FCRL3, an Autoimmune Susceptibility Gene, Has Inhibitory Potential on B-Cell Receptor-Mediated Signaling

Yuta Kochi, Keiko Myouzen, Ryo Yamada, Akari Suzuki, Tomohiro Kurosaki, Yusuke Nakamura and Kazuhiko Yamamoto

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A polymorphism that up-regulates the expression of Fc receptor-like 3 (FCRL3) gene has recently been described as predisposing for several human autoimmune diseases. FCRL3 is preferentially expressed on B cells and is unique in displaying both an ITAM and an ITIM in the cytosolic domain, suggesting signaling functions. Herein, we show that FCRL3 potentially inhibits BCR-mediated signaling, using murine FcγRIIB/human FCRL3 chimeric protein. Coligation of the chimeric protein with BCR leads to phosphorylation of tyrosine residues in the cytosolic phosphorylation and calcium mobilization in addition to activation-induced cell death mediated by BCR signaling. Mutational analysis showed the tyrosine residues in two potential ITIMs at 662 and 692 offer the main contributions to this inhibition, which is further supported by strong associations of SH-2 domain-containing phosphatases with the following phosphotyrosine motifs: SHIP with the ITIM-like motif at 662; and SHP-1 and -2 with the canonical ITIM at 692. These results, together with previous genetic data, suggest that augmented inhibition of BCR-mediated signaling by FCRL3 with the disease-risk genotype alter the activation threshold and promote tolerance breakdown in B cells. The Journal of Immunology, 2009, 183: 5502–5510.

1 Laboratory for Autoimmune Diseases, Center for Genomic Medicine, RIKEN, Tokyo, Japan; 2 Laboratory of Functional Genomics, Human Genome Center, the Institute of Medical Science, the University of Tokyo, Tokyo, Japan; 3 Laboratory of Molecular Medicine, Human Genome Center, the Institute of Medical Science, the University of Tokyo, Tokyo, Japan; 4 Laboratory of Lymphocyte Differentiation, Research Center for Allergy and Immunology, RIKEN, Yokohama, Japan; 5 Laboratory for Autoimmune Diseases, Center for Genomic Medicine, RIKEN, 7-3-1 Hongo, Bunkyo-Ku, Tokyo, Japan; 6 Laboratory of Autoimmune Diseases, Center for Genomic Medicine, RIKEN, 7-3-1 Hongo, Bunkyo-Ku, Tokyo, Japan

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2 Address correspondence and reprint requests to Dr. Yuta Kochi, Laboratory for Autoimmune Diseases, Center for Genomic Medicine, RIKEN, 7-3-1 Hongo, Bunkyo-Ku, Tokyo, Japan. E-mail address: ykochi@src.riken.jp

3 Abbreviations used in this paper: RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; FCRL3, Fc receptor-like 3; SHP, Src homology 2 domain-containing phosphatase; HA, hemagglutinin; 7-AAD, 7-amino actinomycin D; CM-WT, chimera wild type; CM-Mt, chimera mutant.

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A polymorphism that up-regulates the expression of Fc receptor-like 3 (FCRL3) gene has recently been described as predisposing for several human autoimmune diseases. FCRL3 is preferentially expressed on B cells and is unique in displaying both an ITAM and an ITIM in the cytosolic domain, suggesting signaling functions. Herein, we show that FCRL3 potentially inhibits BCR-mediated signaling, using murine FcγRIIB/human FCRL3 chimeric protein. Coligation of the chimeric protein with BCR leads to phosphorylation of tyrosine residues in the cytosolic phosphorylation and calcium mobilization in addition to activation-induced cell death mediated by BCR signaling. Mutational analysis showed the tyrosine residues in two potential ITIMs at 662 and 692 offer the main contributions to this inhibition, which is further supported by strong associations of SH-2 domain-containing phosphatases with the following phosphotyrosine motifs: SHIP with the ITIM-like motif at 662; and SHP-1 and -2 with the canonical ITIM at 692. These results, together with previous genetic data, suggest that augmented inhibition of BCR-mediated signaling by FCRL3 with the disease-risk genotype alter the activation threshold and promote tolerance breakdown in B cells. The Journal of Immunology, 2009, 183: 5502–5510.
could modulate BCR signaling and may consequently affect immunological tolerance in B cells. Functional dissection of FCRL3 will thus facilitate an understanding of roles in the pathogenesis of human autoimmune diseases. We report herein that FCRL3 has inhibitory potential on BCR-mediated signaling, possibly through association of ITIMs with phosphatases SHIP, SHP-1, and SHP-2.

Materials and Methods

Cells and Abs

ST486 cells, which represent human germinal center-derived B cell lymphoma and lack expression of FcγRs, and Jurkat cells were obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, and 100 U/ml penicillin/streptomycin. DT40 cells were obtained from the RIKEN Cell Bank and maintained using the medium described above with the addition of 1% chicken serum. Anti-hemagglutinin (HA) Ab (12CA5) was obtained from Roche Diagnostics; anti-phospho-Syk (Tyr352) and anti-phospho-PLCγ2 (Tyr1217) Abs from Cell Signaling Technologies; biotinylated anti-murine FcγRIIb (2.4G2) and HRP or PE-coupled anti-phosphotyrosine Abs (PY20) from BD Biosciences; anti-PLCγ2, anti-SHP-1, anti-SHP-2, anti-SHIP and agarose-conjugated anti-HA Abs from Santa Cruz Biotechnology; anti-ZAP70 Ab from eBioscience, and intact and F(ab′)2 of rabbit anti-human IgM and goat anti-chicken IgY from Jackson ImmunoResearch Laboratories.

FIGURE 1. FCRL3 and murine FcγRIIB chimeric proteins. A, Schematic structures of FCRL3 and murine FcγRIIB chimeric proteins. The black line represents FCRL3 sequence and the gray line represents the murine FcγRIIB sequence. HA (hemagglutinin)-tag was added at the NH2-end of chimeric protein. Each semicircular structure in the extracellular domain represents a single Ig-like domain. Tyrosine residues (Y) of the potential ITAM and ITIMs in FCRL3 and their positions are indicated. In the mutant construct (CM-Mt), tyrosines were displaced by phenylalanines (F). B, Expression of chimeric proteins was measured by flow cytometry after staining with anti-HA. The black line represents anti-HA staining and the gray line represents the isotype-control staining. C, Schematic representation of stimulation strategy. F(ab′)2 of anti-IgM stimulates BCR only (left), while intact anti-IgM stimulates BCR in coligation with the chimeric protein (right).
Generation of chimeric mutants of FCRL3 and stable transfectants

The cDNAs encoding transmembrane and intracellular domains of human FCRL3 were fused to cDNA of the extracellular domain of murine FcγRIIB and cloned into pDisplay vector (Invitrogen). A NotI site was introduced between the extracellular domain of FcγRIIB and the transmembrane domain of FCRL3, translating into a 3-amino-acid spacer. Site-directed mutagenesis was performed by PCR according to standard protocols. For lentiviral transduction, cDNAs encoding the chimeric proteins were excised from pDisplay and cloned into pLent6 vector (Invitrogen). All constructs were verified by DNA sequencing. Transfection of ST486 cells and DT40 cells was performed according to the instructions of the manufacturer. Virus-containing supernatant was mixed with polybrene to a final concentration of 3 μg/ml and added to 5 × 10^5 cells. Lentivirally transduced cells were incubated for 10 days in a medium containing blasticidin S (10 μg/ml) and stably transfected were purified using anti-HA Ab and Pan Mouse IgG Dynabeads (Dynal). Expression of chimeric proteins was determined using a FACScalibur flow cytometer (BD Biosciences).

Calcium measurements

For ST486 cells, 5 × 10^5 cells were loaded with 5 μg of fura-2/AM (Dojin) in RPMI 1640 medium. After incubation for 30 min at 37°C, cells were washed twice and resuspended in 0.5 ml HBSS with 1 mM CaCl_2, 1 mM MgCl_2, and 0.1% BSA. Cytosolic calcium concentrations of 5 × 10^5 cells were measured using a CAF 110 spectrophotometer (Jacso), after addition of 15.0 μg/ml intact anti-IgM, 10.0 μg/ml F(ab’)2 of anti-IgM, or biotinylated 5.0 μg/ml anti-murine FcγRIIB (2.4G2) followed by 10.0 μg/ml streptavidin. For DT40 cells, cells were instead loaded with 5 μg of fura-2/AM (Dojin). Cytosolic calcium concentrations in 1 × 10^5 cells were measured using the FACScalibur flow cytometer, after addition of 50.0 μg/ml intact anti-chicken IgY or 50.0 μg/ml F(ab’)2 of anti-anti-chicken IgY.

Apoptosis assay

Stably transfected ST486 cells were incubated in the presence of anti-IgM Abs (10.0 μg/ml F(ab’)2, and 15.0 μg/ml for intact IgG). Dead cells were determined using 7-amino actinomycin D (7-AAD) staining. Cells were also stained with PE-labeled annexin V to detect those in the apoptotic phase, according to the specifications of the manufacturer for the Guava nexin assay kit (GE Healthcare). Stained cells were counted using a Guava PCA96 analytical system (GE Healthcare).

Cellular activation, Western blotting, affinity precipitation, and immunoprecipitation

To monitor the effects of transduced receptors on BCR-induced signaling, 5 × 10^6 cells were washed twice and incubated for 2 h in medium lacking FCS and supplemented with 20 nM HEPEs (pH 7.2), before stimulation with intact anti-IgM (15 μg/ml) or anti-IgM F(ab’)2 (10 μg/ml). Western blotting of affinity precipitation and immunoprecipitation were performed as described (18) with some modifications. In brief, cells were spun down before incubation in RIPA buffer containing 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, and the addition of complete protease inhibitor mixture (Roche Diagnostics) and phosphatase inhibitors Na_2VO_3 (1 mM) and NaF (1 mM). Whole-cell lysates were quantitated using BCA reagent (Pierce) and resolved by SDSPAGE before transfer to PDVF membranes (Millipore), which were then probed with the indicated Abs, visualized using ECL reagent (GE Healthcare), and detected by LAS-3000 (Fuji Film). For affinity precipitation, cells were stimulated with 100 μM pervanadate (Sigma-Aldrich) prepared as described (19). Whole-cell lysates of ST486 and Jurkat cells in 1 ml RIPA buffer were incubated at 4°C for 6 h with 20 μl of NeutrAvidin-agarose beads (Pierce) saturated by biotinylated phosphor-peptides (Oropen Bio-technology). For immunoprecipitation of chimeric proteins, 4 × 10^5 stable transfectants were lysed with RIPA buffer after stimulation with intact anti-IgM (50 μg/ml) for 3 min. The lysate was then treated with 10 μg of agarose-conjugated anti-HA Ab and incubated at 4°C for 4 h. Beads were then washed three times with 1 ml of lysis buffer and boiled, and Western blotting was performed as described above.

Flow cytometric analysis of cellular phosphorylation

DT40 stable transfectants were incubated at 37°C for 2 h before stimulation. The cells (5 × 10^5) were then stimulated with 50.0 μg/ml intact anti-chicken IgY or 50.0 μg/ml F(ab’)2 of anti-anti-chicken IgY. Finally, treated cells were fixed with 200 μl of Phosflow Fix Buffer (BD Biosciences) at 37°C for 10 min, permeabilized with BD Phosflow Perm Buffer II (BD Biosciences) for 30 min at on ice, and then stained with PE-conjugated anti- phosphotyrosine Abs (PY20) before FACS analysis.

Results

Construction of chimeric proteins

To investigate the function of FCRL3 in B cells, we constructed a chimeric protein which comprised the extracellular domain of murine FcγRIIB and the transmembrane and intracellular domains of FCRL3 (designated as chimera wild type (CM-WT)) (Fig. 1A). In addition, the intracellular domain of the chimeric protein was mutated by replacing all 4 tyrosine residues with phenylalanine (designated as chimera mutant (CM-Mt)) to analyze the potential function of motifs around the residues. These constructs were stably transfected to ST486, which is a germinal-center derived B cell line and lacks expression of FcγRs. Surface expression of the chimeric proteins was confirmed by flow cytometer (Fig. 1B). Transfectants were stimulated with intact anti-IgM Abs, resulting in colligation of BCR and the chimeric proteins. As a control, the F(ab’)2 of anti-IgM Abs was used to stimulate BCR alone (Fig. 1C).

FCRL3 engagement inhibits BCR-stimulated tyrosine phosphorylation and Ca_2+ influx

To elucidate the function of FCRL3 in B cells, and associations with BCR signaling in particular, we first examined BCR-stimulated tyrosine phosphorylation in ST486 transfectants of chimeric proteins. Although few proteins were phosphorylated at the tyrosine residues under resting status, BCR ligation with F(ab’)2 anti-IgM strongly induced tyrosine phosphorylation in all transfectants (Fig. 2A). BCR-induced phosphorylation was modestly inhibited in CM-WT transfectants, but not in mock transfectants, when intact anti-IgM was used. Phosphorylation of adapter proteins in BCR complex, such as Syk and PLCγ2, was also inhibited when BCR was stimulated with intact anti-IgM in CM-WT transfectants (Fig. 2B). The same inhibition was not observed in CM-Mt transfectants, in which all tyrosine residues had been replaced, suggesting that tyrosine motifs in CM-WT have inhibitory effects on tyrosine phosphorylation in BCR signaling.

We then examined whether the chimeric proteins themselves were phosphorylated through BCR stimulation. Chimeric proteins were immunoprecipitated after BCR stimulation, then immunoblotted to detect phosphorylation status at the tyrosine residues (Fig. 2C). Whereas CM-WT proteins were not phosphorylated in resting status, BCR stimulation alone by F(ab’)2 anti-IgM induced slight phosphorylation in tyrosine residues in chimeric proteins. When CM-WT transfectants were stimulated with intact anti-IgM, tyrosine phosphorylation of CM-WT protein was greatly enhanced. This suggests that chimeric proteins were culligated to BCR, and that co-localization with BCR allowed tyrosine kinases in the BCR complex to phosphorylate tyrosine residues of CM-WT chimeric proteins. This phosphorylation of tyrosine residues may have led to recruitment of adapter proteins to the tyrosine motifs of chimeric proteins, in turn reducing phosphorylation of proteins in the cells. Ligation of chimeric proteins alone did not induce cell tyrosine phosphorylation (data not shown), although tyrosine residues at 650 and 662 display strong similarity to ITAM and may have activation potential. Together, these results suggest that the cytoplasmic domain of FCRL3, in total, displays inhibitory potential for tyrosine phosphorylation when colligated with BCR.

Next, we examined Ca_2+ mobilization as a downstream event of the phosphorylation of PLCγ2 in the BCR complex (Fig. 2D). Although BCR ligation with the F(ab’)2 of anti-IgM resulted in a characteristic calcium influx in all transfectants, this was partially inhibited by intact anti-IgM in CM-WT transfectants but not in...
mock transfectants. When BCR was coaggregated with CM-Mt, no inhibition of Ca\(^{2+}\)/H\(_{11001}\) influx was observed, suggesting the inhibitory effects of tyrosine motifs in CM-WT on Ca\(^{2+}\)/H\(_{11001}\) mobilization. We also examined whether the chimeric proteins could induce Ca\(^{2+}\)/H\(_{11001}\) mobilization by ligating the chimeric proteins alone (Fig. 2D). No Ca\(^{2+}\) influx was observed for any of the transfectants, indicating the absence of ability to mobilize Ca\(^{2+}\)/H\(_{11001}\) in FCRL3.

To further confirm the inhibition of BCR-induced signaling by the chimeric proteins, another set of stable transfectants was examined using DT40 cells (Fig. 3 and supplemental Fig. 1B). Tyrosine phosphorylation induced by BCR cross-linking in DT40 cells was evaluated by flow cytometry using anti-phosphotyrosine Abs. Inhibition of tyrosine phosphorylation in CM-WT transfectants was again observed when coaggregated with BCR, as in ST486 cells (Fig. 3A). We also examined BCR-induced Ca\(^{2+}\)/H\(_{11001}\) influx in DT40 cells (Fig. 3B). Inhibition of Ca\(^{2+}\) influx in CM-WT transfectants was even more obvious in DT40 cells when coaggregated with chimeric proteins, while moderate inhibition was also observed in CM-Mt transfectants, unlike ST486 cells. To evaluate each individual effect of tyrosine motifs on Ca\(^{2+}\)/H\(_{11001}\) influx, additional mutational constructs were made by replacing three tyrosine residues with phenylalanine and leaving a single tyrosine residue intact (designated as CM-Y650, CM-Y662, CM-Y692, and CM-Y722). We also evaluated the effect of a potential ITAM motif

**FIGURE 2.** Coaggregation of FcγRIIB/FCRL3 chimeric protein inhibits BCR-induced tyrosine phosphorylation and Ca\(^{2+}\)/H\(_{11001}\) influx in ST486 cells. **A**, ST486 transfectants of chimeric proteins were stimulated with F(ab\(^{\prime}\))\(_2\) of anti-IgM (10.0 \(\mu\)g/ml) or intact anti-IgM (15 \(\mu\)g/ml) for 3 min before analysis of whole-cell phosphorylation of tyrosine residues detected by antiphosphotyrosine Ab (PY20). Blots were stripped and reprobed using anti-β-actin Ab. The densities of phosphotyrosine proteins were quantified and shown on the top of figure, where each transfectant stimulated with F(ab\(^{\prime}\))\(_2\) anti-IgM for 3 min is set to be 1.00. **B**, Whole lysates from A were immunoblotted against phospho-Syk (Tyr352) and phospho-PLC\(_{\gamma2}\) (Tyr1217). Blots were stripped and reprobed with anti-Syk and PLC\(_{\gamma2}\) Ab, respectively. The densities of phospho-Syk and phospho-PLC\(_{\gamma2}\) were quantified and shown on the top of figure, where each transfectant stimulated with F(ab\(^{\prime}\))\(_2\) anti-IgM is set to be 1.00. **C**, Whole lysates from A were immunoprecipitated with anti-HA Ab and immunoblotted with anti-phosphotyrosine Ab. Blots were stripped and reprobed with anti-HA Ab. Data are representative of four separate experiments. **D**, ST486 transfectants of indicated chimeric proteins were loaded with fura-2 and stimulated with 10.0 \(\mu\)g/ml F(ab\(^{\prime}\))\(_2\) of anti-IgM (solid thick line) or 15 \(\mu\)g/ml intact anti-IgM (dotted line). Chimeric proteins were also ligated alone with biotinylated 5.0 \(\mu\)g/ml anti-murine FcγRIIB (2.4G2) followed by 10.0 \(\mu\)g/ml streptavidin (solid thin line). The results are representative of three independent experiments.
comprising tyrosines at 650 and 662, by mutating the remaining tyrosines (named CM-Y650/Y662). Among these constructs, inhibition of BCR-induced Ca\textsuperscript{2+} influx was observed in CM-Y662, CM-Y692, and CM-Y650/Y662. Intriguingly, and contrary to our expectations, Ca\textsuperscript{2+} influx was enhanced when colligated with chimeric proteins in transfectants of CM-Y650 and CM-Y722.

**FCRL3 engagement inhibits BCR-induced cell death**

Activation-induced cell death occurs in germinal center B cells, in which FCRL3 is highly expressed, following prolonged ligation of BCR by Abs. This mimics the negative selection process of self-reactive B-cells induced by strong BCR ligation with self-Ags (20). This also occurs in germinal center-derived ST486 cells (21). We therefore examined BCR-induced cell death in ST486 transfectants of chimeric proteins. BCR was stimulated alone or with coligation to chimeric proteins. Compared with mock transfectants, coligation to chimeric proteins significantly reduced cell death in CM-WT transfectants (Fig. 4A, Annexin V\textsuperscript{+} 7-AAD\textsuperscript{+} cells). Interestingly, coligation to CM-Mt chimeric proteins enhanced BCR-induced cell death. To evaluate individual effects of the four tyrosine residues (Y650, Y662, Y692, and Y722), additional mutated constructs of CM-WT were created (supplemental Fig. S1; each tyrosine residue was replaced with phenylalanine and constructs were designated CM-F650, CM-F662, CM-F692, and CM-F722, respectively). In comparison with CM-WT, inhibition of cell death was reduced in CM-F662 and partially reduced in CM-F692, while mutations in CM-F650 and CM-F722 had little effect (Fig. 4B). This suggests that tyrosine motifs at 662 and 692 play critical roles in the inhibitory function of FCRL3 on activation-induced cell death.

**Association of tyrosine kinases and phosphatases with FCRL3**

Given that the intracellular domain of FCRL3 can inhibit tyrosine phosphorylation of cell proteins, calcium mobilization, and cell
death induced by BCR ligation, we sought to identify the adaptor proteins that can mediate these inhibitory effects. To precipitate adaptor proteins from cell lysates, phosphopeptides corresponding to the ITAM and ITIMs in the FCRL3 cytosolic domain were used (Fig. 5A). As tyrosines at 650 and 662 could potentially be components of a single ITAM or two separate ITIMs, a peptide extending from 644 to 678 was synthesized (designated Y650Y662). Two other peptides corresponding to potential ITIMs at 692 and

![Graph A](image1.png)

**FIGURE 4.** Coligation of FcγRIIB/FCRL3 chimeric protein inhibits activation-induced cell death by BCR stimulation. A, ST486 transfectants were incubated in medium alone ( ), with 10.0 μg/ml F(ab′)2 of anti-IgM ( ), or with 15 μg/ml intact anti-IgM ( ) for 24 h. Cells were then stained with PE-labeled annexin V and 7-AAD to detect early apoptotic cells (annexin V+, 7-AAD−, and dead cells (annexin V+ 7AAD+). B, ST486 transfectants with the chimeric protein and mutants (each tyrosine residue was replaced with phenylalanine and designated as CM-F650, CM-F662, CM-F692, and CM-F722, respectively) were incubated with 10.0 μg/ml F(ab′)2 of anti-IgM or with 15 μg/ml intact anti-IgM for 48 h. Reduction in the proportion of dead cells (7-AAD positive cells) in the latter (intact anti-IgM) compared with the former (F(ab′)2 of anti-IgM) is shown with a solid bar. Data represent mean ± SD of quadricate cultures (*, p < 0.01), and are representative of three independent experiments.
were also used (designated Y692 and Y722, respectively). In addition, peptides phosphorylated in tyrosine residues were also prepared (designated, for example, as pY650Y662: pY stands for phosphorylated tyrosine). Amino acid “pY” represents phosphorylated tyrosine. Consensus ITAM (italic) and ITIM (underlined) motifs are indicated.

**FIGURE 5.** Tyrosine motifs of FCRL3 associate with kinases and phosphatases. A. Phosphopeptides were synthesized according to the sequences around the tyrosine residues of the FCRL3 cytosolic domain. Amino acid “pY” represents phosphorylated tyrosine. Consensus ITAM (italic) and ITIM (underlined) motifs are indicated. B, ST486 cells and Jurkat cells were activated with pervanadate, then lysed. Using whole cell lysates, proteins were affinity precipitated with phoshopeptide-coated beads, and separated by SDS/PAGE, and blots were probed with specific Abs. C, FcγRIIB/FCRL3 chimeric proteins in ST486 transfectants stimulated with intact anti-IgM (50 μg/ml) for 3 min were immunoprecipitated with anti-HA Abs and separated by SDS/PAGE. The resulting blots were probed with specific Abs. D, FcγRIIB/FCRL3 mutational chimeric proteins in ST486 transfectants were immunoprecipitated under stimulation of BCR for 3 min, as described in C. Results are representative of four independent experiments.

Jurkat cells, while only Syk but not ZAP-70 was associated in ST486 cells (Fig. 5B). When either of two tyrosines in the peptides was not phosphorylated, this association was diminished. Tyrosine phosphatases, in contrast, were also associated with the phosphopeptides of FCRL3 cytosolic domain. SHP-1 and -2 were associated with both Y650pY662 and pY692, although stronger association was observed between SHP-1 and pY692, particularly in ST486 cells, and between SHP-2 and Y650pY662 in both ST486 cells and Jurkat cells. This further supported the finding by Xu et al. (7), in which GST fusion proteins of FCRL3 and Jurkat cell lysates were used for pull-down assays.
et al. that the tyrosine motif at 692 had recruited SHP-1 and might function as an ITIM. Another SH2 domain-containing phosphatase, SHIP, was preferentially bound to Y650pY662 in ST486 cells, but not in Jurkat cells, in which SHIP expression was absent. Because Xu et al. used lysates of Jurkat cells and did not detect binding of SHIP to FCRL3, this is the first report to identify that SHIP binds to the phosphotyrosine motif at 662 of FCRL3.

To confirm the associations of these adaptor proteins with FCRL3, we also examined binding of these adaptor proteins to chimeric protein in ST486 transfectants under the condition of BCR signaling. Chimeric proteins were immunoprecipitated alone or after colligation with BCR using intact anti-IgM Abs. Although SHIP and SHP-1 and -2 were detected binding to chimeric proteins in which tyrosine residues were phosphorylated, Syk association was below detectable levels (Fig. 5C). We also immunoprecipitated mutational proteins (CM-F650, CM-F662, CM-F692, and CM-F722) to examine the role of individual tyrosine motifs. Binding of SHIP and SHP-1 was highly reduced in CM-F662 and CM-F692, respectively, matching the results of peptide experiments. In contrast, binding of SHP-2 was substantially reduced in CM-F692, despite the greatest binding capacity of peptide pY662, indicating that SHP-2 mainly binds to tyrosine motif at 692 under physiological conditions.

**Discussion**

In the present study using B cell transfectants of FCRL3 chimeric proteins, which display the unique property of having both ITAM and ITIM, we demonstrated the inhibitory potential of FCRL3 on BCR-mediated signaling. When colligated with BCR, FCRL3 chimeric protein inhibited BCR-mediated cell tyrosine phosphorylation and calcium mobilization. FCRL3 also inhibited activation-induced cell death followed by prolonged BCR signaling. Analysis with mutational chimeric proteins showed tyrosine residues in the cytosolic domain were essential for these inhibitory effects of FCRL3. Binding assays using phosphopeptides of the tyrosine motifs displayed that SHIP associated with the phosphotyrosine motif at 662, while SHP-1 and SHP-2 bound to phosphotyrosine motifs at 662 and 692 (stronger association at 692 under physiological conditions). Both of the tyrosine motifs at 662 and 692 displayed inhibitory potential on BCR-induced cell death according to the study of the mutational constructs. Taken together, the two tyrosine motifs at 662 and 692 participate in the inhibitory effects of FCRL3 on BCR-mediated signaling through binding of phosphatases SHIP, SHP-1, and SHP2. Although the potential ITAM included tyrosines at 650 and 662 bound with Syk in ST486 cells, which play important roles in initiating BCR-signaling, no activation signaling was observed in ST486 transfectant when the FCRL3 chimeric protein were ligated alone. This may be explained by the functional predominance of phosphatases SHIP, SHP-1, and SHP-2, which could antagonize BCR-signaling initiated by Syk and other phosphoproteins in the signalosome. The lack of detectable levels of Syk association in coprecipitation with FCRL3 chimeric protein further implicates relatively lower activation potential of the ITAM in FCRL3.

The enhanced calcium influx observed in CM-Y650 and CM-Y722 transfectants in DT40 cells implies additional biological effect of FCRL3 cytosolic domain in the absence of tyrosine motifs at 662 and 692, which may be mediated by unknown adaptor molecules binding to FCRL3. As this enhancement was not observed in CM-Mt transfectant, at least one intact tyrosine-motif at either 662 or 692 is needed. In contrast, enhanced apoptosis was observed in the CM-Mt transfectant in ST486 cells. Similar observations were reported for FCGR2B, where a mutant FCGR2B protein lacking SHIP recruitment to the mutated ITIM showed proapoptotic effects on B-cells when coligated with BCR (22). As CM-Mt protein did not show to enhance tyrosine-phosphorylation or calcium influx in ST486 and DT40 cells, this proapoptotic effect may be independent of these signaling events. Although these observations indicate additional biological functions of the intracellular domain of FCRL3 other than the inhibitory potential of tyrosine motifs at 662 and 692, overall potential seems inhibitory. The function of FCRL3, however, may be altered depending on the cell environment, particularly expression levels of adaptor proteins, as suggested by the specific binding of ZAP-70 and SHIP in Jukat and ST486 cells, respectively.

Genes for the six Fc receptor-like molecules (FCRL1–6) lie in the 1q21–23 chromosomal region and share some characteristics (23). The proteins are preferentially expressed in B cell lineages, showing homologous structures consisting of Ig-like extracellular domains and potential ITIM and/or ITAM motifs in the cytoplasmic tails. FCRL1, which has two potential ITAM motifs, is the only member that has been shown to have an activating coreceptor function on B-cells. FCRL4 and FCRL5, in contrast, have been shown to exhibit inhibitory potential on BCR-signaling by recruiting tyrosine phosphatases including SHP-1, SHP-2, and SHIP to the ITIM motifs. Interestingly, FCRL5 has both ITIMs and an ITAM, just like FCRL3, and has been shown to inhibit B cell activation via SHP-1 recruitment of tyrosine phosphatase to the ITIM (24). The dominant effect of ITIM over ITAM in FCRL5 could be explained by the lower binding capacity of Syk binding to ITAM or the dominant antagonistic effect of phosphatases on kinases, as was the case in FCRL3. Together with differential expression of FCRLs in the B cell lineage and the potential for both activatory and inhibitory potential on B cells, these mechanisms provide sophisticated control of the differentiation of B-cells and may influence the fate of individual B cell clones.

Tolerance susceptibility of B cells and thresholds of BCR signaling can be modulated by coreceptors, as demonstrated by several transgenic and knockout mice models (17). For instance, transgenic mice that overexpress human CD19, an activating coreceptor on B cells, exhibit increased levels of autoantibodies (25), while gene targeting of FCGR2B that has an inhibitory potential on BCR signaling renders several murine strains susceptible to autoimmune disorders (26, 27). In humans, polymorphism in the transmembrane region of FCGR2B gene is reportedly associated with susceptibility to SLE (28). As the mutant allele of FCGR2B cannot associate with the lipid rafts where the BCR complex resides, loss of inhibition in BCR signaling may promote autoimmune response (29). These findings indicate enhancement or loss of inhibition in BCR signaling by coreceptors augments autoimmune response in both mice and humans.

Intriguingly, and contrary to this, the results presented in this study show an opposing role for FCRL3, with inhibitory potential on BCR signaling, where gain of gene function due to elevated expression leads to autoimmunity. Supporting evidence for our observation that reduced BCR-signaling leads to autoimmunity has recently been reported by Kumar et al. using the NZM240 murine lupus model (30). By crossing a congenic strain bearing the Sle1 locus of lupus susceptibility with the HEL/Ig-HEL transgenic strain, they demonstrated that B cell tolerance was critically regulated by Ly108.1, a member of the Slam gene family. Lupus-associated allele Ly108.1 reduced the strength of BCR signaling compared with the normal allele Ly108.2. This reduced BCR signaling resulted in incomplete induction of anergy and deletion in autoreactive B cells, leading to breakdown of tolerance. This observation led us to consider that augmented expression of FCRL3 in individuals with the disease-susceptibility genotype may interfere with the induction of B cell tolerance by up-regulating the...
FCRL3 INHIBITS BCR SIGNALING

activation threshold of BCR signaling. More interestingly, parallel situations have been described in T cell-mediated autoimmunities in both mouse and human autoimmune diseases. In SKG mice, a new model for rheumatoid arthritis, inadequate TCR signaling due to a mutation in Zap70 caused tolerance breakdown of T cells (31). In several human autoimmune diseases, a mutation in PTPN22, which encodes tyrosine phosphatases in the TCR complex, reportedly reduces TCR response in T cells and increases the risk of diseases (2, 3, 32). Given that T and B cells show strong similarities in Ag-receptor signaling and mechanisms of tolerance induction, the notion that reduction of Ag receptor signaling may be a common mechanism of tolerance breakdown in both T and B cells is intriguing; autoreactive lymphocytes would be rescued from tolerance mechanisms due to incomplete induction of anergy or deletion that should have been induced by self Ags.

In conclusion, FCRL3 displays inhibitory potential on BCR-mediated signaling, and greater inhibition in cells with the disease-risk genotype may lead to breakdown of tolerance. Blockade of FCRL3 function might thus restore tolerance in autoreactive B cells and could represent a potential therapy for autoimmune diseases. To further clarify the involvement of FCRL3 in autoimmunity and the potency of FCRL3-tageted therapy, identification of the unknown ligands and analysis in animal disease models are needed.

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Disclosures

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