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 *Fcmr* 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. *Fcmr* 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 responses against phosphorylcholine, but not protein, determinants compared with wild-type mice. By contrast, upon immunization with a hapten-carrier conjugate, nitrophenyl-coupled chicken γ-globulin (NP-CGG), the mutant mice had a diminished primary IgG1 response to both NP and CGG. These findings suggest that FcμR has an important role in IgM homeostasis and regulation of humoral immune responses.

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 infections due to lack of serum IgM and an unexpected inefficient induction of a protective IgG Ab response (4–6). Autoimmune pathology associated with IgG autoantibodies is exacerbated in these mutant mice, possibly because of impaired clearance of autoantigen-expressing apoptotic cells (7, 8). Secreted IgM can thus profoundly influence immune responses to pathogens and to self-antigens. The activity of effector proteins that interact with IgM, such as complement, complement receptors, and IgM-binding agglutinins, has failed to fully account for the immune protection and regulation of immune responses mediated by IgM (9, 10). Particularly, the role of the Fc receptor for IgM (FcμR), which is likely a key player in these IgM-mediated effector functions, is completely unknown. Although FcRs for switched Ig isotypes have been extensively characterized at both protein and genetic levels (11), an FcμR has defied identification until our recent functional cloning of the *FCMR* gene (12).

FcμR is a transmembrane sialoglycoprotein of ~60 kDa that contains an extracellular Ig-like domain homologous to two other IgM-binding receptors, the polymeric Ig receptor (pIgR) and the FcR for IgM and polymeric IgA (Fcα/μR). However, unlike these receptors, FcμR exhibits an exclusive binding specificity for the Fc region of IgM (12). Distinct from other FcRs, the major cell types constitutively expressing FcμR in humans are the adaptive immune cells, B and T lymphocytes. natural killer (NK) cells, which are now considered to have features of both adaptive and innate cells (13), also express FcμR, albeit at very low levels, and are the only known example of FcμR expression by cells other than B and T cells (12). In contrast to human FcμR, our initial immunofluorescence analysis of mouse FcμR with a receptor-specific mAb (4B5) revealed that FcμR was expressed by B cells, but not by T cells or NK cells (12, 14). In the present studies we have conducted a comprehensive cellular analysis of FcμR expression in mice with new receptor-specific mAbs and have explored the in vivo function of the receptor by determining the consequences of an *Fcmr* null mutation.

Results

Confirmation of *Fcmr* Ablation. We generated FcμR-deficient mice in which the *Fcmr* 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. *Fcmr* heterozygous mice were backcrossed onto a C57BL/6 background for more than eight generations, and *Fcmr*-deficient homozygous (KO) mice were produced in the expected Mendelian ratio from heterozygous crossbreeding ($Fcmr^{+/+}$: 20%; $Fcmr^{+/-}$: 49%; $Fcmr^{-/-}$: 31%; Fig. S1). *Fcmr* KO mice were indistinguishable from littermates with respect to appearance, general behavior, body and organ weights, and fertility. Ablation of the *Fcmr* was confirmed by the absence of FcμR proteins and full-length FcμR transcripts (Fig. 1 and Fig. S2, respectively). $Fcmr^{+/+}$ 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 *Fcmr* 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. 1*A*), 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 *Fcmr* 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 $\gamma\delta^+$ 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

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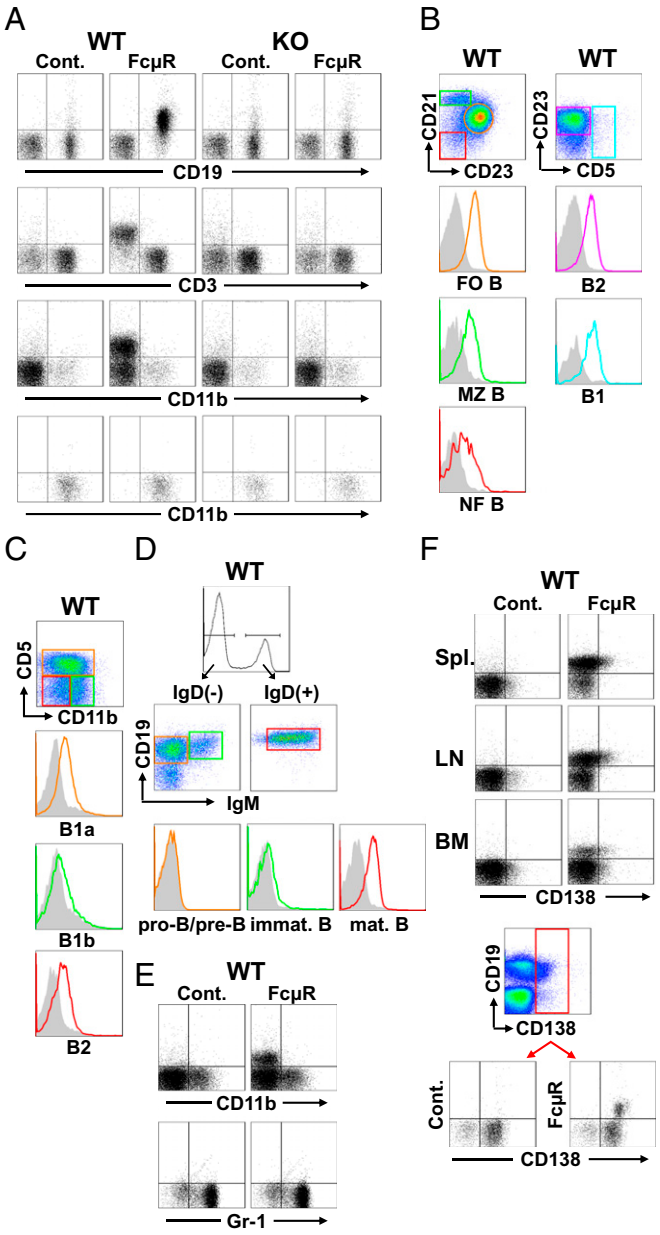

Fig. 1. Immunofluorescence analysis of cells from *Fcmr* KO and WT mice. (*A*) B-cell–restricted expression of FcμR. Splenocytes from *Fcmr* KO (*Right*) and WT control (*Left*) mice of the same age and sex were first incubated with AB93 FcγRII/III and 9E9 FcγRIV mAbs or aggregated human IgG to block FcγRs and then with biotin-MM3 anti-FcμR mAb (FcμR) or biotin-IgG1 control mAb (Cont.). The bound mAbs were identified by addition of PE-streptavidin (SA), before counter staining with FITC anti-CD19, APC anti-CD3ε and PE/Cy7 anti-CD11b mAbs. Stained cells with the light scatter characteristics of lymphocyte/macrophages (*Upper* three panels) or granulocytes (*Lower* panel) were analyzed using an Accuri C6 flow cytometer (BD). (*B* and *C*) Surface FcμR levels on B-cell subsets. $CD19^+$ B cells in each group of mice were analyzed for FcμR expression as: $CD21^{hi}/CD23^-$ MZ, $CD21^{lo}/CD23^+$ FO, and $CD21^-/CD23^-$ NF B cells (*Left* in *B*) and $CD5^-$ B2 and $CD5^+$ B1 cells (*Right* in *B*) in spleen; $CD5^+$ B1a, $CD5^-/CD11b^+$ B1b and $CD5^-/CD11b^-$ B2 cells in peritoneal cavity (*C*). The reactivity with anti-FcμR and control mAbs is indicated by the dark black lines and shaded histograms, respectively. (*D* and *E*) FcμR expression by bone marrow B-lineage and myeloid cells. FcμR expression was analyzed as $CD19^+/sIgM^-/sIgD^-$ pro-B/pre-B, $CD19^+/sIgM^+/sIgD^-$ immature B and $CD19^+/sIgM^+/sIgD^+$ mature B cells in bone marrow (*D*) and as $CD11b^+$ or $Gr\text{-}1^+$ myeloid cells (*E*). (*F*) FcμR expression by plasma cells. FcμR expression was examined by $CD138^+$ cells in spleen, lymph nodes, and bone marrow (*Left*). $CD138^+$ cells in WT bone marrow were examined for the expression of FcμR and CD19 (*Right*). These results are representative of more than 10 different experiments.

prepared, marrow $CD11b^+$ myeloid cells (Fig. 1*E*) or by macrophage colony-stimulating factor (M-CSF)–induced bone marrow macrophages. The lack of FcμR 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 FcμR expression by each B-cell subset in spleen: $CD21^{lo}/CD23^+$ follicular (FO) > $CD21^{hi}/CD23^-$ marginal zone (MZ) > $CD21^-/CD23^-$ newly formed (NF) B cells (Fig. 1*B*). The FcμR MFI was indistinguishable between $CD5^+$ B1 and $CD5^-$ B2 cells. In the peritoneal cavity, the FcμR MFI in each $B220^+$ B-cell subpopulation was, in the order, $CD11b^-/CD5^-$ B2 ≅ $CD5^+$ B1a > $CD11b^+/CD5^-$ B1b cells (Fig. 1*C*). In contrast, the MFI of FcμR on $GL7^+$ germinal center (GC) B cells was much lower than that on $GL7^-$ B cells, suggesting that the FcμR is down-modulated during GC reactions. FcμR 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, FcμR was undetectable in the $CD19^+$/surface (s) $IgM^-/sIgD^-$ pro-B/pre-B-cell compartment, was low on $CD19^+/sIgM^+/sIgD^-$ immature B cells, and higher on $CD19^+/sIgM^+/sIgD^+$ mature or recirculating B cells, indicating that FcμR expression begins at the immature B-cell stage of differentiation (Fig. 1*D*). The majority of $CD138^+$ plasma cells in spleen and lymph nodes expressed FcμR, whereas only a fraction of the $CD138^+$ cells, which express high levels of CD19 or B220, in bone marrow were positive (Fig. 1*F* and Fig. S4), suggesting that FcμR is expressed by plasmablasts rather than plasma cells. Collectively, these findings clearly demonstrate that the expression of FcμR 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 FcμR during the GC reaction.

Alteration of B-Cell Subpopulations in *Fcmr*-Deficient Mice. To examine whether *Fcmr* 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 $CD23^-/CD21^{hi}$ or $CD1d^+/CD5^{lo}$ 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. 2*A* 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 the CD21 and CD23 levels were slightly lower in mutant mice than in WT controls (Fig. 2*B*). 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 *Fcmr* 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 *Fcmr*-Deficient Mice. To determine whether *Fcmr* 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 ±

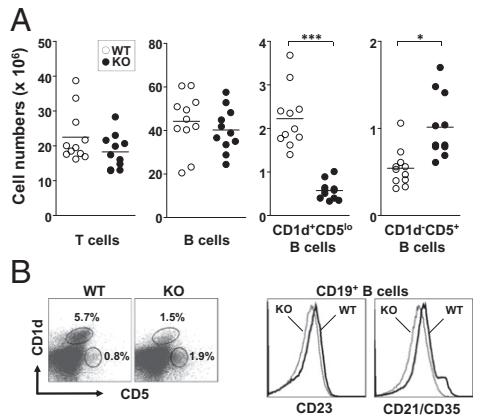

Fig. 2. Cellular compartments and surface protein levels in *Fcmr* KO and WT mice. (*A*) The number of T, B, CD19$^+$/CD1d$^+$/CD5^{lo} MZ (or regulatory) B and CD19$^+$/CD1d$^-$/CD5$^+$ B1 cells in spleen from *Fcmr* KO (●) and WT control (○) mice of the same age and sex was assessed by flow cytometry. Each circle represents data from an individual mouse. The horizontal bar indicates the arithmetic mean and the data were analyzed by Student's *t* test. ***P < 0.001 and *P < 0.05, respectively. (*B*, *Left*) Representative profile of one pair of mice. The splenic B cells were gated and examined for their expression of CD1d and CD5. Note the reduction of CD1d$^+$/CD5^{lo} MZ B cells (1.5 vs. 5.7%) and increase of CD1d$^-$/CD5^{hi} B cells (1.9 vs. 0.8%) in the *Fcmr* KO mouse compared with WT control. (*Right*) Surface levels of CD21 and CD23 on splenic B cells. Experiments were performed with a total of 10–11 different, age- and sex-matched mice from each group.

1292 μg/mL vs. 567 ± 350 μg/mL for IgG3 (P < 0.003) (Fig. 3). To determine whether *Fcmr* ablation affects the half-life of IgM, we first examined the expression of FcμR by liver sinusoidal endothelial cells (LSECs), which are thought to be the primary site of IgM catabolism, at least in the rat (16). Liver and spleen from WT, *Rag1* KO (to avoid contamination with FcμR$^+$ B cells), and *Fcmr* KO were examined by both immunohistological and RT-PCR analyses. There was no expression of FcμR by endothelial cells including LSECs or by phagocytic cells including Kupffer cells (Fig. S2), thus further supporting the B-cell–restricted expression of FcμR. Next, the half-life of injected IgM^a in mutant and WT control mice was assessed by ELISA using a mAb specific for the IgM^a allotype and was found to be comparable (Fig. S5). These findings suggest that the increase of serum IgM levels in naïve *Fcmr* KO mice may be the consequence of FcμR-mediated regulation of IgM production at the B- or plasma-cell stage.

Elevated Natural Antibodies in *Fcmr*-Deficient Mice. Ca^{2+} 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 FcμR-deficient B cells (Fig. S6). We next determined whether the elevated serum levels of IgM and IgG3 in naïve *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 naïve mutant mouse sera displayed various staining patterns including homogeneous or speckled nuclear and cytoplasmic staining (Fig. 4*A*). 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 naïve mice of 10–15 wk by ELISA. Both IgM and IgG anti-dsDNA and antichromatin Abs were significantly elevated in *Fcmr* KO mice (Fig. 4*B*). 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 rage (10^8–10^2 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. 5*A*, IgM and IgG3 PC-specific responses were comparable within both groups of mice when immunized with 10^6 or more bacteria. By contrast, at a suboptimal dose of R36A (10^4 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 10^2 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 (10^4 and 10^2 cfu; Fig. 5*B*). IgG1, IgG2b, and IgG2c Abs developed only in mice receiving higher Ag doses ($\geq 10^6$ 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 *Fcmr* ablation influences the affinity maturation of Abs, we used the well-characterized hapten-carrier conjugate system, nitrophenyl-coupled chicken γ-globulin (NP-CGG), as another TD Ag. Mice were immunized i.p. with

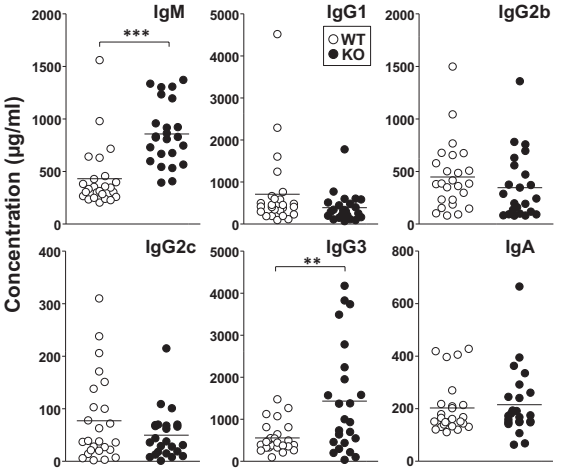

Fig. 3. Serum Ig concentrations in naïve mice. Sera were collected from 24 *Fcmr* KO (●) and 25 WT control (○) mice of the same age (13–18 wk). The concentrations of each Ig isotype were determined by sandwich ELISA in triplicate along with appropriate Ig standards. Each circle represents data from an individual mouse. Horizontal bar indicates arithmetic mean. ***P < 10^{-5} and **P < 0.003, respectively. Note that serum IgM and IgG3 levels are significantly elevated in naïve *Fcmr* KO mice.

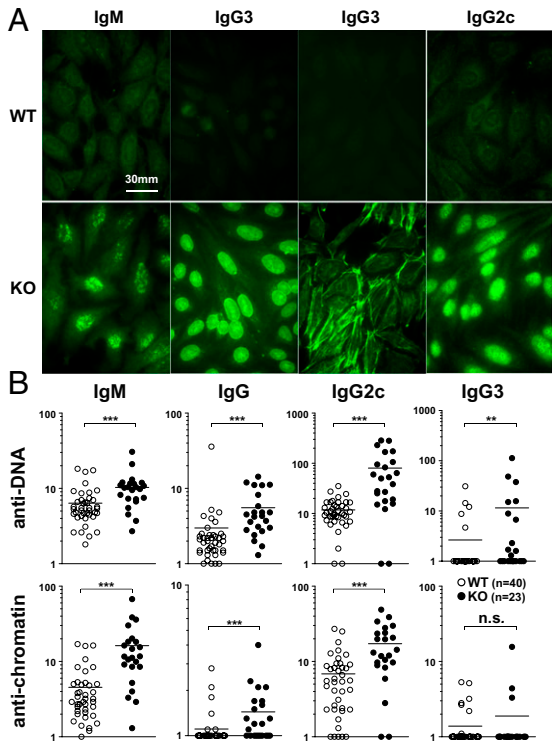

Fig. 4. Natural Abs in naïve mice. (*A*) Representative nuclear and cytoplasmic staining patterns of HEp-2 cells. Indirect immunofluorescent results are depicted of three different sera from each group of naïve animals, WT (*Upper*) and *Fcmr* KO (*Lower*), developed with FITC-goat Abs specific for IgM, 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.

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. 6*A*). 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. 6*B*). For Ab affinity assessment, we first quantified serum Abs reactive with NIP25-BSA and NIP5-BSA at three different time points during immunization and the ratio of anti-NIP25 Ab to anti-NIP5 Ab was used as a relative affinity maturation index. As shown in Fig. 6*C*, 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.

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 FO 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 four-fold 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, pIgR on mucosal epithelial cells or Fcα/μR 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 FcγR, FcγRIIb, 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 $CD19^{hi}$ or $B220^{hi}$ 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 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

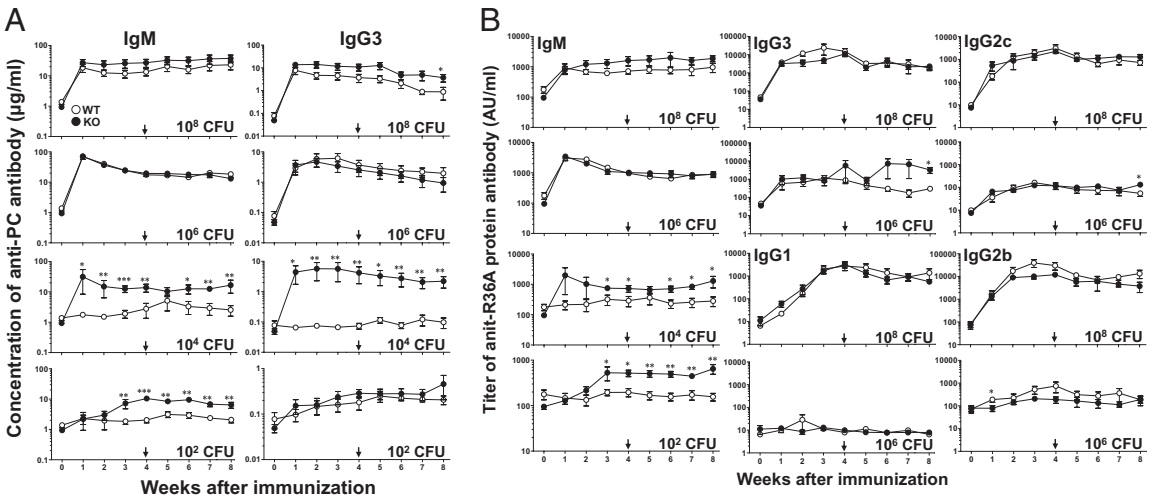

Fig. 5. IgM and IgG3 Ab responses to phosphorylcholine (PC) in *Fcmr* KO and WT mice. Age- and sex-matched KO and WT mice ($n = 5$, each group) were injected i.p. with a wide range (10^8–10^2 cfu) of live R36A, avirulent strain of *S. pneumoniae*, and boosted with 10^2 cfu of live R36A 4 wk later indicated 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 (BH8) and IgG3 (HPCG11) 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.

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 FcμR 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), FcμR 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, FcμR 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 FcμR 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).

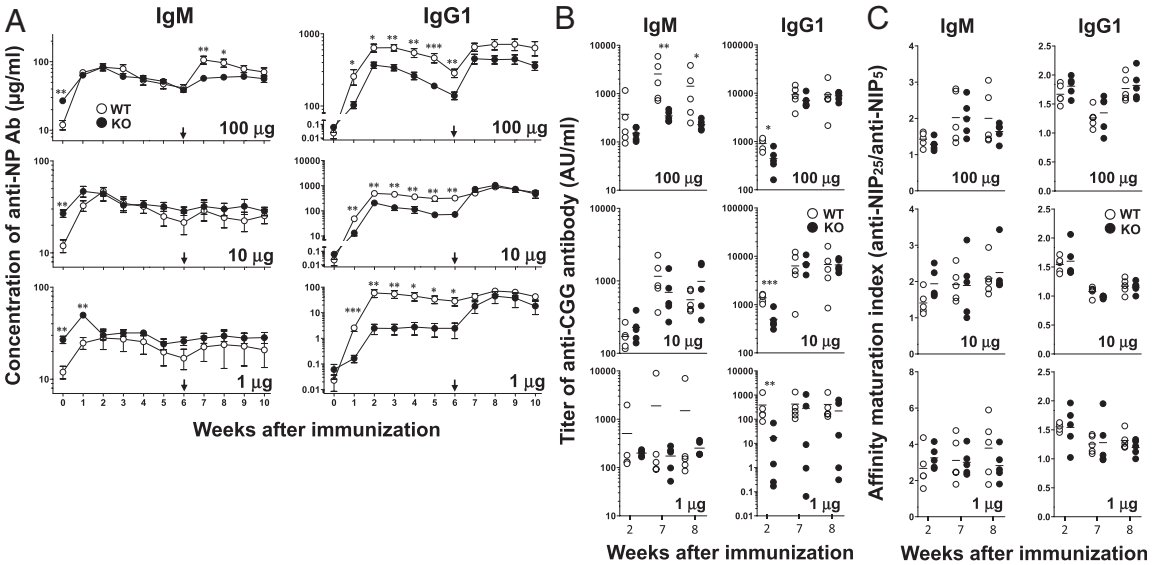

Fig. 6. IgM and IgG1 Ab responses to NP-CGG in *Fcmr* KO and WT mice. *Fcmr* KO (solid circles) and WT control (open circles) mice of the same age and sex ($n = 5$, each group) were immunized i.p. with the indicated doses of NP23-CGG in alum and boosted with the same dose of NP-CGG in PBS 6 wk later (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 NIP25-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 FcμR-deficient mice.

On the other hand, when immunized with NP-CGG precipitated in alum, *Fcmr* 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 (TLRLs) are coexpressed. TLRLs 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-CGG 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

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

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