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IgM-mediated signaling is required for the development of a normal B cell memory response

Linjie Guo, Xuejun Zhang, Biao Zheng, and Shuhua Han*

Department of Immunology, Baylor College of Medicine, Houston, USA

Abstract

Mature B cells co-express both IgM and IgD types of antigen receptors before activation. Our earlier work has shown that the co-expression of IgD and IgM plays an important role in regulating the composition of antibody repertoire during a primary immune response. However, the roles of these two B cell receptors in the development of B cell memory responses remain unclear. The present study shows that during the secondary immune response to (4-hydroxy-3-nitrophenyl)acetyl (NP), IgM^{-/-} mice secreted significant amount of NP-specific IgD antibodies. The kinetics of antigen-specific IgD antibodies produced in IgM^{-/-} mice was similar to that of IgM antibodies in wild type mice during the secondary response. However, the production of antigen specific class-switched antibodies in IgM^{-/-} mice was significantly reduced compared to that in wild type mice, particularly at the early phase of the memory response. In addition, germinal center (GC) reaction was significantly diminished in IgM^{-/-} mice after secondary challenge with soluble antigen. Nevertheless, affinity maturation of antibodies appears largely intact in IgM^{-/-} mice during memory response. Thus, our studies demonstrate that IgM-mediated signaling plays an important role in the development of efficient B cell memory responses.

Keywords

B cells; IgM; memory response; germinal center; antibody

1. Introduction

IgM and IgD expression is strictly controlled during B cell development and differentiation. IgM is one major type of B cell antigen receptors (BCRs) expressed on immature and mature B cells. Newly generated immature B cells in the bone marrow express IgM but not IgD on their surface. IgD expression is first up-regulated in transitional B cells in the spleen at the T2 stage; then the expression of IgD increases as B cells differentiate towards the mature B cell stage (Loder et al., 1999). The vast majority of mature peripheral B cells co-express membrane IgM and IgD. Marginal zone (MZ) B cells and B1 cells express high levels of IgM but down-regulate IgD on their surface (Oliver et al., 1997; Oliver et al., 1999). After activation, B cells rapidly down-regulate the expression of IgD, but not IgM (Bourgeois et al., 1977; Monroe et al., 1983). This strict regulation of IgM and IgD expression suggests functional differences between the two BCRs. However, to date, the biological function of the dual expression of IgM and IgD by most naïve mature B cells is not fully understood.

* Correspondence: Shuhua Han, M.D., Ph.D., Department of Immunology, Baylor College of Medicine, One Baylor Plaza, M929, Houston, TX 77030, USA, Phone: 713-798-8805, Fax: 713-798-3033, E-mail: shan@bcm.edu.

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It has been shown that IgD-deficient mice respond well to both T cell-independent and T cell-dependent antigens, but affinity maturation is delayed in the early primary response compared to control wild type animals (Roes and Rajewsky, 1993), suggesting that there may be differences in the function of IgD and IgM as BCRs. On the other hand, in IgM^{-/-} mice, although B cell development and maturation proceed normally with IgD replacing IgM (Lutz et al., 1998), our earlier work has shown that, during the primary response, the repertoires for both GC B cells and extrafollicular antibody forming cells (AFCs) were significantly altered in IgM^{-/-} mice compared to that in wild type mice (Han et al., 2004). However, it is not known whether IgM-mediated signals play a role in induction of B cell memory response. In this study, we demonstrated that IgM^{-/-} mice exhibit reduced GC formation and decreased production of isotype switched antibodies during memory response. However, antibody affinity maturation was largely intact in the absence of IgM-mediated signaling.

2. Material and methods

2.1. Mice and Immunization

IgM-deficient mice on BALB/c background (Han et al., 2004) were backcrossed to C57BL/6 background for 6 generations. The mice were maintained in microisolator cages on a 12-h light/dark cycle in the Center for Comparative Medicine at Baylor College of Medicine. To induce secondary response, wild type and IgM^{-/-} mice at 8-10 weeks old were immunized intraperitoneally with 100 µg of NP-CGG precipitated in alum (Han et al., 1995a; Han et al., 1995b). Two months later, the mice were challenged *i.v.* with 30 µg of soluble NP-CGG in PBS. Mice were sacrificed at day 12 after secondary immunization. For primary response, wild type and IgM^{-/-} mice were either immunized *i.p.* with 100 µg NP-CGG in alum or *i.v.* with 30 µg NP-CGG in PBS. The mice were sacrificed at day 12 after primary immunization.

2.2. ELISA assays

Mice were bled at various time points post-immunization. Serum specific antibodies against NP hapten were determined by ELISA using two different coupling ratios of NP-BSA as described (Han et al., 2003). Briefly, NP₅-BSA or NP₂₅-BSA was coated onto 96-well plates. Sera with serial dilutions were added and incubated at room temperature for 2 hours. After washing, HRP-conjugated secondary antibodies against mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 were added. The concentrations of anti-NP IgG1 antibodies were determined using standard curves created from the H33Lγ1/λ1 control antibody on each plate (Han et al., 2003). For other isotypes of antibodies, a mixed serum sample from NP-CGG immunized mice was always used to establish standard curves on each plate and antibody levels were expressed as artificial units. For detecting NP-specific IgD antibodies, anti-IgD-FITC was added and followed by anti-FITC-HRP (DAKO, Carpinteria, CA). The TMB peroxidase substrate kit (Bio-Rad Laboratories, Hercules, CA) was used to visualize HRP activity. Optical densities were determined by an ELISA reader at 450 nm.

2.3. Flow cytometry

To estimate the frequency of GC B cells, spleen cells were stained with FITC-labeled GL-7, PE-conjugated anti-Fas and Percp-conjugated anti-B220 (BD Pharmingen) after incubation with anti-FcγIII/IIIR to block FcγR-mediated binding. Samples were collected on a FACScalibur machine (Becton Dickinson) and analyzed using Flow Jo software (Tree Star Inc., San Carlos, CA).

2.4. Histology

6 μ m serial sections of spleen were cut with a cryostat microtome and prepared as previously described (Han et al., 1995b). GCs were identified by staining with PNA-FITC and anti-IgD-PE (BD PharMingen).

3. Results

3.1. IgD antibody production in IgM^{-/-} mice during the secondary response

It is known that wild type mice produce very little IgD antibodies in their sera. To determine whether antigen-specific IgD antibody response can be induced in the absence of IgM during a secondary response and whether the kinetics of IgD antibody production is similar to that of IgM antibodies elicited in wild type mice, we measured the levels of serum NP-specific IgM or IgD antibodies in wild type or IgM^{-/-} mice at 0, 5, and 12 days after secondary immunization by ELISA. Although IgD antibodies were undetectable in the serum of wild-type mice after secondary challenge (not shown), IgM^{-/-} mice secreted high amount of NP-specific IgD antibodies in their sera (Fig. 1). After secondary immunization, NP-specific IgM antibodies in wild type mice and IgD antibodies in IgM-deficient mice quickly increased and declined thereafter, showing similar responding kinetics (Fig. 1A). We have also determined the secondary antibody response to phosphorylcholine (PC) in wild type and IgM^{-/-} mice of Balb/c background that were immunized with PC-keyhole limpet hemocyanin (KLH). Consistently, PC-specific IgM or IgD antibody responses in wild type or IgM mutant mice exhibited similar kinetics (Fig. 1B). Therefore, the data demonstrated that the kinetics of antigen-specific IgD antibody production in IgM^{-/-} mice is comparable to that of IgM antibody production in wild-type mice during memory responses.

3.2. IgM^{-/-} mice exhibited diminished long-term antibody levels and impaired secondary antibody responses

To determine the role of IgM in the maintenance of long-term antibodies and development of memory B cells, we measured class-switched anti-NP antibodies in wild type and IgM mutant mice. Before the secondary challenge (60 days after primary immunization), the levels of serum antigen-specific IgG1, IgG2b, and IgG3 antibodies in IgM^{-/-} mice were significantly lower compared to that in wild type mice (Fig. 2), indicating that IgM-mediated signals are required for optimal generation of long-lived plasma cells.

After the secondary challenge, the production of all isotype-switched antibodies in IgM^{-/-} mice was significantly decreased compared to that in wild type mice (Fig. 2). Although NP-specific IgG1 antibody levels were increased with time in IgM^{-/-} mice after the secondary immunization and reached similar levels as in wild type mice at day 12 (Fig. 2A), IgM^{-/-} mice exhibited significantly delayed kinetics of IgG1 antibody production. Furthermore, NP-specific IgG2a antibody levels in IgM^{-/-} mice were significantly depressed and the increase of IgG2a following secondary immunization was minimum (Fig. 2B). Similarly, the production of IgG2b was low at all time points in IgM^{-/-} mice whereas wild type mice produced significantly higher NP-specific IgG2b antibodies (Fig. 2C). Antigen-specific IgG3 antibody production was increased with time after secondary immunization in both wild type and IgM^{-/-} mice but the levels were significantly lower in the mutant mice (Fig. 3D). Overall, the memory antibody responses to NP was significantly decreased in IgM^{-/-} mice, indicating that there is a defect in the generation of memory B cells in the absence of IgM-mediated signals.

3.3. Affinity maturation is largely intact in IgM-deficient mice

Somatic hypermutation of Ig genes has been considered a hallmark of memory B cells (Berek et al., 1985; Griffiths et al., 1984; Kelsoe, 1995; MacLennan, 1994). Selection of GC B cell

mutants, presumably on the basis of higher affinity for the eliciting antigen, takes place concurrently with memory B cell development (Clarke et al., 1990). To determine whether the process of antibody affinity maturation in IgM mutant mice were functional, we measured the relative affinities of NP-specific antibodies produced during the secondary antibody response in wild type and IgM mutant mice. Serum anti-NP IgG1 present at day 0, 5, 12 of the secondary response was tested by ELISA for binding to NP₅ and NP₂₅. The affinity threshold of antibody binding to NP-BSA conjugates of different NP/BSA ratios has been determined using several monoclonal antibodies with different affinities for NP (Dal Porto et al., 1998; Takahashi et al., 1998).

As expected, the serum anti-NP IgG1 antibodies from wild type mice showed almost equivalent binding to NP₂₅-BSA and NP₅-BSA (Fig. 3), indicating that the IgG1-producing cells had acquired affinity maturation during the primary and secondary responses. Similarly, the NP-specific IgG1 antibodies from IgM^{-/-} mice exhibited similar binding abilities to NP₂₅ and NP₅ (Fig. 3), suggesting that antibody affinity maturation is intact in IgM mutant mice.

We further determined the relative affinities of NP-specific IgM or IgD antibodies elicited in wild type or IgM mutant mice, respectively. At day 0 of the secondary response, NP-specific IgM antibodies produced in wild type mice bound efficiently to NP₂₅-BSA conjugates but showed much weaker binding to NP₅-BSA (Fig. 4, left panel). Similarly, NP-specific IgD antibodies from IgM mutant mice exhibited significantly weaker binding capabilities to NP₅-BSA than NP₂₅-BSA (Fig. 4, right panel). The binding of both IgM and IgD antibodies to NP₅ was somewhat increased at day 5 of the secondary response but quickly declined at day 12 (Fig. 4). These data indicated that IgD antibodies produced in IgM^{-/-} mice, as IgM antibodies elicited in wild type mice, had relatively lower affinities compared to IgG1 antibodies.

3.4. Reduced secondary GC response in IgM^{-/-} mice

GCs are the principal sites of Ig V(D)J hypermutation, affinity-driven clonal selection, and generation of the B cell memory compartment as well as the long-lived plasma cell pool (Gray, 1993). To investigate the mechanisms responsible for the diminished memory antibody responses in IgM-deficient mice, we examined the GC response in wild type and IgM^{-/-} mice. For primary GC response, mice were immunized with 100 µg NP-CGG in alum and analyzed 12 days later. To induce secondary GC response, mice that were primed with 100 µg of NP-CGG in alum 2 month earlier received 30 µg soluble NP-CGG. Mice were analyzed at day 12 after challenge. The magnitude of GC response in wild type and IgM mutant mice was examined by flow cytometry analyses and immunohistology.

By flow cytometry analysis, the GC B cell population after primary immunization was slightly increased in IgM mutant mice compared to that in wild type mice, but the difference was not statistically significant (Figs. 5A & C). Immunohistological results confirmed similar numbers and sizes of GCs in the spleen of wild type and IgM mutant mice (Fig. 5B). Interestingly, IgM^{-/-} GCs were not stained by PNA as brightly as wild type GCs. In addition, unlike wild type GC B cells, substantial numbers of GC B cells in the spleen of IgM^{-/-} mice were IgD⁺ (Fig. 5B).

After secondary immunization with soluble NP-CGG, in contrast to the primary response, the percentages of GC B cells (B220⁺GL-7⁺Fas⁺) in the spleens of IgM mutant mice were significantly lower compared to that in wild type mice (Figs. 5A & E). Both the sizes and the numbers of GCs formed in IgM mutant mice was reduced (Figure 5B).

To further distinguish whether the GCs developed were induced by activating naïve B cells or memory B cells, we also examined GC formation elicited by 30 µg soluble NP-CGG alone in naïve mice. As expected, soluble NP-CG induced very little GC reaction in wild type mice

(Fig. 5D). Comparatively, higher percentages of GL-7⁺ GC B cells were present in IgM^{-/-} mice after injection of soluble antigen. This is consistent with slightly elevated primary GC response induced by antigen with alum in IgM^{-/-} mice (Fig. 5C). However, in the absence of alum, the difference between the GC B cell populations from wild type and IgM^{-/-} mice was statistically significant (Fig. 5D). Thus, the GCs developed in wild type mice truly represented memory response because one time soluble antigen injection gave rise to little GC formation. In contrast, GC reaction elicited via memory response in IgM^{-/-} mice was very minimal. Taken together, these data indicate that the secondary GC response in IgM^{-/-} mice was severely impaired.

4. Discussion

The present study demonstrates that the long-term antibody levels and secondary antibody response induced in IgM^{-/-} mice were significantly diminished compared to that of wild type mice. Thus, the generation of long-lived plasma cells and memory B cells is impaired in the absence of IgM-mediated signaling. IgD expression in IgM^{-/-} mice cannot fully replace the role of IgM in B cell memory responses.

It is known that GC reaction is primarily responsible for generation of memory B cells as well as the long-lived plasma cells (Berek et al., 1991; Gray, 1993; Jacob et al., 1991). Therefore, it is interesting that IgM-deficient mice exhibit vigorous GC formation during the primary response. However, although the magnitude of GC reaction is not reduced in IgM^{-/-} mice, our earlier work has shown that the clonal type of GC B cells is drastically changed in the absence of IgM (Han et al., 2004). The development of an efficient memory B cell compartment and an optimal long-lived plasma cell pool is the end product of many processes during the B cell activation and differentiation, particularly in the GCs, including antigen-driven clonal expansion and selection. It is possible that the development of an effective memory antibody response may primarily depend on the quality of B cell clones (the right clones) that have been activated and survived the GC pathway of B cell differentiation rather than the magnitude of the GC response. The differences in the dominant clonal types between wild type and IgM-deficient mice (Han et al., 2004) suggest that the expression of IgM during primary responses appears to be important for the activation and/or selection of certain B cell clones.

Although the production of class-switched antibody response was significantly depressed compared to that in wild type mice, affinity maturation of class-switched antibodies in a secondary response was largely normal in IgM mutant mice. This result is consistent with our earlier findings that GC B cells in IgM^{-/-} mice are capable of undergoing somatic hypermutation and selection (Han et al., 2004), which are the major mechanisms for antibody affinity maturation. Interestingly, our current study found that both IgM antibodies from wild type mice and IgD antibodies from IgM^{-/-} mice exhibited lower affinities than that of IgG1 antibodies. It is commonly believed that the isotypes of antibodies are associated with effector functions but not with the antibody affinities, which are determined by Ig variable regions. One possible explanation for the low affinities of IgM and IgD antibodies is that IgM- or IgD-producing cells have not gone through GC reaction and/or affinity maturation during the primary or secondary response.

Taken together, our results demonstrated that the absence of IgM-mediated signals led to significantly reduced production of isotype-switched antibodies and decreased secondary GC formation via memory response. Thus, IgM is required for induction of an optimal B cell memory response and IgD cannot fully replace the function of IgM.

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Figure 1.

Comparison between the antigen-specific serum IgM and IgD levels in wild type and IgM^{-/-} mice respectively during secondary responses. Sera from wild type and IgM^{-/-} mice on C57BL/6 background (A) and BALB/c background (B) were used to detect NP-specific or PC-specific IgM or IgD levels by ELISA. Mixed sera were used as a standard and artificial units were calculated according to the standard curves.

Figure 2.

Reduced production of class-switched antibodies in IgM^{-/-} mice. Sera taken at days 0, 5, and 12 after secondary immunization from wild type (open bars) and IgM^{-/-} mice (black bars) were measured for different isotypes of IgG antibodies by ELISA.

*, p<0.05; **, p<0.01; ***, p<0.001. Data are representative of 2 independent experiments with 10 mice in each group.

Figure 3.

Affinity maturation of IgG1 antibodies in wild type and IgM^{-/-} mice during the secondary immune response. ELISA plates were coated with NP₅-BSA (lines with open circles) or NP₂₅-BSA (lines with solid circles). Each figure shows the result of one mouse serum sample but is a representative of 10 samples with similar data in each group.

Figure 4.

Comparison of affinity maturation of NP-specific IgM and IgD antibodies in wild type and IgM^{-/-} mice at day 0, 5 and 12 after secondary immunization. ELISA plates were coated with NP₅-BSA (lines with open circles) or NP₂₅-BSA (lines with solid circles). Each figure shows the result of one mouse serum sample but is a representative of 10 samples with similar data in each group.

B**Primary response** **Secondary response**

Figure 5.

GC responses in IgM-deficient mice after primary and secondary immunization. GC reaction in wild type and IgM^{-/-} mice was analyzed at day 12 after primary and secondary immunization. **A.** GC B cell populations were analyzed by FACS. Splenic cells were stained with anti-B220-Percp, GL-7-FITC and anti-Fas-PE. Numbers shown represent the percentages of GL-7⁺Fas⁺ GC B cells within B cell (B220⁺) gate. **B.** Fluorescence microscopic pictures showing GCs in wild type and IgM^{-/-} mice. Frozen splenic sections were stained with PNA-FITC (green) and anti-IgD-PE (red). Arrows indicate GCs. **C.** The percentages of GC B cells after the primary immunization with NP-CGG in alum were analyzed by FACS. **D.** The percentages of GC B cells after the primary immunization with soluble NP-CGG were analyzed

by FACS. **E.** The percentages of GC B cells after the secondary immunization were analyzed by FACS. 10 mice were used in each group.