Critical role of the IgM Fc receptor in IgM homeostasis, B-cell survival, and humoral immune responses

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Edited by Jeffrey V. Ravetch, The Rockefeller University, New York, NY, and approved August 21, 2012 (received for review June 22, 2012)

IgM antibodies have been known for decades to enhance humoral immune responses in an antigen-specific fashion. This enhancement has been thought to be dependent on complement activation by IgM–antigen complexes; however, recent genetic studies render this mechanism unlikely. Here, we describe a likely alternative explanation; mice lacking the recently identified Fc receptor for IgM (FcμR) on B cells produced significantly less antibody to protein antigen during both primary and memory responses. This immune deficiency was accompanied by impaired germinal center formation and decreased plasma and memory B-cell generation. FcμR did not affect steady-state B-cell survival but specifically enhanced the survival and proliferation induced by B-cell receptor cross-linking. Moreover, FcμR-deficient mice produced far more autoantibodies than control mice as they aged, suggesting that FcμR is also required for maintaining tolerance to self-antigens. Our results thus define a unique pathway mediated by the FcμR for regulating immunity and tolerance and suggest that IgM antibodies promote humoral immune responses to foreign antigen yet suppress autoantibody production through at least two pathways: complement activation and FcμR.

B-cell activation | autoimmune disease | complement receptor

IgM is the first antibody isotype to appear during ontogeny and the only isotype produced by all species of vertebrates (1, 2). It is also the first isotype produced during an immune response and plays a crucial role in front-line host defense against pathogens (3). The significance of natural as well as antigen-specific IgM in humoral immune responses has been well documented. More than 40 y ago, it was reported that the immune response was enhanced by 19S (now known as IgM) antigen-specific antibodies if they were administered before immunization (4). Later, it was shown that the IgM-mediated enhancement of antibody production was dependent on complement activation because mutant IgM unable to activate complement lacked the enhancing activity (5). More recently, mice lacking the secreted form of IgM ($S\mu^-$) were shown to have impaired antibody production against T-dependent (TD) antigens (6, 7). It was proposed that IgM–antigen immune complexes enhanced B-cell survival by interacting with both B-cell receptor (BCR) and the complement receptor (CR) on B cells (2, 8). However, very recently, knock-in mice expressing mutant IgM unable to activate complement were found to have completely normal antibody responses (9). Therefore, IgM-mediated enhancement of antibody production cannot be solely explained by activation of the classic complement pathway, and additional mechanisms are likely involved.

Cellular receptors for the constant Fc region of antibodies mediate a wide range of functions, including phagocytosis of antibody-coated pathogens, induction of cellular cytotoxicity, transcytosis of antibodies through cells, and regulation of hypersensitivity responses and B-cell activation (10–12). The existence of an Fc receptor for IgM (FcμR) was first suggested 40 y ago (13–15), but its gene identity was revealed only recently (16, 17). FcμR is unique among FcRs in that it is expressed only by lymphoid cells, B cells in mice, and B and T cells in humans (16, 17). In the present study, we have established and analyzed FcμR-deficient mice. We found that FcμR specifically enhanced B-cell survival following BCR cross-linking but had no effect when the cells were stimulated by CD40 ligand or LPS. Mice lacking FcμR had impaired germinal center (GC) formation and produced significantly less antibody against protein antigen. The abnormalities found in FcμR-deficient mice largely recapitulated those observed in mice lacking secreted IgM (6–8, 18, 19), suggesting that a significant portion of the effector function of IgM, especially with regard to the enhancement of humoral immune responses and suppression of autoimmunity, is mediated by FcμR.

Results

Cell Surface FcμR Expression During B-Cell Development, Maturation, and Activation. Our previous studies revealed that FcμR was expressed in B cells but not in T cells, dendritic cells, and macrophages in mice (17). We further analyzed FcμR expression during B-cell development, maturation, and activation. FcμR was predominantly expressed by $B220^+$ splenocytes but not by $B220^-$ splenocytes in mice (Fig. S1*A*). Its expression was low in pro-B, pre-B, and immature B cells in the bone marrow and increased in the follicular (FO) B cells and marginal zone (MZ) B cells in the spleen and in the B1a cells in the peritoneal cavity (PC) (Fig. S1*B*). Within the Peyer's patches, its expression was lower in the $B220^+$peanut agglutinin $(PNA)^+$ GC B cells compared with the $B220^+PNA^-$ non-GC B cells (Fig. S1*B*). We further analyzed FcμR expression by antigen-specific B cells following immunization with 4-hydroxy-3-nitrophenylacetyl–coupled chicken γ-globulin (NP-CGG). FcμR levels were reduced on the 4-Hydroxy-3-iodo-5-nitrophenylacetyl $(NIP)^+$ (NP-specific) B cells and further down-modulated on the NIP^+ GC B cells (Fig. S1 *C* and *D*). These results demonstrate that FcμR is predominantly expressed by mature B cells and down-modulated on B-cell activation in vivo.

B-Cell Development and Maturation in *FcμR*$^{-/-}$ Mice. To explore the physiological function of FcμR, we established FcμR-deficient mice (Fig. S2). FACS analysis confirmed the lack of FcμR ex-

Author contributions: H.O., J.Y.W., T.K. and T.T. designed research; R.O., H.M., and K.H. performed research; H.T. and H.O. contributed new reagents/analytic tools; R.O. analyzed data; and J.-Y.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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See Author Summary on page 15989 (volume 109, number 40).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1210706109/-/DCSupplemental.

pression on $Fc\mu R^{-/-}$ splenocytes (Fig. S2*B*). B-cell development appeared normal in the absence of FcμR (Fig. S3), except for a reduction of $CD21^{+}CD23^{low}$ MZ B cells and a partial block in B-cell maturation as revealed by a decrease of the $B220^{+}AA4^{+}CD23^{+}IgM^{low}$ T3 population and the $IgM^{low}IgD^{high}$ B cells in the spleen (Fig. 1 *A*–*C*). FcμR-deficient mice had the normal frequency and numbers of B1a cells in the PC (Fig. S3*C*). Serum IgM levels were significantly elevated in the absence of FcμR in a gene dose-dependent manner (Fig. 1*D*) and inversely correlated with the cell surface levels of FcμR on B cells (Fig. 1 *E* and *F*). These results suggest that some portion of the IgM in WT and FcμR heterozygous mice binds to the FcμR, whereupon it is sequestered or degraded. Consistently, we found that exogenously administered IgM had a similar half-life in WT and FcμR-deficient mice (Fig. 1*G*), suggesting that FcμR in WT mice was already occupied by IgM in vivo and that the bound IgM was not replaced by the exogenous IgM. The serum IgG and IgA levels were similar in WT, $Fc\mu R^{+/-}$, and $Fc\mu R^{-/-}$ mice (Fig. S4).

Reduced Survival of FcμR-Deficient B Cells After BCR Cross-Linking. B-cell proliferation in response to LPS stimulation or CD40 ligation ± IL-4 was unchanged in the absence of FcμR (Fig. S5*A*). In addition, IgG_1 isotype switching induced by CD40 ligand plus IL-4 or LPS plus IL-4 was the same in WT and $Fc\mu R^{-/-}$ B cells (Fig. S5*B*). However, the proliferative response of FcμR-deficient B cells to α-IgM stimulation was consistently less than that of WT cells (Fig. 2*A*), and this decrease was accompanied by reduced survival (Fig. 2*B*). No significant difference was observed in the actual number of cell divisions by the viable WT and FcμR-deficient B cells (Fig. 2*C*), suggesting that the decreased proliferation observed was largely attributable to reduced FcμR-deficient B-cell survival. WT B cells stimulated with a low concentration of the $F(ab')_2$ α-IgM antibodies initially showed a decrease in survival compared with nonstimulated cells, but cell viability gradually increased with increased amounts of α-IgM antibodies (Fig. 2*B*, white bars). By contrast, a higher concentration of the $F(ab')_2$ α-IgM antibodies was required to increase viability to a similar extent in FcμR-deficient B cells (Fig. 2*B*, solid bars). These observations suggest that absence of FcμR increased the threshold of BCR signaling. However, the reduced survival in FcμR-deficient B cells could also reflect differences in the B-cell subpopulations. We therefore further analyzed whether cross-linking FcμR had an effect on BCR-mediated survival. Indeed, the 4B5 α-FcμR antibody, but not an isotype control, enhanced α-IgM–induced proliferation (Fig. 2*D*) and survival (Fig. 2*E*) of WT B cells (Fig. 2 *D* and *E*, compare striped bars with white bars). In contrast to its effect on BCR signaling events, the FcμR antibody did not affect LPS-induced proliferation (Fig. 2*D*) or survival (Fig. 2*E*) of WT B cells. In addition, the 4B5 antibody had no effect on the survival/proliferation of FcμR-deficient B cells (Fig. 2 *D* and *E*, compare black and gray bars), demonstrating that the effect of the 4B5 antibody was indeed mediated by FcμR. Notably, α-FcμR antibody alone in the absence of BCR cross-linking had no effect on B-cell survival or proliferation [Fig. 2 *D* and *E*, 0 μg/mL $F(ab')_2$ α-IgM antibodies]. These results collectively suggest that FcμR by itself was unable to transmit a signal sufficient to induce B-cell survival or proliferation but was specifically able to enhance the survival induced by BCR cross-linking. To investigate whether FcμR contributes to elevated BCR signaling, we analyzed BCR-triggered Ca^{2+} influx. WT and FcμR-deficient B cells showed a similar magnitude of Ca^{2+} influx (Fig. S6*A*). In addition, cross-linking FcμR on WT B cells did not enhance Ca^{2+} influx induced by BCR stimulation (Fig. S6*B*). These results suggest that FcμR did not affect the early signaling events triggered by BCR cross-linking but, rather, contributed to more downstream events, leading to increased survival.

Impaired Humoral Immune Responses in FcμR-Deficient Mice. To explore the in vivo function of FcμR, we analyzed antibody production against the T-independent (TI) antigen NP-Ficoll and the TD antigen NP-CGG. Consistent with the decreased

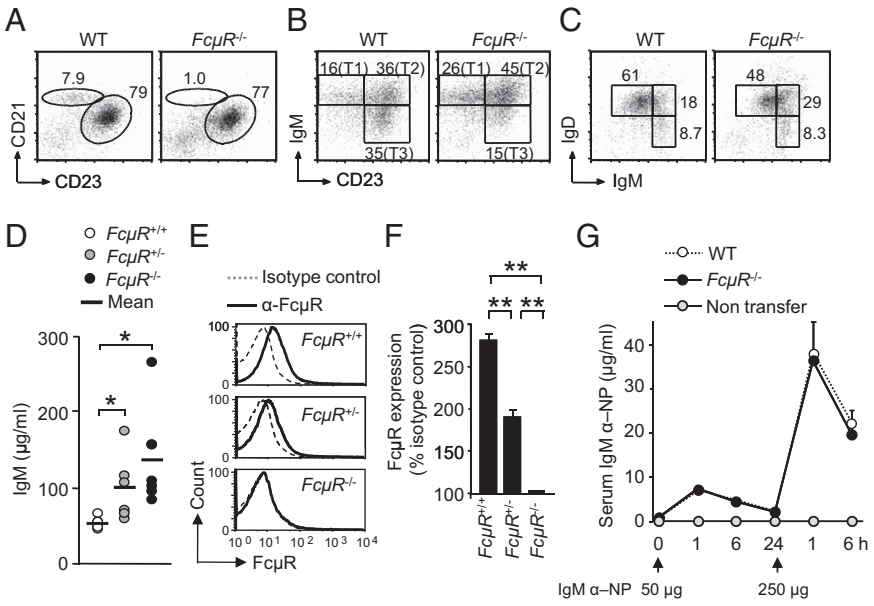

Fig. 1. B-cell development, maturation, and serum Ig levels in FcμR-deficient mice. Reduced proportion of $CD21^{+}CD23^{low}$ MZ B cells (*A*) and a partial block of B-cell maturation as revealed by decreased T3 (*B*) and $IgM^{low}IgD^{high}$ populations (*C*) in FcμR-deficient mice are shown. (*A*–*C*) Results from analysis of three pairs of WT and FcμR-deficient mice are summarized in Fig. S3. (*D*) Serum IgM levels of unimmunized WT, $Fc\mu R^{+/-}$, and $Fc\mu R^{-/-}$ mice. The results for six mice of each genotype are depicted. A horizontal bar indicates the mean. $^{*}P < 0.05$. (*E*) Cell surface FcμR levels on WT, $Fc\mu R^{+/-}$ and $Fc\mu R^{-/-}$ B cells. (*F*) Mean fluorescence intensity ± SD of FcμR expression of two mice of each genotype. $^{**}P < 0.01$. (*G*) Similar half-life of serum IgM in WT and $Fc\mu R^{-/-}$ mice. A monoclonal IgM antibody specific for NP (clone 1A86) was injected i.p., and mice were bled at the indicated time points. The serum concentration of the NP-specific IgM was analyzed by ELISA. The mean values from three pairs of WT and $Fc\mu R^{-/-}$ mice are shown.

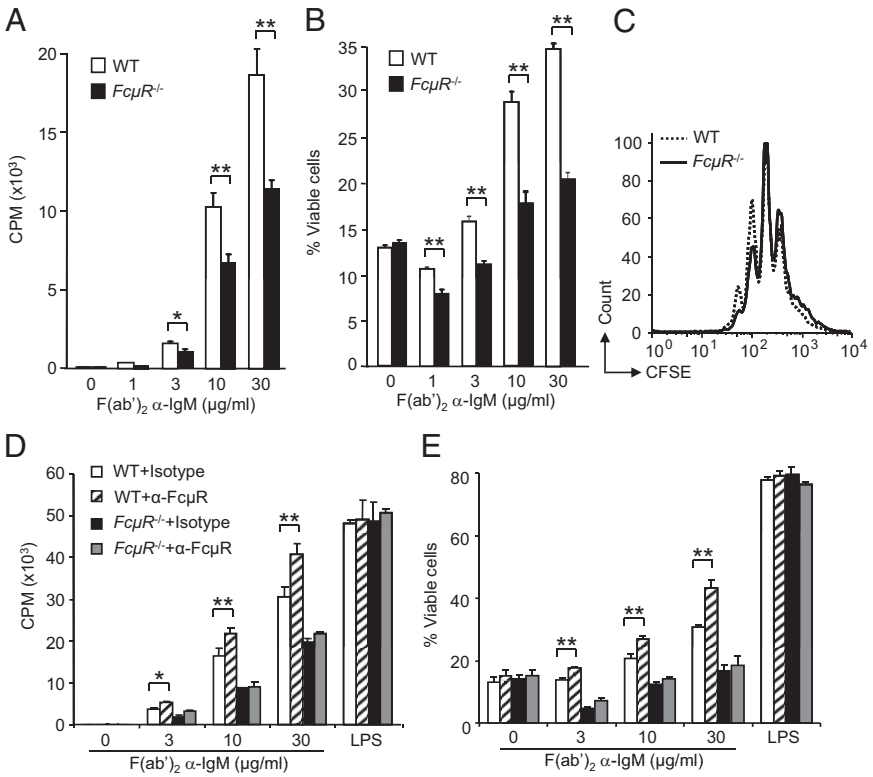

Fig. 2. Reduced proliferation and decreased survival of FcμR-deficient B cells in response to BCR stimulation. Purified spleen B cells were seeded at 5×10^5 cells/mL and stimulated with various doses of F(ab′)$_2$–anti-mouse IgM antibodies. (*A*) Reduced DNA synthesis in FcμR-deficient B cells as measured by thymidine incorporation. B cells were cultured for 48 h and pulsed with 1μCi of ^{3}H-thymidine for the last 6 h. CPM, counts per minute. (*B*) Decreased B-cell survival after anti-IgM stimulation. Cells cultured for 48 h as above were stained with 7AAD to identify dead cells and analyzed by FACS. (*C*) Cell division analysis. Cells were labeled with CFSE and then cultured with 30 μg/mL anti-IgM for 72 h. CFSE intensity was analyzed after gating on live cells. In *A*–*C*, representative results of seven independent experiments are shown. (*D* and *E*) FcμR cross-linking enhanced B-cell survival and proliferation induced by BCR stimulation. Spleen B cells were first incubated with an anti-FcγRIIB antibody (clone 2.4G2) passed through a gel filtration column to remove azide) and then seeded as above. The cells were cultured for 2 d with different doses of F(ab′)$_2$–anti-mouse IgM antibodies in the presence of either the 4B5 anti-FcμR or an isotype control antibody. (*D*) Proliferation as measured by ^{3}H-thymidine uptake. (*E*) Cell survival revealed by 7-aminoactinomycin D (7AAD) staining. Similar results were obtained in three independent experiments for *D* and *E*. The error bars represent means ± SD. $*P < 0.05$; $**P < 0.01$.

survival and proliferation of FcμR-deficient B cells in response to BCR stimulation in vitro, production of both IgM and IgG$_3$ antibodies to NP-Ficoll was reduced in FcμR-deficient mice (Fig. 3*A*). The response to NP-CGG was analyzed after immunization with several different doses of antigen. When mice were immunized with relatively low doses of NP-CGG (2.5 or 10 μg), antibody production was reduced during both the primary and memory responses (Fig. 3*B*, *Left* and *Center*); the production of both high-affinity (anti-NP$_3$) and low-affinity (anti-NP$_{30}$) antibodies was decreased. By contrast, antibody production in response to a high dose (100 μg) of NP-CGG was similar between WT and FcμR-deficient mice (Fig. 3*B*, *Right*), likely because the responses had already reached a plateau. These results collectively demonstrate that FcμR is a positive regulator of antibody production to both TI and TD antigens in vivo.

Impaired GC Formation and Reduced Memory and Plasma Cell Differentiation in FcμR-Deficient Mice. To understand the mechanism of the decreased antibody production in FcμR-deficient mice, we then analyzed GC formation after immunization with 10 μg of NP-CGG. We used expression of the activation-induced cytidine deaminase (AID) to define GC B cells that undergo Ig gene hypermutation and class switch recombination. Immunofluorescent staining of spleen sections revealed fewer and significantly smaller GCs in FcμR-deficient mice compared with WT mice (Fig. 4 *A* and *B*), indicating that GC formation and B-cell expansion in the GC were impaired in the absence of FcμR. We further analyzed the generation of antigen-specific GC B and memory B cells in response to NP-CGG (Fig. 4*C*). The numbers of NIP-binding, NP-specific GC B cells were decreased in FcμR-deficient mice at both 2 wk and 6 wk after immunization (Fig. 4*C*, *Center*). As might be expected, given the decreased serum antibody production during the primary response, NP-specific antibody-forming cells were also reduced in FcμR-deficient mice (Fig. 4*D*). The numbers of NP-specific memory B cells were also reduced in FcμR-deficient mice (Fig. 4*C*, *Right*), possibly as a result of impaired GC B-cell expansion. The reduced secondary response in FcμR-deficient mice (Fig. 3*B*) is thus likely due to a reduction in the numbers of responding NP-specific memory B cells and, based on the outcome with naive B cells, probably their reduced survival following BCR ligation, although this has not been directly tested.

Normal MHC Class II Antigen Presentation by FcμR-Deficient B Cells. One potential function of the FcμR is the endocytosis of IgM–antigen complexes and their processing and presentation to helper T cells. To analyze whether the impaired GC formation is due to impaired antigen presentation by FcμR-deficient B cells, we crossed FcμR-deficient mice with B1-8^{hi} mice, which carry a precombined NP-specific V_H186.2DFL16.1J_H2 antibody gene (20, 21). We then analyzed BCR-mediated internalization of the synthetic NP-Eα-GFP antigen and subsequent presentation of the residue 52–68 Eα-derived peptide on MHC class II molecules by using the Y-Ae monoclonal antibody, which recognizes

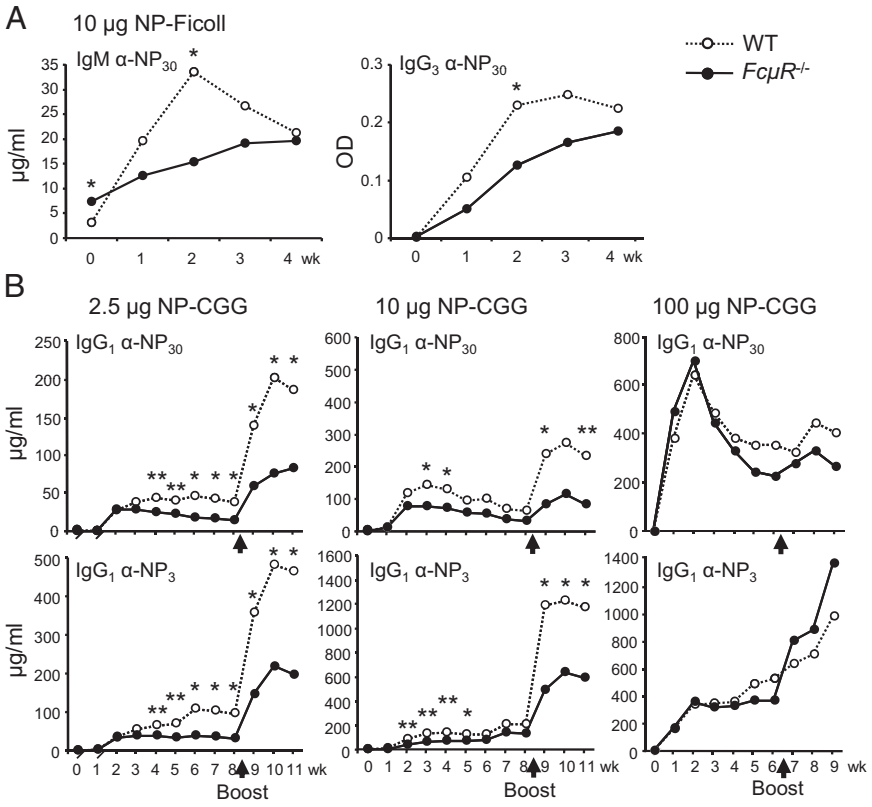

Fig. 3. Impaired humoral immune responses in FcμR-deficient mice. (*A*) Antibody production to a TI antigen, NP-Ficoll. Eight pairs of WT and FcμR-deficient mice were immunized i.p. with 10 μg of NP-Ficoll and analyzed for the production of NP-specific IgM (*Left*) and IgG_3 (*Right*) antibodies in the serum at 1–4 wk after immunization. The mean values of WT and FcμR-deficient mice at the indicated time points are shown. (*B*) Response to the TD antigen NP-CGG. Mice were immunized with 2.5 μg (*Left*), 10 μg (*Center*), or 100 μg (*Right*) of NP-CGG in alum at week 0 and boosted with the same amount of NP-CGG in PBS 8 wk (for 2.5 μg and 10 μg) or 6 wk (100 μg) later. NP-specific serum IgG_1 levels were analyzed each week by ELISA with NP_{30}-BSA or NP_3-BSA as a capture agent, and the mean values of eight pairs (2.5 μg), seven pairs (10 μg), and eight pairs (100 μg) are shown. $*P < 0.05$; $**P < 0.01$.

the complex of MHC II and Eα peptide (22). No significant difference was observed between WT and FcμR-deficient B cells in their ability to internalize and present the NP-Eα-GFP antigen, as assessed by the finding of a similar proportion of $GFP^+Y\text{-}Ae^+$ cells (Fig. S7 *A* and *C*) and the mean fluorescent intensity of GFP and Y-Ae (Fig. S7*D*). Furthermore, administration of NP-Eα-GFP together with an IgM anti-NP monoclonal antibody (1A86) to form antigen–antibody complexes had no effect on antigen incorporation or presentation by either WT or FcμR-deficient B cells (Fig. S7 *B–D*), suggesting that FcμR does not contribute to the internalization or presentation of IgM immune complexes under these experimental conditions.

Autoantibody Production in FcμR-Deficient Mice. We have shown that FcμR is required for efficient humoral immune responses against a foreign antigen. Paradoxically, we found that these mice produced autoantibodies in the absence of additional genetic defects. As shown in Fig. 5, FcμR-deficient mice had normal levels of total serum IgG (Fig. 5*A*) but significantly elevated levels of IgG α-dsDNA (Fig. 5*B*) and IgG rheumatoid factor (Fig. 5*C*) compared with WT mice at 33 wk of age. Furthermore, five of eight FcμR-deficient mice but none of the eight WT mice produced antinuclear antibodies as revealed by immunofluorescent staining of Hep2 cells (Fig. 5*D* and Fig. S8). Both males and females produced similarly increased levels of anti-dsDNA antibodies, rheumatoid factor, and antinuclear antibodies. These results suggest that FcμR is required for suppression of autoantibody production.

Discussion

In the present study, we have elucidated the physiological function of FcμR in mice. FcμR specifically enhanced B-cell survival induced by BCR cross-linking. The mutant mice had impaired GC formation with a concomitant reduction of antigen-specific plasma cells and memory B cells, as well as decreased antibody production to both TI and TD antigens. These results reveal a critical role for FcμR in B-cell survival following antigen stimulation and humoral immune responses.

One unique feature of FcμR is its specific expression on B cells in mice, as shown by FACS analysis in our previous (17) and current studies. Consistently, microarray analysis revealed that *Fcmr* transcripts were only detectable in isolated B cells, as well as in spleen and lymph node tissues, but not in any other mouse tissues or cell types examined. Although we cannot formally exclude the possibility that FcμR is expressed by a minor population of certain cell types and/or tissues, the available data indicate that FcμR predominantly regulates B-cell function in mice. In humans, FcμR was found to be expressed by B cells, T cells, and natural killer cells (16), and it is possible that human FcμR may have additional functions not present in mice. In fact, FcμR has been suggested to regulate Fas-mediated apoptosis in human T and B cells (23, 24).

FcμR-deficient mice had a normal frequency and normal numbers of mature FO B cells in the spleen and B1a cells in the PC. Only MZ B cells were reduced, and there was a partial block of B-cell maturation revealed by an accumulation of the T2 and $IgM^{high}IgD^{high}$ population. The alterations in B-cell differentiation and maturation in FcμR-deficient mice were different from

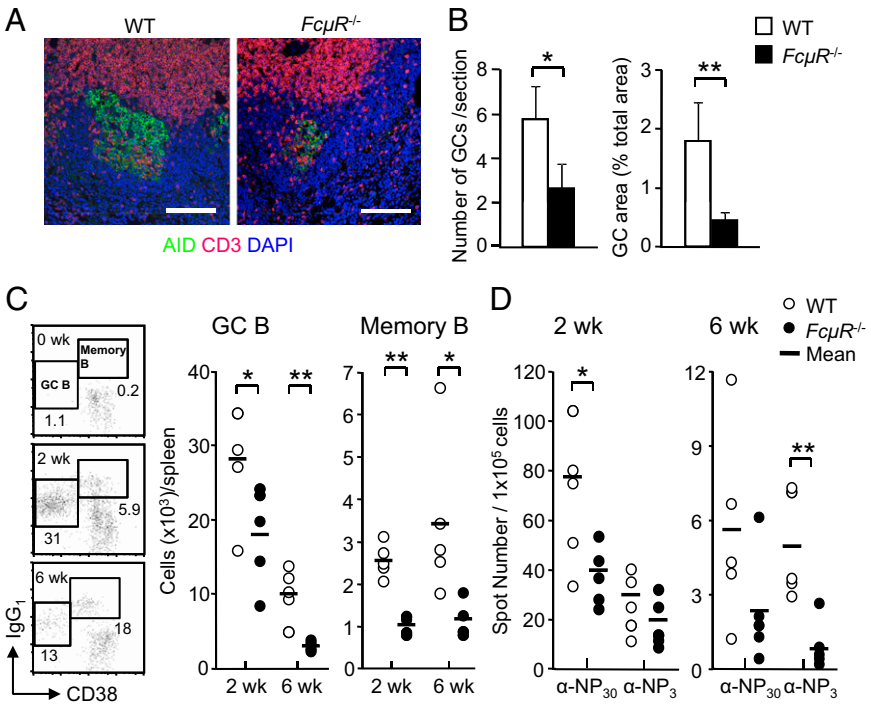

Fig. 4. Impaired GC formation and reduced memory and plasma cell differentiation in response to the TD antigen NP-CGG. (*A*) Representative GCs in WT (*Left*) and FcμR-deficient (*Right*) mice at 2 wk after immunization with 10 μg of NP-CGG in alum. (Scale bar, 100 μm.) (*B*) Numbers of GCs per entire sagittal spleen section (*Left*) and areas of the GCs relative to the total area (*Right*) (mean ± SD of 4 WT and 5 $Fc\mu R^{-/-}$ mice) are indicated. (*C*) Impaired generation of the GC B cells and memory B cells in FcμR-deficient mice. Mice were immunized as above, and the NP-specific GC and memory B cells were identified at 2 and 6 wk after immunization by FACS. (*Left*) Typical FACS profiles of WT mice. Five pairs of WT and FcμR-deficient mice were analyzed, and the total numbers of GC B cells (*Center*) and memory B cells (*Right*) in each mouse are shown. (*D*) Decreased numbers of NP-specific antibody-secreting cells in FcμR-deficient mice. Mice immunized as above were analyzed for the NP-specific antibody-forming cells (AFCs) by enzyme-linked immunospot (ELISPOT) assay. AFCs in the spleen at 2 wk (*Left*) and in the bone marrow at 6 wk (*Right*) after immunization are shown. $*P < 0.05$; $**P < 0.01$.

those found in mice lacking the B-cell activating factor (BAFF) or its receptor (BAFF-R), in which both mature B and MZ B cells were greatly decreased (25–27). An important difference between the function of FcμR and BAFF-R is that FcμR enhanced B-cell survival only after BCR cross-linking, whereas BAFF/BAFF-R is required for the survival of naive B cells by collaborating with the "tonic" survival signals through the BCR. Alterations in the B-cell subpopulations in FcμR-deficient mice also did not correspond to those found in mice with impaired BCR signaling, including mice lacking Bruton's tyrosine kinase (28), B cell linker protein (29), or B-cell adaptor for phosphoinositide 3-kinase (30). In these mice, both spleen mature B cells and peritoneal B1a cells were decreased. These observations suggest that the absence of FcμR did not impair the tonic signals through the BCR, such that the development and maintenance of mature B and B1a cells were not affected.

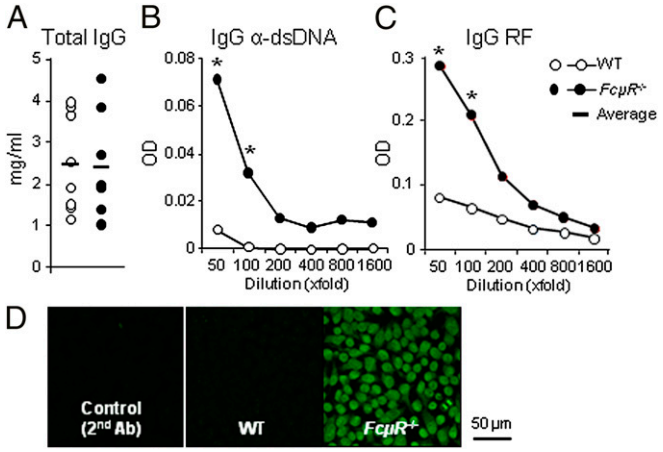

Fig. 5. $Fc\mu R^{-/-}$ mice produce α-dsDNA, rheumatoid factor, and α-nuclear antibodies. Results of eight WT (1 male and 7 female) mice and eight $Fc\mu R^{-/-}$ (2 male and 6 female) mice are shown. (*A*) Total serum IgG. (*B*) IgG α-dsDNA antibodies. (*C*) IgG rheumatoid factor (RF). $*P < 0.05$ (unpaired *t* test). (*D*) Hep2 cells were stained with sera (100-fold dilution) from WT and $Fc\mu R^{-/-}$ mice, followed by FITC–anti-mouse IgG as described (22). Original magnification: 200 ×. Five (1 male and 4 female) of eight $Fc\mu R^{-/-}$ mice, but none of the WT mice, produced α-nuclear antibodies (Fig. S8).

Serum IgM levels were elevated by approximately twofold in FcμR-deficient mice compared with WT mice. One possible explanation is that the production of natural IgM was increased in FcμR-deficient mice. However, B1 cells, which are considered to be the major source of natural IgM, were not increased in the PC of FcμR-deficient mice. In addition, FcμR heterozygous mice showed increased serum IgM levels, and there was an inverse correlation between serum IgM levels and the cell surface FcμR levels on B cells. We consider it more likely that some fraction of serum IgM in WT mice binds to the FcμR, leading to decreased levels of serum IgM. Serum IgM levels were not affected in mice lacking the pIg receptor (31) or Fcα/μR (32), both of which can bind IgM. These observations suggest that only FcμR is involved in the homeostasis of serum IgM. Exogenously administered IgM had a similar half-life in WT and FcμR-deficient mice, suggesting that FcμR is not involved in the clearance of IgM under steady-state conditions. However, it is possible that during an immune response, the IgM–antigen complexes may bind FcμR, resulting in their catabolism. Further studies are required to elucidate the

dynamics of the interaction between IgM and FcμR in vivo during an immune response.

FcμR specifically enhanced B-cell survival induced by BCR cross-linking. Notably, in the absence of α-IgM stimulation, WT and FcμR-deficient B cells survived equally well. In addition, cross-linking FcμR on WT B cells with the α-FcμR antibody in the absence of α-IgM stimulation had no detectable effects on B-cell survival and proliferation. These observations suggest that FcμR does not affect steady-state B-cell survival and, by itself, is unable to transmit a signal strong enough to trigger a biological response. The survival function of FcμR only became evident in conjunction with BCR cross-linking. We would propose that this mechanism ensures that FcμR only functions in antigen-stimulated B cells, allowing it to enhance the activation of antigen-specific B cells. The cytoplasmic tail of FcμR contains potential phosphorylation motifs for protein kinase C and casein kinase C, as well as several conserved tyrosine residues (16). It remains to be elucidated how FcμR-mediated signals cooperate with BCR-derived signals to promote B-cell survival. Interestingly, it was recently reported that the malignant B cells from patients with chronic lymphocytic leukemia (CLL) express much higher levels of FcμR than normal B cells from healthy donors (33). It is widely believed that the initial expansion and prolonged survival of the CLL B-cell clone are driven by self-antigen (34). Thus, the CLL B cells may experience chronic BCR stimulation in vivo, and FcμR may further enhance their survival.

FcμR-deficient mice had impaired GC formation and a decreased TD antibody response. A possible explanation for the decreased antibody response is that there are fewer MZ B cells in the FcμR-deficient mice. However, a decrease in MZ B cells does not necessarily lead to impaired antibody responses (35, 36). Moreover, MZ B cells are more notable for their participation in TI responses than in TD responses. A successful TD humoral immune response requires intimate interactions between helper T cells and B cells, and antigen presentation by B cells is a critical step to initiate T–B interactions. Using B1-8^{hi} mice, however, we found that FcμR did not contribute to antigen internalization or subsequent presentation on MHC class II molecules. Nevertheless, because the antigen presentation experiment was performed under conditions quite different from typical immunization, the possibility remains that FcμR may play a role in antigen presentation during the actual GC reaction. Notably, the phenotype of FcμR-deficient mice is quite similar to, although not as severe as, that in CD19-deficient mice (37) in terms of decreased MZ B cells, impaired GC formation, reduced antibody production to TI and TD antigen, and impaired memory response. It was shown that defective CD19 signaling caused an accumulation of the IgD^{+} early GC B cells in the FO dendritic cell zone of GC and inhibited GC development (38). It is unclear at this point whether the reduced GC response in FcμR-deficient mice is only due to decreased B-cell survival or due to additional defects during GC development, such as fewer mature B cells entering the GC or impaired maturation of GC B cells as observed in CD19 deficiency. In this regard, we found that the GC area was reduced by 75% in FcμR-deficient mice compared with WT mice. This reduction is even more dramatic than the reduction in B-cell survival observed under in vitro culture conditions. It is thus possible that additional mechanisms other than the decreased B-cell survival after BCR cross-linking are responsible for the dramatically decreased GC formation in vivo in FcμR-deficient mice.

Although FcμR-deficient mice were impaired in antibody production against TI and TD antigens, we also found that these mice produced elevated IgG autoantibodies as they aged in the absence of any additional genetic defects. The seemingly paradoxical role for FcμR suggests that it is required for tolerance to self-antigens as well as immunity against foreign antigens. Although the precise mechanisms for autoantibody production remain to be discovered, one likely possibility is that IgM–autoantigen complexes may cross-link FcμR and BCR on autoreactive B cells and trigger their deletion/anergy. The phenotype of FcμR-deficient mice resembles that observed in mice lacking secreted IgM (Sμ^{-}) in terms of impaired GC formation, reduced antibody production against protein antigens, and development of autoantibodies (2, 6–8, 18, 19). Earlier studies suggested that IgM-mediated enhancement of antibody production was dependent on activation of the classic complement pathway (5). However, a very recent report demonstrated that mice expressing IgM unable to activate complement (Cμ13 mice) had completely normal antibody production (9). Collectively, these observations suggest that much of the effector function of IgM, especially with regard to the enhancement of humoral immune responses and suppression of autoimmunity, is mediated through FcμR. Nevertheless, several differences in the phenotypes of FcμR-deficient and Sμ^{-} mice were observed. Antibody production to TI antigen was reduced in FcμR^{-} mice but enhanced in Sμ^{-} mice. In addition, FcμR-deficient mice showed reduced MZ B cells in the spleen but a normal B1a population in the PC, whereas Sμ^{-} mice had increased MZ B and B1a cells. The differences in the phenotypes between the Sμ^{-} and FcμR^{-} mice could be explained by the fact that serum IgM can function through both FcμR and CR. Our results suggest that IgM antibodies promote humoral immune responses to foreign antigen yet suppress autoantibody production through at least two pathways: complement activation and FcμR. It remains to be investigated whether FcμR and CR on B cells work independently or cooperate/compete with each other to regulate distinct aspects of B-cell functions under different circumstances.

Among Fc receptors, B cells also express FcγRIIB that contains the immunoreceptor tyrosine-based inhibition motif (10, 12). This receptor can inhibit B-cell activation on binding immune complexes containing IgG and the cognate antigen, which then results in coligation of FcγRIIB and the BCR. Therefore, B cells express two types of Fc receptors of opposing functions, with FcμR positively and FcγRIIB negatively regulating B-cell activation. As long as 44 y ago, it was shown that the immune response was inhibited by 7S (now known as IgG) but enhanced by 19S (IgM) antigen-specific antibodies if they were administrated before immunization (4). Furthermore, 7S and 19S antibodies were competitive in their effect. During a typical humoral immune response, IgM is first produced and then followed by IgG. We propose that during the early phase of the response, when IgM production is dominant, B-cell activation is augmented by both FcμR- and CR-mediated positive signals. However, at a later phase of the response, when IgG production is dominant, further B-cell activation is attenuated by FcγRIIB-mediated inhibition. B cells can thus sense the presence of antigen-specific IgM and IgG in the local environment and regulate their own activation. Our findings also have significant implications in understanding the etiology of immunological disorders in which there is altered production of IgM vs. IgG antibodies. Patients with hyper-IgM syndrome are immunodeficient due to defects in the production of class-switched antibodies. Paradoxically, these patients frequently develop autoimmune diseases (39), and the underlying mechanisms remain elusive. Our results suggest that higher levels of IgM relative to IgG may predispose these patients to sustained B-cell activation that can ultimately lead to autoantibody production. Blocking the IgM/FcμR interaction with specific antibodies and/or inhibitors may provide therapeutic benefit for the treatment of autoimmunity in these patients.

Materials and Methods

Generation of FcμR-Deficient Mice. A targeting vector was designed to delete exons 2–4 of the *FcμR* gene in 129/Sv-derived R1 ES cells by homologous recombination (Fig. S2*A*). The correctly targeted cells were screened by long-range PCR, and the KO was further confirmed by Southern blot analysis. Chimeric mice were bred with C57BL/6 mice to obtain heterozygotes, which

were then backcrossed to C57BL/6 mice for >10 generations before intercrossing to obtain homozygotes. Analysis of microsatellite markers on chromosome 1 revealed that although an ~30-Mb region surrounding the targeted *Fcmr*/Faim3/Toso gene was still in the 129 background, all the remaining regions of chromosome 1, including the region containing the FcγRIIB gene known to be associated with autoantibody production, were completely replaced by B6 genome. FACS analysis confirmed the lack of FcμR expression on splenocytes of FcμR-deficient mice (Fig. S2*B*). All the animal experiments were approved by the Animal Facility Committee of RIKEN Yokohama Institute (Permission number 20-025).

Antibodies and FACS Analysis. A monoclonal rat IgG_{2a} antibody (clone 4B5) against mouse FcμR was obtained by immunizing rats with the recombinant extracellular portion of FcμR. The binding of 4B5 to mouse FcμR on B cells was not inhibited by preincubation of the cells with IgM, indicating that it does not recognize the ligand binding site. For analyzing FcμR expression in various B-cell subpopulations, cells were first incubated with a rat IgG_{2b} anti-mouse CD16/CD32 monoclonal antibody (clone 2.4G2; BD Biosciences) to block FcγR and then stained with either anti-FcμR (4B5) or an isotype control antibody (clone eBR2a; eBioscience), and further stained with phycoerythrin (PE)-conjugated anti-rat IgG_{2a} (clone RG7/1.30; BD Biosciences). After washing, the cells were further stained with FITC-, allophyocyanin (APC)-, or PE/Cy7-conjugated antibodies against various surface molecules expressed during B-cell development, maturation, activation, and differentiation as described (22, 40). Memory and GC B cells were identified essentially as described (41). Briefly, splenocytes were first incubated with anti-CD16/CD32; stained with NIP-PE, PE/Cy7–anti-mouse CD45R/B220 (clone RA3-6B2; Biolegend), APC–anti-mouse CD38 (clone 90; Biolegend), and Pacific blue anti-mouse IgG_1 (clone A85-1; BD Biosciences); and then analyzed using a Becton Dickinson FACS CANTO II flow cytometer.

B-Cell Survival and Proliferation Assay. Mature B cells were purified from the spleen using an IMAG negative sorting kit (BD Biosciences). The purity of the B cells was >95% as judged by staining with anti-B220. The cells were seeded and analyzed for proliferation by ^{3}H-thymidine incorporation as described previously (40). For the survival assay, the cells were stained with 7-amino-actinomycin D and analyzed by FACS. For the cell division assay, purified B cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and then stimulated with $F(ab')_2$–anti-IgM antibodies. CFSE intensity was analyzed 3 d later after gating on live cells.

Immunization, ELISA, Enzyme-Linked Immunospot Assay, and Detection of Autoantibodies. These experiments were performed essentially as described elsewhere (22, 42).

Immunofluorescent Staining. Mice were immunized with 10 μg of NP-CGG precipitated with alum. Two weeks later, spleens were fixed in 4% (wt/vol) paraformaldehyde in PBS for 2 h on ice with rocking followed by soaking in 30% (wt/vol) sucrose in PBS for 20 min, 2 h, 2 h, and then overnight on ice with rocking. The spleens were then embedded in optimal cutting temperature compound; cryosections were fixed with Cytofix (BD Biosciences), blocked with 2% goat serum in blocking buffer, and stained with a rat anti-human/mouse AID (clone mAID-2; eBiosciences, final concentration of 16.7 μg/mL) and hamster anti-CD3ε (clone 145-2C11; BD Biosciences, final concentration of 10 μg/mL) at 4 °C overnight. After washing, the sections were stained for 90 min with Alexa Fluor 488 anti-rat IgG and Cy3–anti-hamster IgG (Invitrogen). The specimens were mounted in VECTASHIELD mounting medium containing DAPI (Vector Laboratories) and analyzed using a BIOREVO BZ-9000 fluorescence microscope (KEYENCE) and a DM-IRE2 confocal laser-scanning microscope (Leica Microsystems). The numbers and area of GCs were analyzed by means of BZ-H1C Dynamic Cell Count software (KEYENCE).

Statistical Analysis. Statistical significance was assessed by an unpaired Student *t* test ($*P < 0.05$; $**P < 0.01$).

ACKNOWLEDGMENTS. We thank Prof. Michel Nussenzweig for providing the B1-8^{hi} mice; Drs. Hiromi Kubagawa, Yoshimasa Takahashi, Sidonia Fagarasan, Takaharu Okada, Kohei Kometani, and Akemi Sakamoto for helpful advice; the RIKEN Research Center for Allergy and Immunology Animal Facility for maintaining and breeding the mice; and the FACS Facility for cell sorting. This work was supported in part by Grant-in-Aid 22590445 for scientific research from the Japan Society for the Promotion of Science (to J.-Y.W.).

1. Fellah JS, Wiles MV, Charlemagne J, Schwager J (1992) Evolution of vertebrate IgM: Complete amino acid sequence of the constant region of *Ambystoma mexicanum* mu chain deduced from cDNA sequence. *Eur J Immunol* 22:2595–2601.
2. Ehrenstein MR, Notley CA (2010) The importance of natural IgM: Scavenger, protector and regulator. *Nat Rev Immunol* 10:778–786.
3. Murphy K, Travers P, Walport M (2008) *Janeway's Immunobiology* (Garland Science, New York), 7th Ed, pp 400–420.
4. Henry C, Jerne NK (1968) Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. *J Exp Med* 128:133–152.
5. Heyman B, Pilström L, Shulman MJ (1988) Complement activation is required for IgM-mediated enhancement of the antibody response. *J Exp Med* 167:1999–2004.
6. Ehrenstein MR, O'Keefe TL, Davies SL, Neuberger MS (1998) Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci USA* 95:10089–10093.
7. Boes M, et al. (1998) Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* 160:4776–4787.
8. Notley CA, Baker N, Ehrenstein MR (2010) Secreted IgM enhances B cell receptor signaling and promotes splenic but impairs peritoneal B cell survival. *J Immunol* 184:3386–3393.
9. Rutemark C, et al. (2011) Requirement for complement in antibody responses is not explained by the classic pathway activator IgM. *Proc Natl Acad Sci USA* 108: E934–E942.
10. Nimmerjahn F, Ravetch JV (2008) Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* 8:34–47.
11. Kaetzel CS (2005) The polymeric immunoglobulin receptor: Bridging innate and adaptive immune responses at mucosal surfaces. *Immunol Rev* 206:83–99.
12. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV (1996) Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 379:346–349.
13. Basten A, Warner NL, Mandel T (1972) A receptor for antibody on B lymphocytes. II. Immunochemical and electron microscopy characteristics. *J Exp Med* 135:627–642.
14. Moretta L, Ferrarini M, Durante ML, Mingari MC (1975) Expression of a receptor for IgM by human T cells *in vitro*. *Eur J Immunol* 5:565–569.
15. Lamon EW, Andersson B, Whitten HD, Hurst MM, Ghanta V (1976) IgM complex receptors on subpopulations of murine lymphocytes. *J Immunol* 116:1199–1203.
16. Kubagawa H, et al. (2009) Identity of the elusive IgM Fc receptor (FcmuR) in humans. *J Exp Med* 206:2779–2793.
17. Shima H, et al. (2010) Identification of TOSO/FAIM3 as an Fc receptor for IgM. *Int Immunol* 22:149–156.
18. Boes M, et al. (2000) Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proc Natl Acad Sci USA* 97:1184–1189.
19. Ehrenstein MR, Cook HT, Neuberger MS (2000) Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies. *J Exp Med* 191:1253–1258.
20. Shih TA, Roederer M, Nussenzweig MC (2002) Role of antigen receptor affinity in T cell-independent antibody responses in vivo. *Nat Immunol* 3:399–406.
21. Shih TA, Meffre E, Roederer M, Nussenzweig MC (2002) Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat Immunol* 3:570–575.
22. Ouchida R, Kurosaki T, Wang J-Y (2010) A role for lysosomal-associated protein transmembrane 5 in the negative regulation of surface B cell receptor levels and B cell activation. *J Immunol* 185:294–301.
23. Hitoshi Y, et al. (1998) Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. *Immunity* 8:461–471.
24. Nguyen X-H, et al. (2011) Toso regulates the balance between apoptotic and nonapoptotic death receptor signaling by facilitating RIP1 ubiquitination. *Blood* 118:598–608.
25. Schiemann B, et al. (2001) An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293:2111–2114.
26. Sasaki Y, Casola S, Kutok JL, Rajewsky K, Schmidt-Supprian M (2004) TNF family member B cell-activating factor (BAFF) receptor-dependent and -independent roles for BAFF in B cell physiology. *J Immunol* 173:2245–2252.
27. Shulga-Morskaya S, et al. (2004) B cell-activating factor belonging to the TNF family acts through separate receptors to support B cell survival and T cell-independent antibody formation. *J Immunol* 173:2331–2341.
28. Khan WN, et al. (1995) Defective B cell development and function in *Btk*-deficient mice. *Immunity* 3:283–299.
29. Pappu R, et al. (1999) Requirement for B cell linker protein (BLNK) in B cell development. *Science* 286:1949–1954.
30. Yamazaki T, et al. (2002) Essential immunoregulatory role for BCAP in B cell development and function. *J Exp Med* 195:535–545.
31. Shimada S, et al. (1999) Generation of polymeric immunoglobulin receptor-deficient mouse with marked reduction of secretory IgA. *J Immunol* 163:5367–5373.
32. Honda S, et al. (2009) Enhanced humoral immune responses against T-independent antigens in Fc alpha/muR-deficient mice. *Proc Natl Acad Sci USA* 106:11230–11235.
33. Li FJ, et al. (2011) Enhanced levels of both the membrane-bound and soluble forms of IgM Fc receptor (FcμR) in patients with chronic lymphocytic leukemia. *Blood* 118:4902–4909.
34. Ghia P, Scielzo C, Frenquelli M, Muzio M, Caligaris-Cappio F (2007) From normal to clonal B cells: Chronic lymphocytic leukemia (CLL) at the crossroad between neoplasia and autoimmunity. *Autoimmun Rev* 7:127–131.
35. Samardzic T, et al. (2002) Reduction of marginal zone B cells in CD22-deficient mice. *Eur J Immunol* 32:561–567.
36. Sato S, et al. (1996) CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: Altered signaling in CD22-deficient mice. *Immunity* 5:551–562.
37. Wang Y, et al. (2002) The physiologic role of CD19 cytoplasmic tyrosines. *Immunity* 17: 501–514.

38. Wang Y, Carter RH (2005) CD19 regulates B cell maturation, proliferation, and positive selection in the FDC zone of murine splenic germinal centers. *Immunity* 22:749–761.
39. Jesus AA, Duarte AJ, Oliveira JB (2008) Autoimmunity in hyper-IgM syndrome. *J Clin Immunol* 28(Suppl 1):S62–S66.
40. Ouchida R, et al. (2008) A lysosomal protein negatively regulates surface T cell antigen receptor expression by promoting CD3ζ-chain degradation. *Immunity* 29:33–43.
41. Takahashi Y, Ohta H, Takemori T (2001) Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* 14:181–192.
42. Goëb V, et al. (2007) Clinical significance of autoantibodies recognizing Sjögren's syndrome A (SSA), SSB, calpastatin and alpha-fodrin in primary Sjögren's syndrome. *Clin Exp Immunol* 148:281–287.