show such inhibitory function. These results suggest that a sole constitutive lipid-raft–residing TM cannot inhibit the colocalization of BCR and CD19 microclusters. There could be two explanations for this result: first, the sequence of the TM domain of FcγRIIB is also important in exerting its inhibitory function to impair BCR and CD19 colocalization. Second, there is an early report by Sohn et al. (29) showing that FcγRIIB is not.
located in lipid raft regions in quiescent B cells but is translocated to the lipid rafts upon BCR and FcγRIIB co-ligation. Thus, it is possible that the co-ligation-induced interaction of the TM domain of FcγRIIB with lipid rafts might play an important role in inhibiting the colocalization of BCR and CD19. Regardless, together, these data suggest that the complex regulatory network by FcγRIIB and CD19 functions to balance the interaction of BCR and lipid rafts. Obviously, such a sophisticated network shall be important to maintain an appropriate response of B cells upon Ag recognition, and by doing so, secures a perfect homeostasis, balancing immunoprotection and immunopathology.

Three important points emerge from our results using advanced three-color time-lapse TIRF imaging to interpret the inhibitory function of FcγRIIB TM domain. First, FcγRIIB WT or FcγRIIB-I232T mutant shows comparable recruitment into the B cell IS in response to BCR and FcγRIIB co-ligation, signifying that the inability of FcγRIIB-I232T to inhibit is not because of its impaired synaptic recruitment; moreover, PCI analyses showed that BCRs similarly codistribute with either FcγRIIB-WT or FcγRIIB-I232T after BCR and FcγRIIB co-ligation, suggesting a similar physical cross-linking effect in both cases. Second, FcγRIIB-I232T can still partially impair the growth feature of BCR microclusters, but fully loses the ability to inhibit the growth of pCD19, pSyk, and p-P13K microclusters. These results indicate that synaptic recruitment of BCRs is subjected to the regulation of the TM and the cytoplasmic tail domain of FcγRIIB, whereas the recruitment of signaling molecules is more likely to be controlled by the TM domain of FcγRIIB. Third, PCI analyses on the dynamic pattern of the synaptic codistribution of BCR and CD19 in human ST486 B cells demonstrated an increase of colocalization of these molecules within 5 min of B cell activation. Starting at 5 min under co-ligation conditions, FcγRIIB WT but not the FcγRIIB-I232T mutant impaired the colocalization. These results indicate that a certain responding time is needed for FcγRIIB to inhibit the synaptic colocalization of BCR and CD19 microclusters through its TM domains. This point is also supported by the results from human Ramos B cells (10 min responding time) and from primary human B cells (10 min responding time). We do not have a clear mechanism to precisely explain why FcγRIIB needs such a responding time to inhibit the synaptic colocalization of BCR and CD19 microclusters. We assume that FcγRIIB can only execute its ability to inhibit the colocalization of BCR and CD19 microclusters upon the accumulation of sufficient amount of FcγRIIB molecules after BCR and FcγRIIB co-ligation. Our hypothesis is partially supported by the first two points as discussed earlier. Our hypothesis is also supported by two extra independent analyses: first, the synaptic accumulation of FcγRIIB plateaued in 5–8 min after BCR and FcγRIIB co-ligation; second, Pearson’s correlation coefficient for BCR and FcγRIIB plateaued around 5 min after co-ligation, suggesting the existence of a responding time to accumulate sufficient amount of FcγRIIB molecules near BCR microclusters. Because FcγRIIB WT and FcγRIIB-I232T show no differences in these two analyses, we propose that such an inhibitory effect also requires the ability of FcγRIIB to associate with lipid rafts. Thus, our model is that only when a sufficient amount of FcγRIIB-WT, but not the loss-of-function mutant FcγRIIB-I232T, is accumulated to the IS could FcγRIIB-WT block the colocalization of BCR and CD19 microclusters by destabilizing the association of CD19 and/or BCR with lipid rafts. It is our current endeavor to examine these hypotheses by using mathematical calculation–based molecule simulation and super-resolution imaging approaches.

In this report, we also showed that colocalized BCR and CD19 microclusters are more frequently observed at the periphery of the B cell IS, consistent with the published work of Batista and colleagues (17). Their study showed that BCR and CD19 microclusters highly colocalize as the B cell spreads, such that when the B cell reaches its maximal spreading phase, >45% of Ag-associated BCR microclusters are colocalized with CD19 microclusters. But, as Ag-associated BCR microclusters contract into the central supramolecular activation cluster (cSMAC), that is, the central region of the B cell IS, colocalization of BCR and CD19 microclusters decreases. However, in this report, we observed that the colocalization of BCR and CD19 microclusters increases within 5 min and then quickly plateaus. Over the next 10 min, only a very mild decrease of colocalization efficiency occurs. This discrepancy can be reconciled by the fact that there is a lack of an obvious contraction phase of BCR microclusters into a cSMAC in human B cells in our experimental system. Indeed, a TIRF imaging study by Davey et al. (35) showed that in contrast with mouse B cells, human B cells display only subtle contraction responses after maximal spreading, and thus do not form a well-organized cSMAC even 10 min after encountering Ags. Similarly, we observed a continuous spreading response after 15 min for Ramos and ST486 human B cell lines and 30 min for human primary B cells. The spreading dynamics of human B cells on Ag-containing PLBs, therefore, may explain why the colocalization of BCR and CD19 microclusters increases and then plateaus over time in our experiments. From this point of view, the observations in this report are not inconsistent with data from Batista and colleagues (17) that BCR and CD19 microclusters highly colocalize during the B cell spreading phase. In conclusion, the inhibitory role of the FcγRIIB TM domain in BCR signaling may functionally explain the severely hyper-reactive features of SLE patient B cells (4–6, 21).

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Disclosures

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