Enhanced humoral immune responses against T-independent antigens in Fcα/μR-deficient mice

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IgM is an antibody class common to all vertebrates that plays a primary role in host defenses against infection. Binding of IgM with an antigen initiates the complement cascade, accelerating cellular and humoral immune responses. However, the functional role of the Fc receptor for IgM in such immune responses remains obscure. Here we show that mice deficient in Fcα/μR, an Fc receptor for IgM expressed on B cells and follicular dendritic cells (FDCs), have enhanced germinal center formation and affinity maturation and memory induction of IgG3$^+$ B cells after immunization with T-independent (TI) antigens. Moreover, Fcα/μR-deficient mice show prolonged antigen retention by marginal zone B (MZB) cells and FDCs. In vitro studies demonstrate that interaction of the IgM immune complex with Fcα/μR partly suppress TI antigen retention by MZB cells. We further show that downregulation of complement receptor (CR)1 and CR2 or complement deprivation by in vivo injection with anti-CR1/2 antibody or cobra venom factor attenuates antigen retention by MZB cells and germinal center formation after immunization with TI antigens in *Fcα/μR$^{-/-}$* mice. Taken together, these results suggest that Fcα/μR negatively regulates TI antigen retention by MZB cells and FDCs, leading to suppression of humoral immune responses against T-independent antigens.

Fc receptor | IgM | follicular dendritic cells (FDCs) | memory B cells | affinity maturation

IgM is an antibody class common to all vertebrates that constitutes most of the natural antibodies in the pleural and peritoneal cavities of naive hosts (1, 2). In addition, IgM is the first antibody to be produced by naive B cells upon antigen recognition. Therefore, IgM is believed to play an important role in innate immunity against variable bacterial and viral infections (3, 4). Binding of IgM with an antigen initiates the complement cascade, resulting in the acceleration of cellular and humoral immune responses (1). Mice lacking complement 3 or complement 4, or their receptors, complement receptor 1 (CR1) and complement receptor 2 (CR2) (CD35/21), show impaired IgG production in response to T-dependent (TD) antigens (5, 6). Mice lacking the secreted form of IgM (sIgM) also show markedly impaired antibody production and germinal center (GC) formation against TD antigens (7, 8). Thus IgM plays pivotal roles both in innate immunity and in the linkage between innate and adaptive immunity. However, the role of IgM in humoral immune responses against T-independent (TI) antigens is incompletely understood.

During humoral immune responses against TD antigens, an increase in GC size and number is induced in the lymphoid organs; the GC is a principal site for antibody class switching, affinity maturation, and memory B-cell generation (9–11). Follicular dendritic cells (FDCs) play a pivotal role in GC formation by retaining and presenting antigens to follicular B cells (12, 13). In contrast to TD antigens, TI antigens such as polysaccharides and glycolipids of encapsulated bacteria quickly stimulate marginal zone B (MZB) cells in the marginal zone of the follicle to produce IgM and IgG3 class antibodies (14, 15). Although antibody production against TI antigens occurs outside the follicular region, without GC formation (16), several reports have demonstrated that under certain conditions GCs are induced against TI antigens (17, 18). However, the regulation and the immunological consequences of GC formation against TI antigens remain unclear.

We previously identified an Fc receptor for IgM and IgA that we designated Fcα/μR (19, 20). The *Fcα/μR* gene has been mapped to chromosome 1 (1F in mice and 1q32.3 in humans) (19, 21) near several other Fc receptor genes, including *FcγR-I, II, III,* and *IV, FcεRI*, and the *polymeric IgR* (22–24), and Fc receptor homologues (25). Fcα/μR is expressed on the majority of follicular B cells and macrophages but not on granulocytes or T and natural killer cells. Although, unlike other immunoglobulin isotypes, IgM is present in all the vertebrate classes, Fcα/μR is the only receptor for IgM that thus far has been identified on hematopoietic cells of humans and rodents.

We show here that Fcα/μR is expressed preferentially on FDCs and MZB cells, as well as on follicular B cells, in the spleen. Interaction of IgM with Fcα/μR negatively regulates humoral immune responses against TI antigens.

Results

Increased GC Formation in Response to T-Independent Antigen in *Fcα/μR$^{-/-}$* Mice. To investigate the role of Fcα/μR in humoral immune responses in vivo, we established *Fcα/μR*-deficient (*Fcα/μR$^{-/-}$*) mice (see *SI Materials and Methods* and Fig. S1). Because Fcα/μR is expressed on B cells and FDCs (Fig. S2), we examined whether lack of Fcα/μR expression affected B-cell differentiation. Naive *Fcα/μR$^{-/-}$* mice had lymphocyte populations of normal composition in the spleen and showed no differences from their control littermates (*Fcα/μR$^{+/+}$*) in each population of B-cell subsets in the spleen, bone marrow (BM), or peritoneal cavity (data not shown), suggesting that Fcα/μR is not involved in the development of B cells or other lymphocyte lineages. We also observed no differences in the titers of each subclass of IgG and IgM in the sera of naive *Fcα/μR$^{-/-}$* mice (Fig. S3*A*).

Fcα/μR$^{-/-}$ mice demonstrated normal antibody responses

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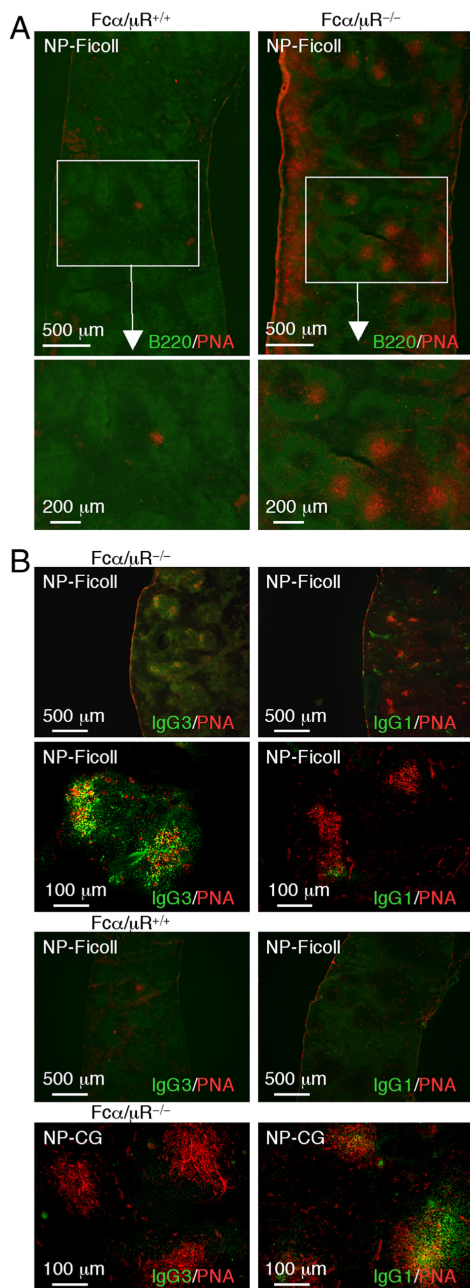

Fig. 1. GC reaction against T-independent (TI) antigens in $Fc\alpha/\mu R^{-/-}$ mice. $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice were immunized i.p. with the TD antigen (NP-CG) emulsified with alum or with the TI antigen NP-Ficoll. A week (for NP-Ficoll) or 2 weeks (for NP-CG) after the immunization, spleen sections were stained with biotinylated PNA, followed by Alexa594-conjugated streptavidin and FITC-conjugated anti-B220 (*A*) or with anti-mouse IgG3 or IgG1 (*B*). Data are representative of 3 independent experiments.

after immunization with the TD antigen 4-hydroxy-3-nitrophenylacetyl-chicken gamma globulin (NP-CG), the TI type 1 antigen NP-LPS, or the TI type 2 antigen NP-Ficoll (Fig. S3*B*). Unexpectedly, however, immunization with either of the TI antigens significantly increased the number and size of GCs in the spleen in $Fc\alpha/\mu R^{-/-}$mice but not $Fc\alpha/\mu R^{+/+}$ mice (Fig. 1*A* and data not shown). Although these GCs were Bcl-6$^+$, B220^{dull}, and IgD^{low}, similar to those induced after immunization with TD antigens (data not shown), they preferentially produced IgG3 (Fig. 1*B*), rather than the IgG1 detected in the GC B cells induced by the TD antigen NP-CG. Analyses by flow cytometry showed that the numbers of GC B cells, defined as B220$^+$, GL7$^+$ cells (26), increased significantly in $Fc\alpha/\mu R^{-/-}$ mice after immunization with the TI antigens NP-Ficoll and NP-LPS but not after immunization with the TD antigen NP-CG (Fig. 2*A* and Table S1). Similar results were obtained by flow cytometry when GC B cells were defined more specifically as PNA^{high}, GL7$^+$ cells (Fig. 2*B*). Depletion of CD4$^+$ T cells by in vivo injection of an anti-CD4 monoclonal antibody diminished the number of GC B cells after immunization with NP-CG but not after immunization with NP-Ficoll in both $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice (Fig. 2*A*), demonstrating that the increased GC reaction to NP-Ficoll in $Fc\alpha/\mu R^{-/-}$ mice was a T-cell-independent event.

To determine the cell types expressing Fcα/μR responsible for the increased GC formation in response to TI antigens in $Fc\alpha/\mu R^{-/-}$ mice, we established BM chimeric $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice reconstituted with either $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ BM cells. Because FDCs are radio resistant and therefore are not replaced by donor cells following BM transfer (27), these BM chimeric mice would have donor-derived B cells and recipient-derived FDCs. Immunization with NP-Ficoll increased the number of GC B cells in both $Fc\alpha/\mu R^{+/+}$ and $Fc\alpha/\mu R^{-/-}$ mice reconstituted with $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ BM cells, respectively, to a significantly greater degree than in $Fc\alpha/\mu R^{+/+}$ mice reconstituted with $Fc\alpha/\mu R^{+/+}$ BM cells (Fig. 2*C*). These results suggest that lack of Fcα/μR expression on both FDCs and B cells is responsible for the enhanced GC formation in response to TI antigens.

Generation of Memory B Cells in Response to TI Antigens in *Fcα/μR*$^{-/-}$ Mice. We then examined whether enhanced GC formation led to memory B-cell generation after immunization with TI antigens in $Fc\alpha/\mu R^{-/-}$ mice. $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice were immunized with NP-Ficoll and were re-challenged with the same antigen 12 weeks after the first immunization. Although the antibody titers again increased in both $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice 1 week after the second immunization, they did not exceed those at 1 week after the first immunization in either group of mice (Fig. S4). These results suggest that memory B cells specific to NP-Ficoll had not been generated. However, it also was possible that the large amount of anti-NP IgG3 that was still detectable in the sera of both groups of mice before the second immunization and that might have been produced by long-lived plasma cells was veiling a small recall response by memory B cells in response to NP-Ficoll.

To dissect further the antibody responses by memory B cells to the second immunization, spleen cells or BM cells from $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice before or 7 weeks after immunization with NP-Ficoll were transferred into SCID mice, which then were challenged with the same antigen on the following day (Fig. 3*A*). A week after the immunization, NP-specific IgG3 production in the SCID mice that had been given the $Fc\alpha/\mu R^{-/-}$ BM cells was significantly greater than that in the SCID mice given the $Fc\alpha/\mu R^{+/+}$ BM cells (Fig. 3*B*). We barely detected NP-specific IgG1 in either group of SCID mice (Fig. 3*B*). In contrast, we did not observe any difference in NP-specific IgG3 production between SCID mice transferred with spleen cells from $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice (data not shown). Because NP-specific IgG3 was not detected in SCID mice that received BM cells from primed mice without the second immunization (Fig. 3*B*), long-lived plasma cells may absent or very rare in BM cells. Collectively, these results suggest that enhanced GC formation in response to TI antigens in $Fc\alpha/\mu R^{-/-}$ mice led to the generation of memory B cells producing IgG3.

Affinity Maturation of IgG3$^+$ B Cells Specific to TI Antigens in *Fcα/μR*$^{-/-}$ Mice. We next examined whether increased GC formation also was associated with affinity maturation of B cells in $Fc\alpha/\mu R^{-/-}$ mice. $Fc\alpha/\mu R^{-/-}$ mice and $Fc\alpha/\mu R^{+/+}$ mice were

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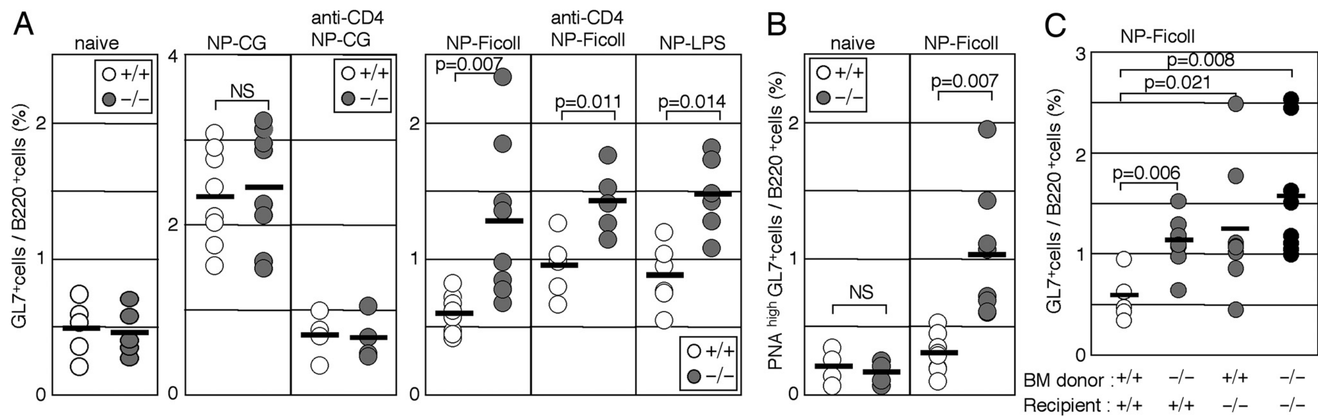

Fig. 2. GC B cells after immunization with T-dependent (TD) or T-independent (TI) antigens in $Fc\alpha/\mu R^{-/-}$ mice. (*A*, *B*) $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice (n = 5–9) were immunized i.p. with TD antigen (NP-CG) emulsified with alum or with TI antigens NP-Ficoll or NP-LPS. A week (for NP-Ficoll and NP-LPS) or 2 weeks (for NP-CG) after the immunization, spleen cells were stained with FITC-conjugated anti-GL-7 and PE-conjugated anti-B220 (*A*) or biotinylated PNA, followed by APC-conjugated streptavidin (*B*). To deplete $CD4^+$ T cells, mice were injected with anti-CD4 (GK1.5) mAb before immunization. Data are representative of 3 independent experiments. (*C*) We injected 5×10^6 BM cells from $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice via the tail vein into lethally irradiated $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice (n = 6–8/group). BM chimeric mice were immunized with NP-Ficoll 6 to 8 weeks later, and $GL7^+$ cells among $B220^+$ cells in the spleen were analyzed 1 week after immunization, as described previously. Data are representative of 2 independent experiments.

immunized with NP-Ficoll or NP-LPS and then were re-challenged with the same antigens 12 weeks later. Sera were collected 1 week and 12 weeks after the first antigen challenges and 1 week after the second antigen challenges and were measured for affinity of NP-specific antibody (Fig. 4*A*). The affinity for NP-specific IgG3 was comparable between $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice 1 week and 12 weeks after the first antigen challenges (data not shown). Although in $Fc\alpha/\mu R^{+/+}$ mice the affinity for NP-specific IgG3 after the second immunization was comparable to that after the first immunization, $Fc\alpha/\mu R^{-/-}$ mice showed significantly higher affinity for NP-specific IgG3 after the second immunization than after the first immunization (Fig. 4*B* and Fig. S5). In contrast, affinities for NP-specific IgG1 were low after the first immunization and were not altered after the second antigen challenge in both $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice (Fig. 4*B* and Fig. S5). These results indicate that $Fc\alpha/\mu R^{-/-}$ mice showed affinity maturation of NP-specific IgG3 after the second challenge with the TI antigens NP-Ficoll or NP-LPS.

To elucidate the molecular basis of the affinity maturation in $Fc\alpha/\mu R^{-/-}$ mice, we examined whether NP-Ficoll induced somatic hypermutation (SHM) in NP-specific $V_H186.2$ $IgG3^+$ B cells in $Fc\alpha/\mu R^{-/-}$ mice. $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice were immunized with NP-Ficoll; then PCR was performed to amplify $V_H186.2$ IgG3 clones from the spleen. We analyzed more than 10 amplified clones and barely detected the $V_H186.2$ clone from the spleen of naive $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice. However, more than half the clones amplified from $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice 1 week after immunization were $V_H186.2$ clones, suggesting that the $V_H186.2$ clone was expanded in response to the immunization. We observed only a few mutations in the $V_H186.2$ clones from $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice 1 week after immunization (Fig. S6). However, in recall responses, $Fc\alpha/\mu R^{-/-}$ mice had higher frequencies of SHM in

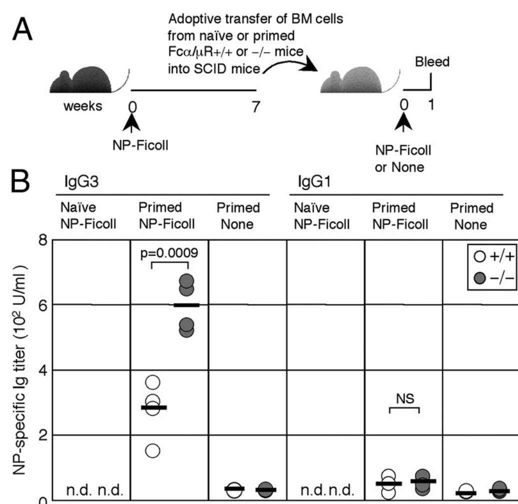

Fig. 3. Recall response against TI antigen in $Fc\alpha/\mu R^{-/-}$ mice. (*A*) $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice were immunized with NP-Ficoll or were not immunized (naive mice). BM cells from naive mice or from the $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice primed with NP-Ficoll were transferred to SCID mice 7 weeks after immunization. The SCID mice then were re-challenged with the same antigen or were not re-challenged. (*B*) Anti-NP IgG3 or IgG1 titers in SCID mice were determined 1 week after the second challenge. n.d., not detected.

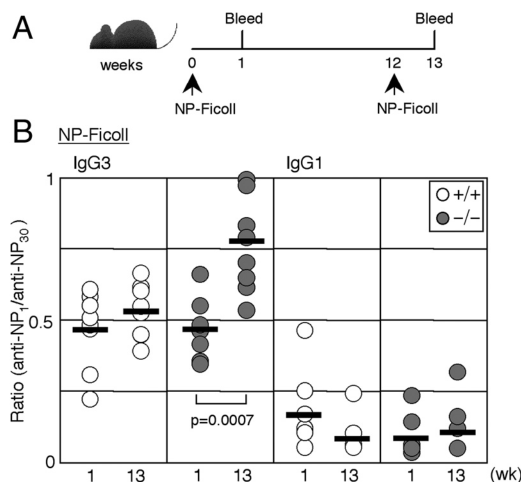

Fig. 4. Affinity maturation after immunization with TI antigen in $Fc\alpha/\mu R^{-/-}$ mice. (*A*) $Fc\alpha/\mu R^{+/+}$ or $Fc\alpha/\mu R^{-/-}$ mice were immunized with NP-Ficoll and were re-challenged with the same antigen 12 weeks later. (*B*) The affinities of anti-NP IgG3 and IgG1 in the sera were determined 1 week after the first immunization and after the second immunization, as described in the experimental procedures.

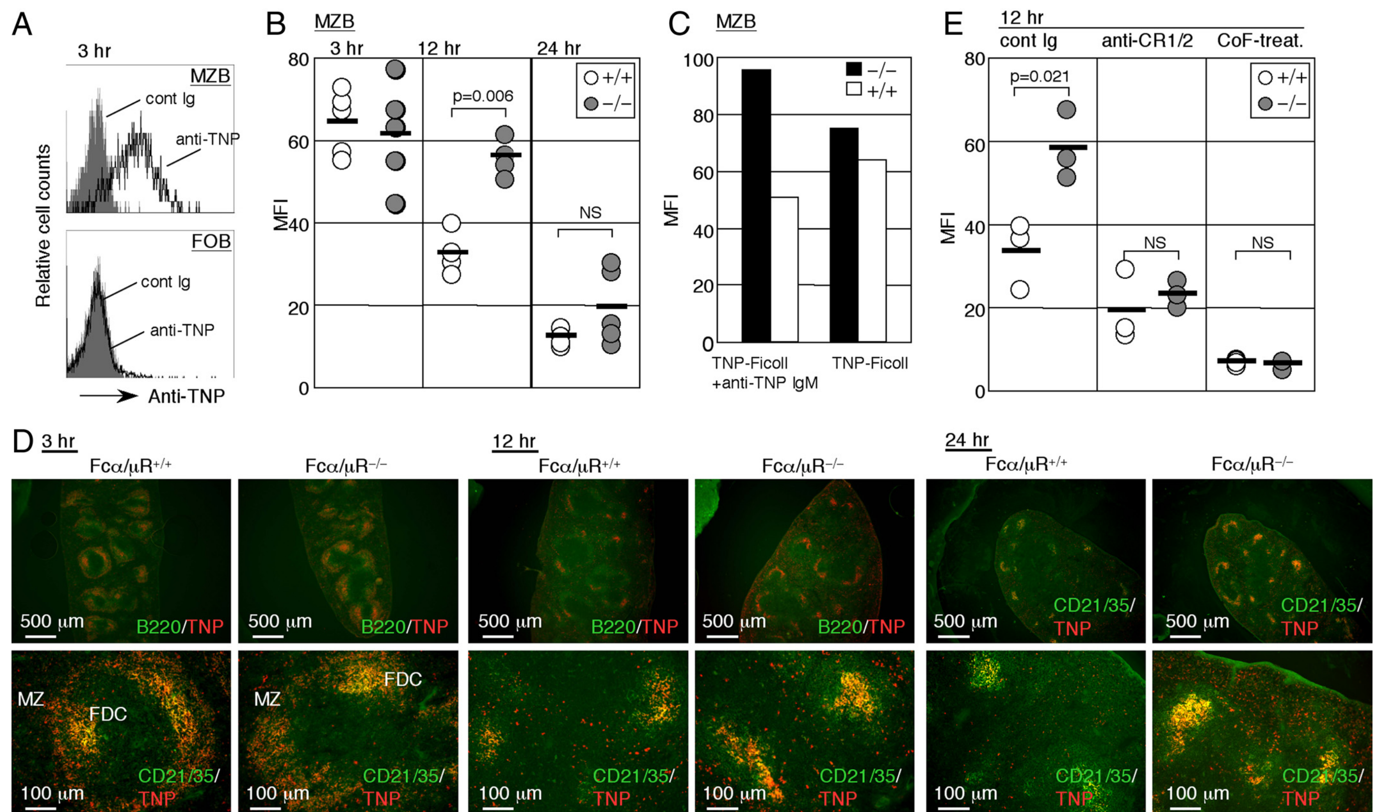

Fig. 5. TI antigen retention on MZB cells and FDCs in *Fcα/μR*$^{-/-}$ mice. (*A*, *B*) *Fcα/μR*$^{+/+}$ or *Fcα/μR*$^{-/-}$ mice were injected i.v. with 50 μg of TNP-Ficoll. Spleen cells were stained with biotin-conjugated anti-TNP, followed by APC-conjugated streptavidin, FITC-conjugated anti-CD21/35, and PE-conjugated anti-CD23 3, 12, or 24 hours after injection. (*A*) Representative flow cytometry profiles of TNP signal on MZB cells (CD21/35^{high}, CD23^{-}) and FOB cells (CD21/35^{+}, CD23^{+}) are shown. (*B*) The mean fluorescence intensity (MFI) of the TNP signal on MZB cells is shown. (*C*) Spleen cells from *Fcα/μR*$^{+/+}$ or *Fcα/μR*$^{-/-}$ mice were incubated with TNP-Ficoll alone or with anti-TNP IgM (IgM IC) in the presence of *RAG*-deficient mouse serum. Cells were stained with biotin-conjugated anti-TNP, followed by APC-conjugated streptavidin, FITC-conjugated anti-CD21/35, and PE-conjugated anti-CD23 for flow cytometry analysis. The MFI of TNP signals on MZB- and FOB-gated cells is shown. (*D*) After antigen injection, the spleen sections were stained with biotin-conjugated anti-TNP, followed by Alexa 594-conjugated streptavidin and FITC-conjugated anti-CD21/35 or anti-B220. (*E*) *Fcα/μR*$^{+/+}$ or *Fcα/μR*$^{-/-}$ mice were injected i.v. with anti-CR1/2, control antibody, or CoF before immunization with TNP-Ficoll. Spleen cells were stained with biotin-conjugated anti-TNP 12 hours after the immunization, followed by APC-conjugated streptavidin, FITC-conjugated anti-CD1d, and PE-conjugated anti-CD23. The MFI of TNP signal on MZB-gated cells (CD1d^{high}, CD23^{-}) is shown.

the V_H genes of anti-NP IgG3 than did *Fcα/μR*$^{+/+}$ mice (Fig. S6). These increased SHM might cause affinity maturation of NP-specific IgG3 in *Fcα/μR*$^{-/-}$ mice.

Prolongation of TI Antigen Retention on MZB Cells and FDCs in *Fcα/μR*$^{-/-}$ Mice. Our next goal was to determine how Fcα/μR expressed on FDCs and B cells is involved in GC formation. Because MZB cells and FDCs capture antigens efficiently, we speculated that Fcα/μR was involved in TI antigen retention on MZB cells and/or FDCs. To test this hypothesis, we injected 2,4,6-trinitrophenyl (TNP)-Ficoll i.v. into *Fcα/μR*$^{-/-}$ or *Fcα/μR*$^{+/+}$ mice and analyzed antigen retention by using anti-TNP antibody. In both *Fcα/μR*$^{-/-}$ mice and *Fcα/μR*$^{+/+}$ mice, flow cytometry analyses detected the TNP signal on MZB cells (CD21/35^{high}, CD23^{-} cells) but not on follicular B (FOB) cells (CD21/35^{+}, CD23^{+} cells) 3 hours after TNP-Ficoll injection (Fig. 5*A* and data not shown). The extent of antigen retention on MZB cells, as determined by the intensity of the TNP signals, was comparable in *Fcα/μR*$^{-/-}$ mice and *Fcα/μR*$^{+/+}$ mice 3 hours after antigen injection. Although the TNP signal on MZB cells gradually declined with time, its diminution was much slower in *Fcα/μR*$^{-/-}$ mice than in *Fcα/μR*$^{+/+}$ mice (Fig. 5*B*).

To examine whether IgM is involved in TI antigen retention by MZB cells, we incubated MZB cells derived from *Fcα/μR*$^{-/-}$ mice and *Fcα/μR*$^{+/+}$ mice with either TNP-Ficoll alone or an immune complex (IC) of TNP-Ficoll and anti-TNP IgM in a culture medium containing sera from *RAG*$^{-/-}$ mice (the medium contained complement but not IgM antibody) and analyzed antigen binding by flow cytometry using anti-TNP antibody. TNP-Ficoll retention (as determined from the mean fluorescence intensity of anti-TNP antibody) by *Fcα/μR*$^{-/-}$ cells and *Fcα/μR*$^{+/+}$ MZB cells in the absence of anti-TNP IgM was comparable, but the *Fcα/μR*$^{-/-}$ MZB cells captured the IgM IC to a greater degree than did the *Fcα/μR*$^{+/+}$ MZB cells (Fig. 5*C*). In contrast, antigen retention was not observed, even in the presence of anti-TNP IgM, on either *Fcα/μR*$^{-/-}$ or *Fcα/μR*$^{+/+}$ FOB cells (data not shown). These results indicated that the interaction of Fcα/μR with IgM IC inhibited TI antigen retention by MZB cells.

Immunohistochemical analyses showed that the antigen was preferentially localized in the marginal zone area 3 hours after antigen injection in both *Fcα/μR*$^{-/-}$ and *Fcα/μR*$^{+/+}$ mice (Fig. 5*D*). Notably, the TNP signal also was detected on FDCs in the follicles in both *Fcα/μR*$^{-/-}$ and *Fcα/μR*$^{+/+}$ mice (Fig. 5*D*), suggesting that TNP-Ficoll is captured by the FDCs as early as 3 hours after antigen injection. Although 12 hours after antigen injection the TNP signal was scarcely detected in the marginal zone area in both *Fcα/μR*$^{-/-}$ and *Fcα/μR*$^{+/+}$ mice and on the FDCs of *Fcα/μR*$^{+/+}$ mice, it still was readily detectable on the FDCs of *Fcα/μR*$^{-/-}$ mice (Fig. 5*D*). These results indicate that the retention of TI antigen on MZB cells and FDCs is prolonged in *Fcα/μR*$^{-/-}$ mice.

Involvement of the Complement Pathway in Humoral Immune Responses against TI Antigens in *Fcα/μR*$^{-/-}$ Mice. Previous reports have demonstrated that humoral immune responses against TI antigens are defective in the absence of complement (28, 29). To determine whether the complement pathway is involved in the increased humoral immune response against TI antigens in *Fcα/μ*R$^{-/-}$ mice, we injected *Fcα/μ*R$^{-/-}$ and *Fcα/μ*R$^{+/+}$ mice with the anti-CR1/2 monoclonal antibody 7G6, which is able to downmodulate the expression of CR1 and CR2 (CD35/21) (30). These mice were injected with the TI antigen TNP-Ficoll 2 days later. Flow cytometric analyses showed that, 12 hours after the antigen injection, antigen retention on the MZB cells of *Fcα/μ*R$^{-/-}$ mice had returned to the level in *Fcα/μ*R$^{+/+}$ mice (Fig. 5*E*). Because antigen retention by MZB cells is associated with GC formation, we next examined whether the presence of 7G6 antibody also affected GC formation in *Fcα/μ*R$^{-/-}$ mice. *Fcα/μ*R$^{-/-}$ and *Fcα/μ*R$^{+/+}$ mice were immunized with NP-Ficoll 2 days after injection with 7G6 antibody. A week after the immunization, flow cytometric analyses demonstrated that the number of GC B cells in the spleen of *Fcα/μ*R$^{-/-}$ mice had returned to the level in *Fcα/μ*R$^{+/+}$ mice (Fig. S7*A*). Supporting these results, complement deprivation by injection with cobra venom factor (CoF) attenuated antigen retention and GC formation in response to TI antigens in *Fcα/μ*R$^{-/-}$ mice (Fig. 5*E* and Fig. S7*A*). Moreover, the reduction of GC formation by CoF in *Fcα/μ*R$^{-/-}$ mice resulted in the diminution of affinity maturation of IgG3 antibody (Fig. S7*B*). Taken together, these results suggest that the increased antigen retention on MZB cells and enhanced GC formation in *Fcα/μ*R$^{-/-}$ mice depends on CR1/2.

Discussion

Our results represent the characterization of mice deficient in Fcα/μR. We demonstrated that TI antigens induce enhanced GC formation (Figs. 1 and 2), IgG3 memory response (Fig. 3), and affinity maturation (Fig. 4) in *Fcα/μ*R$^{-/-}$ mice. In contrast, we observed no differences between *Fcα/μ*R$^{+/+}$ mice and *Fcα/μ*R$^{-/-}$ mice in humoral immune response against TD antigens. Although an increase in GC number and size obviously is induced in response to TD antigens (9–11), several lines of evidence have demonstrated that GCs also can be induced against TI antigens, although the response is not as marked as the response to TD antigens (17, 18). However, the structural and functional characteristics of TI antigen-induced GCs have not been elucidated. Although GC reaction was enhanced in *Fcα/μ*R$^{-/-}$ mice after immunization with TI antigens, antibody titers in the sera were not elevated, an observation that is in line with previous findings (31). Instead, we observed increased SHM in the V_H genes of IgG3 and affinity maturation of IgG3 against NP-conjugated TI antigens in *Fcα/μ*R$^{-/-}$ mice (Fig. S6), consistent with previous reports that SHM occurs within GCs, albeit at low levels, in response to TI antigens (18, 32). We observed increased SHM of anti-NP IgG3 in *Fcα/μ*R$^{-/-}$ mice, and this increase might have been associated with affinity maturation of IgG3 after the second challenge by TI antigens. Moreover, we showed that BM cells from *Fcα/μ*R$^{-/-}$ mice (but not from *Fcα/μ*R$^{+/+}$ mice) primed with TI antigens produced enhanced amounts of anti-NP IgG3 in SCID mice in response to the second antigen challenge (Fig. 3). We indeed detected IgG3, rather than IgG1, localized within enlarged GC areas after immunization with NP-Ficoll (Fig. 1*B*). These results collectively suggest that memory B cells that produced high-affinity IgG3 specific to TI antigens were generated as part of the enhanced GC formation in response to TI antigens in *Fcα/μ*R$^{-/-}$ mice.

We also demonstrated prolonged retention of TI antigen on MZB cells and FDCs in *Fcα/μ*R$^{-/-}$ mice (Fig. 5). Retention of TI antigens by MZB cells is diminished in complement 3-deficient mice (28, 33), indicating that this process is complement dependent. Here, we found that modulation of complement receptor expression by in vivo injection with anti-CR1/2 antibody or complement deprivation by CoF injection abrogated the TI antigen retention on MZB cells in both *Fcα/μ*R$^{-/-}$ and *Fcα/μ*R$^{+/+}$ mice (Fig. 5*E*). Moreover, this modulation also diminished the number of GC cells produced in response to TI antigens in *Fcα/μ*R$^{-/-}$ mice (Fig. S7*A*). Collectively, these results indicate that increased complement-dependent TI antigen retention on MZB cells leads to enhanced GC reaction in response to TI antigens in *Fcα/μ*R$^{-/-}$ mice.

Because the *SLAM* gene, which maps closely to the *Fcα/μ*R on chromosome 1, has been shown to affect lymphocyte activity in mice of the 129 strain (34), it is possible that the phenotype observed in the *Fcα/μ*R$^{-/-}$ mice might result from the remaining *SLAM* gene derived from E14 ES cells with the 129 genetic background. To address this issue, we subsequently established a new line of *Fcα/μ*R$^{-/-}$ mice by using BALB/c ES cells (Fig. S8 *A* and *B*). We also observed increased GC formation and antigen retention on MZB cells and FDCs after TI-antigen challenge in BALB/c Fcα/μR-deficient mice (Fig. S8 *C–F*). Thus, we concluded that the phenotype in the Fcα/μR-deficient mice of B6/129 background resulted from the deficiency of Fcα/μR.

An important question that has remained unanswered is the molecular mechanism of the functional association of the complement cascade with Fcα/μR. Our data suggest that Fcα/μR negatively regulates CR1/2-mediated antigen retention by MZB cells. The classic complement cascade is initiated by C1q binding to the Fc portion of IgM bound to an antigen (35). Therefore, a simple explanation would be that the binding of IgM IC to Fcα/μR prevents C1q from binding to the Fc portion of IgM of the IC, resulting in downregulation of the complement cascade on MZB cells. However, this scenario seemed unlikely, because we did not observe such competition between Fcα/μR and C1q for binding to the Fc portion of IgM in IC (data not shown). Another hypothesis that could explain the Fcα/μR-mediated negative regulation of complement-dependent antigen retention by MZB cells is that Fcα/μR and CR1/2 compete for IgM- or complement-mediated binding of antigen to each receptor. An alternative explanation would be that interaction of IgM IC with Fcα/μR mediates signals that affect CR1/2 function in complement binding. In the present study, we also showed that antigen retention is prolonged on FDCs that markedly express both Fcα/μR and CR1/2 (Fig. 5*D*). Therefore, it also is essential that we clarify whether and how Fcα/μR suppresses CR1/2-mediated antigen retention by FDCs. Further analyses are required to determine the molecular and functional association between Fcα/μR and CR1/2 on MZB cells and FDCs.

We demonstrated that the interaction of IgM with Fcα/μR is primarily involved in antigen retention in vitro (Fig. 5*C*). Therefore, mice deficient in sIgM might be similar to *Fcα/μ*R$^{-/-}$ mice. In fact, sIgM-deficient mice show increased IgG2a and IgG2b (*7*) or IgG3 (8) production in response to NP-Ficoll. We observed increased IgG3 production after the second NP-Ficoll challenge in *Fcα/μ*R$^{-/-}$ mice, which demonstrated a phenotype similar to that of sIgM mice in the context of enhanced humoral immune responses against TI antigens. On the other hand, sIgM-deficient mice also show markedly impaired antibody production and GC formation in response to TD antigens (7, 8) that was not observed in *Fcα/μ*R$^{-/-}$ mice, suggesting that IgM acts not only as a ligand for Fcα/μR but also has other important roles in the immune response. Of note, mice deficient in sIgM show accelerated development of IgG autoantibodies and autoimmune diseases (36, 37). These results suggest that interaction of the IgM immune complex with Fcα/μR suppresses the humoral immune response against self-antigens. Because most natural antibodies are polyreactive to TI antigens, including a variety of foreign antigens and self-antigens (1, 38), our results shed light on humoral immune responses against both infections and self-antigens.

Materials and Methods

Materials and methods for mice, antibodies, ELISA, immunohistochemistry, generation of BM chimeric mice, and the SHM assay used here are described in *SI Materials and Methods*.

Immunization. For every immunization, wild-type ($Fc\alpha/\mu R^{+/+}$) littermate mice were used as controls for $Fc\alpha/\mu R^{-/-}$ mice. $Fc\alpha/\mu R^{-/-}$ and $Fc\alpha/\mu R^{+/+}$ mice (8 to 12 weeks old) were immunized i.p. with 10 μg NP-CG emulsified with alum as a TD antigen. For TI antigen immunization, 10 μg NP-Ficoll or NP-LPS (Biosearch Technologies) was injected i.p. For recall response, mice were rechallenged with either TI antigen 12 weeks after primary immunization. Sera were collected 1 week after each immunization and were used for ELISA analysis. For in vivo depletion of CD4 T cells, mice were given 100 μg of anti-CD4 (GK1.5) mAb i.p. 2 days before immunization. For downmodulation of CR1/2 in vivo, mice were given 200 μg anti-CR1/2 (7G6) mAb i.p. 2 days before immunization. For complement depletion, 3 μg of CoF was injected into each mouse via the tail vein 1 day before immunization. For the BM cell transfer experiment, 2×10^7 BM cells obtained from $Fc\alpha/\mu R^{-/-}$ or $Fc\alpha/\mu R^{+/+}$ mice 7 weeks after NP-Ficoll immunization were transferred into CB17.SCID mice that were immunized with the same antigen 1 day later.

Affinity Measurement. Antibody affinity was measured by using plates coated with either NP_{30}-BSA or NP_1-BSA. Sera were diluted serially to determine the dilution multitude to the absorbance 2 times greater than background, by using HRP-conjugated goat Abs specific for each mouse immunoglobulin isotype. The ratios of dilution multitudes determined by each plate coated by NP_{30}-BSA or NP_1-BSA were calculated for individual sera.

Antigen Retention Analysis. Mice were injected with 50 μg of TNP-Ficoll (Biosearch) via the tail vein. The splenocytes then were stained with biotin-conjugated hamster anti-TNP IgG, followed by allophycocyanin (APC)-conjugated streptavidin in combination with FITC-conjugated anti-CD21/35 and phycoerythrin (PE)-conjugated anti-CD23 (PharMingen) for flow cytometric analyses. For immunohistochemical analyses, frozen sections of the spleen were incubated with biotin-conjugated hamster anti-TNP IgG (PharMingen), followed by Alexa 594-conjugated streptavidin (Invitrogen) in combination with FITC-conjugated anti-CD21/35 or anti-B220. For the in vitro experiment, TNP-Ficoll was incubated with anti-TNP mouse IgM (PharMingen) for 30 min at 37 °C and then cultured for 15 min at 37 °C with splenocytes in 1% $RAG^{-/-}$ mouse-derived serum containing TC buffer [140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Tris (pH 7.5), supplemented with 1% BSA]. The cells were stained for flow cytometric analysis as described earlier in the article.

Statistics. Statistical analyses were performed by using Student's unpaired *t* test.

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