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Enhanced B-1 Cell Development, But Impaired IgG Antibody Responses in Mice Deficient in Secreted IgM[1]

Marianne Boes,* Christine Esau,* Michael B. Fischer,† Tara Schmidt,* Michael Carroll,† and Jianzhu Chen[2]*

The role of endogenous natural IgM in promoting the adaptive Ab response was investigated in newly constructed mutant mice in which B cells do not secrete IgM but still express surface IgM and IgD and undergo class switching to express other Ig isotypes. While the mutant mice had relatively normal numbers of conventional B (B-2) cells in all tissues examined, unexpectedly, B-1 cells in the peritoneum and spleen were approximately threefold more abundant. The elevated levels of B-1 cells were already detectable at 4 wk of age and were stably maintained throughout life. The levels of serum IgG2a, IgG3, and IgA were also elevated in the mutant mice at an early age. IgG2a response to a T cell-independent Ag was augmented, whereas IgG Ab responses to suboptimal doses of a T cell-dependent Ag were impaired. The latter defect was associated with fewer splenic germinal centers, impaired Ab affinity maturation, and less Ag trapping on follicular dendritic cells. Together, these findings demonstrate a physiologic role of natural IgM in the feedback regulation of B-1 cell development, the regulation of IgG2a production, and the promotion of efficient B-2 cell Ab responses. ***The Journal of Immunology*****, 1998, 160: 4776–4787.**

Among the five classes of Igs in humans and mice, IgM is unique in its physiologic and structural properties. IgM is the first class of Abs produced during ontogeny. Because it is present in human cord blood, in "antigen-free" mice, and in normal individuals in the absence of apparent stimulation by specific Ags, the spontaneously produced IgM is referred to as natural Ab (reviewed in Refs. 1 and 2). Natural IgM is usually encoded by germline V gene segments with no or very few N-region nucleotide additions or somatic hypermutation (3–5). As a result, it tends to be polyreactive to both endogenous and exogenous components including Ig molecules and the conserved bacterial surface structures such as phosphatidyl choline (6–10). It also tends to have relatively low Ag-binding affinities. However, the secreted form of IgM is a pentamer, and a stable interaction with polymeric Ags is readily achieved through multiple binding sites. Moreover, the multimeric structure makes IgM a potent complement activator; a single bound IgM molecule can activate complement to lyse a RBC, while one thousand IgG molecules are required (11). Due to its natural presence, its polyreactivities with high avidities, and its exquisite ability to activate complement, natural IgM is thought to bind to invading pathogens immediately upon their entry, activating complement to provide a first line of defense against the infection (1, 2, 12). The resulting immune complex of Ag, IgM, and the activated complement component C3 (Ag-IgM-C) has also been postulated to augment the ensuing Ab response (12, 13–16). Supporting this notion, simultaneous administration of Ag and exogenous Ag-specific IgM has often yielded an enhanced Ab response compared with administration of Ag alone (17–21). However, the effect of exogenous IgM varies widely with different concentrations of Ag and Ab, and occasionally, the coinjected IgM has inhibited the Ab response (22).

To clarify the ambiguities associated with the use of exogenous IgM and to examine the role of endogenous IgM in the Ab response, we have constructed a novel mouse strain that is deficient in secreted IgM. In these mutant mice, B cells are still capable of expressing surface IgM and IgD and secreting other Ig isotypes. Unexpectedly, mutant mice have elevated levels of B-1 cells in both the peritoneum and spleen. B-1 cells differ from the conventional B (B-2) cells in their differentiation during fetal and neonatal development, in a capacity for self renewal, and in their characteristic localization in lamina propria and pleural and peritoneal cavities in adult mice (6–8). Because B-1 cells secrete the major portion of natural IgM, our finding in the mutant mice suggests a physiologic role of natural IgM in the feedback regulation of B-1 cell differentiation and/or maintenance. In addition, our data demonstrate that natural IgM secreted by B-1 cells promotes the T cell-dependent (TD)[3] Ab response by conventional B cells.

Materials and Methods

Construction of the mutant mouse strain

A mutant mouse strain that does not secrete IgM but still expresses membrane-bound IgM was made by targeted mutagenesis in embryonic stem (ES) cells. In the targeting vector, the μ_s exon and its three downstream polyadenylation (poly(A)) sites were replaced by a cDNA fragment encoding the Cμ4 and μ_m exons (Fig. 1). The positive selectable marker neomycin phosphotransferase (*neo*) gene was inserted at the *Sph*I site 200 bp downstream of the μ_m poly(A) site so that the μ_m RNA processing was not affected by the inserted *neo* gene. The vector contained 2 kb and 1.5 kb

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[3] Abbreviations used in this paper: TD, T cell-dependent; ES, embryonic stem; poly(A), polyadenylation; CD40L, CD40 ligand; μ_m, membrane-bound form of μ heavy chain; μ_s, secreted form of μ heavy chain; FDC, follicular dendritic cells; AP, alkaline phosphatase; HRP, horseradish peroxidase; PNA, peanut agglutinin; TI, T cell-independent; NP-KLH, (4-hydroxyl-3-nitrophenyl)acetyl-keyhole limpet hemocyanin; *neo*, neomycin phosphotransferase gene; *tk*, thymidine kinase gene.

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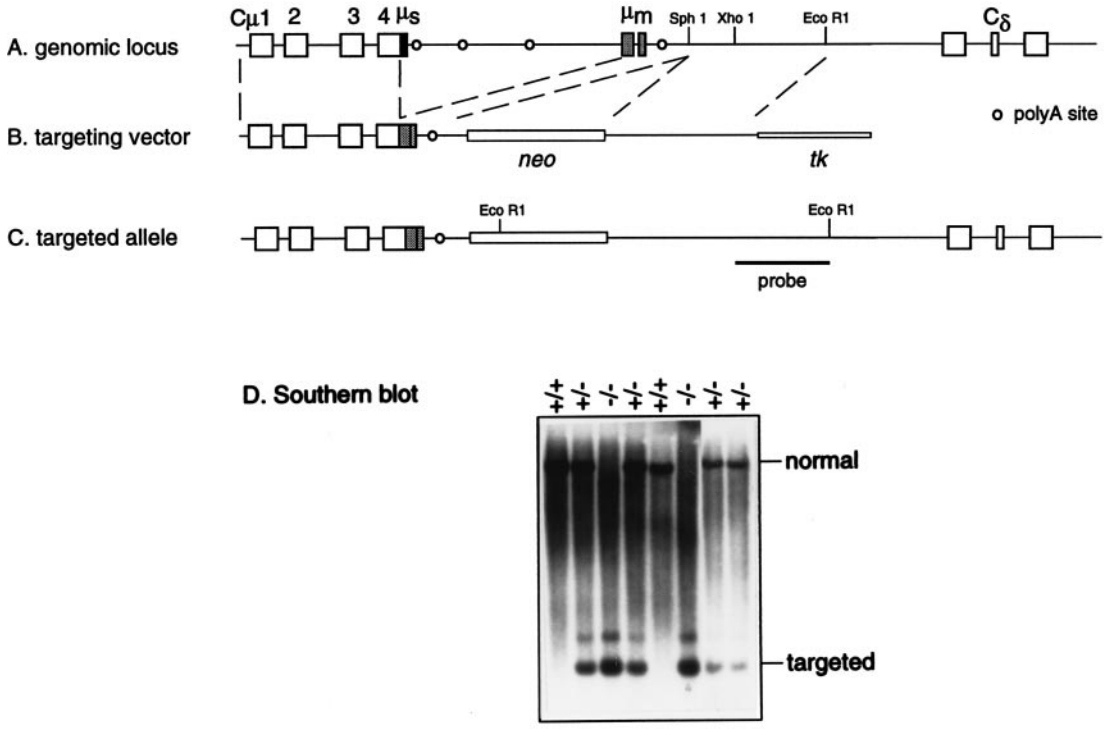

FIGURE 1. Targeted mutagenesis at the Cμ locus. *A*, Schematic diagram of the Cμ-Cδ locus. *B*, Schematic diagram of the targeting vector. *C*, Targeted replacement of μ_s exon and poly(A) sites with μ_m exons and poly(A) site. *D*, Southern blot analysis of genotypes of mutant mice. Tail DNA was digested with *Eco*RI, and the filter was hybridized with an 0.9-kb *Xho*I-*Eco*RI probe as depicted in *C*. The 13-kb fragment indicates the normal allele (+) and the 2-kb fragment indicates the targeted allele (−).

of homologous sequences at the 5′ and 3′ end of the *neo* gene, respectively. The negative selectable marker thymidine kinase (*tk*) gene was placed just outside of the 3′ homologous region (Fig. 1). After homologous recombination, only μ_m heavy chain can be expressed from the Cμ locus.

The linearized vector was transfected into J1 ES cells by electroporation. Transfectants were enriched for homologous recombinants by selection with G418 for the presence of the *neo* gene and gancyclovir for the absence of the *tk* gene (23). Resistant clones were screened for homologous recombinants by Southern blot with various probes (data not shown) and 2 clones (of 200) were found to have the properly targeted allele. Both mutant ES cell clones were injected into blastocysts from C57BL/6 mice. Resulting chimeras gave germline transmission of the mutated allele. Heterozygous mutant mice were intercrossed to obtain homozygous mutant mice. Mice were housed in specific pathogen-free facilities, and studies were performed according to institutional guidelines for animal use and care.

Flow cytometry analyses

Lymphocyte development in the mutant mice was analyzed by flow cytometry. Fluorescein- and phycoerythrin-conjugated mAbs specific for cell surface markers, including CD45R (B220), CD40, CD19, CD5, CD11b (Mac-1), CD23, MHC IA, CD4, CD8, CD90 (Thy-1.2), TCR$\alpha\beta$, and TCR$\gamma\delta$, were from PharMingen (San Diego, CA). Conjugated anti-IgM and -IgD were generously provided by Dr. Leonore Herzenberg (Stanford University, Stanford, CA). Single-cell suspensions were prepared from the spleen, lymph node, and thymus. Bone marrow cells were obtained by flushing femurs with cold PBS plus 2% FCS. Peritoneal cells were obtained by lavage. Erythrocytes in splenocyte and bone marrow cell suspensions were removed by lysis with 0.14 M NH_4Cl and 17 mM Tris · Cl, pH 7.4. Cells (5×10^5) were stained with conjugated Abs and analyzed by FACScan (Becton Dickinson, Mountain View, CA). Ten thousand live cells were collected for each sample.

B cell stimulation assay

Splenocytes prepared as described above were incubated with rat mAbs specific for CD90 (Thy-1.2), CD4, and CD8. The bound T cells were removed by magnetic immunoselection using goat anti-rat IgG-coated beads (Dynal, Oslo, Norway). The resulting B cells (>95%) were cultured in quadruplicates at 4×10^6/ml in RPMI 1640 plus 5% FCS, 10 mM HEPES, pH 7.2, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. B cells were stimulated with 1 μg/ml or 10 μg/ml of $F(ab')_2$ fragments of goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA); soluble CD40L-CD8α fusion proteins (as developed by Peter Lane (24)) cross-linked by 1 μg/ml anti-CD8 Ab; the combination of anti-IgM and cross-linked CD40L-CD8α fusion proteins; or different concentrations of LPS (Sigma, St. Louis, MO). After 48 h, cultures were pulsed with 0.5 μCi/ml of [^{3}H]thymidine, and cell proliferation as indicated by [^{3}H]thymidine incorporation was determined 6 h later.

Immunization

Eight- to twelve-week-old homozygous mutant mice and wild-type or heterozygous controls were used for immunizations with both TD and TI (T cell-independent) Ags. For TD Ag, different amounts of hapten (4-hydroxyl-3-nitrophenyl)acetyl-conjugated keyhole limpet hemocyanin (NP-KLH) in 0.1 or 0.2 ml of HBSS were injected i.v. through the tail vein on day 0 and again on day 22. Mice were bled from the tail vein at day 7, 14, 21, and 29 after primary immunization. For TI Ag, different amounts of NP-Ficoll in 0.2 ml of HBSS were injected i.p. Mice were bled on day 7 and 14 after immunization.

ELISA assay

Serum Ig levels were measured by ELISA (Southern Biotechnology Associates, Birminghan, AL). Plates were coated with goat anti-mouse Ig(M+G+A) and developed with horseradish peroxidase (HRP)-conjugated goat Abs specific for each mouse Ig isotype. The concentrations were calculated using the linear ranges of the dilution and purified mouse Ig isotypes as standards. NP-specific IgG Abs were measured by ELISA with NP-BSA-coated assay plates and developed with HRP-conjugated anti-IgG Abs. Ab affinity maturation was also determined by ELISA. Briefly, plates were coated with either NP_5-BSA or NP_{15}-BSA (generously provided by Dr. Garnett Kelsoe, University of Maryland, College Park, MD). Serial dilutions of serum were added to the coated plates, and the relative binding of total IgG Abs was determined using HRP-conjugated anti-IgG. The ratios of binding to NP_5-BSA and NP_{15}-BSA were calculated for individual mice using OD_{405} in the linear ranges of the assays.

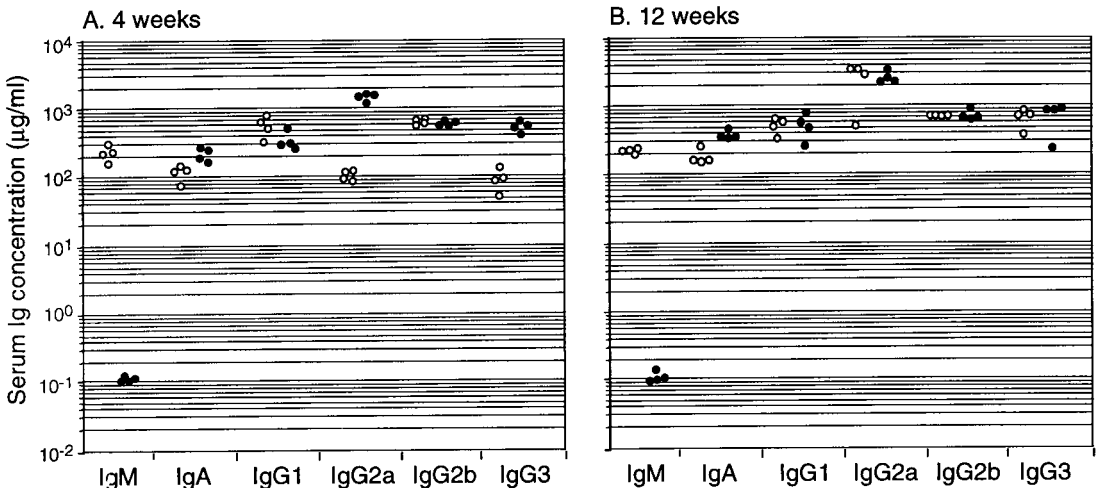

FIGURE 2. Serum Ig levels in wild-type (open circle) and mutant (closed circle) mice. Sera were collected from 4-, 8-, and 12-wk-old mice. The concentrations of individual Ig isotypes were determined by ELISAs in which the assay plates were coated with goat anti-mouse Ig(M+G+A) Abs and developed with HRP-conjugated isotype-specific goat Abs. Each point represents an individual mouse. Data from 8-wk-old mice were similar to those from 4-wk-old mice and are not shown. Serum Ig levels in heterozygous mutant mice were similar to those of age-matched wild-type mice. Serum IgE levels were not measured.

Immunohistochemistry

Spleens were harvested 7 days after secondary immunization and snap frozen in OCT embedding media. Serial, 4-μm-thick frozen sections were cut in a cryostat microtome, thaw mounted onto polyL-lysine-coated slides, air dried, fixed in ice-cold acetone for 10 min, and stored at −80°C. For two-color immunolabeling of germinal centers, spleen sections were incubated with biotin-conjugated anti-CD45R (B220) and HRP-conjugated peanut agglutinin (HRP-PNA, E-Y Laboratories, San Mateo, CA), followed by incubation with streptavidin-alkaline phosphatase (ExtrAvidin-AP, Sigma). Bound AP and HRP were visualized using naphthol AS-MX phosphate/Fast blue BB base and 3-aminoethylcarbazole, respectively (25). For Ag trapping, mice were immunized with either 1 or 10 μg of NP-KLH on day 0 and were then injected with 50 μg of NP-BSA-biotin on day 12. Spleens were harvested 18 h later. To visualize the trapped NP-BSA-biotin on the surface of follicular dendritic cells (FDCs), spleen sections were incubated with streptavidin-AP and HRP-PNA. Bound AP and HRP were visualized as described above.

Results

Construction of mutant mice that do not secrete IgM

Ig μ heavy chains are expressed in both membrane-bound (μ_m) and secreted (μ_s) forms from the same Cμ gene during B cell development (26). In association with either surrogate light chains λ_5 and VpreB or κ and λ light chains, the μ_m heavy chain is required for the stepwise progression from pro-B cells to Ab-secreting plasma cells (27). Therefore, to construct a mutant mouse strain in which B cells are rendered incapable of expressing μ_s, μ_m expression has to be preserved. The expression of μ_s and μ_m results from the differential utilization of poly(A) sites and alternative splicing of the same precursor RNA (Fig. 1) (28, 29). Utilization of poly(A) sites downstream of the μ_s exon produces a mature RNA that will be translated into the μ_s heavy chain. Utilization of the poly(A) site downstream of the μ_m exon leads to the deletion of the μ_s exon by splicing and to production of a mature RNA encoding the μ_m heavy chain. To engineer the locus so that only μ_m heavy chain is expressed, we employed homologous recombination to replace the μ_s exon and its three downstream poly(A) sites with a cDNA fragment encoding the μ_m exons already spliced to the Cμ4 exon (Fig. 1; see *Materials and Methods* for details). Two properly targeted ES cell clones were obtained and used to derive germline mutant mice. Mutant mice were identified by Southern blot analysis of tail DNA after *Eco*RI digestion and hybridization with an 0.9-kb *Xho*I-*Eco*RI probe (Fig. 1*C*). The normal allele gives rise to a 13-kb fragment, and the targeted allele gives rise to a 2-kb fragment (Fig. 1*E*).

Targeted mutation specifically blocks IgM secretion

To determine the effect of the targeted mutation on IgM secretion, sera were collected from wild-type and homozygous mutant mice at 4, 8, and 12 wk of age, and the concentrations of IgM, IgA, IgG1, -2a, -2b, and -3 were determined by ELISA. An average of 250 μg/ml of IgM was detected in the sera of wild-type mice at all three ages, whereas in the sera of mutant mice, IgM was only 0.1 μg/ml (Fig. 2). This level of IgM was <10-fold above the background in the recombination activating gene (*RAG*)-2 deficient mice (data not shown). Furthermore, the level of IgM did not change in mutant mice as they aged (up to 1 yr, data not shown). The residual serum IgM detected in mutant mice probably represents membrane IgM released from the B cell surface as a result of proteolytic degradation and/or B cell death. Thus, in practical terms, the targeted mutation renders mutant mice deficient in secreted IgM.

In contrast to the virtual absence of serum IgM, mutant mice had normal levels of serum IgG1 and -2b. The levels of IgG2a and -3 were significantly higher in mutant mice than in wild-type mice at 4 wk of age (Fig. 2*A*), but the differences disappeared when mice matured (Fig. 2*B*). Mutant mice also had slightly elevated levels of IgA. The elevated levels of serum IgG3 and IgA at early ages in mutant mice are probably due to the increased numbers of B-1 cells, while the elevated levels of IgG2a are probably due to other effects of the absence of natural IgM on the immune system (see below and *Discussion*). Nevertheless, in adult mice, the targeted mutation specifically abolishes IgM secretion without significantly affecting the overall levels of serum Ig.

Enhanced B-1 cell differentiation in mutant mice

The normal levels of serum IgG and IgA in adult mutant mice suggest a relatively normal B cell development in these mice. To determine the effect of the absence of natural IgM on lymphocyte development and to rule out any aberrant effect of the targeted mutation, the cell numbers in spleen were enumerated and the phenotypes of splenocytes were extensively characterized by flow

Table I. *Number and frequency of cells in different organs of wild-type and mutant mice*[a]

Organ	+/+ (n = 6)	−/− (n = 6)
Spleen		
Total cell number ($\times 10^{-7}$)	6.2 ± 1.2	6.7 ± 1.1
% B cells ($B220^+$, IgM^+)	59.6 ± 8.3	52.2 ± 5.6
% T cells ($CD5^+$, IgM^-)	32.3 ± 8.6	40.4 ± 7.0
% B-1 cells ($CD5^+$, IgM^+)	2.5 ± 0.4	6.3 ± 0.8
B-1 cell number ($\times 10^{-6}$)	1.6 ± 0.3	4.2 ± 0.8
Bone marrow		
Total cell number ($\times 10^{-6}$)[b]	8.0 ± 3.7	8.0 ± 1.9
% pro-B and pre-B cells ($B220^{low}$, IgM^-)	12.3 ± 1.8	16.4 ± 2.2
% immature and mature B cells ($B220^+$, IgM^+)	13.2 ± 1.1	19.3 ± 3.0
Peritoneum		
Total cell number ($\times 10^{-6}$)	3.2 ± 1.2	5.9 ± 1.5
% B-2 cells ($B220^+$, Mac-1^-)	4.5 ± 1.7	4.4 ± 2.0
% B-1 cells ($B220^+$, Mac-1^{low})	40.3 ± 12.3	66.9 ± 4.9
B-1 cell number ($\times 10^{-6}$)	1.3 ± 0.8	4.0 ± 1.0
% macrophages (Mac-1^{high})	45.9 ± 13.1	20.7 ± 4.2
% T cells ($CD5^+$, IgM^-)	1.7 ± 0.7	2.1 ± 0.8

[a] Six 2- to 3-mo-old mice were anlyzed by FACS in two separate experiments. B-1 cell numbers in the spleen and peritoneum were calculated by multiplying total cell numbers with the percentages of B-1 cells in individual mice. Averages and standard deviations are shown.
[b] Cells were from one femur.

cytometry for the expression of various surface markers. While the total numbers of cells recovered from wild-type and mutant mice were very similar, the percentages of B ($B220^+$, IgM^+) cells showed some variation from mouse to mouse (Table I). However, the differences in the percentages of B cells in the mutant and wild-type mice were not significant ($p = 0.1$). Similarly to wild-type mice, almost all $B220^+$ splenic B cells from mutant mice expressed IgM (Fig. 3*A*), suggesting that targeted mutation did not grossly affect membrane-bound IgM expression. Furthermore, almost all IgM^+ B cells also expressed IgD (Fig. 3*A*), indicating that the introduced *neo* gene did not block significantly the transcription and alternative splicing to the δ locus. In addition, B cells from mutant mice expressed normal levels of CD40, CD19, CD23, CD16, and MHC IA (data not shown). Finally, histologic staining of spleen sections of mutant mice yielded normal numbers and morphologies of lymphoid follicles (data not shown). However, there was a clear difference in the B cell compartment in the spleen between mutant and wild-type mice. The percentage of $CD5^+$ B-1 cells was significantly increased in the spleen of mutant mice (Fig. 3*A*), resulting in an ~2.6-fold increase of B-1 cell numbers (Table I, $p < 0.01$). Consistently, more splenic B cells in the mutant mice were of B-1 cell phenotype: $B220^{low}$, IgM^{high}, IgD^{low}, and Mac-1^+ (Fig. 3*A* and data not shown). The elevated level of B-1 cells in mutant mice was already established by 4 wk of age (data not shown).

In the bone marrow, the total numbers of cells recovered were similar in wild-type and mutant mice, but the percentages of B-lineage cells showed considerable variation at different ages. For example, the percentages of pro-B and pre-B cells ($B220^{low}$, IgM^-) and immature and mature B cells ($B220^+$, IgM^+) had increased in mutant mice at 2 to 3 mo of age (Fig. 3*B* and Table I, $0.05 < p < 0.01$), but no difference was detected in mice at 1 mo of age (data not shown). Staining of bone marrow B cells for IgM and IgD failed to detect any consistent changes in the percentages of immature B cells (IgM^+, IgD^-) and mature B cells (IgM^+, IgD^+) (data not shown). The relatively normal number of B cells in the spleen of mutant mice suggests that the absence of natural IgM does not significantly affect conventional B cell differentiation in the bone marrow.

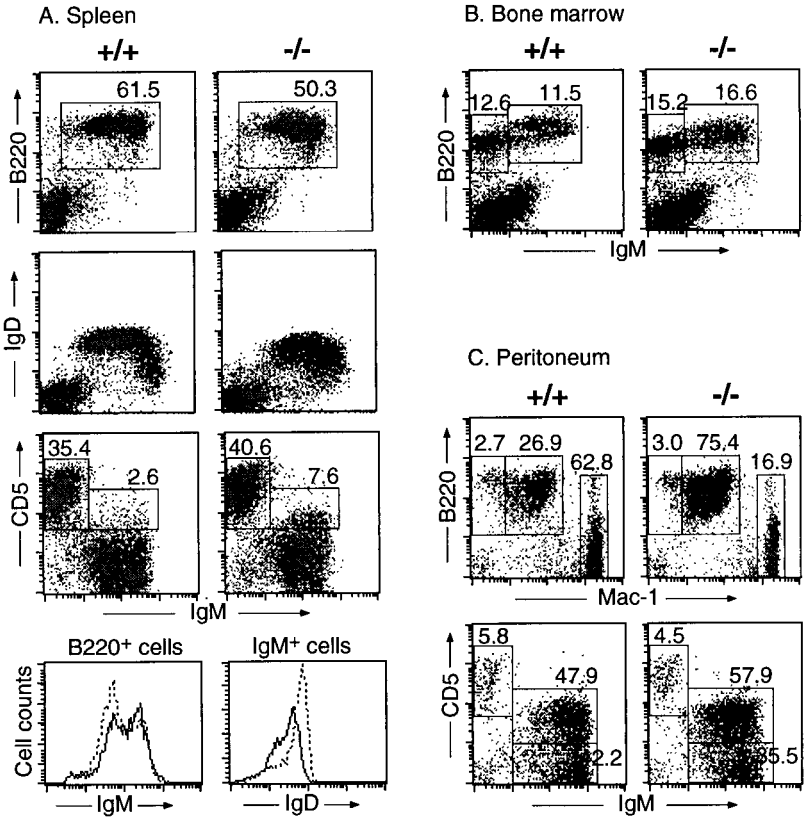

FIGURE 3. Flow cytometry analyses of B cell development in mutant mice. *A*, Flow cytometry analyses of splenocytes. Splenocytes from wild-type (+/+) and homozygous mutant (−/−) mice were stained with Abs specific for IgM and CD45R (B220), IgM and IgD, or IgM and CD5. The numbers indicate the percentages of cells in the gated areas. Histograms show the levels of IgM expression on $B220^+$ cells and IgD expression on IgM^+ cells. +/+, Dotted line; −/−, solid line. *B*, Flow cytometry analysis of bone marrow cells. Cells were stained with anti-IgM and CD45R (B220). The numbers indicate the percentages of cells in the gated areas. Pro-B and pre-B cells, $B220^{low}IgM^-$; immature B cells and mature B cells, $B220^+IgM^+$. *C*, Flow cytometry analyses of peritoneal cells. Peritoneal cells were stained with Abs specific for CD45R (B220) and CD11b (Mac-1), or IgM and CD5. All live cells were shown in Mac-1 vs B220 dot plots. Percentages of conventional B cells ($B220^+$, Mac-1^-), B-1 cells ($B220^+$, Mac-1^{low}), and macrophages (Mac-1^{high}) are indicated. B-1 cells were also $CD23^-$ (data not shown). Cells within the lymphocyte size-gate were shown in the IgM vs CD5 plots. The numbers indicate the percentages of cells within the gated areas. Relative levels of B-1a and B-1b cells in mutant mice are similar to those in wild-type mice. At least 10 homozygous mutant mice and 10 wild-type mice (age and sex matched) were analyzed in four different experiments. Data shown are representatives from one experiment. Data obtained from six mice are summarized in Table I.

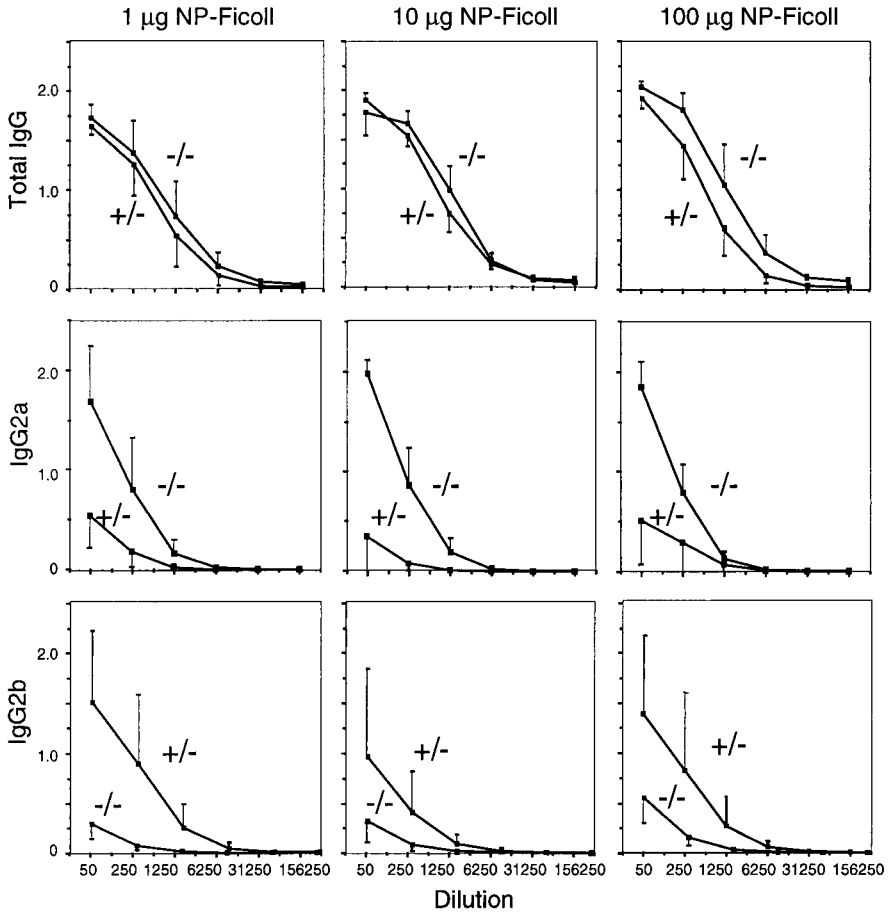

FIGURE 4. Ab responses to different doses of NP-Ficoll immunization. Groups of five mice were immunized with 1, 10, or 100 μg of NP-Ficoll on day 0. Sera were collected on day 7 and 14. Titers of NP-specific total IgG, individual IgG isotypes, and IgA were measured by ELISA with NP-BSA-coated plates. OD_{405} vs serum dilutions were plotted. Error bars indicate the SDs among five mice. Data shown are NP-specific total IgG, IgG2a, and IgG2b 14 days after immunization. Data from day 7 are similar (not shown). NP-specific IgG1, IgG3, and IgA titers were not altered in mutant mice (data not shown). Similar results were obtained from a separate immunization of four wild-type and four mutant mice.

The most apparent effect of the absence of natural IgM on B cell development was on B-1 cells in the peritoneum. More cells were recovered from peritoneal lavage from mutant mice than from wild-type mice (Table I). Significantly higher percentages of these peritoneal cells from the mutant mice were B-1 cells ($B220^+$, IgM^+, IgD^+, $CD5^{+/-}$, Mac-1^{low}, and $CD23^-$) (Fig. 3*C* and data not shown). Quantitation revealed that the numbers of B-1 cells in the peritoneum were increased approximately threefold in the mutant mice (Table I, $p < 0.01$), which was similar to the increase of B-1 cells in the spleen. The elevated levels of B-1 cells were already detectable at 4 wk of age and were stably maintained up to 12 mo of age, in the oldest mice analyzed (data not shown). The increased numbers of B-1 cells in the peritoneum (and spleen) were observed in mutant mice of both the 129 background and the mixed background of C57BL/6 and 129. Together, these observations in the mutant mice suggest a physiologic role of natural IgM in B-1 cell development.

T cells in the spleen, lymph node, thymus, and peritoneum were characterized by surface expression of CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$, CD90 (Thy-1.2), and CD5. In addition to the slight difference in the percentages of T cells in the spleen, no phenotypic difference in T cells was detected in all lymphoid organs analyzed. Thus, the absence of natural IgM has no effect on T cell development.

Enhanced IgG2a response to a TI Ag in mutant mice

In the mutant mice, the elevated levels of serum IgG2a, IgG3, and IgA were correlated with the increased numbers of B-1 cells. B-1 cells are known to secrete IgM, IgG3, and IgA but not IgG2a (30, 31). The increased IgG2a level in the mutant mice suggests that natural IgM may regulate IgG2a production in normal mice. This notion was further supported by the Ab response to TI Ag NP-Ficoll in the mutant mice. Homozygous mutant mice and heterozygous controls were immunized i.p. with 1, 10, or 100 μg of NP-Ficoll. Sera were collected at day 7 and 14 after immunization, and the levels of NP-specific IgG, individual IgG isotypes, and IgA were assayed by ELISA. While the total NP-specific IgG was relatively normal, the levels of NP-specific IgG2a were three- to fourfold higher in the homozygous mutant mice than in the heterozygous controls at all three Ag doses (Fig. 4). In contrast, NP-specific IgG2b levels were reduced in the homozygous mutant mice (Fig. 4). The levels of NP-specific IgG1, IgG3, and IgA were similar in both types of mice (data not shown). Thus, IgG2a response to TI Ag appears to be enhanced in the absence of natural IgM.

Impaired IgG responses to suboptimal doses of a TD Ag in mutant mice

To determine the effect of the absence of natural IgM in Ab response to TD Ag, homozygous mutant mice and heterozygous controls were immunized i.v. with 0.1, 1, 10, and 100 μg of the TD Ag NP-KLH on day 0 and rechallenged with the same doses of Ag on day 22. Sera were collected on day 7, 14, 21, and 29 and assayed for NP-specific IgG Abs by ELISA. Figure 5 shows the relative levels of NP-specific IgG at day 14 (primary response) and 29 (secondary response). When the Ag dose was 1 μg (suboptimal), the levels of NP-specific IgG Abs were ~25-fold lower for both the primary and secondary responses in homozygous mutant mice than in heterozygous controls. Further analyses of the levels of NP-specific IgG1, -2a, -2b, and -3 Abs showed that IgG1 was the most severely reduced (>100-fold; data not shown). In contrast,

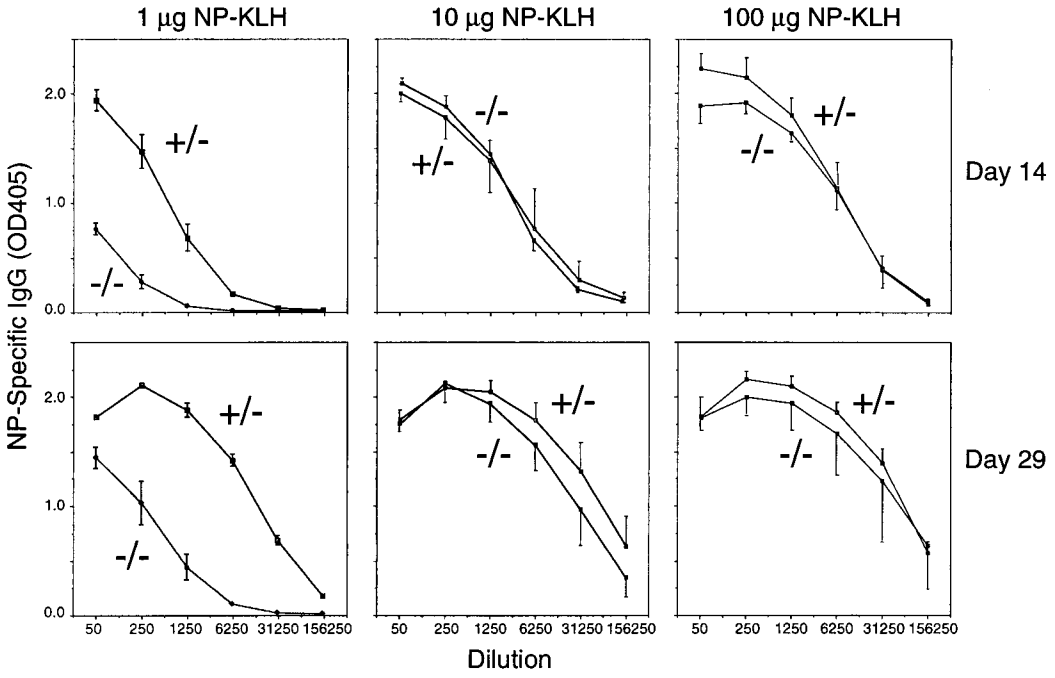

FIGURE 5. IgG Ab responses to different doses of NP-KLH immunization. Groups of four mice were immunized with 0.1, 1, 10, or 100 μg of NP-KLH on day 0 and 22. Sera were collected on day 7, 14, 21, and 29. Titers of NP-specific IgG Abs were measured by ELISA with NP-BSA-coated plates. Average OD_{405} were plotted against fold of dilutions of serum. Error bars indicate SDs among four mice. Day 14, primary response; day 29, secondary response. Data from day 7 and 21 are similar to those from day 14 and are not shown. Most mice did not respond to immunization with 0.1 μg of NP-KLH. In two additional experiments, similar results were obtained with independently immunized mice.

when Ag doses were 10 and 100 μg, homozygous mutant mice generated similar levels of NP-specific IgG Ab responses. Thus, in the absence of natural and/or immune IgM, the IgG Ab response to limiting doses of TD Ag was impaired.

B cells from mutant mice respond normally to in vitro stimulations

To distinguish whether the impaired TD Ab response in mutant mice is due to the absence of endogenous IgM or to an intrinsic defect of B cells, B cell activation and proliferation to various stimulations were assayed in vitro. Splenic B cells were purified from homozygous mutant mice and heterozygous controls and stimulated in vitro with different doses of anti-IgM, cross-linked CD40L-CD8α fusion protein, or both. B cells from homozygous mutant mice proliferated to the same extent as those from control mice in a dose-dependent manner to anti-IgM or CD40L stimulation individually and synergistically to the combination of both (Fig. 6*A*). Similarly, B cells from mutant mice proliferated normally in response to different concentrations of LPS (Fig. 6*B*). Thus, B cells differentiated in the absence of natural IgM do not appear to have any intrinsic defect, suggesting that the impaired Ab response is likely due to the absence of natural and/or immune IgM.

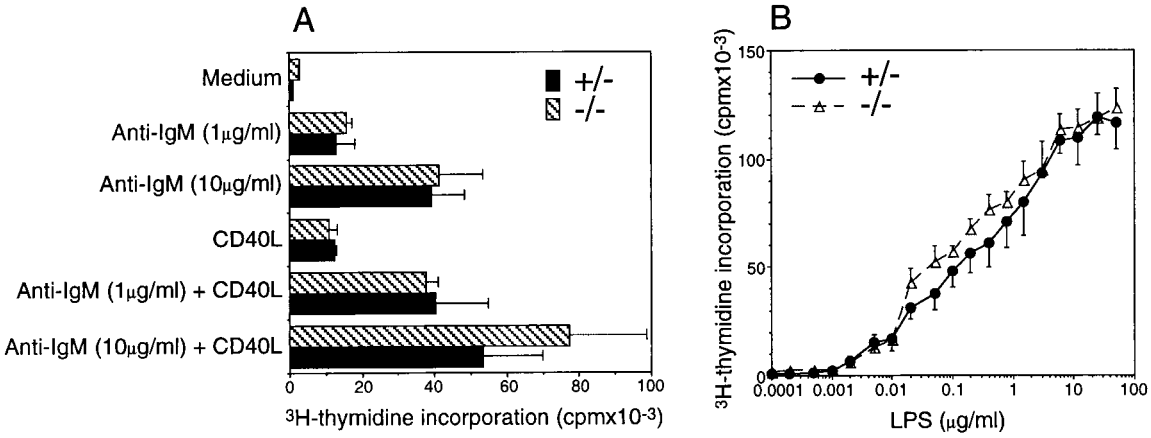

FIGURE 6. Proliferation of splenic B cells to in vitro stimulations. Splenocytes from two homozygous mutant mice and two heterozygous littermate controls were pooled and were then depleted of T cells by anti-CD90 (Thy-1.2), CD4, and CD8 Abs plus magnetic immunoselection. The resulting B cells (>95%) were stimulated with 1 or 10 μg/ml of anti-IgM, CD40L-CD8α fusion protein followed by anti-CD8α or a combination of both, or different concentrations of LPS for 48 h (see *Materials and Methods* for details). Cells were pulsed with [^{3}H]thymidine for 6 h before they were harvested and counted. Quadruplicates were used for each sample point, and SDs are indicated.

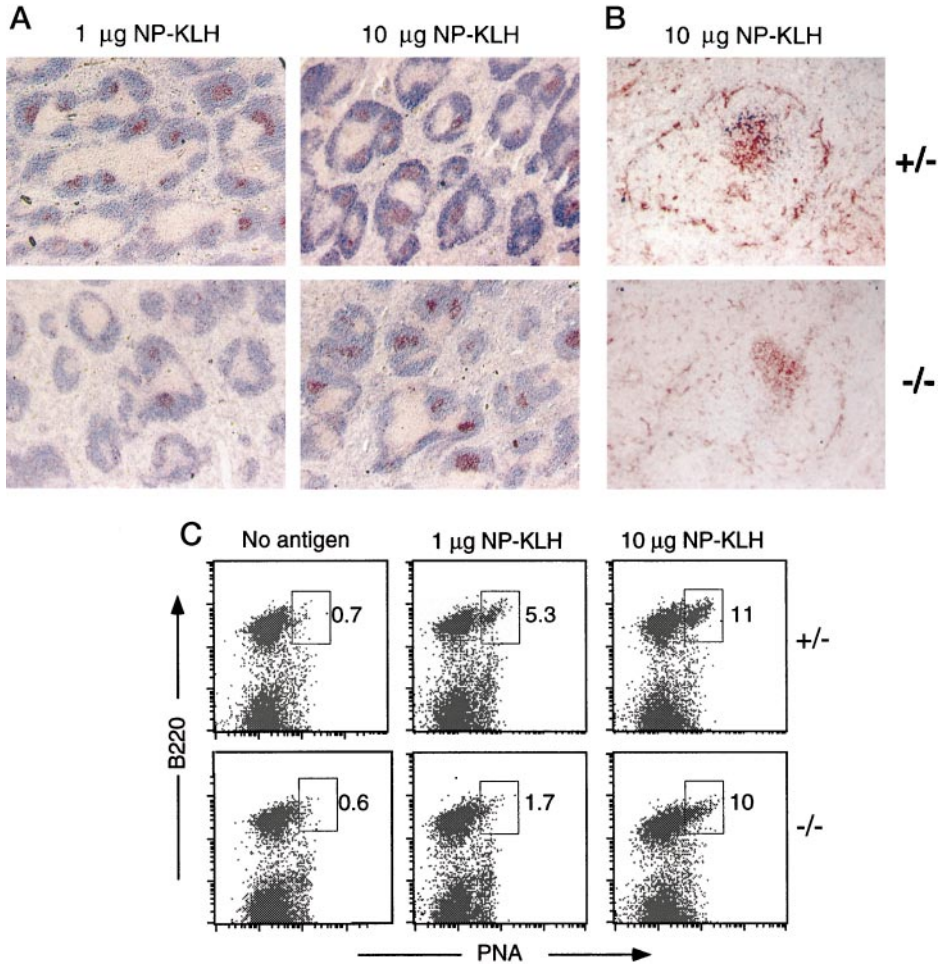

FIGURE 7. Analyses of germinal centers and Ag trapping after immunization with different doses of NP-KLH. *A*, Immunohistochemical staining of germinal centers in spleen sections 7 days after secondary immunization (day 29). Histologic sections were stained with HRP-PNA and biotin-conjugated anti-CD45R (B220) followed by AP-streptavidin. Bound AP and HRP were visualized using naphthol AS-MX phosphate/Fast blue BB base and 3-aminoethylcarbazole, respectively. Red stains indicate germinal centers and blue stains outline B cell follicles (magnification, 40×). Data shown are representatives from many spleen sections from four homozygous mutant mice and four heterozygous controls. There were no differences in the numbers and morphologies of germinal centers, after immunization with 100 μg of NP-KLH, between homozygous and heterozygous mutant mice (data not shown). *B*, Ag trapping on FDC in the germinal centers. Homozygous mutant mice and heterozygous controls were immunized with 1 or 10 μg of NP-KLH on day 0 and were then given 50 μg of NP-BSA-biotin on day 12. Spleens were harvested 18 h later. NP-BSA-biotin trapped on FDC was assayed by staining with AP-streptavidin. Germinal centers were labeled by HRP-PNA. Bound AP and HRP were visualized as above. Red stains indicate the germinal center, and blue stains show Ag trapped on FDC (magnification, 100×). No Ag trapping was detected in spleen sections after immunization with 1 μg of NP-KLH. *C*, Flow cytometry analyses of germinal center B cells after immunization with different doses of NP-KLH. Mice were immunized with 1 or 10 μg of NP-KLH on day 0. On day 12, splenocytes were stained with PE-anti-B220 and FITC-PNA and analyzed on a FACScan. The numbers indicate the percentages of PNA^{high} germinal center B cells among the total splenic B cells ($B220^{+}$). Unimmunized mice were used as controls. At least three mice were analyzed for each category.

Impaired germinal center reaction to suboptimal doses of Ag in mutant mice

Natural IgM is thought to enhance the Ab response by promoting the formation of immune complexes containing activated C3 fragments (13, 15, 32). The immune complexes can be trapped through complement receptors (CR) on FDC, promoting efficient germinal center reactions (16, 33). To determine the underlying cause of the diminished Ab response to suboptimal doses of NP-KLH in the mutant mice, germinal centers in the spleen were assayed 7 days after secondary immunization by immunohistochemical staining of frozen sections with PNA coupled to HRP (Fig. 7*A*). Consistent with the diminished Ab response to 1 μg of NP-KLH, the number and size of germinal centers were dramatically reduced in splenic sections of homozygous mutant mice compared with heterozygous controls. Quantitation of many spleen sections revealed that the reduction of the number of germinal centers was approximately fourfold. In contrast, similar numbers of germinal centers were detected in spleen sections of homozygous mutant mice as in heterozygous controls when 10 and 100 μg of NP-KLH were used (Fig. 7*A* and data not shown), consistent with the relatively normal IgG Ab response at these Ag doses.

Germinal center reactions in the spleens of homozygous mutant mice were also impaired after primary immunization with 1 μg of NP-KLH. Twelve days after immunization, $B220^{+}PNA^{high}$ germinal center B cells were assayed by flow cytometry (Fig. 7*C*). On average, 5% of B cells were PNA^{high} germinal center B cells in heterozygous controls compared with 1.5% in homozygous mutant mice. When 10 μg of NP-KLH was used, the percentages of $B220^{+}PNA^{high}$ germinal center B cells were similar (~10%). Together, these findings suggest that the impaired IgG Ab response in the absence of secreted IgM is associated with an impaired germinal center reaction.

Impaired Ag trapping on FDC in mutant mice

The impaired germinal center reaction in mutant mice could be due to a defect in Ag trapping on FDC. To directly demonstrate this mechanism, Ag trapped on FDC was assayed by immunohistochemical staining of spleen sections (16, 34–36). Homozygous mutant mice and heterozygous controls were immunized with either 1 or 10 μg of NP-KLH on day 0 and then were given 50 μg of NP-BSA-biotin on day 12. Spleens were harvested 18 h later, and NP-BSA-biotin trapped on FDC was visualized by staining with AP-streptavidin in combination with HRP-PNA staining for germinal centers. NP-BSA-biotin trapped on FDC in the germinal center was clearly detectable in spleen sections of control mice after immunization with 10 μg of NP-KLH (Fig. 7*B*). In contrast, no trapped Ag was detected on FDC in the spleen sections of similarly immunized homozygous mutant mice. Thus, Ag trapping was impaired in the mutant mice after immunization with 10 μg of NP-KLH, although the titers of NP-specific IgG Abs to this dose of Ag were similar in homozygous and heterozygous mutant mice (Fig. 5). As shown below, Ab affinity maturation was also impaired in the homozygous mutant mice after immunization with 10 μg of NP-KLH. No Ag trapping was detected in spleen sections after immunization with 1 μg of NP-KLH in either type of mice, probably due to the low dose of the Ag used (data not shown). Thus, in the absence of natural IgM, Ag appears to be less efficiently trapped by FDC.

Impaired Ab affinity maturation in mutant mice

The germinal center is thought to be the major site at which Ab affinity maturation occurs (37–39). The impaired germinal center reaction and Ag trapping on FDC in the mutant mice could compromise Ab affinity maturation. To assay for Ab affinity maturation in the absence of IgM, a plate-binding assay was used (40, 41). Briefly, plates were coated with either NP_5-BSA or NP_{15}-BSA, and the relative binding of NP-specific IgG Abs was determined by ELISA. The ratios of Ab binding to NP_5-BSA and NP_{15}-BSA were calculated. These ratios give a relative measure of Ab affinities, because Abs with high affinities will bind to both NP_5-BSA and NP_{15}-BSA, whereas Abs with low affinities may not bind to NP_5-BSA. In both heterozygous and homozygous mutant mice, the ratios increased from primary to secondary responses (Fig. 8), indicating the occurrence of Ab affinity maturation. However, the ratios of both the primary and secondary responses were significantly lower in homozygous mutant mice after immunization with 1 or 10 μg of NP-KLH. At 100 μg of NP-KLH, the ratios were similar to those of the control mice. In heterozygous controls, the ratios of the primary or secondary responses were similar at all three Ag doses, whereas in homozygous mutant mice, the ratios increased with increasing doses of Ags. Together, these data suggest that in the absence of natural and/or immune IgM, Ab affinity maturation is compromised in response to suboptimal doses of Ag.

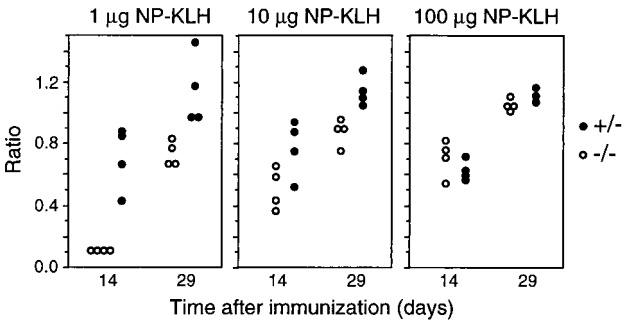

FIGURE 8. Comparison of Ab affinity maturation in homozygous and heterozygous mutant mice. The same sera used in Figure 5 were used to assay for Ab affinity maturation using a plate-binding assay. Plates were coated with either NP_5-BSA or NP_{15}-BSA, and the relative binding of NP-specific IgG Abs was determined by ELISA. The ratios of Ab binding to NP_5-BSA and NP_{15}-BSA were calculated for individual mice using OD_{405} in the linear ranges of the assays. Day 14, primary response; day 29, secondary response.

Discussion

Natural IgM consists of a collection of secreted IgM with different specificities and may function in different biologic processes. The putative functions of natural IgM have not been vigorously examined in vivo due to a lack of adequate animal models. The mutant mouse strain described here provides an ideal model to investigate the role of natural IgM under physiologic conditions in immune processes of interest.

A mutant mouse strain deficient in secreted IgM

The expression of μ_m and μ_s chains is mediated by the differential polyadenylation and alternative RNA splicing. To construct a mutant mouse strain that lacks the secreted IgM, the targeted mutation has to preserve the expression of μ_m. We replaced the μ_s exon and its downstream poly(A) sites with a cDNA fragment encoding the μ_m exons already spliced to the Cμ4 exon (Fig. 1). The constructed mutant mouse strain exhibited the expected phenotype. B cells in the mutant mice expressed IgM on their surface at a level that is at least as high as that on B cells from normal mice (Fig. 3). In contrast, the level of serum IgM in mutant mice was reduced 2,500-fold to a level that is <10-fold above the background (Fig. 2). The residual serum IgM is probably released from the B cell surface and is therefore monomeric. It is unlikely to be effective in binding to the normal endogenous and/or exogenous Ags. Thus, the mutant mice are functionally deficient in secreted IgM.

Multiple molecular events occur at the IgH locus during B cell development, including VDJ rearrangement, transcription, and class switching. In our targeted mutation, the *neo* gene was introduced 200 bp downstream of the poly(A) site of the μ_m exons. Considering that replacement of Eμ or Eκ enhancer with a *neo* gene resulted in a more severe block of recombination at the IgH or Igκ locus, respectively, than the clean deletions (42–45), it was a concern that the presence of the *neo* gene at the IgH locus in our targeted mutation may have artificially biased the observed phenotype. However, findings in the mutant mice do not substantiate the concern. In our mutant mice, the introduced *neo* gene is >10 kb downstream of the Eμ enhancer. Flow cytometry analyses revealed a relatively normal early B cell differentiation in the bone marrow and a relatively normal number of conventional B cells in the spleen (Fig. 3 and Table I), indicating that the *neo* gene did not significantly affect Ig heavy chain gene rearrangement. The presence of the *neo* gene is unlikely to have altered the transcription of the locus because the level of IgM on B cells from mutant mice is at least as high as that on B cells from normal mice (Fig. 3). Similarly, the *neo* gene is unlikely to have affected class switching because mutant mice have normal levels of serum IgG1 and IgG2b. Although the levels of IgG2a, IgG3, and IgA are elevated at early ages in mutant mice, in adults the levels are the same as in wild-type controls (Fig. 2). After class switching, the *neo* gene is deleted from the locus and therefore can not affect the expression of the switched Ig. Thus, if the presence of the *neo* gene affected class switching to IgG2a, IgG3, and IgA, it would have to exert the effect in an age-dependent manner. As discussed below, the elevated levels of IgG2a, IgG3, and IgA at earlier ages were probably

due to the increased numbers of B-1 cells and other effects of the absence of natural IgM in the mutant mice.

Our targeted mutation is very similar to the targeted replacement of the Igδ locus in which the *neo* gene is inserted 2.1 kb downstream of the μ_m exons (41). In contrast to our mutant mice, replacement of the Igδ locus by the *neo* gene did not result in elevated levels of B-1 cells or serum IgG2a, IgG3, and IgA, indicating that introduction of the *neo* gene downstream of Cμ exons did not affect the recombination, transcription, and class switching of the locus. In addition, IgD-deficient mice had almost normal TD and TI Ab responses except that the affinity maturation is delayed in the early primary response (41), indicating that the *neo* gene did not grossly affect Ab response. In our mutant mice, TD Ab response is impaired to suboptimal dose of Ag but not to optimal doses of Ag. It is unlikely that the introduced *neo* gene interfered with the Ab response in an Ag dosage-dependent manner.

In our mutant mice, the level of IgD on B cells is slightly lower than that on B cells from normal mice, indicating that the inserted *neo* gene may interfere with the μ-δ RNA splicing. Alternatively, the observed changes could be due to the increased numbers of B-1 cells in the spleen, which are IgM^{high} and IgD^{low} (6–8). Nevertheless, the subtle change in IgD level on B cells did not grossly affect conventional B cell differentiation and function as indicated by the normal number of B-2 cells in the spleen and their normal activation and proliferation in in vitro stimulation assays (Fig. 6). Further supporting this notion, studies using IgD-deficient mice have shown that IgD is dispensable for B cell development and Ab response (41).

Role of natural IgM in B-1 cell development

The most dramatic effect of the absence of natural IgM on B cell development is the elevated levels of B-1 cells in the mutant mice. At 4 wk, an average of threefold more B-1 cells was already detectable in the peritoneum of mutant mice than in wild-type mice (Table I and data not shown). The increase was also evident in the spleen, where B-1 cells are just barely detectable in normal mice (Fig. 3 and Table I). The elevated levels of B-1 cells were stably maintained when mice became older (1 yr). B-1 cells are known to secrete IgG3 and IgA (30, 31). Correlating with the increased numbers of B-1 cells, mutant mice had elevated levels of serum IgG3 and IgA at early ages (<8 wk old), but the differences disappeared as mice matured (>12 wk old) (Fig. 2). Natural IgM has been postulated to play important roles in the regulation of the immune system (1, 2). Our findings in the mutant mice are probably the first unequivocal demonstration that natural IgM regulates B cell development under physiologic conditions.

The differentiation pathways of B-1 cells are still controversial. The "lineage" hypothesis postulates distinct precursors for B-1 cells and conventional B (B-2) cells (6, 46). B-1 cells are differentiated predominantly from precursors in fetal omentum and liver but rarely from B cell precursors in adult bone marrow, which give rise to B-2 cells (31, 47, 48). The differentiation of B-1 cells in the bone marrow of adult mice is inhibited by the existing B-1 cells through an undefined feedback mechanism (49, 50). In the absence of continuous differentiation, the relatively constant level of the B-1 cell population is thought to be maintained by the capacity for self-renewal of B-1 cells (47, 51). In contrast, the "specificity or differentiation pathway" hypothesis proposes that both B-1 and B-2 cells are differentiated from a common precursor pool. The acquisition of the B-1 cell phenotype is due to the cross-linking of surface Ig receptors in the absence of a cognate T cell help (52–54). Fetally derived B cells are preferentially selected into the long-lived B-1 cell compartment on the basis of germline-encoded self-reactivity (7, 8). Various cell surface receptors and signaling molecules that mediate B cell activation have been implicated in B-1 cell differentiation and maintenance (55). For example, mice deficient in B cell co-receptor components CD19 or CD21 or signal transduction molecules Btk or Vav have reduced levels of B-1 cells (56–59). Transgenic mice overexpressing CD19 or mice deficient in CD22 or SHP-1 have elevated levels of B-1 cells (30, 60–62). Considering that B-1 cells secrete the major portion of natural IgM, and given our findings that mutant mice lacking natural IgM have increased numbers of B-1 cells, we propose that natural IgM is involved in the feedback regulation of B-1 cell differentiation and/or maintenance.

In mutant mice, the effect of the absence of natural IgM on the levels of B-1 cells is already established by 4 wk of age, suggesting that natural IgM may regulate B-1 cell differentiation at fetal and neonatal stages. Potentially, in the absence of natural IgM as a competitor, fetally derived B cells have an increased probability to bind to the normal self-Ag through surface Ig receptors, leading to an enhanced B-1 cell differentiation (52–54). Cytokines such as IL-5 and IL-10 can promote B-1 cell proliferation, while IFN-γ inhibits B-1 cell growth (63–67). Natural IgM may exert its effect on B-1 cell differentiation through regulating the secretion and/or function of these cytokines. Consistent with this notion, natural Abs have been shown to be capable of binding to cytokines and their receptors (2, 68, 69). IL-5 also augments IgA secretion (64), and the IgA level was elevated in the mutant mice (Fig. 2). It is also possible that natural IgM may mediate the maintenance of B-1 cell levels in adults including the feedback inhibition of further B-1 cell differentiation in adult bone marrow by the existing B-1 cells (49, 50). For example, natural IgM tends to be self-reactive (9, 10, 70), so it is possible that natural IgM may directly interact with Ag receptor or other receptors on B-1 cells and inhibit B-1 cell proliferation. Supporting this notion, cross-linking of surface IgM on B-1 cells usually leads to apoptosis rather than proliferation, as in conventional B cells (71–73). In addition, evidence from Ig transgenic mice suggests that B-1 cells can be activated by gut bacterial Ags such as LPS in the lamina propria and are able to traffic between the lamina propria and peritoneum (74, 75). IgM is secreted into the lumen of the gut and may bind to bacterial and/or food Ags, thereby neutralizing their ability to stimulate B-1 cell proliferation. The involvement of natural IgM in the feedback regulation of B-1 cell homeostasis is consistent with the close association of B-1 cell levels and serum IgM levels. The expression of μ_m has been shown to be required for the stepwise progression of B cells (27, 76). Our observation in mutant mice demonstrates that the secreted IgM is also utilized for regulating B-1 cell differentiation and/or maintenance.

Role of natural IgM in IgG2a production

The elevated level of serum IgG2a in the mutant mice was not expected (Fig. 2). It is unlikely that the targeted insertion of the *neo* gene specifically affected class switching to IgG2a but not to other isotypes. Neither is it due to the increased numbers of B-1 cells, because B-1 cells are not known to secrete a large portion of serum IgG2a (30, 31). Furthermore, IgG2a response to the TI Ag NP-Ficoll was also elevated in mutant mice (Fig. 4), whereas IgG3 and IgA, which are known to be secreted by B-1 cells, were not affected (data not shown). Thus, the increased IgG2a production in the mutant mice represents a specific effect due to the absence of natural IgM. One possible mechanism by which natural IgM may modulate IgG2a production may be through its regulation of cytokine secretion and/or function, which in turn affects class switching to IgG2a (2, 68, 69, 77, 78). Consistent with this notion, IFN-γ induces class switching to IgG2a and IgG3 (78), both of which were elevated in mutant mice at early ages (Fig. 2). Investigation

of cytokine profiles in the mutant mice at early ages or after challenge with a TI Ag may help to elucidate the mechanisms underlying the increased IgG2a production.

Role of natural IgM in B cell Ab response

The mutant mice had an impaired IgG Ab response to a limiting dose of the TD Ag NP-KLH (Fig. 5). This effect is not due to an intrinsic defect of the B cells differentiated in the absence of natural IgM. First, mutant mice have relatively normal numbers of B-2 cells, and these B cells respond normally to in vitro stimulation by anti-IgM, CD40L, and LPS (Table I and Fig. 6). Second, at high doses of Ag, IgG Ab responses are normal in the mutant mice (Fig. 5), suggesting that B cells are intrinsically capable of mounting a normal Ab response. Thus, the impaired IgG response is likely caused by the absence of natural and/or immune IgM. IgM is the first class of Ab produced during a primary Ab response. The absence of the immune IgM in the mutant mice could also contribute to the observed defect. While the resolution of the two possibilities awaits further experimentation in mutant mice that have been reconstituted with normal serum IgM, existing evidence supports a more important role of natural IgM in the process. Natural IgM that binds KLH is readily detectable in normal human sera (79). KLH-binding natural IgM is probably also present in normal mouse serum. Coinjection of Ag and Ag-specific IgM usually results in augmented Ab responses (17, 18, 20, 21, 80). However, injection of IgM just 1 or 2 h after the Ag suppresses rather than augments the Ab response (81), indicating that the immune IgM may not play an important role in enhancing the ongoing Ab response. Similarly, since adult mutant mice have normal levels of IgGs (Fig. 2), natural IgG Abs are clearly not sufficient to compensate for the IgM deficiency in promoting the Ab response. Together, these findings suggest that natural IgM is most likely required for efficient Ab responses to limiting doses of TD Ags.

Similar to mice and guinea pigs deficient in C3, C4, or CD21 (CR2) (82–86), IgM-deficient mice had impaired TD IgG responses to limiting doses but not to optimal doses of Ags (Fig. 5), suggesting that natural IgM promotes Ab response through the same pathways as C3, C4, and CD21. C3, C4, and CD21 can potentially augment Ab responses through two common pathways. First, C3-containing immune complexes can cross-link B cell Ag receptor (BCR) and CD19/CD21 complex and reduce the threshold dose of Ag required to activate B cells (56, 57, 87, 88). Second, immune complexes are more efficiently trapped on FDC through the expressed complement receptors (CD21 and CD35 in mice), leading to an effective germinal center reaction during the TD Ab response (13, 32, 89). Natural IgM could augment the Ab response through the same pathways by promoting an efficient formation of immune complexes. Supporting this notion, Ag trapping on FDC was impaired in the mutant mice (Fig. 7) indicating a role of natural IgM in immune complex formation. Furthermore, we found that germinal center formation and Ab affinity maturation were impaired in the mutant mice (Fig. 7 and 8). Thus, natural IgM, through its associated immune complexes, stimulates germinal center reactions and Ab affinity maturation (16, 34, 35, 90). In addition, natural IgM may also be involved in the initial B cell activation by promoting immune complex formation.

Increasing evidence suggests a critical role of the innate immune system in the priming of adaptive immune responses (12, 33). One mechanism that promotes efficient Ab response is the formation of immune complexes containing activated C3 fragment (87). Under physiologic conditions, complement can be activated by the classical, alternative, and lectin pathways (91). Both the alternative and lectin pathways require specific structures of the Ag such as bacterial surface and carbohydrates. Most protein Ags such as KLH do not contain such structural determinants. They have to rely on the classical pathway to activate complement and generate C3-containing immune complexes. However, before B cell activation and the Ab response, where does the Ab that can bind to an Ag come from? Natural IgM is ideally suited for this purpose due to its natural presence, polyreactivities with high avidities, and exquisite ability to activate complement. Our findings in mutant mice demonstrate a physiologic role of endogenous natural IgM in this process. Through natural IgM secreted by B-1 cells, the adaptive immune system appears to have evolved a built-in mechanism that interacts with the innate immune system to promote efficient adaptive Ab responses by conventional B cells.

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