Altered Ig levels and antibody responses in mice deficient for the Fc receptor for IgM (FcμR)

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Cell surface Fc receptor for IgM antibody (FcμR) is the most recently identified member among FcRs. We determined the cellular distribution of mouse FcμR and the functional consequences of FcμR disruption. Surface FcμR expression was restricted to B-lineage cells, from immature B to plasma cells, except for a transient down-modulation during germinal center reactions. FcμR ablation had no significant effect on overall B- and T-cell development, but led to a reduction of marginal zone B cells and an increase in splenic B1 B cells. Preimmune serum IgM in mutant mice was significantly elevated as were natural autoantibodies. When immunized with live attenuated pneumococci, mutant mice mounted robust antibody elevation as were natural autoantibodies. When immunized with a reduction of marginal zone B cells and an increase in splenic B1 B cells. Preimmune serum IgM in mutant mice was significantly elevated as were natural autoantibodies. When immunized with live attenuated pneumococci, mutant mice mounted robust antibody responses against phosphorylcholine, but not protein, determinants.

Results

Confirmation of FcμR Ablation. We generated FcμR-deficient mice in which the FcμR gene was disrupted by replacing exons 2–4 (corresponding to a part of the signal peptide and the most extracellular region including the IgM-binding Ig-like domain) with a Neo gene. FcμR heterozygous mice were backcrossed onto a C57BL/6 background for more than eight generations, and FcμR-deficient homozygous (KO) mice were produced in the expected Mendelian ratio from heterozygous crossbreeding (FcμR+/-: 20%; FcμR-/-: 49%; FcμR−/-: 31%; Fig. S1). FcμR KO mice were indistinguishable from littermates with respect to appearance, general behavior, body and organ weights, and fertility. Ablation of the FcμR was confirmed by the absence of FcμR proteins and full-length FcμR transcripts (Fig. 1 and Fig. S2, respectively). FcμR+/− littermates were used as WT controls in this study.

FcμR Expression Within B-Cell Subsets. In our previous studies with the 4B5 rat mAb, the expression of FcμR in mice, unlike humans, was found to be restricted to B cells (14). The precise distribution of FcμR within B-cell subsets, however, was not addressed, because this mAb loses activity after conjugation with biotin or other tags. A new panel of five mAbs specific for mouse FcμR was made by immunizing FcμR KO mice with cells stably expressing mouse FcμR (Fig. S3). The immunofluorescence assessments with the use of the biotin-labeled MM3 anti-FcμR mAb showed the expression of FcμR on CD19+ B cells, but not on CD3+ T, CD11b+ macrophages, CD11b+ granulocytes (Fig. L4), and CD11c+CD11b+ dendritic cells (DCs) in spleen. These results were confirmed with other FcμR-specific mAbs from our panel. As expected, cells specifically reactive with anti-FcμR mAbs were not observed in FcμR KO mice. The restricted expression of FcμR to B cells was also confirmed in lymph nodes, blood, and peritoneal cavity. Neither splenic CD3−/−/DX5− NK/NKT cells nor intestinal intraepithelial y6+ T cells expressed FcμR on their cell surface. FcμR expression by T cells and macrophages was not induced after treatment with various stimuli including anti-CD3 (for T cells), phorbol myristate acetate (PMA), mixed lymphocyte culture supernatants, and LPS (for both T cells and macrophages). FcμR expression was not observed by freshly

natural antibody | B-cell tolerance | B-cell subset | autoimmunity

IgM is the first Ig isotype to appear during phylogeny, ontogeny, and the immune response (1). The importance of both preimmune “natural” IgM and antigen (Ag)-induced immune IgM Abs in protection against infection and autoimmune diseases have been established through studies of mutant mice deficient in IgM secretion (2, 3). Naïve B cells in these mice express membrane-bound IgM and, following Ag challenge, can undergo Ig isotype switching to other Ig isotypes that can be secreted. However, these animals are unable to control viral, bacterial, and fungal infection due to lack of serum IgM and an unexpected inefficient induction of a protective IgG CLASS AB response (4–6). Autoimmune pathology associated with IgG autoantibodies is exacerbated in these animals are unable to control viral, bacterial, and fungal infection due to lack of serum IgM and an unexpected inefficient induction of a protective IgG CLASS AB response (4–6). Autoimmune pathology associated with IgG autoantibodies is exacerbated in these mice. FcμR KO mice, with or without FcμR expression by T cells


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prepared, marrow CD11b⁺ myeloid cells (Fig. 1E) or by macrophage colony-stimulating factor (M-CSF)-induced bone marrow macrophages. The lack of FcγR expression by non-B-cell populations was also confirmed by RT-PCR analysis (Fig. S2).

There was a hierarchy of mean fluorescence intensity (MFI) of surface FcγR expression by each B-cell subset in spleen: CD21⁺/CD23⁺ follicular (FO) > CD21⁺/CD23⁻ marginal zone (MZ) > CD21⁻/CD23⁺ newly formed (NF) B cells (Fig. 1B). The FcγR MFI was indistinguishable between CD5⁺ B1 and CD5⁻ B2 cells. In the peritoneal cavity, the FcγR MFI in each B220⁺ B-cell subpopulation was, in the order, CD11b⁺/CD23⁺ B2 > CD5⁺ B1 > CD11b⁻/CD23⁺ B1b cells (Fig. 1C). In contrast, the MFI of FcγR on GL7⁺ germinal center (GC) B cells was much lower than that on GL7⁻ B cells, suggesting that the FcγR is down-modulated during GC reactions. FcγR was expressed not only by IgM⁺, but also by IgG⁺ or IgA⁺ B cells, suggesting that the receptor expression is not directly linked with IgM production and that it is maintained on the switched memory B cells. In bone marrow, FcγR was undetectable in the CD19⁺/surface (s) IgM⁺/IgD⁺ pro-B/pre-B-cell compartment, was low on CD19⁺/IgM⁺/IgD⁺ immature B cells, and higher on CD19⁺/IgM⁺/IgD⁺ mature or recirculating B cells, indicating that FcγR expression begins at the immature B-cell stage of differentiation (Fig. 1D). The majority of CD138⁺ plasma cells in spleen and lymph nodes expressed FcγR, whereas only a fraction of the CD138⁺ cells, which express high levels of CD19 or B220, in bone marrow were positive (Fig. 1F and Fig. S4), suggesting that FcγR is expressed by plasmablasts rather than plasma cells. Collectively, these findings clearly demonstrate that the expression of FcγR in mice is restricted to B-lineage cells, beginning at the early immature B-cell stage in bone marrow and continuing through to the terminally differentiated plasma cell stage of differentiation, accompanied by down-modulation of FcγR during the GC reaction.

Alteration of B-Cell Subpopulations in FcμR-Deficient Mice. To examine whether FcμR deficiency leads to alterations in the development of B and T cells, each cell compartment of mutant or WT control mice of the same age and sex was evaluated. The total number of splenic T and B cells was indistinguishable in both groups of mice (Dataset S1). However, the number of CD23⁻/CD21⁺ or CD1d⁺/CD5⁺ MZ [or regulatory (15)] B cells, which constitute 5–8% of the splenic B cells in WT mice, was reduced by fourfold in the mutant mice (P < 0.01; Fig. 2A and Dataset S1). Splenic B1 cells were increased by approximately twofold in mutant mice (P < 0.01). In the peritoneal cavity, the total numbers of B1a, B1b, and B2 cells were comparable in both groups of mice, but intriguingly, the number of T cells was increased by approximately twofold in mutant mice compared with WT controls (P < 0.01; Dataset S1). The numbers of pro-B/pre-B, immature B, and recirculating B cells as well as of myeloid cells in bone marrow were identical in both groups of mice. In addition to the changes in cell numbers, there were some differences in the density of certain cell surface markers between mutant and WT controls. The CD19 levels on splenic B cells were indistinguishable, but interestingly, the number of T cells was increased by approximately twofold in mutant mice compared with WT controls (P < 0.01). The CD19 and CD5 levels on spleen were identical in both groups of mice. In addition to the changes in cell numbers, there were some differences in the density of certain cell surface markers between mutant and WT controls. The CD19 levels on splenic B cells were indistinguishable, but in bone marrow the CD19 and CD5 levels on peritoneal B1a cells were lower in mutant mice than in WT controls (Fig. 2B). A similar trend was also observed with the CD11b and CD5 on peritoneal B1a cells. The surface density of IgM or IgD on splenic B cells was comparable in both groups of mice. Collectively, these findings suggest that FcμR ablation does not significantly affect overall B- and T-cell development, but alters numbers of B-cell subpopulations, accompanied by a change in the density of surface expression of certain markers on B cells.

Elevated Serum IgM Levels in Naïve FcμR-Deficient Mice. To determine whether FcμR deficiency affects preimmune Ig isotype levels, sera from age-matched, naïve mutant, and WT control mice were examined by ELISA. Both IgM and IgG3 levels were twofold higher in mutant mice than WT controls: 857 ± 298 µg/mL vs. 431 ± 297 µg/mL for IgM (mean ± 1 SD; P < 10⁻⁵) and 1434 ±
To determine whether Fcm mab or coligation of BCR and Fcm mediated regulation of IgM production at the B- or plasma-cell stage.

### Elevated Natural Antibodies in Fcmr-Deficient Mice

Ca2+ mobilization upon either ligation of B-cell Ag receptor (BCR) with anti-μ mAb or coligation of BCR and FcμR in vitro was comparable between WT and Fcmr-deficient B cells (Fig. S6). We next determined whether the elevated serum levels of IgM and IgG3 in naive Fcmr KO mice are accompanied by an increase in natural Abs. Immunofluorescence analysis for the presence of antinuclear and cytoplasmic autoantibodies with the use of HEp-2 cells showed that naive mutant mouse sera displayed various staining patterns including homogeneous or speckled nuclear and cytoplasmic staining (Fig. 4A). Most (4/5) mutant sera contained IgM and IgG2c Abs reactive with either the nucleus or cytoplasm of HEp-2 cells even at a 32-fold dilution or more, whereas none of WT sera displayed any reactivity at a 1:16 dilution. All mutant mouse sera also contained variable titers of IgG3 Abs with similar staining patterns as IgM and IgG2c Abs. To further assess the natural Abs, we determined the levels of autoantibodies to dsDNA and chromatin in naive mice of 10–15 wk by ELISA. Both IgM and IgG anti-dsDNA and antichromatin Abs were significantly elevated in Fcmr KO mice (Fig. 4B). This increase was observed with IgG2c Abs to both autoantigens and IgG3 Abs to dsDNA. These findings suggest that FcμR deficiency leads to an elevation of IgM and IgG natural Abs.

### Altered Antibody Responses in FcμR-Deficient Mice

To determine whether FcμR deficiency affects humoral immune responses, both groups of age- and sex-matched mice were immunized i.p. with a wide dose range (10^5–10^10 cfu) of a live nonencapsulated (avirulent) strain of *Streptococcus pneumoniae* (R36A), and 4 wk later were boosted i.p. with 10^2 live R36A. Serum Abs against the following three different Ags were assessed weekly by ELISA for 8 wk: as a T-cell independent type 2 (TI-2) Ag, phosphorylcholine (PC), and as T-cell–dependent (TD) Ags, R36A-associated crude proteins and recombinant PspA proteins (17). As shown in Fig. 5A, IgM and IgG3 PC-specific responses were comparable within both groups of mice when immunized with 10^2 or more bacteria. By contrast, at a suboptimal dose of R36A (10^5 cfu) both IgM and IgG3 PC-specific Abs were markedly elevated in mutant mice. Notably, a large fraction of anti-PC Abs in both groups of mice was positive for the T15 idiotype, suggesting that typical PC responses were being induced. Prolonged and significantly higher levels of IgM anti-PC were observed in mutant mice immunized even with 10^3 cfu. In contrast to PC responses, IgM and IgG responses against R36A-associated protein determinants were generally indistinguishable between mutant and WT control mice, except that a slight increase of IgM Abs was observed in mutant mice at suboptimal Ag doses (10^2 and 10^3 cfu; Fig. 5B). IgG1, IgG2b, and IgG2c Abs developed only in mice receiving higher Ag doses (≥10^3 cfu) and the booster injection with 10^2 cfu did not induce a recall response to protein determinants in either group of mice. Unlike the IgG3 anti-PC response, protein-specific IgG3 Abs were also comparable in both groups of mice at all four different Ag doses. IgM and IgG Ab responses to PspA were essentially the same as those to R36A crude proteins (Fig. S7). Thus, these results demonstrate that after immunization with live attenuated bacteria, Fcmr ablation affects PC-specific Ab responses more profoundly than protein-specific ones, suggesting a preferential regulatory role of FcμR on B and/or plasma cells in TI type 2 immune responses.

Next, to determine whether FcμR ablation influences the affinity maturation of Abs, we used the well-characterized hapten-carrier conjugate system, nitrophenyl-coupled chicken y-globulin (NP-CCG), as another TD Ag. Mice were immunized i.p. with...
three different doses (100, 10, and 1 μg) of NP23-CGG precipitated in alum, and 6 wk later the immunized animals were boosted i.p. with the same dose of NP23-CGG in PBS as the primary injection. Notably, preimmune or natural IgM anti-NP Ab was significantly higher in mutant mice than WT controls (Fig. 6A). Primary IgM anti-NP responses to all three different Ag doses were mostly comparable in both groups of mice, but the recall IgM responses were impaired in mutant mice receiving the highest Ag dose. In contrast to IgM responses, primary IgG1 anti-NP responses were significantly impaired in mutant mice for all three different Ag doses, but secondary IgG1 anti-NP responses were indistinguishable from WT controls. Essentially similar results were obtained with Ab responses to the carrier protein CGG: namely, impairment of both primary IgG1 and secondary IgM anti-CGG responses (Fig. 6B). For Ab affinity assessment, we first quantified serum Abs reactive with NIP25-BSA and NIPS-BSA at three different time points during immunization and the ratio of anti-NIP25 Ab to anti-NIPS Ab was used as a relative affinity maturation index. As shown in Fig. 6C, there were no significant differences in the affinity indices of anti-NP Abs between mutant and WT mice, suggesting that Fcmr disruption did not influence Ab affinity maturation. Thus, these findings demonstrated that: (i) upon immunization with NP-CGG, Fcmr KO mice had diminished primary IgG1 and secondary IgM responses to both NP and CGG compared with WT control mice, suggesting different outcomes of Fcmr ablation in TD immune responses depend on the form of the antigen, isolated (NP-CGG) versus particulate, bacteria-associated proteins (R36A proteins/PspA), and (ii) Fcmr ablation does not influence antibody affinity maturation.

Figure 4. Natural Abs in naïve mice. (A) Representative nuclear and cytoplasmic staining patterns of Hep2 cells. Indirect immunofluorescent results are depicted of three different sera from each group of naïve animals, WT (upper) and Fcmr KO (lower), developed with FITC-goat Abs specific for IgM, IgG3, or IgG2c. The serum dilution was 32-fold for IgM and IgG2c and 64-fold for IgG3. Note various nuclear and cytoplasmic staining patterns with the Fcmr KO sera. (B) Titers of Abs against dsDNA or chromatin in preimmune sera. Levels of natural Abs reactive with dsDNA or chromatin in Fcmr KO (●) and WT (○) mice were assessed by ELISA.

Discussion
The goal of these studies was to define the precise cellular distribution of mouse FcμR using new receptor-specific mAbs and to explore its in vivo function by examining the phenotype of FcμR-deficient mice. Unlike in humans, the FcμR in mice was only expressed by B-lineage cells, beginning at the immature B-cell stage in bone marrow and continuing through to the terminally differentiated plasma-cell stage. The surface receptor expression was highest on FDC B cells and was transiently down-modulated during the GC reactions, before returning to starting levels on memory B and plasma cells. FcμR ablation (i) did not grossly affect overall B- and T-cell development, but resulted in a fourfold reduction of MZ B cells and a twofold increase in splenic B cells; (ii) led to an approximately twofold increase in the concentration of IgM in preimmune sera accompanied by an increase in natural autoantibodies; (iii) resulted in robust and long-lasting anti-PC Ab responses, especially when suboptimal doses of live avirulent pneumococci were injected i.p.; and (iv) caused impaired TD Ab responses, especially primary IgG1 responses, when immunized with a non–bacteria-associated form of TD Ag.

The finding of elevated serum IgM in naïve Fcmr KO mice is remarkable. Because serum IgM was not affected in mice with null mutations of other IgM-binding receptors, plgR on mucosal epithelial cells or FceRI on follicular dendritic cells (18, 19), the FcμR appears to be the sole receptor in this family that may be involved in maintenance of serum IgM levels within the physiological range. Because the half-life of the injected IgM is the same in Fcmr KO and WT control mice, FcμR does not appear to be involved in IgM catabolism likely mediated by LSECs, but rather is involved in the production and/or secretion of IgM by B and/or plasma cells. In humans, chronic lymphocytic leukemia (CLL) B cells overexpress cell surface FcμR and rapidly ingest IgM ligands through this receptor (20, 21). Curiously, as many CLL patients have reduced serum Ig levels including IgM, it would be worthwhile to determine whether this reduction is related to the enhanced expression of FcμR on the leukemic B cells. The concomitant increase in serum IgG3 levels in naïve Fcmr KO mice may suggest the possibility of FcμR-mediated regulation in IgM production and/or secretion by B or plasma cells, probably down-regulating more selectively TI Ab responses, as discussed below. Notably, serum IgM levels in mice raised under germ-free conditions are similar to those in mice held under conventional or specific pathogen-free housing conditions (22, 23). The preimmune natural IgM might be the consequence of exposure to self-Ags associated with cell corpses and B1 cells are a major source of natural IgM Abs (24), consistent with our findings that the Fcmr KO mice have a twofold increase over WT controls of B1 cells in spleen and elevated levels of natural autoantibodies to nuclear or cytoplasmic components. It will be important to determine whether this increase facilitates autoimmune processes in such mutant mice. The inhibitory FcyR, FcyRIIB, is expressed on long-lived plasma cells in bone marrow and its cross-linking induces their apoptosis, thereby controlling their homeostasis (25). Because FcμR is expressed by CD19hi or B220hi plasmablasts, the functional role of FcμR in these marrow Ab-producing cells remains to be elucidated.

Another remarkable finding is an enhanced PC-specific, but not protein-specific, Ab response in Fcmr KO mice upon i.p. administration of live R36A. PC is an immunodominant epitope on the pneumococcal cell wall polysaccharide (26, 27), and it is generally considered as a TI-2 Ag, although a distinct type of T-cell help, different from that in classical TD Ab responses, has been proposed to influence this type of response (28). The molecular basis for the selective enhancement of the anti-PC response in the Fcmr KO mice remains to be elucidated; however, several possibilities are worth consideration. First, given such a persistent Ab response, PC-containing polysaccharides may be retained and stimulate B1 or MZ B cells for a longer period in mutant mice than in WT controls. Upon i.p. injection of R36A, phagocytes in the peritoneal cavity of both groups of mice must ingest bacteria after opsonization at an equivalent level, because FcμR is not
mAbs and mouse antisera against R36A were used as Ig standards. Data are plotted as mean ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively.

Fig. 5. IgM and IgG3 Ab responses to phosphorylcholine (PC) in Fcmr KO and WT mice. Age- and sex-matched KO and WT mice (n = 5, each group) were injected i.p. with a wide range (10^2–10^6 cfu) of live R36A, avirulent strain of S. pneumoniae, and boosted with 10^2 cfu of live R36A 4 wk later indicated by an arrow. Levels of PC-specific IgM and IgG3 Abs (A) and protein-specific Abs of the indicated Ig isotypes (B) were quantified weekly by ELISA in triplicate using plates precoated with PC-BSA or R36A-derived crude proteins and AP-labeled goat Abs specific for each Ig isotype. PC-specific IgM (B8) and IgG3 (HPCG11) mAbs and mouse antisera against R36A were used as Ig standards. Data are plotted as mean ± 5E. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively.

expressed by these cells. Among the phagocytes, dendritic cells (DCs) are known to be crucial for priming TI-2 immune responses by interacting locally with peritoneal B1 cells or by migrating to and interacting with splenic MZ B cells (29). As naturally occurring anti-PC Abs are present in both humans and mice (30, 31), IgM-opsonized, PC-containing particulates, probably in the context of membrane vesicles or exosomes, could be cleared by the B-cell FcR in WT control mice, but not in mutant mice. Second, because the transmembrane activating calcium modulator and cytophilin ligand interactor (TACI) receptor on mature B cells is essential for TI-2 immune responses (32, 33), FcR might negatively regulate TACI-mediated signal transduction, because it was recently shown that TACI could activate NFκB in a toll-like receptor (TLR)-like MyD88/IRAK4 pathway (34). Third, FcR might negatively regulate a TI-2 B-cell memory response. Unlike TD immune responses to protein Ags, TI-2 responses to bacteria-associated polysaccharides confer long-lasting humoral immunity without recall responses. However, B cells from mice immunized with TI-2 Ags can respond to secondary challenge when adoptively transferred into naïve irradiated recipients. Notably, Ag-specific IgM or IgG3 Abs were shown to inhibit this TI-2 memory response (35–37), and thus, it is possible that FcR may control the TI-2 B-cell memory. It is also worth noting that similar selective enhancement of TI-2 immune responses was also observed in mice with null mutations of components of the BCR complex such as CD19 (38), CD81 (39), and the secretory exon of IgM (3).

Fig. 6. IgM and IgG1 Ab responses to NP-CGG in Fcmr KO and WT mice. Fcmr KO (solid circles) and WT control (open circles) mice of the same age and sex (n = 5, each group) were immunized i.p. with the indicated doses of NP23-CGG in alum and boosted with the same dose of NP-CGG in PBS 6 wk later (arrows). NP-specific IgM and IgG1 Ab titers were measured weekly by ELISA in triplicate using plates precoated with NP25-BSA (A) or CGG (B). The affinity measurement of anti-NP Abs (C) was assessed by ELISA using plates precoated with NP25-BSA or NIP5-BSA. NP-specific IgM (B1-8), low-affinity IgG1 (N1G9), and high-affinity IgG1 (H33L) mAbs were used as standards. *P < 0.05 and **P < 0.01, respectively. Note diminished primary IgG1 responses to NP and CGG in FcR-deficient mice.
On the other hand, when immunized with NP-CCG precipitated in alum, FcμR KO mice had impaired primary IgG1 and secondary IgM NP-specific responses, even though they had elevated IgM natural anti-NP Abs compared with WT controls. Notably, mice deficient in IgM secretion also displayed a reduced primary IgG1 anti-NP Ab response, which could be corrected by administration of pooled serum IgM (3). Thus, FcμR-mediated signals might be crucial for potentiating the initial IgG1 anti-NP Ab responses. It has also been shown that there are fundamental differences in immune responses to an isolated protein or polysaccharide Ag versus an intact extracellular bacterium (28, 40). Intact bacteria are complex particulate immunogens on which multiple proteins and polysaccharide Ags and TLR ligands (17–20) are coexpressed. TLRs are well known to enhance immune responses, although the role of TLRs in B-cell activation and Ab production is not fully understood with conflicting results (41–43). The differences in TD Ab responses to R36A proteins versus NP-CCG in mutant mice might be due to the different antigenic forms, bacteria associated versus isolated.

In this study, we show that FcμR is expressed only by B-lineage cells in mice. The null mutation of Fcmr leads to elevation in preimmune serum IgM levels accompanied by an increase in natural autoantibodies and to altered humoral immune responses characterized by robust and long-lasting PC-specific, but not protein-specific, Ab responses after i.p. immunization with live avirulent S. pneumoniae. This mutant strain of mice would thus be valuable for investigating IgM-mediated immune protection and regulation of immune responses. Although FCMR deficiency has not yet been identified in humans, it seems likely that the phenotype will be much more complex and profound than that of the FcμR deficiency described here, because the human FcμR is expressed by additional cell types, namely T and NK cells (12). However, the results from the present studies provide some hints regarding the potential phenotype of FCMR deficiency.

Materials and Methods

Materials and Methods provides complete experimental methods. These include genotyping of offspring from breeding FcμR−/− mice, production of anti-FcμR mAbs, and other mAbs and reagents, flow cytometric analysis, immunization of Abs, ELISA, detection of natural Abs, IgM catabolism, and calcium mobilization analysis. Statistical analysis of results is also included.

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Supporting Information

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Materials and Methods

Mice. Breeding pairs of Fc\textsuperscript{mr}+/− heterozygous mice, which were generated after crossing of Fc\textsuperscript{mr}−/− homozygous mice to C57BL/6 mice for eight generations, were obtained from one of the authors (H.O.) and were bred and housed at University of Alabama at Birmingham. The Fc\textsuperscript{mr} genotype of the offspring from breeding Fc\textsuperscript{mr}+/− mice was determined by genomic PCR using tail DNA and diagnostic sets of primers: 5′-cagaggctgcatgccttgcaggg-3′ (forward), 5′-cagagctcatgcatgtatgtggtggtgg-3′ (reverse), and 5′-ctctctctcccatagtgtgggccattggggc-3′ (reverse) corresponding to the 3′-flanking and 3′-flanking Fc\textsuperscript{mr} exons 2 and 5, respectively. Littermate Fc\textsuperscript{mr}−/− mice were used as WT controls. All studies involving animals were conducted in accordance with and after approval of the University of Alabama at Birmingham institutional animal care and use committee.

Monoclonal Anti-Fc\text{R} \text{\gamma} Antibodies. Fc\text{R} \text{\gamma} KO mice were hyperimmunized with the BW5147 thymoma cell stably expressing mouse Fc\text{R} \text{\gamma} and the regional lymph node cells were fused with the Ag8.653 plasmacytoma line (1). Culture supernatants from the resultant hybridomas were screened by flow cytometry using an equal mixture of BW5147 cells stably expressing both mouse Fc\text{R} \text{\gamma} and GFP and control BW5147 cells and a mixture of PE-labeled goat Abs specific for each mouse IgG subclass (SouthernBiotech) as described (1). After subcloning by limiting dilutions, five different mAbs with specificity for mouse Fc\text{R} \text{\gamma} were thus obtained: MM1 and MM3 (\gamma\text{1c}), MM2 (\gamma\text{2b}), MM4 (\gamma\text{2c}), and MM6 (\gamma\text{2c}).

Flow Cytometry. A single-cell suspension was prepared from spleen, lymph nodes, peritoneal cavity, and long bones as described (2). Cells were first incubated with AB93 anti-Fc\text{R} \text{II}/III \text{I} (3) and 9E9 anti-Fc\text{R}IV mAbs (4) and with 100 μg/ml mouse IgG2b as blocking reagent. The cells were then incubated with the indicated mAbs and with an FITC-conjugated mouse IgG2b isotype control and with PE-labeled goat anti-mouse IgG antibodies. The stained cells were analyzed with a FACSCalibur (BD Biosciences). The stained slides were examined with an Optronics DE-1470 CCD video camera (Optronics) under a Leica/Leitz DMRB microscope (14). Images were acquired from naïve mice, before developing with FITC-labeled goat anti-mouse IgG antibodies. Controls included isotype-matched irrelevant mAbs labeled with appropriate fluorochromes. The detailed method will be published elsewhere.

Antigens, Immunization, and Serum Collection. For bacterial Ags, a single colony of the nonencapsulated strain of Streptococcus pneumoniae (R36A) was grown in Todd-Hewitt medium containing 0.5% yeast extract and the log phase bacteria with an optical density at 600 nm (OD\text{600}) of ≤0.6 were harvested by centrifugation, suspended in 10% (vol/vol) glycerol in Ringer’s lactate at 10\textsuperscript{6} cfu/mL as determined by OD\text{600} of 1 = 8 × 10\textsuperscript{6} cfu/mL, and stored at –80°C as frozen stocks. For immunization, the bacteria death rate and sterility of frozen stocks were reevaluated by thawing a vial and plating onto blood agar plates. A wide dose range (10\textsuperscript{5}, 10\textsuperscript{6}, and 10\textsuperscript{7} cfu) of live R36A in 100 μL of PBS was injected i.p. into five mice per each dose, followed 4 wk later by another i.p. injection with 10\textsuperscript{6} cfu of live R36A in 100 μL of PBS. For the hapten-carrier conjugate Ag, three different doses (100, 10, and 1 μg) of nitrophenyl 23-coupled chicken γ-globulin (NP23-CGG) (Biosearch Technologies) precipitated in alum were injected i.p. into five mice per each dose, followed 6 wk later by a second i.p. injection of the same dose of NP23-CGG in PBS as the primary one. Sera were collected weekly from retroorbital plexus and were stored at –80°C until assessment by ELISA.

ELISA. For determination of the concentrations of the major Ig isotypes (μ, γ\text{1}, γ\text{2b}, γ\text{2c}, γ\text{3}, and α) in preimmune sera, serial dilutions of sera from naïve mice were added in triplicate into 96-well half-area polystyrene plates (Corning) precoated with polyvalent goat Ab against mouse Ig heavy and light chains (SBA). The bound Igs were detected by addition of alkaline phosphate (AP)-labeled goat Abs specific for each mouse Ig isotype (SBA), followed by the substrate p-nitrophenyl phosphate. The enzyme reaction was measured at 405 nm with an Epoch spectrometer microplate. The results were analyzed by microplate data collection and analysis software (Gen5 version 1.11.4, BioTek). The purified, mouse mAbs with irrelevant specificity were used as an Ig isotype standard: IgM antichicken class II (CIA) (5), IgG1 antihuman Fc\text{R} \text{\mu} (AM9.9), IgG2b anti-human Fc\text{R} \text{\mu} (AM9.4) (6), IgG2c anti-TdT (6.3) (SBA); and IgG3 anti-human Fc\text{R} (HM2.4) mAbs. The assay of natural Abs was performed as described (7). For assessment of serum Abs against PspA (8), R36A crude proteins prepared from freeze-thaw extracts by salt fractionation, phosphorylcholine (PC) or nitrophenyl (NP) in immunized animals, serial dilutions of immune sera were added in triplicate into 96-well half-area plates precoated with PspA (a generous gift from Susan Hollingshead, Department of Microbiology, University of Alabama at Birmingham) (8), R36A crude proteins, PC-BSA, or NP25-BSA (Biosearch Technologies). Pooled antisera from C57BL/6 WT mice hyperimmunized s.c. with R36A plus Freund’s adjuvants served as a standard antiserum to determine the arbitrary units (AU) of Abs to PspA or R36A crude protein determinants as follows. At a given optical density value in the linear range, the reciprocal dilutions of the serum samples were divided by the reciprocal dilution of the standard antiserum. Affinity-purified, PC-specific mAbs [BSH (μκ) and HPCG11 (γ\text{3c})] (9, 10) and NP/NIP-specific mAbs [B1-8 (μκ), N1G9 (γ\text{1b}), and H33L (γ\text{1b})] (11–13) were used as standard mAbs. For affinity measurement of anti-NP Abs, serial dilutions of immune sera and H33L anti-NP/NIP mAb as a standard were added in triplicate into the NIP25-BSA– or NIP5-BSA–coated plates and Abs reactive with NIP25 or NIP5 were quantified and the ratio of anti-NIP25 Abs to anti-NIP5 Abs was defined as the affinity maturation index.

Detection of Natural Antibodies. To determine the serum titers of autoantibodies, HEp-2 cell smears (BioRad) were incubated with 20 μL of twofold serial dilutions (1:4–1:128) of sera collected from naïve mice, before developing with FITC-labeled goat Abs specific for mouse IgM or IgG3. The stained slides were examined under a Leica/Leitz DMRB microscope (14). Images were acquired with an Optronics DE-1470 CCD video camera (Optronics) and processed with IP LAB Spectrum software (Signal Analytics).
IgM Catabolism. To determine the half-life of IgM Abs, Fcmr KO and WT control C57BL/6 mice of the same age and sex (n = 5) were injected i.v. with 200 μg of highly purified, BALB/c-derived IgM. After injection, blood samples were collected at 5 min and then daily from the retroorbital plexus, before assessment of the concentration of the injected IgM by ELISA using a mAb (RS3-1 clone) specific for an IgM* allotype as described (15). The concentration of IgM* in sera at 5 min after injection was defined as 100% of IgM* injected.

Calcium Mobilization Analysis. Intracellular Ca2+ concentration was measured after cross-linkage of B-cell Ag receptor (BCR) and/or FcγR on splenic B cells as described (16). Briefly, B cells enriched from splenocytes of Fcmr KO and WT control mice by depleting CD43+ cells by MACS were loaded with Fluo 4-AM (0.25 μM; Molecular Probes), rested, and then treated with F(ab')2; fragments of rat anti-mouse μ mAb (B76 clone; 3–30 μg/mL) (17) or with a mixture of biotin-B76 antismouse μ (5–20 μg/mL) and biotin-MM3 antismouse FcγR (5–20 μg/mL) mAbs (plus SA). Changes in Fluo4 fluorescence in the labeled cells following treatment were measured over real-time with FACS Calibur flow cytometer and analyzed by CellQuest software (BD Biosciences).

Statistical Analysis. All data comparisons were performed using Student’s t test, and a P value of <0.05 was defined as statistically significant.

Fig. S1. Genotyping of offspring from FcmrKO/C57BL/6 mice. Tail DNA from the offspring breeding from FcmrKO/C57BL/6 mice was subjected to genomic PCR using diagnostic sets of Fcmr primers. HindIII-digested λ DNA was used as a size marker (kb). Note that the normal allele (WT) gives rise to an ~0.57-kb product and the targeted allele (KO) is ~0.73 kb.
Fig. S2. Lack of full-length FcμR transcripts in Fcmr-deficient mice. Total RNAs isolated from liver and spleen tissues from Rag1−/− and CD19+ B and CD19− non-B cells enriched by FACS from Fcmr+/+ or Fcmr−/− mice were converted to first-strand cDNA before PCR amplification using a set of primers corresponding to the translation start and termination sites of the FcμR cDNA (forward, 5′-cagggaaccatggacttt-3′ and reverse, 5′-tcattggcatgaagatctg-3′). Amplification of paired Ig-like receptors of activation isoform with a set of primers (forward, 5′-cctgtggagctcacagtctcag-3′ and reverse, 5′-cccagtgtagaacattgaagatg-3′) was performed as a control. Amplified products were electrophoresed in 0.7% agarose and stained with ethidium bromide. Lane cont. is a PCR control without a first-strand cDNA template. HindIII-digested λ DNA was used as a size marker. Note lack of full-length FcμR transcripts in Fcmr KO splenic B and non-B cells, Fcmr WT splenic non-B cells, and Rag1 KO liver and splenic tissues. Experiments were performed three times.

Fig. S3. Generation of anti-FcμR mAbs. (A) Mixture of control BW5147 thymoma cells and BW5147 cells stably expressing mouse FcμR and green fluorescent protein (GFP) was incubated with five different mouse mAbs specific for mouse FcμR [MM1 and MM3 (γ1κ), MM2 (γ2bκ), MM6 (γ2cκ), and MM4 (γ3κ)], the corresponding isotype-matched control mAbs, or an IgMκ myeloma protein (TEPC183), before developing with PE-labeled goat antimouse Ig Ab. Note that (i) anti-FcμR mAbs recognize FcμR-bearing cells but not control cells, (ii) the mAb reactivity and the GFP intensity (as an indicator of FcμR transgene expression) are well correlated, and (iii) the mAb reactivity is more sensitive in the detection of FcμR than IgM ligand binding. (B and C) SDS/PAGE analysis of cell surface FcμR. Plasma membrane proteins on control or mouse FcμR cDNA-transduced BW5147 cells (B) and the IgG1κ-bearing A20 B-cell line (C) were labeled with biotin, quenched, and incubated with IgG purified from pooled normal mouse sera (IgG), FcμR-specific mAbs (MM1, MM2, MM3, or MM6), mouse γ1κ control mAb (Cont.), or TEPC183 myeloma IgMκ, before washing and solubilization in 1% Nonidet P-40 lysis buffer containing protease inhibitors. The mAb- or ligand-bound plasma membrane proteins were captured by addition of rat antimouse κ mAb-coupled beads as described (1). Bound materials were resolved on SDS-10% PAGE under nonreducing and reducing conditions, followed by transfer onto membranes, blotting with HRP-SA, and visualization by ECL. The same results were obtained with other anti-FcμR mAbs. Arrow indicates the ~60 kDa FcμR protein, which is specifically precipitated from lysates of surface biotinylated FcμR transductants and the A-20 B-cell line with FcμR-specific mAbs and IgM ligand. Results are representative of three different experiments.
Fig. S4. FcμR expression by bone marrow plasma cells. Marrow nucleated cells were stained sequentially with biotin-MM3 anti-FcμR or isotype-matched control mAb (and SA-PE), FITC anti-B220 mAb, and APC anti-CD138 or isotype-matched control mAb, and CD138+ cells were examined for their expression of FcμR and B220.

Fig. S5. Half-life of the injected IgM in FcμR-deficient and WT control mice. The serum concentrations of injected IgM in Fcμr KO (closed circles) or WT control (open circles) C57BL/6 mice (n = 5) were assessed by ELISA using an IgM allotype-specific mAb and are expressed as the percentage of the concentration at 5 min after injection. Results are shown as mean ± SD.

Fig. S6. Ca²⁺ mobilization after BCR cross-linkage. Fluo4-loaded B cells from spleen of Fcμr KO (Right) and WT control (Left) mice were treated with F(ab')₂ fragments of rat anti-μ mAb (B76 clone; Upper) at the indicated protein concentrations or with a mixture of biotin-labeled mAbs: B76 anti-μ and MM3 anti-FcμR mAbs (red lines), B76 anti-μ and mouse γ1κ control mAbs (green lines), or rat γ1κ control and MM3 anti-FcμR mAbs (blue lines) at the protein concentration of 30 μg/mL each along with SA as a cross-linker (20 μg/mL; Lower). X and y axes indicate time (in seconds) and intracellular Ca²⁺ levels (fluorescence intensity).
Fig. S7. Antibody responses to a PspA recombinant protein in FcμR-deficient and WT control mice. Sera collected from Fcμr KO (closed circles) or WT control (open circles) mice at the indicated weeks after immunization with variable doses (10^8–10^9 cfus) of live R36A bacteria were assessed for titers of IgM and IgG Abs specific for PspA by ELISA as described in the legend of Fig. 5. Each circle represents data from an individual mouse. *P < 0.05. Note that the titers of anti-PspA Abs of all isotypes are indistinguishable between mutant and WT mice.

Dataset S1. Number of lymphocytes in each subset of FcμR-deficient and WT control mice

Dataset S1