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Mouse IgM Fc Receptor, FCMR, Promotes B Cell Development and Modulates Antigen-Driven Immune Responses

Seung-Chul Choi,*1 Hongsheng Wang,†,1 Linjie Tian,* Yousuke Murakami,* Dong-Mi Shin,‡,2 Francisco Borrego,‡ Herbert C. Morse, III, † and John E. Coligan*

FcR specific for pentameric IgM (FCMR) is expressed at high levels by B cells. Although circulating IgM has profound effects on responses to pathogens, autoimmunity, and B cell homeostasis, the biologic consequences of its binding to FCMR are poorly understood. We interrogated FCMR contributions to B cell function by studying mice that lack FCMR. FCMR transcripts are expressed at different levels by various B cell subsets. FCMR-deficient mice have reduced numbers of developing B cells, splenic follicular and peritoneal B-2 cells, but increased levels of peritoneal B-1a cells and autoantibodies. After immunization, germinal center B cell and plasma cell numbers are increased. FCMR-deficient B cells are sensitive to apoptosis induced by BCR ligation. Our studies demonstrate that FCMR is required for B cell differentiation and homeostasis, the prevention of autoreactive B cells, and responsiveness to antigenic challenge. The Journal of Immunology, 2013, 190: 987–996.

Immunoglobulin M is the first Ab isotype produced by all vertebrates after initial Ag encounter (1). It is present as a membrane-bound form on the surface of B cells and as a secreted form (secreted IgM [sIgM]) that is mainly found in the blood. sIgM is composed of two classes: natural and immune IgM. Natural IgM, characterized by polyreactivity and low affinity, is found in normal quantities in mice raised under germ-free or specific pathogen-free conditions (2, 3). Immune IgM is secreted after exposure to specific pathogens.

The study of mice deficient in sIgM (Sµ−/−) has provided unexpected insights into its role in diverse processes, ranging from B cell survival to atherosclerosis (3, 4), as well as in autoimmunity and protection against infection (5). In addition, Sµ−/− mice also show increased levels of serum IgA, have elevated humoral immunity to T-dependent (TD) Ag, have an increased propensity to develop IgG autoantibodies and autoimmune disease, and have an expanded population of B-1a cells (6–9).

Peritoneal B-1a cells and, to a lesser extent, marginal zone (MZ) B cells have been identified as the major sources of natural IgM, with spleen and bone marrow (BM) being the major sites of natural IgM production by B-1 cells (10, 11). Interestingly, Sµ−/− mice have increased numbers of both B-1a and MZ B cells, suggesting that B cells sense the presence of sIgM (12). The mechanisms governing expansion of these populations could be related either to modulation of the antigenic environment by natural IgM or its interaction with specific FcRs on the B cell itself. Indeed, it was recently reported that sIgM enhances BCR signaling and regulates B cell homeostasis in different peripheral compartments (13).

Although several ligands and receptors for IgM have been characterized—Clq (14), mannose-binding lectin (15), polymeric Ig receptor (16), and Fcγ/µR (17)—a long-postulated FcR specific for pentameric IgM, the FCMR (18, 19), had proved to be remarkably elusive. Nonetheless, recent elegant studies have provided definitive evidence for the presence of FCMR on human and mouse lymphocytes, and have characterized the genes encoding the proteins (20–22). Notably, however, other studies have suggested that this molecule does not bind IgM but instead confers resistance to cell death mediated by TNFR1 and CD95 signaling (23–25). A clear definition of the function of the receptor in the biology of normal B cells has not been developed.

In this study, we took advantage of FCMR-deficient (Fcmr−/−) mice to understand the contributions of the receptor to B cell development, differentiation, and function. These experiments identified essential roles played by FCMR in B lymphopoiesis in the BM, the population of mature B cell subsets, responses to TD and T-independent (TI) Ags, signaling through the BCR, and inhibition of autoreactivity. We determine that FCMR is critically involved in...
multiple aspects of B cell differentiation, homeostasis, and function.

Materials and Methods
Mice and cell line
Fcmr−/− mice on a C57BL/6 (B6) genetic background were provided by the University Health Network (Toronto, Ontario, Canada). In brief, to generate the mice, we replaced exons 2–8 of the Fcmr gene by a neo-mycin resistance gene cassette that was assembled using a 7.5-kbp fragment found within an intron located in the 5′ leader sequence of the gene and a 0.65-kbp fragment that was synthesized downstream of the last methionine codon in the gene by PCR (Supplemental Fig. 1). After electroporating this construct into embryo stem cells, homologous recombinant cells were injected into blastocysts and implanted into pseudopregnant mice. The chimeras produced were bred until germline transmission occurred in the progeny. Mice were analyzed for homozygosity of the rearranged allele; then homozygous mice were bred together to obtain homozygosity of the rearranged allele. Sp−/− mice (7) were provided by Dr. Troy Randall (University of Rochester), Wild type (+/+; controls) were littermates generated by crosses of mutant heterozygotes. Mice were used in this study under protocol LIG-5E approved by the National Institute of Allergy and Infectious Diseases Institutional Animal Care and Use Committee. The human YTS NK cell line and methods used for infection with a control lentivirus or a lentivirus-expressing full-length mouse Fcmr were described previously (20).

Flow cytometry (FACS)
Single-cell suspensions were prepared from BM of the tibia and femur from one leg, spleen, and peritoneum using standard procedures. After red cell lysis, cells were blocked with anti-CD16/32 Ab (2.4G2) and stained for FACS in 0.5% FBS in PBS. Abs used were to CD3 (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD6 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (MB91-1), CD21 (eBio8D9), CD23 (B3B4), CD24 (30-F1), CD43 (eBioR2/60), CD138 (28-1-2), B220 (RA3-6B2), Gr-1 (RB6-8C5), Ly-77 (GL7), NK1.1 (PK136), CD25 (7D4), pre-BCR (SL156), Igs (187.1), BP 1 (6C3), AA4.1 (AA4.1), IgM (APF-78), and IgD (217-170). Data were collected on a FACSort or LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star). Ab-stained cells were also sorted by a FACSaria (BD Biosciences) with a purity ≥95%.

RNA isolation and quantitative real-time RT-PCR
Splenic CD4+ T, CD8+ T, NK, and B cells were purified by negative selection using MACS MicroBeads kits. Splenic Gr-1+, CD11b+, CD11c+, and BM c-KIT+ cells were purified by positive selection using MACS MicroBeads kits. B cell subsets were purified by FACS. Total RNA isolated from purified cells using the RNeAguous-4PCR kit (Ambion) was treated with DNase I according to the manufacturer’s recommendations. cDNA was synthesized with Qscript CDNA synthesis kit (Quanta Biosciences). Quantitative real-time PCR (qRT-PCR) was performed using Lightcycler 480 SYBR green I master supermix, according to the manufacturer’s instructions (Roche Diagnostics). Oligonucleotide primers for Lightcycler 480 SYBR green I master supermix, according to the manufacturer’s instructions (Roche Diagnostics). Quantitative real-time PCR (qRT-PCR) was performed using ELISA using NP (27)-BSA (Biosearch Technology) precoated plates, followed by incubation with diluted serum samples, and developed with HRP-conjugated mouse-isotype specific Ab (Southern Biotech) and p-nitrophenyl phosphate substrate (MP Biomedical). Serum anti-dsDNA IgG levels were analyzed with a mouse anti-dsDNA IgG-specific ELISA kit (Alpha Diagnostic International). The plates were read at 450 nm using an ELISA plate reader.

ELISPOT
Spleen and BM Ab-secreting cells were quantified by an NP-specific IgG ELISPOT assay. Aliquots of 1.25–5.0 × 106 spleen and BM cells were plated in duplicate in NP-BSA precoated 96-well polyvinylidene difluoride membrane plates (Millipore) and were incubated for 1 d at 37°C in 5% CO2. The plates were washed with PBS containing 0.05% Tween 20 and incubated with HRP-conjugated anti-mouse IgM or IgG1 (Jackson ImmunoResearch Laboratory), followed by reaction with FAST 5-bromo-4-chloro-3-indolyl phosphate/NBT chromogen substrate (Sigma). The spots were detected with a CTL-ImmuNoSpot S5 Core Analyzer (Cellular Technology) and analyzed by ImmunoSpot Software 4.0 (Cellular Technology).

Reconstitution and serum transfer
Lethally irradiated (5 Gy) Rag1−/− mice were reconstituted i.v. 1 d later with 2 × 107 hematopoietic stem cells (HSCs) purified from BM cells of 2-mo-old +/- and Fcmr−/− mice using lineage cell depletion kits (Miltenyi Biotec). After 2 wk, 100 µl sera collected from 2-mo-old B6 and Sp−/− mice were injected i.v. three times per week for 2 wk. BM cells were harvested and B cell subpopulations were analyzed by FACS.

Assays for cell proliferation and apoptosis
Splenic B cells were purified by negative selection using a MACS MicroBeads kit (Miltenyi Biotec), according to the manufacturer’s instructions. For in vitro B cell proliferation assays, cells seeded at 2 × 105 cells/well were cultured in 96-well flat-bottom plates with LPS (1 µg/ml; Sigma), CpG-DNA (2.5 µg/ml; InvivoGen), anti-CD40 mAb (1 µg/ml; Alexis), recombinant mouse IL-4 (10 ng/ml; R&D Systems), or F(ab′)2 anti-mouse IgM Ab (10 µg/ml; Jackson Immunoresearch Laboratory) for 3 d. Cells were pulsed with 1 µCi 3H-thymidine for the last 16 h of culture, and incorporation was measured by liquid scintillation counting.

For analyses of apoptosis, purified B cells seeded at 5 × 105 cells/well in 24-well plates were stimulated with LPS, anti-CD40 mAb, or F(ab′)2 anti-mouse IgM Ab for 24 h. Cells were then stained with Annexin V (BD Biosciences) and 7-aminoactinomycin D (7-AAD; eBioscience) for FACS analysis.

Statistical analysis
Significance of differences between groups was evaluated by Student t test. A p value <0.05 was considered significant.

Results
Fcmr is highly expressed by B lineage cells
Previous studies using qRT-PCR and flow cytometry indicated that Fcmr, the gene encoding FCMR, is expressed primarily by cells of the adaptive immune system (20, 21, 24). Because there is no commercially available source of Abs to FCMR to study cell-surface protein expression, we extended these analyses by using qRT-PCR to quantify Fcmr transcript levels in purified subpopulations of hematopoietic cells from spleen, peritoneum, and BM. Splenic CD4+ and CD8+ T cells expressed very low levels of

Effects of FCMR on B cell development and function
Fcmr transcripts with slightly higher levels in NK cells, whereas expression in B cells was 500-fold higher than in T cells. Transcripts in follicular (FO) B cells were 2-fold higher than in MZ B cells, whereas expression in GC B cells and plasma cells (PCs) was markedly lower (Fig. 1B). Transcript levels in peritoneal B-1 and B-2 cells were comparable with those of MZ B cells (Fig. 1C). These findings indicate that FCMR is differentially expressed in distinct subsets of mature peripheral B cells and may differentially affect their functions.

The finding of high levels of Fcmr transcripts in peripheral B cells prompted us to investigate whether differing levels of expression were associated with progression of B cell development in the BM. Studies of sort-purified B lineage subsets demonstrated that transcript levels in pro-B and pre-B cells were low but were significantly higher in more differentiated immature B cells (Fig. 1D), suggesting that FCMR influences early B cell development.

Studies of splenic myeloid cells revealed low levels of transcripts in CD11b+ macrophages with higher levels in CD11c+ dendritic cells (DCs) and Gr-1+ granulocytes (Fig. 1A). Parallel studies of BM cells (Fig. 1A) revealed only very low levels of transcripts among BM-derived DCs and by c-Kit+ cells that include HSCs, multipotent progenitors, common myeloid progenitors, and common lymphoid progenitors.

Fcmr−/− mice exhibit altered B cell differentiation in the BM and distribution in peripheral compartments

To determine whether differential expression of Fcmr by subsets of developing B lineage cells and peripheral B cell subsets might affect B cell differentiation and function, we first quantified B cell subsets in the BM, spleen, and peritoneum of 3-mo-old mice (Fig. 2). Using the gating parameters shown in the left panels of Fig. 2A, FACS analyses of BM cells from wild type (+/+ ) and Fcmr−/− mice showed that mutant mice had modestly reduced numbers of pro-B (Hardy fraction [Fr.] A–C) and large pre-B cells (Fr. D, determined by size) but more significant reductions in small pre-B (Fr. D, determined by size) and immature B cells (Fr. E). The numbers of mature recirculating B cells (Fr. F) were similar for both genotypes (Fig. 2A, right panel).

Parallel studies of splenic B cell subsets showed that the total numbers of MZ and transitional B cells were the same for mice of both genotypes, but that the numbers of FO B cells were significantly lower for Fcmr−/− mice (Fig. 2B). Analyses of peritoneal B cell subsets (Fig. 2C) showed that the total numbers of B cells were significantly higher for Fcmr−/− mice. Although numbers of B-1b cells were similar for both sets of mice, the numbers of B-1a cells were significantly higher and the numbers of B-2 cells were significantly lower for Fcmr−/− mice.

Interestingly, the patterns of B cell development and distribution of peripheral B cell subsets seen with Fcmr−/− mice had many similarities to those reported for two strains of mice deficient in slgM (Sμ−/− mice) (6, 7, 9). The fact that both knockout strains were generated using 129 ES cells and were studied at different ages as F2 (7, 9) or N6 (6) mice in crosses with B6 mice complicates reliable comparisons with our Fcmr−/− mice. To avoid problems associated with relating data on mice on different genetic backgrounds and in different environmental conditions, we elected to perform direct comparisons of mice bred in our colony that included Fcmr−/− and Sμ−/− knockouts serially backcrossed onto the B6 background using +/- littermate mice as controls (Supplemental Fig. 2). These studies demonstrated that the numbers of cells in BM Fr. A–C, D, and E, and of peritoneal B-1a and B2 cells were not significantly different for the two mutant strains. However, the numbers of peritoneal B-1b cells were significantly higher for Sμ−/− than Fcmr−/− mice, and in spleen, the FO B cell numbers for Sμ−/− mice were significantly lower than Fcmr−/− mice, and both were significantly lower than for +/- mice. Finally, the numbers and proportions of both MZ and transitional B cells were significantly higher for Fcmr−/− than +/- mice (Supplemental Fig. 2). These results suggest that much of the Sμ−/− B cell phenotype can be ascribed to the absence of FCMR-slgM receptor–ligand interactions. We conclude that expression of FCMR significantly influences pre-B and immature B cell development in the BM and the distribution of mature B cells into distinct subsets in the spleen and peritoneum, and suggest that these effects are mediated, in large part, by binding of slgM.

We also quantified basal levels of serum Ig isotypes of 3- and 6-mo-old +/- and Fcmr−/− mice (Fig. 3A). For 3-mo-old mice, the levels of serum IgM, IgA, and all IgG isotypes except IgG1 (lower in Fcmr−/− mice) were similar for both sets of mice. For 6-mo-old mice, the levels of both IgG3 and IgA were increased in sera from Fcmr−/− mice, whereas levels of the other isotypes were comparable between the cohorts. The increased levels of IgG3 and IgA in sera of 6-mo-old Fcmr−/− mice could reflect the increased numbers of B-1a cells that characterize the strain (Fig. 2C). The increased serum levels of IgG3 in Fcmr−/− mice and the prior demonstration...
of increased levels of class-switched anti-DNA Ab in sera of Sm2/2 mice (6) prompted us to test sera from 6-mo-old Fcmr2/2 mice for autoantibodies. Levels of IgG anti-dsDNA Ab were significantly increased in Fcmr−/− mice (Fig. 3B). Serum ANAs giving a diffuse staining pattern were present in sera from three of seven 6-mo-old Fcmr−/− mice (Fig. 3C), but none of seven +/+ age-matched con-

**FIGURE 2.** Abnormal B cell development in Fcmr−/− mice. Cells from BM (A), spleen (B), and peritoneum (C) of 3-mo-old +/+ and Fcmr−/− mice were stained and analyzed by FACS. (Left panels) FACS profiles of (A) were gated on lymphocytes, (B) on B220+ cells, and (C) on CD19+ cells. Pre-B cells were further subdivided into large and small subsets based on forward scatter (FSC) and side scatter (SSC) (not shown). Numbers indicate percentages of cells falling in each gate. (Right panels) Graphs of the absolute numbers of each B cell population. Each symbol represents a mouse. *p < 0.05, **p < 0.01, ***p < 0.005.

**FIGURE 3.** Basal levels of serum Ig in Fcmr−/− mice. (A) Serum Ig levels of 3- and 6-mo-old +/+ and Fcmr−/− mice were determined by ELISA. (B) The serum levels of IgG anti-dsDNA in 6-mo-old mice were measured by ELISA. Each symbol represents a mouse. (C) Representative patterns of cellular staining of HEp-2 cells by sera diluted at 1:200 prepared from 6-mo-old +/+ (n = 7) and Fcmr−/− mice (n = 7). Original magnification ×20. *p < 0.05, **p < 0.01, ***p < 0.005.
controls. We conclude that FCMR may negatively regulate autoimmunity by functioning as a receptor for sIgM.

Early B cell development is affected by FCMR and sIgM

The reduced numbers of pre-B cells in Fcmr<sup>−/−</sup> mice prompted us to examine whether certain B cell–intrinsic or –extrinsic mechanisms might contribute to this effect. The transcription factor PAX5 was selected as one candidate because it regulates the expression of a number of genes encoding proteins that promote B cell differentiation (26). However, qRT-PCR analyses of Pax5 transcript levels showed that they were equivalent for both cohorts in sorted early B cell populations of increasing maturity (data not shown). We then asked whether early B cell development of Fcmr<sup>−/−</sup> mice could be rescued by IL-7 in vitro because IL-7 acts in concert with the pre-BCR to promote expansion of pre-B cells. BM cells of +/+ and Fcmr<sup>−/−</sup> were cultured with IL-7 for 6 d with +/+ BM cells yielding slightly higher frequencies of B220<sup>+</sup> cells than Fcmr<sup>−/−</sup> BM cells (Fig. 4A). The frequencies of pre-B cells expressing CD25 or the pre-BCR were also equivalent between +/+ and Fcmr<sup>−/−</sup> mice (Fig. 4A). These data argue that heightened IL-7 signaling may compensate for deficient FCMR signaling resulting in the generation of normal frequencies of pre-B cells in Fcmr<sup>−/−</sup> cultures. Surprisingly, IL-7 failed to rescue immature B cell development; 36% fewer immature B cells were generated from cultures of Fcmr<sup>−/−</sup> as compared with +/+ cells (Fig. 4A). Interestingly, the levels of cell-surface Ig expressed by Fcmr<sup>−/−</sup> immature B cells were slightly higher than for cells from +/+ mice, even though the sizes of cells in both populations were essentially identical (Fig. 4A, right panel). This finding suggested that FCMR might influence the levels of BCR expression during B cell development in the BM, thereby affecting positive and negative selection events.

To further evaluate the function of FCMR in pre-B cell development, we reconstituted sublethally irradiated Rag1<sup>−/−</sup> mice using HSC-enriched lin<sup>−</sup> BM cells isolated from +/+ and Fcmr<sup>−/−</sup> mice. Beginning 2 wk after transplantation, the recipients were injected three times a wk for 2 wk with sIgM-containing (sIgM<sup>+</sup>) sera from young B6 mice or sIgM-deficient (sIgM<sup>−</sup>) sera from young S<sub>μ</sub>−/− mice. This allowed us to evaluate the effect of sIgM on early B cell

FIGURE 4. FCMR and sIgM affect pre-B and immature B cell differentiation. (A) BM cells were cultured with 10 ng/ml IL-7 for 6 d and analyzed by FACS. All cells are gated on 7-AAD<sup>−</sup> single cells. The numbers are percentages of cells falling in each gate. (B) Irradiated Rag1<sup>−/−</sup> mice were transplanted with HSC-enriched lin<sup>−</sup> cells purified from BM cells of +/+ and Fcmr<sup>−/−</sup> mice, followed by injections with sIgM<sup>+</sup> or sIgM<sup>−</sup> sera prepared from young +/+ and S<sub>μ</sub>−/− mice, respectively. The injection schedule was based on the understanding that the half life of serum IgM is ~2 d and the amount on the demonstration that the biologic readout of arthritis could regularly be induced in B cell–deficient K/BxN mice with as little as 100 μl arthritic K/BxN serum (27). BM cells of recipient mice were analyzed by FACS. Top diagram shows injection frequencies. Middle panel shows the frequencies of total B cells (left) and B cell subpopulations (right). Bottom panel shows the absolute cell numbers per femur of total B cells (left) and B cell subpopulations (right). Each symbol represents a mouse. *p < 0.05, **p < 0.01, ***p < 0.005.
development. As shown in Fig. 4B, recipients of +/+ HSCs and sIgM+ sera generated significantly more pre-B cells than recipients of +/+ HSCs and sIgM2 sera. The numbers of immature B cells also tended to be higher in recipients of sIgM+ sera than those receiving sIgM2 sera. As expected, the presence or absence of sIgM in sera given to recipients of Fcmr2 HSCs had no effect on the generation of pre-B cells. From this, we conclude that FCMR functions as a gatekeeper controlling pre-B and immature B cell development, and that binding of sIgM to the receptor has a positive effect on development of these cells. These results are fully consistent with the observations that natural IgM influences B cell development and survival (3, 6, 13).

To more rigorously examine the suggested associations between sIgM and FCMR, we incubated the human YTS cell line expressing full-length mouse FCMR with pentameric mouse IgM in serum-free medium. The results showed clear binding of IgM to the transfected line (Supplemental Fig. 3), strongly supporting earlier reports of receptor–ligand interactions between FCMR and IgM on both mouse and human cells (20, 22).

Fcmr2 mice respond to TD and TI Ags with increased GC formation and generation of Ag-specific PCs

We next asked whether FCMR might influence the development of humoral immune responses to either TI or TD Ags by assessing GC development, the generation of total PCs, as well as Ag-specific cells in spleen and BM, and the levels of Ag-specific IgM in sera of immunized mice. We first studied responses to the TI type I Ag, NP-LPS, and the TI type II Ag, NP-Ficoll. TI Ags have been shown to generate short-lived GCs that fail to engage in somatic hypermutation and affinity maturation (28, 29), whereas still leading to the generation of long-lived PCs in spleen and BM (30). At 1 wk after immunization with either TI Ag, the total numbers of splenic GC B cells (PNA+GL7+) were higher for Fcmr2 than for +/+ mice (Fig. 5A). In addition, the numbers of splenic PCs, including fully mature CD138+B220+ PCs and CD138+B220+ plasmablasts, were significantly higher for Fcmr2 mice than their +/+ counterparts (Fig. 5A). Ag-specific, IgM-secreting cells in the spleen and BM were analyzed by ELISPOT, and serum NP-specific IgM Abs were measured by ELISA. After immunization with NP-LPS, the numbers of NP-specific, IgM-secreting cells in both the spleen and BM, and the levels of NP-specific serum IgM were significantly higher for Fcmr2 mice than their +/+ counterparts (Fig. 5B, 5C). This result is in keeping with the increased numbers of B-1a cells in Fcmr2 mice that dominate responses to TI type I Ags. Parallel studies of mice immunized with NP-Ficoll showed that the numbers of Ag-specific splenic PCs and levels of NP-specific serum IgM were similar for mice of both genotypes, although the frequencies of

**FIGURE 5.** Ab responses to TI or TD Ags by Fcmr2 mice. (A) Splenic GC B cells (B220+GL7+PNA+) and PCs (B220+/−7-AAD−CD138+) were analyzed by FACS 1 wk after immunization with NP-Ficoll or NP-LPS. FACS gating schemes are shown in Fig. 6A and 6B. Data are absolute cell numbers. (B) IgM-secreting cells in the spleen and BM were determined by ELISPOT 1 wk after immunization with NP-Ficoll or NP-LPS. (C) Serum Ab titers of NP-specific IgM were determined by ELISA 1 wk after immunization with NP-Ficoll or NP-LPS. (D) GC B cells and PCs were analyzed by FACS 2 wk after immunization with NP-KLH in alum (refer to Fig. 6 for gating schemes). (E) IgG- or IgM-secreting cells in spleen and BM were analyzed by ELISPOT 2 wk after immunization. (F) The serum levels of NP-specific Ab were measured by ELISA 2 wk after immunization. Each symbol represents a mouse. All data are pooled from two experiments. *p < 0.05, **p < 0.01, ***p < 0.005.
Ag-specific PCs were significantly higher in the BM of Fcmr<sup>−/−</sup> than +/+ mice (Fig. 5B, 5C). These data are consistent with the observation that the numbers of MZ B cells, which are primarily responsible for responses to TI type II Ags, are comparable in FCMR-deficient and +/+ mice.

We next examined the responses of +/+ and Fcmr<sup>−/−</sup> mice to immunization with the TD Ag, NP-KLH, in alum. Two weeks after immunization, the total numbers of splenic PCs were significantly higher in Fcmr<sup>−/−</sup> than +/+ mice, even though the numbers of GC B cells were comparable for mice of both genotypes (Fig. 5D). ELISPOT analyses showed that the numbers of Ag-specific IgM-secreting PCs were ~40% higher in spleens of Fcmr<sup>−/−</sup> mice but comparable in the BM. However, studies of NP-specific IgG1-secreting cells showed that their numbers were comparable in spleens of mice of both genotypes but were lower in the BMs of Fcmr<sup>−/−</sup> mice (Fig. 5E). Analyses of NP-specific serum Iggs showed that the levels of IgM Ab were higher and the levels of IgG2b were lower for Fcmr<sup>−/−</sup> mice, whereas the levels of the other isotypes were comparable (Fig. 5F). Taken together, these results suggest that FCMR negatively regulates humoral immune responses against both TI and TD Ags.

Secondary responses to TD Ags by Fcmr<sup>−/−</sup> mice

We next examined the secondary responses of +/+ and Fcmr<sup>−/−</sup> mice to NP-KLH. The mice were boosted 12 wk after a primary immunization and assayed 1 wk later for PCs and serum Abs. The numbers of splenic PCs detected by flow cytometry were significantly higher for Fcmr<sup>−/−</sup> than +/+ mice (Fig. 6A), similar to

**FIGURE 6.** Secondary immune responses to TD Ags by Fcmr<sup>−/−</sup> mice. +/+ and Fcmr<sup>−/−</sup> mice were immunized i.p. with NP-KLH in alum and then boosted with same amount of NP-KLH in alum 12 wk later. (A, B) Splenocytes were analyzed by FACS for GC B cells and PCs 10 d after the second immunization. Left panel shows FACS profiles with the numbers indicating the percentages of cells falling in each gate. Right panel shows absolute cell numbers. (C) Formalin-fixed, paraffin-embedded sections of spleen were stained with PNA to show GCs (dark brown). The first column shows the original images of spleen; second column shows the corresponding markup images with IR (positive pixels; red). The computerized morphometric analysis shows the mean values of PNA-IR per square millimeter. A representative image of one of eight mice per group is shown, and the graph is representative of two independent experiments. (D) IgG1- or IgM-secreting cells in spleen and BM were analyzed by ELISPOT 10 d after the second immunization. (E) The serum levels of NP-specific Abs were determined by ELISA 2 wk after the primary immunization and 1 wk after boosting. Each symbol represents a mouse. All data are pooled from two experiments. *p < 0.05, **p < 0.01, ***p < 0.005.
what was observed in the primary response. In contrast with the primary response, however, the numbers of GC B cells assessed by FACS (Fig. 6B) or by quantitative immunohistochemistry (Fig. 6C) were significantly higher for FCMR-deficient than +/- mice. ELISPOT analyses of cells secreting NP-specific Ab showed that the numbers of cells producing IgM in spleen or BM were similar for mice of both genotypes. However, the numbers of IgG1-producing cells were increased in the spleen and somewhat in the BM of Fcmr-/- mice (Fig. 6D), suggesting that FCMR had a negative influence on class-switch recombination in this secondary response.

Analyses of NP-specific serum Abs revealed that except for IgG3, the levels for other isotypes were similar for mice of both genotypes at 1 wk after the boost. However, similar to the previous study (Fig. 5F), there were small but significant differences in IgM and IgG2b levels after primary immunization (Fig. 6E). Thus, although the numbers of GC B cells and PCs were increased in Fcmr-/- mice after a secondary challenge with NP-KLH, the production of Ag-specific Ab was not significantly increased, arguing against a major role for FCMR during a recall response.

FCMR influences B cell proliferation and survival, but not BCR ligation-induced signaling

To examine the biological functions of FCMR, we tested splenic B cells from +/- and Fcmr-/- mice for their ability to proliferate and undergo cell death upon activation in vitro. We found that transcript levels for Fcmr were markedly reduced after stimulation of purified B cells with LPS or anti-CD40, as well as after BCR ligation with anti-IgM Ab (Fig. 7A). Proliferative responses to stimulation by LPS, Cpg, and anti-CD40 Ab by B cells from both cohorts of mice were similar (Fig. 7B). In contrast, the response of cells from Fcmr-/- mice to BCR ligation with F(ab)2 anti-IgM Ab was significantly reduced in a dose-dependent manner (Fig. 7B, 7C). Parallel studies of cell viability showed that survival of cells from Fcmr-/- mice after a secondary challenge with NP-KLH, the production of Ag-specific Ab was not significantly increased, arguing against a major role for FCMR during a recall response.

Discussion

The results of this study demonstrate that FCMR plays a role in B cell differentiation, function, and homeostasis. Mice deficient in FCMR exhibit multiple alterations in early B cell development, the distribution of mature B cell subsets, responses to antigenic challenge, and are predisposed to autoimmunity. The fact that many of these changes mirror those observed in mice lacking slgM indicates that FCMR is a critical sensor of circulating IgM and provides a basis for understanding a number of its functions in health and disease. Understanding the phenotypes that distinguish FCMR-/- mice from slgM-deficient mice may be helpful in defining the activities of the other IgM receptor, FcεRI/μ receptor (FCAMR), expressed in the surface of B cells. FCMR is but one of three receptors capable of binding IgM. The polymeric Ig receptor is expressed primarily by epithelial cells and mediates the transcytosis of J chain–associated polymeric IgM and IgA across mucosal surfaces (16). The FCAMR is expressed by B cells, macrophages, and FO DCs, but not by granulocytes, T cells, or NK cells. FCAMR does not require the presence of the J chain to bind and endocytose IgM and IgA. Interestingly, mice deficient in FCAMR resemble Fcmr-/- mice in exhibiting enhanced GC responses to TI Ags (31). It has been suggested that FCAMR negatively regulates responses to TI Ags by inhibiting Ag retention by MZ B cells and FO DCs, a concept worthy of further investigation for Fcmr-/- mice. It is important to note that because FCAMR is expressed to some extent by neutrophils, NK cells, macrophages, and DCs, not all the biologic changes associated with deficient expression of the receptor can be attributed to changes in FCRM–slgM interactions on B cells.

The impaired B cell development in Fcmr-/- mice occurred as early as the pre-B cell stage. How FCMR affects pre-B cell survival and development remains unclear. The generation of pre-B cells is marked by clonal proliferation for ~6 divisions driven by signaling through the pre-BCR and the IL-7R (32), followed by cell-cycle arrest and differentiation to small pre-B cells. Deficiencies in pre-BCR signaling caused either by poor expression of the pre-BCR at the cell surface or by disruptions of the signaling machinery downstream of the pre-BCR could severely impact pre-B and subsequent immature B cell development (33–35). Although the numbers of pre-B cells in Fcmr-/- mice were moderately reduced, the subsequent development of large pre-B cells was not significantly affected, indicating that the pre-BCR functions normally in these cells. In fact, pre-BCR expression levels measured by FACS in IL-7–cultured BM pro-B/pre-B cells revealed comparable levels between +/- and Fcmr-/- mice. Two issues remain to be determined. First, the levels of FCMR protein expressed on the surface of pre-B cells are not known because of a lack of commercially available anti-FCMR–specific Abs. Sec-

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**FIGURE 7.** FCMR inhibits BCR-mediated B cell apoptosis. (A) qRT-PCR analysis of Fcmr transcripts in splenic B cells stimulated with LPS, anti-CD40 Ab, or F(ab)2 anti-IgM for 24 h. (B) Proliferative responses of splenic B cells stimulated as in (A) for 3 d. (C) Dose-dependent proliferative responses to BCR ligation induced by F(ab)2 anti-IgM. Splenic B cells were cultured for 3 d. (D) Freshly isolated splenic B cells stimulated for 16 h were stained with 7-AAD and Annexin V, and analyzed by FACS. Live cells were gated as 7-AAD–Annexin V–. *p < 0.05, **p < 0.01, ***p < 0.005. MFI, mean fluorescence intensity.
ond, it is unclear whether FCMR-triggered signaling requires engagement with soluble IgM. However, our analyses of \( \text{S}^{\mu} {\rightarrow}^- \) mice that lack soluble IgM but express FCMR revealed deficiencies in pre-B cell development similar to those seen in \( \text{Fcmr} {\rightarrow}^- \) mice. This argues that pre-B cells are very sensitive to FCMR signaling, and that a certain type of interaction between slgM and FCMR is required to generate a signaling cascade of major importance for development of pre-B and immature B cells. This view is further supported by the observation that administration of slgM enhanced production of pre-B cells in \( \text{Rag}2^{-/-} \) mice reconstituted with \( \text{Fcmr} {\rightarrow}^- \) BM cells (Fig. 4B). Because a previous study of \( \text{S}^{\mu} {\rightarrow}^- \) mice (7) did not identify defects in early B cells like those seen in our analyses of \( \text{Fcmr} {\rightarrow}^- \) mice, the nature of FCMR-mediated signaling requires further investigation.

Analyses of the effects of FCMR deficiency on subsets of mature B cells revealed two contrasting pictures, with the numbers of splenic FO B cells being significantly reduced, whereas the numbers of peritoneal B-1a cells were significantly increased. Remarkably, these observations again mirror those made in slgM-deficient mice (13) implicating FCMR–slgM interactions as critical to both phenotypes. FO B cells, similar to resting small pre-B cells, are not in cell cycle and they differentiate with minimal signaling from the BCR (36). The lack of FCMR-augmented tonic signaling might reduce the level of FO BCR signaling below the threshold required for survival, whereas also enhancing their apoptotic response to BCR ligation. This concept is in keeping with the known importance of tonic signaling for B cell survival demonstrated in studies of Cre-mediated ablation of BCRs in mature B cells (37, 38) and in mice expressing engineered BCRs without extracellular domains (36, 39). It would also be consistent with the observation that \( \text{S}^{\mu} {\rightarrow}^- \) mice, which are unable to activate the FCMR, have more apoptotic splenic B cells (13).

The contrasting increase in peritoneal B-1a cells in FCMR-deficient mice may relate to the greater intensity of BCR signaling associated with their selection and survival than occurs with FO B cells (40, 41) and is associated with their anergic phenotype (42). As suggested by Ehrenstein and Notley (3) from studies of slgM-deficient mice, this strength of signaling may poise B-1a B cells at the threshold for apoptosis such that a reduction in tonic signaling would promote their survival and the observed increases in cell numbers.

The known contribution of natural IgM to complement-dependent clearance of apoptotic cells (43) provides a basis for understanding how expanded populations of B-1a cells, as a source of slgM, could predispose mice lacking FCMR to autoimmunity. Sera from 6-mo-old \( \text{Fcmr} {\rightarrow}^- \) mice had significantly increased levels of IgG anti-DNA Abs, and sera from nearly half of these mice were positive for ANAs with staining patterns characteristic for Abs to dsDNA or histones. Previous studies of \( \text{S}^{\mu} {\rightarrow}^- \) mice documented spontaneous production of IgG anti-dsDNA Abs and glomerular deposition of IgG and C3 in about a third of 12- to 18-mo-old mice (9). Additional studies of autoimmune MRL-\( \text{Fas} {\rightarrow}^- \) mice deficient in slgM documented accelerated appearance of IgG Ab to dsDNA and histones, greater renal pathology, and shortened survival (8). IgG3 anti-dsDNA Abs were also produced by most \( \text{S}^{\mu} {\rightarrow}^- \) mice, but only when 12 mo old. Taken together, these results indicate that interactions of FCMR with natural IgM act to suppress the development of IgG-secreting autoreactive B cells most likely by promoting the clearance of apoptotic cells (43).

Previous studies of \( \text{S}^{\mu} {\rightarrow}^- \) mice revealed that class-switch recombination was impaired in primary immune responses to TD Ags (7, 44), a phenotype that was explained by the lack of an adjuvant effect afforded by IgM–immunogen complexes (45). Interestingly, our studies of \( \text{Fcmr} {\rightarrow}^- \) mice identified enhanced IgM responses to TD Ags (Fig. 5), arguing for a negative rather than a positive regulatory effect on TD immune responses. Nonetheless, IgG responses to TD Ags were not similarly enhanced; indeed, IgG2b production was significantly decreased in \( \text{Fcmr} {\rightarrow}^- \) mice. The lack of enhanced class switching in \( \text{Fcmr} {\rightarrow}^- \) mice is associated with reduced expression of Fcmr by GC B cells and PCs (Fig. 1). It is worth noting that the observed changes in TD immune responses by FCMR-deficient mice could be influenced by other cell types lacking FCMR and/or expression of the FCAMR that was previously shown to influence responses to different Ags (31).

Given the abundance of natural IgM Abs and the broad expression of FCMR, we envision that this receptor could play important roles in innate immunity. IgM immune complexes aggregated by FCMR at the cell surface are readily internalized and transported through the endocytic pathway to lysosomes where they are degraded (46). Engagement of this pathway to dispose of IgM-bound pathogens and extracellular debris could result in synergistic activation of B cells stimulated through their BCRs (47). Both our study and previous studies of \( \text{S}^{\mu} {\rightarrow}^- \) mice demonstrated the critical roles of slgM and FCMR in the development of innate-like B-1a and MZ B cells that are critical to responses to fungal and parasitic infections. This role is exemplified by the finding that \( \text{S}^{\mu} {\rightarrow}^- \) mice respond poorly to infection with \textit{Cryptococcus neoformans} (5). Interestingly, natural IgM is reported to bind malarial parasites, and FCMR is implicated in innate immunity against malaria infection (48).

The identification of FCMR as a receptor for slgM, supported by results presented in this article, need not be an either/or proposition regarding its role in apoptosis. Indeed, our studies suggest a protective role against cell death after BCR ligation of splenic B cells and possibly during early B cell development as well. Understanding how these differing potentials might play out in the biology of individual cells will certainly be a focus of future studies.

In conclusion, the development and function of B cells in FCMR-deficient mice is remarkably similar to that of \( \text{S}^{\mu} {\rightarrow}^- \) mice, highlighting critical roles for the slgM-FCMR axis in regulating normal B cell differentiation, modulating innate and adaptive immunity, and protecting against the development of humoral autoimmunity. Further studies are required to characterize the molecular features of FCMR-triggered signaling and its contributions to these differing phenotypes.

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Disclosures
The authors have no financial conflicts of interest.

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