FcR-Like 2 Inhibition of B Cell Receptor-Mediated Activation of B Cells

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Abstract

FcR-like (FCRL) 2 is a transmembrane protein with immunomodulatory potential that is preferentially expressed by memory B cells in humans. It has two consensus ITIMs in addition to a putative ITAM sequence in its cytoplasmic domain. We have confirmed the cellular distribution of FCRL2 and analyzed its functional potential to show that coligation with the BCR leads to tyrosine phosphorylation of its ITIM motifs and subsequent Src homology region 2 domain-containing phosphatase-1 recruitment to facilitate inhibition of BCR signaling. Mutational analysis indicates that the tyrosine residues in both inhibitory motifs of FCRL2 are required for complete inhibition of BCR signaling, whereas tyrosines in the putative activation motif are dispensable for signal modulation. These findings suggest a negative immunomodulatory function for FCRL2 in the regulation of memory B cells.

The FcR-like (FCRL) molecules are encoded by an ancient family of genes with sequence similarity to the FcγRs (1–5). The transmembrane FCRL1–6 molecules are highly conserved in humans, dogs, and opossums, but not in mice. Mice have only three FCRL orthologs that have limited homology, thereby limiting the usefulness of the murine model for study of the FCRL family (5). Furthermore, despite the FCRL and FcR sequence

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conservation, gene structural similarity, and neighboring genomic localization and organization, none of the human FCRL family members have been shown to bind Igs, and the physiological ligands of all except FCRL6 remain unknown (5). The FCRL molecules are exclusively expressed by lymphocytes, and most of them, FCRL1, −2, −4, and −5, have been found only on B lineage cells. FCRL2 [previously called Src homology 2 domain-containing phosphatase anchor protein (SPAP) (3), FcRH2 (2), IRTA4 (6), BXMAS2 (7) and IFGP4 (4)] has four extracellular C2 type Ig domains, an uncharged transmembrane segment, and a cytoplasmic tail that contains a potential ITAM and two consensus ITIMs (2). Other members of the FCRL family have been shown to have either activating or inhibitory signaling potential (8–10).

Signals transduced by the BCR are pivotal for B cell activation and differentiation (11, 12). Cross-linking of the BCR by Ag or anti-Ig Ab induces a transient increase in cytosolic-free calcium, Src family kinase activity, and enhanced protein tyrosine phosphorylation (13). The noncovalently associated Igα/β components of the BCR play essential signaling roles in B cell signaling. Following ligand recognition, tyrosine residues in the Igα/β ITAMs are phosphorylated by Src family protein tyrosine kinases (14). Once the ITAM tyrosines are phosphorylated, they serve as docking sites for Src homology 2 (SH2) domains of Syk. Upon activation, Syk phosphorylates its target proteins, such as the scaffolding protein BLNK, leading to the activation of effector molecules including phospholipase Cγ2 (PLCγ2) and release of intra-cellular calcium (13). Activation of MAPK signaling cascades leads to phosphorylation and activation of transcription factors, which results in new patterns of gene expression.

Coreceptors on B cells balance competing activating and inhibitory signals to regulate the BCR-mediated responses to antigenic stimulation. The inhibitory FcγRIIb binds to the Fc portion of secreted IgG Abs in complex with Ag to regulate the threshold at which immune complexes will trigger BCR-induced signaling (15). FcγRIIb engagement results in phosphorylation of its ITIM tyrosine, recruitment of the SHIP inositol-phosphatase, and the consequent dampening of BCR-mediated signal transduction events (16). Other ITIM-bearing coreceptors expressed on B cells that negatively regulate BCR signaling include CD22 and CD72, which recruit the tyrosine phosphatase Src homology region 2 domain-containing phosphatase (SHP)-1 (17, 18), PECAM-1, which recruits SHP-1 and −2 (19), and leukocyte-associated Ig-like receptor-1, which associates with both SHP-1 and −2 (20). Similar to those receptors, FCRL molecules also have the potential to be important regulators of BCR signaling. ITIMs in FCRL3 can recruit SHIP, SHP-1, and −2 following coligation with the BCR, FCRL4 recruits SHP-1 and −2 to its membrane distal ITIMs, and FCRL5 recruits SHP-1 to its ITIMs to inhibit calcium mobilization and cellular tyrosine phosphorylation mediated by BCR signaling under in vitro experimental conditions (8, 10, 21).

The FCRL2 signaling potential has not been previously investigated. The *FCRL2* gene is located within the region of chromosome 1q21–25, a hot spot for mutations detected in patients with autoimmune diseases, including systemic lupus erythematosis and insulin-dependent diabetes (1). Moreover, FCRL2 over-expression by B cells has been observed in patients with indolent chronic lymphocytic leukemia (22). These findings suggest potential

roles for FCRL2 dysfunction in autoimmune diseases and cancer and highlight the necessity of a deeper understanding of immunomodulatory mechanisms employed by FCRL2. The two ITIMs in FCRL2 fit the consensus V/L/IXYXXL/V sequence, whereas the ITAM-like sequence is not a perfect fit with the consensus ITAM sequence (D/EXXYXXL/IX$_{6-8}$ YXXL/I) (20, 23). The ITAM-like sequence in FCRL2 contains a proline instead of a leucine or isoleucine at the first Y+3 position and a valine residue at the second Y+3 position instead of a leucine or isoleucine (6). Because the two ITIMs and potential ITAM in the FCRL2 cytoplasmic domain suggest that it may have dual signaling properties, we examined its regulatory influence on BCR signaling and show that FCRL2 exhibits potent inhibitory function via the recruitment of SHP-1 to the two ITIMs in its cytoplasmic tail.

Materials and Methods

Cell lines and Abs

The murine B cell line A20 IIA1.6 was maintained in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5.0% CO_2. BOSC23 cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Anti-hemagglutinin (HA) Ab clone 12CA5 was obtained from Roche (Mannheim, Germany); anti–SHP-1, anti–SHP-2, anti-SHIP, anti–p-ERK (Tyr^{204}), anti–phospho-p38, and anti-eukaryotic translation initiation factor 4E (eIF4E) from Cell Signaling Technologies (Beverly, MA); HRP-coupled anti-phosphotyrosine Abs 4G10 from Millipore (Billerica, MA); anti-SYK, anti-PI3K, anti-PLCγ2, and anti-BLNK from Santa Cruz Bio-technology (Santa Cruz, CA); whole Ig and F(ab′)$_2$ fragments from Jackson ImmunoResearch Laboratories (West Grove, PA); and anti–phospho-ERK R-PE–conjugated for phospho-specific flow cytometry, anti-human IgD-FITC, anti-human CD38-PE, anti-human CD27-APC, anti-human CD19-PE-Cy7, and anti-human CD3-APC-Cy7 were all from BD Biosciences (San Jose, CA).

Preparation and immunofluorescence analysis of blood, splenic, and tonsillar B cells

Tonsil samples obtained from Children's Healthcare of Atlanta and spleen samples from Emory University Hospital (Atlanta, GA) were processed at the Human Tissue Procurement Service in accordance with policies established by the Emory University Institutional Review Board and with informed consent according to the Declaration of Helsinki. Tissue samples were minced through a steel mesh tissue sieve to make single-cell suspensions. Mononuclear blood cells were isolated by Ficoll density gradient centrifugation. Adult peripheral blood samples were collected from healthy individuals, all of whom gave written informed consent, and processed by Ficoll density gradient centrifugation.

Cells were stained for CD19, CD3, IgD, and CD38 expression to separate tonsillar cells into naive (IgD$^+$/CD38$^-$), pregerminal center (IgD$^+$/CD38$^+$), germinal center (IgD$^-$/CD38$^+$), plasma cells (IgD$^-$/CD38^{++}), and memory B cell subpopulations (IgD$^-$/CD38$^-$) using the Abs listed above. Cells were analyzed using flow cytometry (CyAn, DakoCytomation, Copenhagen, Denmark). Biotinylated anti-human intact FCRL2 primary Ab (a generous gift of Dr. Andrew Polson [Genentech, South San Francisco, CA]) and a streptavidin-APC–

conjugated secondary agent (BD Biosciences) were used for indirect staining for FCRL2 expression. The diffuse large B cell lymphoma cell line SUDHL-6, which expresses FCRL2, was stained as a positive control, and primary B cells were stained with an isotype-matched control IgG1 Ab as a negative control (Supplemental Fig. 1). A MoFlo Cell Sorter (DakoCytomation) was used to separate the heterogeneous mixture of tonsillar B cells into subpopulations and collect the cells for RNA isolation.

Quantitative RT-PCR

RNA was isolated from the different tonsillar B cell subpopulations using RNeasy Mini kits (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed using random hexamers according to the SuperScript first-strand synthesis system for reverse transcription PCR (Invitrogen, Carlsbad, CA). Quantitative analysis of FCRL2 mRNA was performed by real-time PCR using an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA). Primers were designed (Invitrogen) to overlap an exon–intron border to avoid amplification of potential genomic DNA contamination: FCRL2 forward primer sequence: 5′-TTGCTGGTCATCTTTGATGCA-3′ and FCRL2 reverse primer sequence: 5′-CAGAAGAGGGCGCCACAA-3′. PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems). Experiments were performed in duplicate on tonsil samples from three donors. Values obtained for *FCRL2* were normalized to the housekeeping gene *RP2*.

Generation of FCRL2/FcγRIIb fusion proteins

Wild-type (WT) and mutant chimeric proteins were generated by fusing the extracellular and transmembrane domains of murine FcγRIIb to the intracellular domain of FCRL2 as described (8). Site-directed mutagenesis was performed by PCR according to the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) protocol converting tyrosine residues to phenylalanines. WT and mutated cDNAs were subcloned into pBluescript (Stratagene) and sequenced. For retroviral transduction, cDNAs encoding the chimeric proteins were excised from pDisplay (Invitrogen), adding the Ig κ-chain leader sequence and HA tag to the FCRL2 chimeric protein, and cloned into pMX-PIE, a retroviral expression vector that expresses the gene of interest upstream of an internal ribosomal entry site element and the enhanced GFP.

Transfection and transduction of cell lines

BOSC23 cells were transfected with vector-only and chimeric FCRL2 pMX-PIE constructs along with the pCL-Eco packaging vector using Lipofectamine 2000 (Invitrogen) according to standard protocols. Virus-containing supernatants were passed through a 0.45-μm filter, mixed with 5 μg/ml Polybrene (Sigma-Aldrich, St. Louis, MO), and added to 2×10^6 A20 IIA1.6 cells, an IgG-expressing mouse B cell line that lacks endogenous FcγRIIb. The retrovirally transduced cells were incubated for 4 d in media containing 1.0 μg/ml puromycin. Cells were sorted by flow cytometry to eliminate cells expressing only GFP or the chimeric receptor.

Analysis of BCR signal transduction

The WT and mutant chimeric FCRL2 expressing A20 IIA1.6 cells (5×10^6) were washed to remove serum Ig and starved for 2 h at 37°C in RPMI 1640 with L-glutamine and 25 mM HEPES (Cellgro, Manassas, VA) prestimulation with 25 μg/ml intact affinity purified rabbit anti-mouse IgG (H+L) and 17.2 μg/ml F(ab′)$_2$ fragment rabbit anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories). Poststimulation for 0, 1, 3, 5, 10, or 20 min, cells were pelleted and lysed in lysis buffer containing 1% Nonidet P-40 (Sigma-Aldrich), PMSF (40 μg/ml), and Na_3VO_4 (0.2 mM) to terminate stimulation.

Protein was quantified using bicinchoninic acid for colorimetric detection and quantitation of total protein (Thermo Scientific, Waltham, MA). Lysates were boiled for 10 min postaddition of an equal volume of SDS sample buffer containing 2-ME. Equal amounts protein per well were resolved by SDS-PAGE (7.5, 10, or 12% acrylamide) and transferred to nitrocellulose membranes using semidry transfer. For immunoprecipitation analysis, cell lysates were incubated at 4°C for 30 min with 4 μg anti-HA Ab. Protein G-coupled Sepharose beads (GE Healthcare, Piscataway, NJ) were added to lysates to precipitate the Ab–Ag complex. Beads were washed a minimum of three times with lysis buffer to reduce nonspecific binding and resuspended in an equal volume of SDS sample buffer. The membranes were probed with the following Abs: anti–phospho-ERK, anti–phospho-p38, anti-HA, anti-eIF4E, anti-PLCγ2, anti-BLNK, anti-PI3K, anti-SYK, and anti–phospho-tyrosine 4G10 and visualized using the ECL Western blotting detection reagent (GE Healthcare).

Calcium flux assays

A20 IIA1.6 cells (6×10^7) were washed twice in HBSS without phenol red, with calcium and magnesium, then resuspended in 1 ml HBSS containing 2 μM fluo-4 calcium indicator (Invitrogen) and incubated for 30 min at 37°C. Cells were washed twice in HBSS and resuspended at a concentration of 5×10^6 cells/ml. Calcium mobilization was assayed using an FACScan flow cytometer (BD Biosciences) after cells were stimulated with Ab as described above by measuring relative fluo-4 fluorescence (FL1-H) on a linear scale over a 300-s time interval.

Phosphopeptide binding assay

A20 IIA1.6 cells were lysed with lysis buffer as previously described. For immunoprecipitations, cell lysates were incubated at 4°C for 30 min with biotinylated peptides that were either phosphorylated or nonphosphorylated (Alpha Diagnostic International, San Antonio, TX). To precipitate the peptides in complex with Ag, 20 μl 50% slurry of streptavidin Sepharose beads (GE Healthcare) was added to the lysates to precipitate the peptides in complex with Ag. Peptides were washed a minimum of three times with lysis buffer and boiled for 10 min postaddition of an equal volume of SDS sample buffer. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with the following Abs: anti-SHIP, anti–SHP-1, anti–SHP-2, anti-SYK, and anti-PLCγ2 and visualized by ECL.

Pervanadate stimulation

HA-tagged transductants expressing WT or mutant FCRL2, at a concentration of 1×10^7, were washed and incubated in RPMI 1640 with 0.1 mM Na_3VO_4 (Sigma-Aldrich) for 25 min at 37°C. Cells were lysed and immunoprecipitated with anti-HA Abs as described above. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Tyrosine phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine Ab 4G10 (Millipore) and anti-HA as a loading control.

Phosphospecific flow cytometry

Phosphospecific flow cytometry was performed according to a previously reported protocol (24). Briefly, PBMCs were starved at 37°C for 2 h before being aliquoted at a density of 1×10^6 cells/ml and incubated for 15 min with or without $F(ab')_2$ fragments of anti-FCRL2 Ab. Cells were stimulated with affinity purified $F(ab')_2$ fragments of rabbit anti-mouse IgG (H +L) (Jackson ImmunoResearch Laboratories) for 30 min at 37°C. Cells were fixed with BD Phosflow Fix Buffer I (BD Biosciences) at 37°C for 10 min and permeabilized with methanol for 30 min at −20°C. Cells were stained with R-PE–conjugated anti–p-ERK1/2 and surface stained with anti-CD19 FITC- and anti-CD27 APC-conjugated Abs for 10 min at 37°C and analyzed using a CyAn flow cytometer (DakoCytomation). The FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR). Mean fluorescence intensity (MFI) was calculated using FlowJo software.

Statistical analysis

The Student *t* test was used to evaluate the significance of differences in experimental results where appropriate. Statistical significance was determined with *p* values <0.05. Data were analyzed using GraphPad Prism Pro (GraphPad, San Diego, CA).

Results

Expression of FCRL2 as a function of B cell differentiation

Earlier reports have indicated FCRL2 expression on memory B cells in blood and tonsil (25). To re-examine the pattern of FCRL2 expression as a function of B cell differentiation, we used differential expression patterns for CD38 and IgD to define the B cell subpopulations in the tonsils. After FACS sorting of the CD19+ B cells into naive, pregerminal center, germinal center, memory, and plasma cell subpopulations, RNA was isolated from each of the purified subpopulations for quantitative analysis of *FCRL2* transcripts. This analysis indicated that *FCRL2* transcripts were most abundant in tonsillar memory B cells (Fig. 1*A*). We then analyzed FCRL2 protein expression on different B cell sub-populations from blood, spleen, and tonsils by flow cytometry using an FCRL2-specific Ab. This analysis indicated relatively high levels of FCRL2 on memory B cells from all of these tissues (Fig. 1*B*). As previously reported (25), FCRL2 expression levels were high on tonsillar memory B cells, low on naive B cells, and absent on germinal center cells. The increased level of FCRL2 surface expression on memory B cells thereby renders these cells prime subjects for FCRL2 immunoregulation.

Construction and expression of WT and mutant FcγRIIb/FCRL2 chimeras

To determine the functional potential of the putative ITAM-like and ITIM sequences of FCRL2, we generated a panel of chimeric FCRL2 molecules in which the intracellular domain of FCRL2 was fused to the extracellular and transmembrane regions of FcγRIIB. To investigate the involvement of the ITIM and ITAM-like sequences of FCRL2, tyrosine-to-phenylalanine mutations were introduced by site-directed mutagenesis (Fig. 2*A*). The cytoplasmic domain of FCRL2 contains four tyrosines at positions Y448, Y462, Y474, and Y502, the first two being in the ITAM-like region and the last two being in the two consensus ITIMs. The tyrosine-to-phenylalanine mutants were numbered according to their amino acid positions, except for the chimeric FCRL2 mutant, wherein all cytoplasmic tyrosines were mutated to phenylalanines, which was designated FFFF (Fig. 2*A*). The constructs for the different chimeric receptors were stably expressed in a mouse B cell line, and their expression levels were shown to be comparable following selection of the transduced cells by puromycin treatment and FACS (Supplemental Fig. 2*A*). Expression of the FCRL2 chimeric receptors by the A20 II A1.6 cells did not alter the IgG2b BCR expression levels (Supplemental Fig. 2*B*). Importantly for this model system, the heterologous transfectants belong to the variant mouse B cell subline, A20 II A1.6, which lacks expression of endogenous FcγRIIb. Coligation of the BCR with the chimeric FcγRIIb/FCRL2 molecules therefore could be achieved by incubation of the cells with intact anti-mouse IgG Abs, whereas ligation of the BCR with F(ab′)$_2$ fragments exclusively induces BCR signaling (Fig. 2*B*).

Examination of FCRL2 immunomodulatory potential

Because the release of calcium from intracellular stores is an early event following BCR ligation, we initially determined whether FCRL2 engagement influenced mobilization of intracellular calcium. The effect of BCR coligation with the chimeric FcγRIIb/FCRL2 protein was evaluated by measuring the calcium flux induced by intact anti-Ig Abs compared with that induced by F(ab′)$_2$ fragments. The results of these experiments demonstrated that BCR coligation of the chimeric receptor with the WT FCRL2 intracellular region effectively inhibited calcium mobilization (Fig. 3). BCR coligation of chimeric receptors with either one or both ITAM-like tyrosines mutated (Y448F, Y462F, and Y448F/Y462F) also resulted in complete inhibition of calcium mobilization. In contrast, mutations in either ITIM, Y474F or Y502F, resulted in only partial inhibition of calcium mobilization. The inhibitory capacity was abolished for chimeric FCRL2 in which both ITIM tyrosines were mutated (Y474F/Y502F); in fact, calcium mobilization appeared to be slightly enhanced by coligation of this chimeric receptor (Fig. 3). These findings indicated that FCRL2 has the potential to modulate calcium signaling and that the tyrosines in both ITIMs contribute to optimal inhibition of calcium mobilization.

To monitor the effects of the chimeric FCRL2 receptor on BCR-induced tyrosine phosphorylation of intracellular signaling components, A20 IIA1.6 cells expressing WT or FFFF chimeric FCRL2 mutants were stimulated with intact and F(ab′)$_2$ fragments of the rabbit anti-mouse IgG Abs. When the BCR was coligated with the WT FcγRIIb/FCRL2 chimera by treatment with intact anti-BCR Abs, tyrosine phosphorylation of multiple intracellular proteins was reduced as a function of time in comparison with the B cells

stimulated with F(ab′)$_2$ fragments of the BCR Ab (Fig. 4, *left panel*). This inhibition of tyrosine phosphorylation was not seen when the FFFF mutant chimera was coligated with the BCR (Fig. 4, *right panel*). Upon immunoprecipitation of chimeric FCRL2 with anti-HA Abs and immunoblotting with antiphosphotyrosine, an increase in tyrosine phosphorylation of the FCRL2 cytoplasmic domain itself was observed when the BCR was coligated with WT chimeric FcγRIIb/FCRL2, but not when the BCR alone was ligated (Fig. 4, *bottom panels*). These results suggest a role for FCRL2 in the inhibition of BCR-induced signaling pathways involving phosphorylation of multiple downstream signaling molecules and that tyrosine phosphorylation of the intracellular region of FCRL2 is crucial for the inhibition of BCR signaling.

MAPK family members are well-characterized components of BCR signaling. To examine the effect of FCRL2 coligation on BCR-mediated activation of these signaling intermediates, B cells expressing the WT or FFFF mutant FCRL2 were stimulated with either the intact or F(ab′)$_2$ fragment of the anti-BCR Ab. Coligation of FCRL2 and the BCR resulted in the inhibition of both p38 and ERK MAPKs (Fig. 5*A*, *left panel*). As expected, stimulation of the BCR with F(ab′)$_2$ fragments increased phosphorylation of p38 and ERK1/2. This BCR-mediated MAPK activation was unaffected by coligation of the FFFF FCRL2 mutant (Fig. 5*A*, *right panel*). Ligation of FCRL2 alone on transfected cells did not result in phosphorylation of the intracellular domain of FCRL2 itself, nor could we observe phosphorylation of intracellular signaling intermediates (data not shown). These findings indicate the potential for FCRL2 to negatively regulate transduction pathways downstream of the BCR, which include p38 and ERK MAPKs.

When we analyzed the contributions of the individual intra-cellular tyrosine residues of the intracellular domain of FCRL2 on the inhibition of ERK MAPK activation, we noted that the ITIM sequences (Y474 and Y502) were required for its immunoregulatory affect, whereas the two membrane proximal tyrosine residues (Y448 and Y462) were dispensable (Fig. 5*B*). These results are in agreement with our earlier analysis of the effect of FCRL2 on calcium mobilization (Fig. 3). These data demonstrate the strong inhibitory effect of FCRL2 on proximal and distal components of BCR signal transduction elements and highlight the importance of the tyrosine residues in the ITIM consensus sequences.

FCRL2 recruits SHP-1 post BCR coligation

The requirement of the phosphorylated ITIM tyrosines for inhibition of calcium mobilization and inhibition of p38 and ERK1/2 MAPKs could be explained by the recruitment of SH2-domain containing phosphatases SHIP, SHP-1, or SHP-2. As a first step in examining this possibility, tyrosine-phosphorylated and unphosphorylated peptides were synthesized that correspond to FCRL2 ITAM-like and ITIM motifs and used as affinity reagents to detect signaling molecules that might bind to FCRL2. In these experiments, lysates of the A20 IIA1.6 cells were incubated with the biotinylated peptides, and complexes were precipitated using streptavidin Sepharose beads. The binding of SH2-containing proteins was then examined by Western blot analysis. The phosphorylated peptide matching the amino acid sequence of the FCRL2 membrane distal ITIM, containing Y502, bound the tyrosine phosphatase SHP-1 (Fig. 6*A*). This binding was phosphorylation dependent, as the

corresponding unphosphorylated peptide failed to bind SHP-1. No association was detected with any of the remaining phosphorylated or nonphosphorylated peptides. These results suggested an involvement of SHP-1 in the inhibitory function of FCRL2.

To determine whether SHP-1 interacts with the tyrosine-phosphorylated intracellular domain of FcγRIIb/FCRL2 postcoligation, we stimulated B cells expressing each of the mutants of FCRL2 with the intact anti-BCR Ab. All of the chimeric receptors were tyrosine phosphorylated when coligated with the BCR except for the FFFF mutant, which contains no intracellular tyrosines (Fig. 6*B*). Notably, the Y474F/Y502F mutant, in which the ITIMs were ablated, was modestly tyrosine phosphorylated, indicating that the putative ITAM tyrosines can be phosphorylated. Furthermore, Western blotting of immunoprecipitates from Y474F/Y502F double ITIM mutant cells treated with the tyrosine phosphatase inhibitor sodium pervanadate showed tyrosine phosphorylation of the Y474F/Y502F mutant FCRL2 (Fig. 6*C*).

Because we found SHP-1 bound to a phosphopeptide corresponding to an FCRL2 ITIM sequence, we investigated whether chimeric FcγRIIb/FCRL2 could coimmunoprecipitate the signal transduction regulators SHP-1, SHP-2, and SHIP. SHP-1 was found to coimmunoprecipitate with the chimeric protein that contained the wild-type cytoplasmic region, the ITAM-like Y448F and Y462F single and double mutants, but not with the Y474F, Y502F, and Y474F/Y502F ITIM mutants or the FFFF mutant (Fig. 6*B*). The finding that coligation of chimeric FCRL2 with the BCR induces the phosphorylation of tyrosine residues within the two ITIM motifs (Y474 and Y502) and the recruitment of SHP-1 suggests the inhibitory activity of FCRL2 on BCR signaling is mediated by SHP-1.

Role for FCRL2 in BCR-mediated signaling in primary memory B cells

The possible effect of FCRL2–BCR cross-linking in IgG-bearing CD19^{+}CD27^{+} memory B cells from healthy donors was examined by use of anti–phospho-ERK1/2 Abs in phospho-flow cytometry analysis. In these experiments, BCR cross-linking alone with anti-Ig F(ab′)$_2$ fragments or BCR cross-linking combined with treatment with an irrelevant control Ab resulted in an increase in ERK1/2 phosphorylation over that observed for the unstimulated control (Fig. 7*A*, 7*B*). Whereas stimulation with the anti-FCRL2 alone did not affect ERK phosphorylation status, the coligation of the BCR and FCRL2 resulted in inhibition of ERK1/2 phosphorylation. Analysis of the MFI of ERK1/2 phosphorylation indicated a statistically significant reduction of ERK phosphorylation in response to FCRL2 coligation with the BCR when compared with BCR ligation alone (Fig. 7*B*). These data recapitulate the inhibitory activity of FCRL2 on Ag receptor signaling that we observed using the A20-IIA1.6 cell line and indicate the potential for FCRL2 to inhibit BCR mediated signaling in primary memory B cells.

Discussion

This study elucidates the immunoregulatory potential of FCRL2. When coligated with the BCR, tyrosine residues 474 and 502 in the ITIMs of FCRL2 become phosphorylated and recruit the inhibitory tyrosine phosphatase SHP-1. The recruitment of SHP-1 is accompanied

by inhibition of BCR-triggered calcium mobilization and dephosphorylation of a number of proteins, including the p38 and ERK MAPKs.

FCRL2 expression is limited to hematopoietic cells, specifically the B cells. Northern blot analysis and gene array analysis indicated that *FCRL2* expression is most abundant in peripheral lymphoid tissues (spleen and lymph node) and is also evident in blood, thymus, and bone marrow (5). The stage at which *FCRL2* is expressed during B cell differentiation has been more difficult to resolve. Using in situ hybridization of human hyperplasic tonsillar tissue, *FCRL2* mRNA expression was detected in the mantle zone of tonsils, which typically contain naive B cells, leading to the conclusion that *FCRL2* is expressed almost exclusively in naive B cells (6). However, immunofluorescence analysis of FCRL2 expression using an anti-FCRL2 Ab indicated its expression on IgD$^-$/CD38$^-$ memory B cells from tonsils and on CD138$^+$ CD38^{++} plasma cells, albeit at lower levels. FCRL2 was also detected on CD20$^+$/CD27$^+$ cells in the circulation, leading to the conclusion that FCRL2 is a marker for memory B cells (25). In addition to these flow cytometric analyses, FCRL2 expression was observed by immunohistochemical staining in the marginal zone of the spleen and tonsil specimens and in the intra- and subepithelial areas adjacent to the mantle zone in the tonsils (25). Our analysis of FCRL2 transcript and protein expression by spleen, tonsillar, and blood cells supports the notion that of FCRL2 is preferentially expressed by memory B cells.

FCRL2 was reported earlier as SPAP1a containing a single Ig-like domain, transmembrane region, and two ITIMs (3). rSPAP1a was produced in 293 cells and shown to be tyrosine phosphorylated upon pervanadate stimulation and to recruit SHP-1, but not SHP-2. In agreement with this analysis, our experiments indicate that FCRL2 coligation with the BCR triggers the phosphorylation of its two ITIM tyrosines and its association with SHP-1, but not SHP-2 or SHIP, to attenuate B cell activation. Mutational analysis of the intracellular tyrosines in the cytoplasmic domain of FCRL2 demonstrates the requirement of the ITIM tyrosines Y474 and Y502 for recruitment of SHP-1. Interestingly, only phosphopeptides corresponding to the amino acid sequence bordering the membrane distal ITIM could be shown to bind SHP-1, implying a dominant role for Y502 in the distal ITIM over the Y474 in the membrane proximal ITIM. This result may reflect in part a greater affinity of the SH2 domains of SHP-1 for the phosphopeptide equivalent to the Y502 containing ITIM or that the Y474 peptide is not folded in a way that allows SHP-1 binding. Optimal activation of the SHP-1 phosphatase has been shown to require both the N-terminal and C-terminal SH2 domains of SHP-1 for binding to phosphorylated ITIMs (26). The data presented in this study demonstrate that phosphorylated Y474 and Y502 in the ITIM sequences of FCRL2 are necessary for optimal SHP-1 binding to inhibit BCR-mediated calcium mobilization and MAPK activation.

The balance between activating and inhibitory B cell surface receptors controls B cell activation. Because the FCRLs have inhibitory and activating signaling potential, they may serve important regulatory roles in normal and neoplastic B cell development (2). Functional analysis of the FCRL family of immunoreceptors indicates that some members may operate as activating receptors, inhibitory receptors, or even bifunctional receptors. Whereas FCRL1 appears to function as coactivator on BCR signaling (9), FCRL3, −4, and −5 all strongly inhibit Ag receptor signaling upon coligation with the BCR (10, 21, 27). The activation-

induced association of FCRL2 with the SHP-1 phosphatase, which results in the inhibition of calcium mobilization and MAPK activation, together with its preferential expression on memory B cells suggests that FCRL2 may have a complementary inhibitory function with that of FCRL4 and FCRL5 to play a role in modulating recall immune responses. Conceivably, FCRL2, FCRL4, and FCRL5 may provide the redundancy needed to preserve memory B cell quiescence, limit B cell responses, or terminate signaling when it is no longer required. The presence of these inhibitory receptors on effector B cells underscores the importance of carefully regulating B cell activation.

FCRL molecules share homology with classical Ig binding FcRs, but none of the members of the FCRL family have been shown to bind Igs. Recently, FCRL5 was reported to be the receptor for the orthopoxvirus MHC class I-like protein (28), and FCRL6 was identified as a receptor for HLA-DR (29). No ligands for FCRL2 have been identified thus far. We therefore employed a system that allows us to investigate the biochemical properties of the intra-cellular domain of FCRL2 on BCR signaling in the absence of FCRL2 ligand. Although the conditions of coligating chimeric FcγRIIb/FCRL2 and the BCR in these studies do not equate with physiologic conditions, this model allows pairing of the chimeric FCRL2 with the ITAM-containing Igα/β of the BCR complex. This system therefore mimics the engagement of ITIM- or ITAM-bearing coreceptors by their natural ligands. The observed inhibition of MAPK signaling postcoligation of endogenous FCRL2 with the BCR on tonsilar memory B cells supports the suitability of our cell line-based approach. The experimental system employed in these studies is thus useful for understanding the function of an orphan receptor with no mouse ortholog.

Sequence analysis of the intracellular domain of FCRL2 suggests a possible context-dependent bifunctional role of this receptor. Cells expressing FCRL2 WT fusion proteins displayed elevated levels of calcium mobilization post BCR ligation for prolonged durations in the absence of coligated fusion proteins, although this was not accompanied by detectable tyrosine phosphorylation of the intracellular domain of FCRL2. However, the peak calcium signal of coligated fusion proteins observed with inactivated ITIM sequences was reproducibly higher than the signal observed with ligation of the Ag receptor without FCRL2 fusion proteins; this corresponded with a weak but detectable tyrosine phosphorylation of the ITAM sequences of FCRL2. Indeed, robust ITAM phosphorylation could be detected in response to pervanadate stimulation, demonstrating the presence of tyrosine kinases that recognize the ITAM of FCRL2 as substrate. These findings thus raise the possibility that FCRL2 could function as an activating receptor. Although the present data are not conclusive regarding the role of the putative ITAM, they suggest that FCRL2 may deserve further consideration as one of the few receptors, like CD22 (30), that may function in both activating and inhibitory roles.

B cell chronic lymphocytic leukemia (CLL), a malignancy of mature B lymphocytes, is the most common form of adult leukemia in the Western world (31). The disease has a variable clinical course, with 50% of patients having progressive disease and short survival, and the other 50% having a relatively stable disease and a normal life span (32). CLL cells resemble activated memory-like B cells, but can be categorized by the mutation status of their *IGHV* gene and expression of ZAP70 and CD38 (22). Patients with mutated IgV_H and

ZAP70^{−}CD38^{−} CLL cells have a more indolent disease course, whereas patients with unmutated CLL and ZAP70^{+} CD38^{+} cells experience a more aggressive course of disease (22). Higher levels of FCRL2 are found on ZAP70^{−}CD38^{−} CLL cells containing mutated *IGHV* genes than on unmutated CLL cells or CD19^{+} polyclonal B cells (22). FCRL2 expression levels have strong predictive value for determining *IGHV* gene mutation status and clinical progression in CLL, thereby suggesting that FCRL2 could affect disease progression and survival (33–35). In light of our demonstration of the inhibitory potential of FCRL2, it will be interesting to investigate whether a causal relationship can be established between increased FCRL2 expression and a more favorable prognosis in CLL.

B cell activation is necessary for optimal responses to Ags. However, unchecked B cell activation can lead to inflammation and autoimmunity. The inhibitory receptors on B cells for which functions have been well characterized include FcγRIIb and CD22, both of which are critical for restraining B cell activation (36). Many other receptors whose functions are less well understood may function to inhibit BCR signaling, including CD72, CD5, and FCRL family members (37). The results of our studies suggest that FCRL2 may serve as a negative regulator of memory B cell response to recall Ags and emphasize the need for identification of the physiological ligands for FCRL2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this paper

CLL	chronic lymphocytic leukemia
eIF4E	eukaryotic translation initiation factor 4E
FCRL	FcR-like
HA	hemagglutinin
MFI	mean fluorescence intensity
PLCγ2	phospholipase Cγ2
SH2	Src homology 2
SHP	Src homology region 2 domain-containing phosphatase
SPAP	Src homology 2 domain-containing phosphatase anchor protein
WT	wild-type

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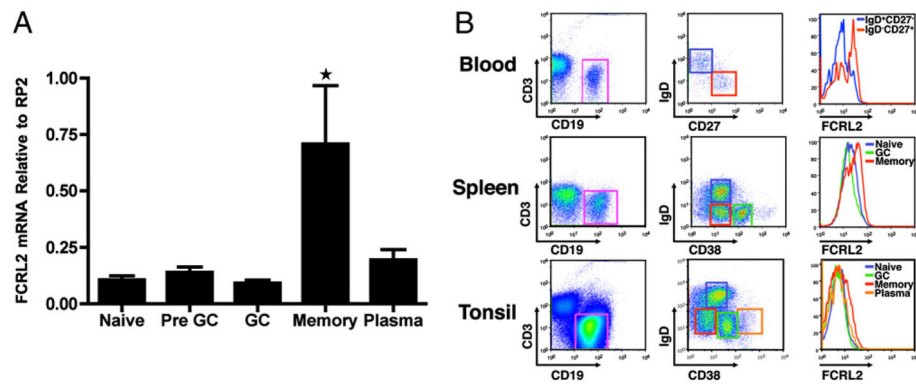
FIGURE 1.
Analysis of *FCRL2* transcripts and protein expression in B cell subsets. *A*, *FCRL2* message, relative to transcript levels of the housekeeping gene RNA polymerase II (*RP2*), was examined as a function of B cell differentiation by real-time PCR. Data are representative of three patients and seven independent experiments. *B*, Flow cytometric analysis of FCRL2 surface protein expression in PBMCs, splenic B cell populations, and tonsillar B cell populations. Data are representative of samples analyzed from three individuals. *$p < 0.05$.
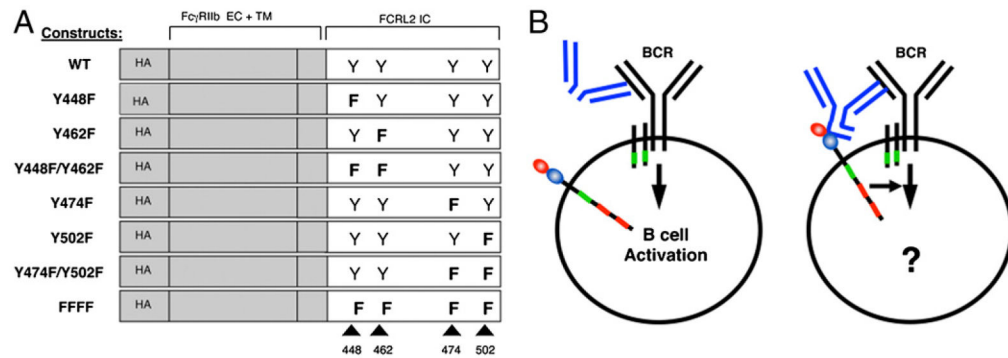
FIGURE 2.

FcγRIIb/FCRL2 chimeric constructs used to assess FCRL2 function. Illustration of FCRL2/FcγRIIb chimeric constructs expressed in A20 IIA1.6 cells. *A*, FcγRIIb extracellular and transmembrane domains are shaded in gray. Tyr^{448} and Tyr^{462} correspond to a noncanonical ITAM. Tyr^{474} and Tyr^{502} correspond to two canonical ITIMs. Tyrosine-to-phenylalanine mutations are indicated by the letter F (boldface). *B*, Model used to evaluate the FCRL2 activity in the A20 IIA1.6 B cell line using F(ab′)$_2$ fragments to activate the BCR alone (*left panel*) or with intact anti-IgG Ab to coligate the chimeric molecule with the BCR (*right panel*). ITAM sequences are represented in green, and ITIM sequences are represented in red.
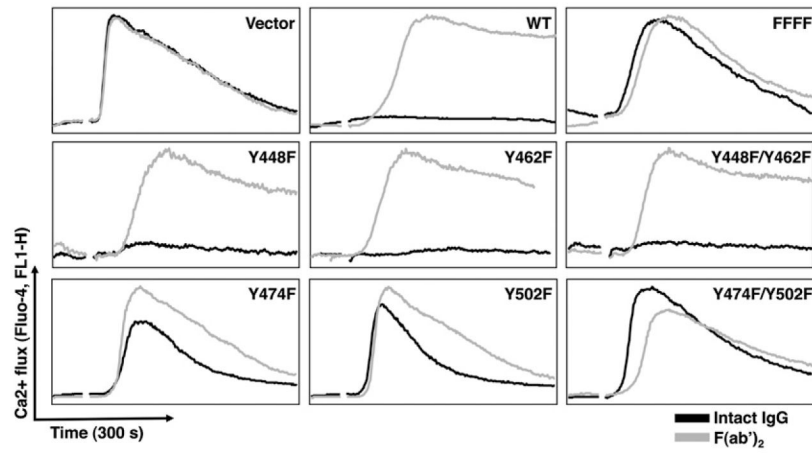
FIGURE 3.
Coligation of the FcγRIIb/FCRL2 chimeric receptor with the BCR inhibits calcium mobilization. A20 IIA1.6 cells expressing the indicated mutant constructs were stained with fluo-4 for calcium flux evaluation. Cells were stimulated with either intact (gray) or F(ab′)$_2$ fragments (black) of anti-IgG Abs. Data are a representative of six independent experiments.
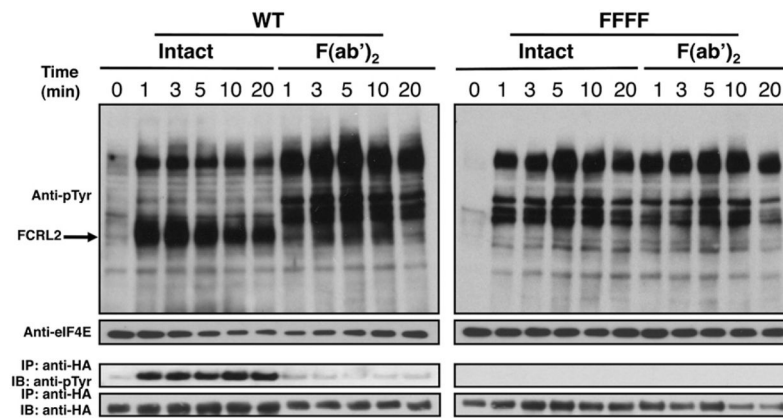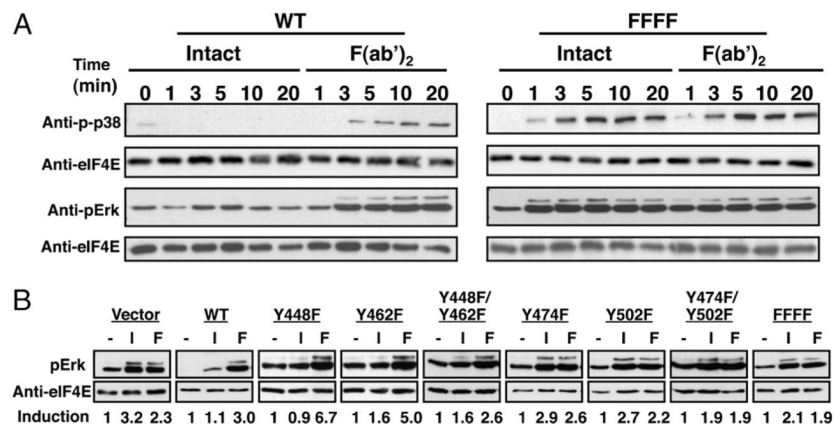
FIGURE 4.
Coligation of chimeric FcγRIIb/FCRL2 with the BCR inhibits whole-cell tyrosine phosphorylation. WT or FFFF cells were treated with intact or F(ab′)$_2$ fragments of anti-IgG Abs, and whole-cell tyrosine phosphorylation was examined at serial time points by Western blot using an anti-phosphotyrosine Ab. Equal protein loading was verified with anti-eIF4E Abs. Immunoprecipitates of chimeric FcγRIIb/FCRL2 were analyzed by antiphosphotyrosine, and equal protein loading was verified with anti-HA Abs.

FIGURE 5.

Coligation of the WT FcγRIIb/FCRL2 chimeric receptor inhibits tyrosine phosphorylation of MAPK p38 and ERK1/2. *A*, WT and FFFF cells were stimulated with intact or F(ab′)$_2$ fragments of anti-IgG Abs over time. The blots were probed with anti–phospho-p38 and anti–phospho-ERK1/2 Abs. Anti-eIF4E Abs were used to verify equivalent protein loading. *B*, ERK1/2 activation in cells from the various FCRL2 mutants. Cells expressing WT or mutant FCRL2 were left untreated (−) or stimulated with intact anti-IgG Abs (I) or F(ab′)$_2$ fragments of anti-IgG Abs for 5 min (F). The lysates were probed with anti–phospho-ERK1/2 Abs and eIF4E Abs to verify equivalent protein loading. ERK induction was measured by densitometry and indicated as fold induction over the corresponding untreated control.
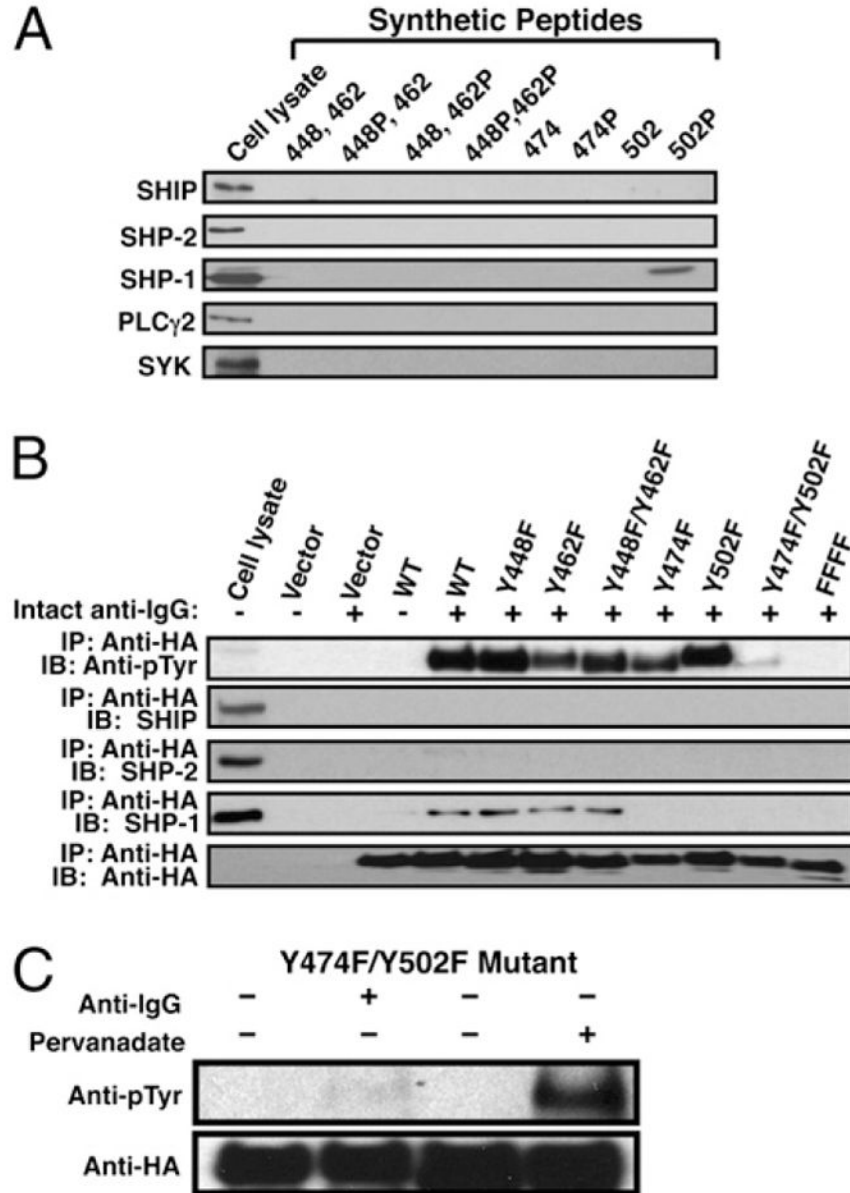
FIGURE 6.
SHP-1 binds to tyrosine phosphorylated ITIMs in FCRL2. *A*, The indicated phosphorylated or unphosphorylated peptides correspond to each ITAM-like and ITIM region of FCRL2. The phosphopeptide corresponding to the membrane distal ITIM associates with SHP-1. SHP-1 binding to chimeric FCRL2/FcγRIIb post BCR coligation. *B*, Post-stimulation of A20 IIA1.6 cells transduced with the indicated constructs with intact anti-IgG Abs (+) or left untreated (−), cell lysates were immunoprecipitated by anti-HA Ab and analyzed for FcγRIIb/FCRL2 interaction with candidate signaling components by Western blotting. The blots were probed with antiphosphotyrosine to assess phosphorylation of the individual receptors and also with anti-HA to verify equal protein loading. *C*, Western blot analysis of extracts from Y474F/Y502F chimeric mutant immunoprecipitated with anti-HA Abs. Cells
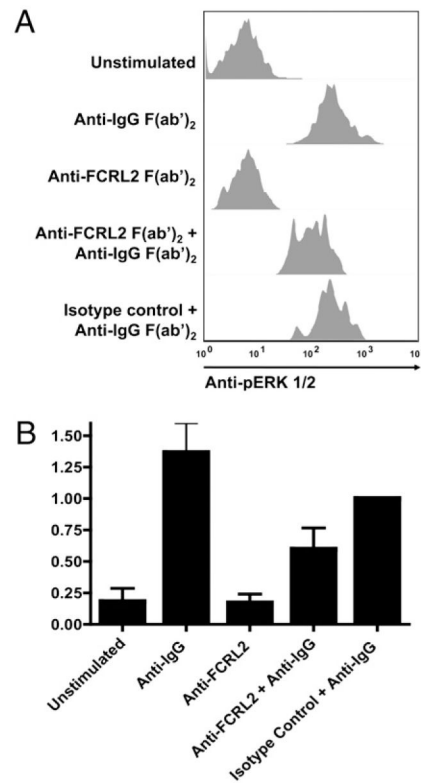
were untreated (−) or treated (+) with anti-IgG Abs or Na_3VO_4 to detect tyrosine phosphorylation.

FIGURE 7.

Human primary memory B cells exhibit attenuated ERK1/2 phosphorylation when FCRL2 is coligated with the BCR. *A*, CD19^{+}CD27^{+} peripheral blood memory B cells were stimulated with the indicated Ab combinations, and ERK phosphorylation was assessed by flow cytometry. Shown is a representative of three independent experiments. *B*, Analysis of MFI values of ERK1/2 phosphorylation in memory B cells relative to isotype control and anti-IgG F(ab′)$_2$ Ab stimulation. The reduction of ERK1/2 phosphorylation is significantly decreased in memory B cells when FCRL2 is coligated with the BCR compared with isotype control and anti-IgG–treated memory B cells ($n = 3$). $p < 0.05$.