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FcμR Interacts and Cooperates with the B Cell Receptor To Promote B Cell Survival

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The IgM FeR (FcμR) promotes B cell survival, but the molecular mechanism remains largely unknown. We show using FcμR−/− and wild-type mice that FcμR specifically enhanced B cell survival induced by BCR cross-linking with F(ab')2-anti-IgM Abs while having no effect on survival when the B cells were activated by CD40 ligation or LPS stimulation. FcμR expression was markedly upregulated by anti-IgM stimulation, which may promote enhanced FcμR signaling in these cells. Immunofluorescence and confocal microscopy analyses demonstrated that FcμR colocalized with the BCR on the plasma membrane of primary B cells. Coimmunoprecipitation analysis further revealed that FcμR physically interacted with the BCR complex. Because NF-κB plays a prominent role in B cell survival, we analyzed whether FcμR was involved in BCR-triggered NF-κB activation. FcμR did not affect BCR-triggered IκBα phosphorylation characteristic of the canonical NF-κB activation pathway but promoted the production of the noncanonical NF-κB pathway component p52. Consistent with the elevated p52 levels, FcμR enhanced BCR-triggered expression of the antiapoptotic protein BCL-ⅩL. Importantly, FcμR stimulation alone in the absence of BCR signaling had no effect on either IκBα phosphorylation or the expression of p52 and BCL-ⅩL. Therefore, FcμR relied on the BCR signal to activate the noncanonical NF-κB pathway and enhance B cell survival. These results reveal a cross-talk downstream of FcμR and BCR signaling and provide mechanistic insight into FcμR-mediated enhancement of B cell survival after BCR stimulation. The Journal of Immunology, 2015, 194: 000–000.

Peripheral B cell survival relies on signals from the BCR and the BAFFR (1). The BCR is a heterotrimeric complex consisting of Ag binding Ig and the signaling Igα/Igβ heterodimers. In vivo ablation of surface Ig (2) or inactivation of Igα (3) causes rapid death of B cells, indicating that BCR transmits essential "tonic" survival signals in the absence of Ag ligands. Crosslinking the BCR on mature B cells with Ag or anti-IgM Abs initiates multiple intracellular signaling cascades, which eventually lead to the activation of ERK, NF-κB, and NFAT pathways. Among these, NF-κB appears to play a prominently protective role in the survival of Ag-stimulated B cells by inducing the expression of several antiapoptotic genes such as Bcl-2, Bcl-xL, and Bfl-1/A1 (4–6). BCR signaling activates the canonical NF-κB pathway, which is characterized by the phosphorylation and ubiquitin-mediated degradation of IκB inhibitory proteins, in particular IκBα. This leads to the translocation of NF-κB into the nucleus to activate target gene transcription. BAFFR is a member of the TNFR family. Deficiency of BAFF or BAFFR results in an almost complete loss of follicular and marginal zone (MZ) B cells (7–9), demonstrating a critical role for BAFFR-mediated signaling in B cell survival. In contrast to BCR, BAFFR activates the noncanonical NF-κB pathway, which depends on the proteolytic processing of p100 to p52 to generate p52/ReLB (NF-κB2) nuclear complexes (10–13). Both BCR and BAFFR are required for the maintenance of peripheral B cell homeostasis. It has been shown that signals from the BCR and BAFFR cooperate to allow B cell survival at multiple stages of peripheral B cell differentiation and during immune responses. BAFF promotes BAFFR-mediated signals through at least two mechanisms by upregulating the expression of BAFFR and by supplying the noncanonical NF-κB pathway substrate p100 for BAFFR-mediated degradation (14–16).

The recently identified IgM FeR (FcμR) (17, 18) has been shown to play a critical role in IgM homeostasis, B cell development and survival, germinal center formation, and humoral immune responses as well as in prevention of autoantibody production (19–22). It remains unclear, however, how FcμR regulates B cell development and function. An intriguing clue came from the in vitro analysis, which revealed a specific defect for FcμR−/− B cells in anti-IgM-induced survival and proliferation (19, 21). These observations suggested a possible functional link between FcμR and BCR. In the current study, we addressed the molecular mechanisms of FcμR-mediated enhancement of anti-IgM-induced B cell survival. We show that FcμR and BCR physically interact on the plasma membrane of primary B cells and functionally cooperate to promote the activation of the noncanonical NF-κB pathway and BCL-xL expression. Importantly, FcμR alone in the absence of BCR signaling had no effect on either B cell survival or NF-κB activation. These

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; B-CLL, B cell chronic lymphocytic leukemia; GC, germinal center; IC, immune complex; MZ, B, marginal zone B; WT, wild-type.

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Materials and Methods

Mice

C57BL/6 mice were purchased from CLEA Japan (Tokyo). Fc<sub>γ</sub>R-deficient mice have been described previously (19). The mice were maintained in specific pathogen-free conditions and all experimental procedures were approved by the Animal Experiment Committee of RIKEN.

B cell survival assay

Primary B cells were purified from the spleen of C57BL/6 and Fc<sub>γ</sub>R-deficient mice using an IMAG negative sorting kit (BD Biosciences). Purified B cells were cultured for 48 h under various conditions as described (19). The cells were stained with 7-aminoactinomycin D (7-AAD) and the percentages of viable (7-AAD<sub>low</sub>FSC<sub>high</sub>) and dead (7-AAD<sub>high</sub>) cells were analyzed by FACS (BD Biosciences). The anti-mouse IgD<sub>A</sub> Ab (clone AMS-9.1; catalog number 406108) and its isotype control (clone MG2b-57; catalog number 401212, LEAF purified) were purchased from BioLegend. The AMS-9.1 mAb was passed through a gel filtration column (PD MidiTrap G-25; GE Healthcare) to remove azide. The anti-mouse IgM Abs (LE/AF goat F(ab')<sub>2</sub>-anti-mouse κ, catalog number 1052-14) were purchased from Southern Biotechnology Associates. Spleen B cells purified from BALB/c mice (a allotype) were used to investigate the effect of Fc<sub>γ</sub>R cross-linking on anti-IgD and anti-IgG-induced B cell activation.

Analysis of Fc<sub>γ</sub>R expression after B cell activation

Purified spleen B cells were cultured in the presence of F(ab')<sub>2</sub>-anti-IgM Abs (5 μg/ml), soluble CD40L, or LPS (10 μg/ml) for 6, 24, and 48 h. The cultured cells were first incubated with a rat IgG<sub>2b</sub> anti-mouse CD16/CD32 PE-conjugated anti-rat IgG<sub>2a</sub> (clone RG7/1.30; BD Biosciences). The cells were stained with 7-aminoactinomycin D (7-AAD) and the percentages of viable (7-AAD<sub>low</sub>FSC<sub>high</sub>) and dead (7-AAD<sub>high</sub>) FSC<sub>high</sub>) cells were analyzed by FACS (BD Biosciences). The anti-mouse IgD<sub>A</sub> Ab (clone AMS-9.1; catalog number 406108) and its isotype control (clone MG2b-57; catalog number 401212, LEAF purified) were purchased from BioLegend. The AMS-9.1 mAb was passed through a gel filtration column (PD MidiTrap G-25; GE Healthcare) to remove azide. The anti-mouse IgM Abs (LE/AF goat F(ab')<sub>2</sub>-anti-mouse κ, catalog number 1052-14) were purchased from Southern Biotechnology Associates. Spleen B cells purified from BALB/c mice (a allotype) were used to investigate the effect of Fc<sub>γ</sub>R cross-linking on anti-IgD and anti-IgG-induced B cell activation.

Immunofluorescence and confocal microscopy

Ten thousand wild-type (WT) B cells were seeded on poly-L-lysine (Sigma-Aldrich)–treated coverslips and allowed to adhere for 15 min at 37°C. Cells were fixed for 15 min at room temperature in 3% paraformaldehyde (Electron Microscopy Sciences), washed with PBS and incubated in staining buffer (0.05% saponin, 10 mM glycine, 5% FBS, and PBS) for 15 min. Cells were incubated with rabbit IgG α-Fc<sub>γ</sub>R (original Ab, 1/500 dilution) together with one of the following Abs: FITC-rat IgG<sub>2a</sub> anti-CD79A (clone 5A5; Cell Signaling Technology), rabbit IgG anti-CD40L or LPS (10 μg/ml) for 6, 24, and 48 h. Consplen B cells cultured in the presence of F(ab')<sub>2</sub>-anti-IgM Abs (Supplemental Fig. 1C, 1D), anti-IgM stimulation reflected by the increased cell sizes and cell division (Supplemental Fig. 1A). However, cross-linking FcγR with the 4B5 mAb did not enhance the anti-IgD–mediated B cell activation (Supplemental Fig. 1B). In contrast, the 4B5 mAb was able to enhance B cell survival following CD40L ligation of CD40 (Fig. 1B). Moreover, cross-linking FcγR on WT B cells with an anti-FcγR Ab enhanced anti-IgM– but not LPS- or CD40L–induced B cell survival (Fig. 1C). Mature B cells express both IgM and IgD on the cell surface. We further investigated whether cross-linking FcγR was able to enhance B cell survival or activation induced by anti-IgD Abs. The anti-IgD Ab AMS-9.1 induced B cell activation as reflected by the increased cell sizes and cell division (Supplemental Fig. 1A). However, cross-linking FcγR with the 4B5 mAb did not enhance the anti-IgD–mediated B cell activation (Supplemental Fig. 1B). In contrast, the 4B5 mAb was able to enhance B cell survival induced by anti-IgD Abs (Supplemental Fig. 1C, 1D), which cross-link both IgM and IgD. These observations collectively demonstrate that FcγR specifically enhances IgM BCR-mediated B cell survival/activation.

Uregulation of FcγR expression on primary B cells by BCR cross-linking

BCR cross-linking upregulates BAFFR expression, which is one mechanism by which the BCR promotes BAFFR-mediated B cell survival (14–16). To analyze how FcγR expression is regulated, splenic B cells were cultured in the presence of F(ab')<sub>2</sub>-anti-IgM Abs, soluble CD40L, or LPS for different times and their FcγR levels were compared with that before culture (0 h). As shown in Fig. 2, FcγR cell surface expression was markedly upregulated after BCR cross-linking with anti-IgM Abs but only moderately increased by CD40L or LPS stimulation. The upregulation of FcγR expression by anti-IgM stimulation may in part contribute to the FcγR-mediated enhancement of BCR-triggered B cell survival. However, FcγR was also moderately upregulated by treatment with CD40L or LPS without affecting B cell survival. Therefore, additional mechanisms likely exist to allow FcγR to specifically enhance B cell survival induced by α-IgM stimulation. Although we found that FcγR protein levels were upregulated upon B cell activation, Choti et al. (21) found that transcript levels for FcγR were reduced after stimulation with LPS or anti-CD40 or F(ab')<sub>2</sub>-anti-IgM Abs. This discrepancy might be due to differential regulation of FcγR transcription and protein expression.
Therefore, the colocalization between FcμR and B cells, rather than real colocalization of FcμR on splenic myeloma B cells, appeared that some BCR were not associated with FcμR and IgM could simultaneously interact, we immunoprecipitated FcμR under a mild detergent condition and analyzed the coprecipitation of the BCR components. As shown in Fig. 3B, immunoprecipitation of FcμR from splenic B cells pulled down IgH (Igμ) and its associated Igα (upper panel). Conversely, FcμR and Igα were coprecipitated with IgM (lower panel). These results collectively indicate that FcμR constitutively associates with the BCR on primary B cells.

Normal BCR internalization in FcμR-deficient B cells

BCR is known to undergo constitutive and Ag-induced internalization, which serves as an important mechanism to regulate surface BCR levels and BCR signaling. The physical association of FcμR and BCR suggested that FcμR might affect BCR internalization and thereby regulate BCR signal strength. We first analyzed constitutive (Ag-independent) BCR internalization by incubating splenic B cells in the presence of brefeldin A, which inhibits the trafficking of the internalized BCR, or by using F(ab’2) anti-IgM Abs, which are unable to trigger BCR signaling. In both cases, WT and FcμR-deficient B cells exhibited very similar kinetics of BCR internalization (Fig. 4, left and middle panels). We next analyzed ligand-dependent BCR internalization using F(ab’2) anti-IgM Abs, which initiate BCR signaling. F(ab’2) anti-IgM Abs induced a much more rapid BCR internalization (Fig. 4, right panel) compared with that induced by F(ab’) anti-IgM Abs (middle panel), but again, this ligand-dependent BCR internalization was unaffected by FcμR deficiency (Fig. 4, right panel). These observations complement our previous finding that FcμR does not contribute to the internalization of Ag and IgM/Ag immune complexes (IC) by B cells and the subsequent presentation on MHC class II molecules (19). Therefore, FcμR does not seem to regulate BCR signaling through modulating its internalization processes.

**FcμR cooperates with BCR to promote p52 and BCL-xL expression**

BCR signaling activates canonical NF-κB pathway to induce the expression of anti-apoptotic genes and enhance B cell survival. This prompted us to examine the effect of FcμR signaling on anti-IgM–induced NF-κB activation. We first analyzed anti-IgM–induced IkBα phosphorylation, which is known to correlate with NF-κB activation in the canonical pathway (1). No difference was observed in the magnitude and kinetics of pIkBα between WT B cells treated with an isotype control or the 4B5 anti-FcμR Ab (Fig. 5A) or between WT and FcμR-deficient B cells (Supplemental Fig. 3A), indicating that FcμR does not contribute to canonical NF-κB activation. We further analyzed the activation of the noncanonical NF-κB pathway. BCR signaling does not activate the noncanonical NF-κB pathway but produces the noncanonical NF-κB substrate p100. Activation of the noncanonical NF-κB pathway results in the processing of p100 to generate p52, which associates with RELB to form NF-κB2 and activates the expression of antiapoptotic proteins such as BCL-xL (14–16). Intriguingly, we found that p52 levels were elevated at later time points in WT B cells stimulated with both anti-IgM and anti-FcμR as compared with those stimulated with anti-IgM and an isotype control Ab (Fig. 5B, 5C, Supplemental Fig. 3D). Moreover, consistent with the elevated levels of p52, anti-FcμR Ab also enhanced the expression of BCL-xL at later points.
of anti-IgM stimulation (Fig. 5B, 5C). Conversely, $Fc_{\mu}R^{-/-}$ B cells showed a reduction in BCR-triggered BCL-xL expression compared with WT B cells (Supplemental Fig. 3B, 3C), which supports a role for $Fc_{\mu}R$ in promoting BCL-xL expression. However, it should be noted that the decreased BCL-xL expression in $Fc_{\mu}R^{-/-}$ B cells also could be due to the differences in B cell subsets and maturation status between WT and $Fc_{\mu}R^{-/-}$ mice.

Intriguingly, although $Fc_{\mu}R$ promoted p52 accumulation and BCL-xL expression after BCR stimulation, cross-linking $Fc_{\mu}R$ alone in the absence of BCR signaling had no detectable effect on either IκBα phosphorylation (Fig. 5D) or the induction of p100, p52, or BCL-xL (Fig. 5E). These observations suggest that $Fc_{\mu}R$ by itself is unable to activate NF-κB1 or NF-κB2 but relies on BCR signaling to promote NF-κB2 activation.

**Discussion**

In the current study, we have demonstrated that $Fc_{\mu}R$ physically associates with BCR in primary B cells and specifically enhances B cell survival induced by anti-IgM but not CD40L or LPS stimulation. $Fc_{\mu}R$ cooperates with BCR to promote the induction of p52 and its target BCL-xL. Importantly, $Fc_{\mu}R$ alone in the absence of BCR signaling has no effect on either B cell survival or NF-κB activation. In other words, $Fc_{\mu}R$ relies on BCR signaling to elicit its survival function.

The cooperation between $Fc_{\mu}R$ and BCR in enhancing B cell survival to some extent resembles the relationship between BAFFR and BCR (14–16). As is the case for BAFFR, $Fc_{\mu}R$ is upregulated by BCR cross-linking, which likely contributes to the $Fc_{\mu}R$-mediated enhancement of BCR-triggered cell survival. However, one critical difference between BAFFR and $Fc_{\mu}R$ is that BAFFR signaling by itself is able to generate p52 and promote BCL-xL expression in B cells by collaborating with BCR “tonic” signals, whereas $Fc_{\mu}R$ alone in the absence of BCR cross-linking is unable to activate either the canonical or the noncanonical NF-κB pathway to induce B cell survival. This difference predicts that BAFFR and $Fc_{\mu}R$ contribute to Ag-independent and -dependent B cell survival, respectively. In agreement with this prediction, mice lacking BAFF/BAFFR have almost a complete loss of mature B cells (7–9), whereas $Fc_{\mu}R^{-/-}$ mice have relatively normal sizes of the follicular B cell pool but show reduced B cell survival after BCR stimulation and impaired germinal center (GC) formation and Ab production against a T-dependent Ag (19). Therefore, the dependence of $Fc_{\mu}R$ function on BCR signaling allows $Fc_{\mu}R$ to specifically enhance the survival of Ag-stimulated B cells. Although we have shown that cross-linking $Fc_{\mu}R$ with the 4B5 anti-$Fc_{\mu}R$ mAb could enhance B cell survival induced by F(ab′)2 anti-IgM Abs, it remains to be investigated whether $Fc_{\mu}R$ signaling by its bona fide ligand soluble IgM has the same effect. Further studies are required to clarify this issue by using BCR-transgenic B cells in which one can simultaneously cross-link BCR with specific Ag and $Fc_{\mu}R$ with soluble IgM.

It is intriguing to note that the MZ B cell population was significantly reduced in $Fc_{\mu}R^{-/-}$ mice (19, 20). It has been suggested that self-reactive B cells may be driven to become MZ B cells (25). An interesting hypothesis would be that MZ B cells may be stimulated by self-Ag to generate a relatively strong survival signal.
by cooperating with the FcµR-mediated signal. Absence of FcµR would thus result in a reduced self-Ag–triggered BCR signal in MZ B cells that is required for maintaining their survival.

During an immune response, Ag–specific B cells are activated in the B cell follicles of the secondary lymphoid organs in response to IC bound to follicular dendritic cells. In this way, the complement receptor (CD21/CD19 complex) coclusters with BCR upon interaction with Ags bearing complement C3d, resulting in efficiently lowering the activation threshold of B cells in comparison of stimulation by BCR alone (26). Notably, the phenotype of FcµR-deficient mice has a marked similarity to that of CD19-deficient mice in terms of decreased MZ B cells, impaired GC formation, reduced Ab production to T-independent and T-dependent Ags, and impaired memory responses (27, 28). The close correspondence in the phenotype of FcµR- and CD19-deficient mice suggests that, similar to CD21/CD19 coreceptor complex, FcµR may function as a positive regulator in B cell responses to IgM-ICs in GCs. Indeed, similar to CD19, the presence of FcµR reduced the dose of BCR stimulation needed for sustaining B cell viability (19). Our present findings also indicate that integration of BCR and FcµR signaling at the level of BCL-xL upregulation by IgM-ICs may help overcome anergy- or apoptosis-inducing effects of the BCR alone and promote the survival and expansion of B cells to initiate GC reactions.

Engagement of the BCR initiates two concurrent processes, signaling and receptor internalization. The latter is an important mechanism to regulate the BCR signal strength and prevent excessive B cell activation. Using WT and FcµR-deficient B cells, we found that FcµR did not affect ligand-dependent and -independent BCR internalization. Therefore, although FcµR associates with BCR, it does not elicit its function through modulating BCR internalization. It remains to be investigated how signals downstream of BCR and FcµR cross-talk to promote p52 induction and BCL-xL expression. Earlier studies have shown that multiple tyrosine and serine residues in the cytoplasmic tail of FcµR are phosphorylated upon ligand binding (17). Given the physical association between FcµR and BCR, one possible scenario is that after BCR stimulation, these residues might be phosphorylated by BCR-activated protein tyrosine kinases and thereby recruit more signaling molecules, participating in and amplifying the BCR-mediated signal cascades.

FcµR-deficient mice produce elevated IgG autoantibodies as they age (19–22), suggesting that FcµR is required for maintaining self-tolerance. The results of the current study demonstrate that FcµR promotes BCR-triggered survival of mature B cells. BCR ligation in different contexts can lead to different biological outcomes, and immature B cells in the bone marrow have been shown to undergo apoptosis upon BCR cross-linking. A reduction in BCR signaling due to the absence of FcµR may lead to insufficient elimination of autoreactive immature B cells in the BM. In addition, autoreactive B cells can be generated in the GC by Ig gene somatic hypermutation (29), and some autoreactive GC B cells might escape the deletion mechanism because of reduced BCR signaling. Studies are in progress to investigate the role of FcµR in the deletion of autoreactive B cells in the BM and during the GC reaction.

BCR signaling also plays an important role in neoplasia. Malignant B cells from patients with chronic lymphocytic leukemia (B-CLL) express much higher levels of FcµR than normal B cells from healthy donors (30, 31). Antigenic stimulation through the BCR is thought to promote the outgrowth of B-CLL (32, 33), and our results suggest that elevated FcµR expression may enhance a BCR-triggered survival signal and contribute to the pathogenesis of B-CLL. Further elucidation of the precise molecular details by which FcµR cooperates with BCR to regulate B cell survival should accelerate our understanding of the etiology of immunological disorders and B cell malignancies associated with altered BCR signals.

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Disclosures
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