B cell–intrinsic TLR signals amplify but are not required for humoral immunity

Almut Meyer-Bahlburg,[1] Socheath Khim,[1] and David J. Rawlings[1,2]

[1]Seattle Children's Hospital Research Institute, Seattle, WA 98101
[2]Department of Pediatrics and Department of Immunology, University of Washington, School of Medicine, Seattle, WA 95195

Although innate signals driven by Toll-like receptors (TLRs) play a crucial role in T-dependent immune responses and serological memory, the precise cellular and time-dependent requirements for such signals remain poorly defined. To directly address the role for B cell–intrinsic TLR signals in these events, we compared the TLR response profile of germinal center (GC) versus naive mature B cell subsets. TLR responsiveness was markedly up-regulated during the GC reaction, and this change correlated with altered expression of the key adaptors MyD88, Mal, and IRAK-M. To assess the role for B cell–intrinsic signals in vivo, we transferred MyD88 wild-type or knockout B cells into B cell–deficient μMT mice and immunized recipient animals with 4-hydroxy-3-nitrophenylacetyl (NP) chicken gamma globulin. All recipients exhibited similar increases in NP-specific antibody titers during primary, secondary, and long-term memory responses. The addition of lipopolysaccharide to the immunogen enhanced B cell–intrinsic, MyD88-dependent NP-specific immunoglobulin (Ig)M production, whereas NP-specific IgG increased independently of TLR signaling in B cells. Our data demonstrate that B cell–intrinsic TLR responses are up-regulated during the GC reaction, and that this change significantly promotes antigen-specific IgM production in association with TLR ligands. However, B cell–intrinsic TLR signals are not required for antibody production or maintenance.

CORRESPONDENCE
David J. Rawlings:
drawling@u.washington.edu

The central role for B cells in initiating or sustaining human autoimmune diseases has recently been underlined by the effectiveness of B cell depletion therapies in a broad range of antibody-mediated disorders (1). Although the signals that promote autoantibody production are multifactorial, a key group of stimuli is Toll-like receptor (TLR) ligands. TLRs specifically recognize molecular patterns of microbial pathogens such as bacteria and viruses and are expressed on a wide variety of cells of the immune system (2, 3). All TLRs, except TLR3, use the downstream adaptor molecule MyD88, whereas TLR3, and also TLR4, in part, signal via the adaptor TRIF. Upon activation, MyD88 initiates signaling cascades that promote NF-κB and AP-1 activation and subsequent inflammatory responses.

In addition to their role in innate immunity, TLRs are also critically involved in the initiation and enhancement of adaptive immune responses (4). Although several studies have addressed the involvement of DC and T cell activation in these processes (5, 6), the role of B cell–intrinsic TLR signals in regulating T-dependent (TD) immune responses is less well known. Among the cells of the immune system, B cells uniquely express both germline-encoded TLRs and a clonally rearranged antigen-specific receptor, the B cell receptor. Based on tissue distribution, receptor specificity, phenotypic, and functional characteristics, the marginal zone (MZ) and B1 subsets of mature B cells have been classified as innate immune cells. In contrast, follicular mature (FM) B cells function primarily within the adaptive immune system (7), and the role for TLR signals in activation of this naive B cell population is largely undefined.

A recent study concluded that TLR signals in B cells are required for TD immune responses (8). However, by demonstrating normal TD immune responses in mice lacking both MyD88 and TRIF, other authors have questioned this finding (9). Most notably, a previous study has suggested that polyclonal activation of human memory B cells via TLR signals is essential for maintenance of serological memory (10). This idea, however, has not been directly tested.

The present study was designed to directly investigate whether B cell–intrinsic TLR signals influence either antibody production or maintenance in response to a TD antigen. Based on gene expression and in vitro functional data, we

The online version of this article contains supplemental material.

show that TLR signaling in B cells is enhanced during the germinal center (GC) reaction. Consistent with this, MyD88-dependent B cell–intrinsic signals promote a more rapid increase in antibody production after TD immunization in vivo. However, our data clearly demonstrate that B cell–intrinsic MyD88-dependent TLR signals are not required for either long-term maintenance of antibodies or for B cell memory responses.

RESULTS AND DISCUSSION

The GC reaction enhances TLR signaling in B cells

Purified murine and human FM B cells proliferate only weakly, if at all, in response to TLR ligands (10, 11). Upon antigenic stimulation, FM B cells enter the GC and differentiate into either antibody-secreting plasma or memory B cells. Human memory B cells, in contrast to naive B cells, proliferate to polyclonal stimulation via TLRs (10). This observation suggests that TLR responsiveness might change in association with B cell differentiation. We therefore sought to determine whether FM B cells gain TLR responsiveness during the GC reaction using a murine model system.

GC B cells were isolated from immunized mice and stimulated in vitro using different TLR ligands. Because GC B cells die rapidly ex vivo, we used short-term stimulation (20 h). GC B cells proliferated similarly to MZ B cells in response to LPS. In contrast, FM B cells did not proliferate at this time point (Fig. 1 A) yet proliferated to BCR engagement after 48–72 h (not depicted). Co-stimulation of GC B cells with an antibody against CD40 and LPS led to a further increase in proliferation that was less robust in MZ B cells. GC B cells also proliferated in response to TLR2 and, less robustly, TLR3 ligands, Pam3, and polyIC, respectively (Fig. 1 B).

In an effort to understand the mechanism governing differential TLR responsiveness, we first evaluated surface expression of TLR4 and its coreceptors RP105 and MD-1 that are required for LPS responsiveness in B cells (12). TLR4/MD-2 protein expression was nearly undetectable, whereas RP105 and MD-1 were expressed on all B cell subsets (Fig. 1 C). RP105 and MD-1 expression was highest on MZ B cells, whereas expression on GC was comparable to FM B cells. Because these expression patterns were unlikely to explain the differential LPS response in GC versus FM B cells, we next analyzed expression of TLR signaling effectors by quantitative PCR. Strikingly, mRNA levels for the TLR adaptors MyD88 and Mal (but not TRIF) were increased fourfold in GC compared with FM and MZ B cells (Fig. 1 D). In contrast, expression of IRAK-M, a negative regulator for TLR signaling (13), was reduced threefold in GC B cells (Fig. 1 E). No significant differences were observed with regard to other effectors, including IRAK-1, IRAK-4, Tollip, TRAF6, or SIGIRR (not depicted). Consistent with the increased expression of MyD88, GC B cells deficient in MyD88 failed to respond to LPS, yet proliferated to anti-CD40 stimulation (Fig. 1 B).

We next studied which signals could induce TLR responsiveness in FM B cells. To mimic the GC reaction in vitro, FM B cells were co-stimulated with either anti-IgM or anti-CD40 in association with a TLR ligand. Co-stimulation led

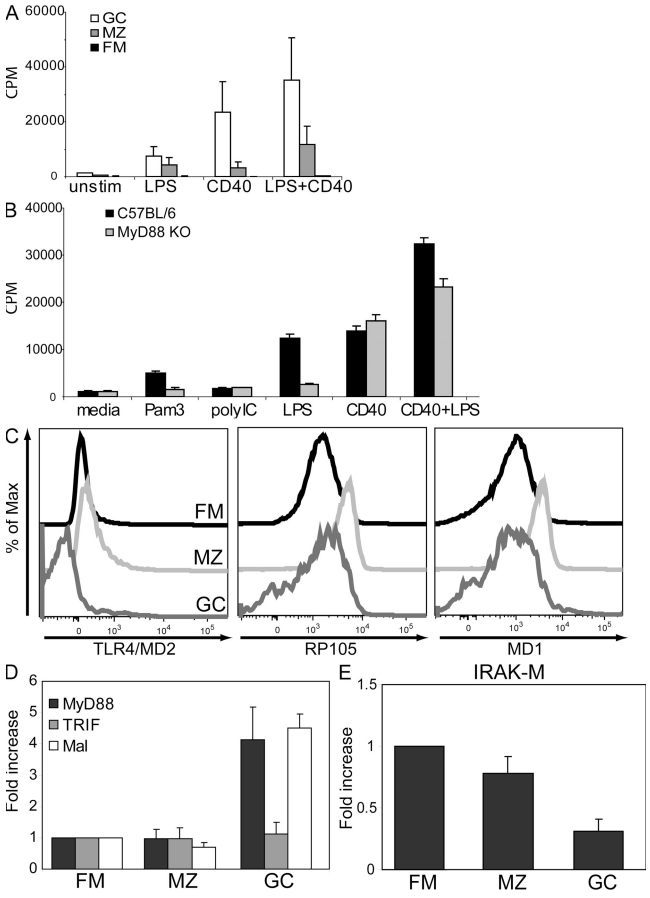

Figure 1. TLR signaling is enhanced during the GC reaction. Proliferation of GC, MZ, and FM B cells after stimulation with (A) 10 µg/ml LPS and/or 10 µg/ml anti-CD40 or (B) 50 µM polyIC or 1 µg/ml Pam3 for 20 h. (C) Surface expression of TLR4/MD-2, RP105, and MD-1 in B cell subsets as determined by FACS. Relative expression of (D) MyD88, TRIF, Mal, and (E) IRAK-M in purified B cells. Expression was determined by quantitative PCR and is shown as fold change in each subset relative to FM B cells. Average data from three independent experiments with SD are shown.

to a marked increase in the proliferative response to LPS or Pam3 (Fig. 2 A). To test if TLR responsiveness could be directly induced by either anti-IgM or anti-CD40 antibodies alone, FM B cells were prestimulated with these signals for 24 h, washed extensively, and then stimulated with LPS or LPS and anti-CD40 for 48 h. Prestimulated FM B cells proliferated robustly to TLR4 engagement, and this response correlated with the relative dose of prestimulating antibody (Fig. 2, B and C). In addition, B cell receptor or CD40 stimulation of FM B cells led to alterations in MyD88 and IRAK-M transcript levels that paralleled their expression in purified GC B cells (Fig. 2, D and E, and not depicted). Consistent with the increase in MyD88 mRNA levels, stimulation of splenic B cells with either anti-IgM or anti-CD40 lead to a marked increase in MyD88 protein expression (Fig. 2 F).

These data demonstrate that, similar to humans, where memory but not naive B cells proliferate after TLR engagement (10), an analogous difference in TLR responsiveness is

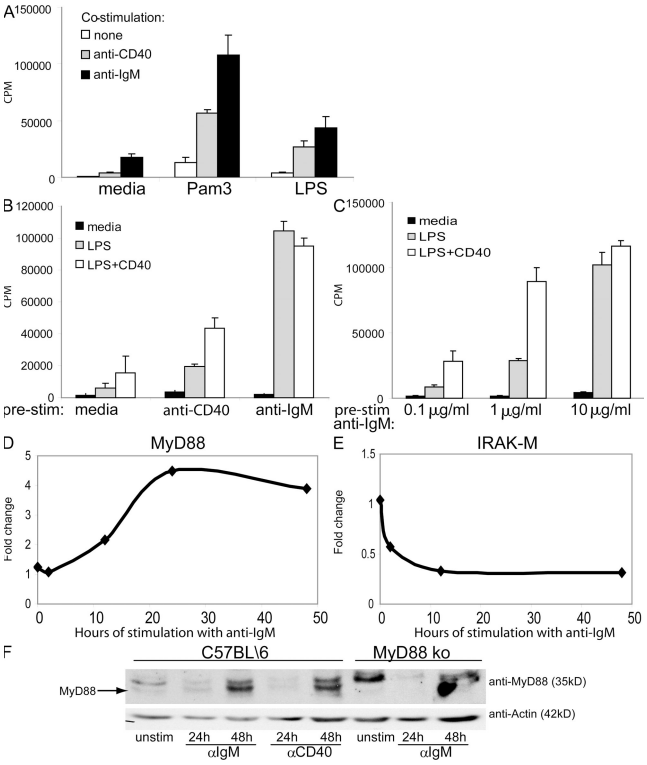

Figure 2. Modeling the GC reaction in vitro leads to increased TLR responsiveness. (A) Proliferation of FM B cells stimulated with 10 µg/ml LPS or 1 µg/ml Pam3 with or without 10 µg/ml anti-IgM or 10 µg/ml anti-CD40 for 48 h. Sorted FM B cells were preincubated with either (B) anti-IgM or anti-CD40, or (C) different doses of anti-IgM for 24 h, washed, and stimulated with LPS or Pam3 with or without anti-CD40 for an additional 48 h. mRNA expression of (D) MyD88 or (E) IRAK-M in FM B cells stimulated with anti-IgM. Data from one of three independent experiments are shown. (F) MyD88 protein expression in stimulated B cells from C57BL/6 versus MyD88 KO mice.

present in murine GC versus FM B cells. Our data also suggest that changes in the expression profile of MyD88 and IRAK-M may control this process. Consistent with this, MyD88 and IRAK-M expression can be modulated by a range of stimuli (14–16), and MyD88 KO B cells fail to proliferate and only partially up-regulate MHC class II in response to LPS (17). Although expression of IRAK-M was previously reported to be restricted to myeloid cells (18), our data suggest that IRAK-M is also present in B cells. Consistent with its suggested negative regulatory role on TLR signal transduction, GC B cells expressed significantly lower levels of IRAK-M. However, we could not detect IRAK-M protein expression by Western blotting and did not observe differences in TLR responses in IRAK-M KO versus WT murine FM or GC B cells (unpublished data). Thus, further studies are required to define the precise role for this protein in B cells.

B cell–intrinsic MyD88 signals are not required for TD immune responses

We next studied the physiological role for B cell–intrinsic TLR signals during TD immune responses. Persistence of antigen is not necessary for maintenance of humoral memory (19). Instead, polyclonal stimulation via TLR signaling has been proposed as a key mechanism to maintain human B cell memory (10). If this prediction is also correct in mice, TLR signaling should be important for maintenance of memory even in animals raised under specific pathogen-free conditions and without the delivery of exogenous TLR ligands because memory is maintained under such experimental circumstances.

Splenic B cells from MyD88 WT, heterozygote (Het), or KO mice were transferred into B cell–deficient µMt mice (20). This allowed us to generate chimeric animals containing only MyD88 WT, Het, or KO B cells with all other hematopoietic cells derived from the WT host. Recipient animals were immunized with the TD immunogen 4-hydroxy-3-nitrophenylacetyl (NP)-chicken gamma globulin (CGG) in aluminiumhydroxide (alum) as adjuvant 1 wk after B cell transfer. As an indirect measure for B cell engraftment, total Ig levels were analyzed in all animals before primary immunization (Fig. 3, A and B).

Immune responses were assessed by determining NP-specific antibody titers before and after immunization. All recipient animals exhibited similar levels of NP-specific IgM and IgG (Fig. 3, C and D). Further, no significant differences were observed in NP-specific IgG1 and IgG2a (Fig. 3, E and F), indicating that MyD88 signals in B cells are not essential for isotype switching. However, antigen-specific IgG2a levels were low under these experimental conditions, making assessment of relative levels difficult. We also observed no differences in Ig isotypes after reimmunization after 4 wk (not depicted).

Our results directly contrast recent findings suggesting that TD immune responses require activation of TLRs in B cells (8). To exclude that the requirement for TLR signals in such TD responses might depend upon the specific antigenic challenge, we also immunized µMT mice (after transfer of B cells from WT or MyD88 KO mice) with human serum albumin (HSA) in alum. In contrast to previous findings (8), we observed a significant and similar increase in HSA-specific antibody titers in both groups of recipient mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071250/DC1).

B cell memory is provided by (a) protective antibodies that are produced by long-lived plasma cells and (b) memory B cells that, upon antigenic challenge, rapidly generate plasma cells secreting high-affinity antibodies. Antigen-specific Ig levels in humans and mice are stable over very long periods (21, 22). Although serological memory can be maintained in the absence of persisting antigen (19), it remains unclear if this is achieved by persistence of terminally differentiated long-lived plasma cells alone, or via continuous differentiation of memory B cells (22). Previous work has suggested a model whereby memory B cells must be triggered via polyclonal stimuli including TLR signals to maintain humoral memory (10). In contrast to this idea, we observed that long-term maintenance (>3 mo after secondary immunization) of specific antibody titers was comparable in all recipient mice (Fig. 3, G and H, wk 0).

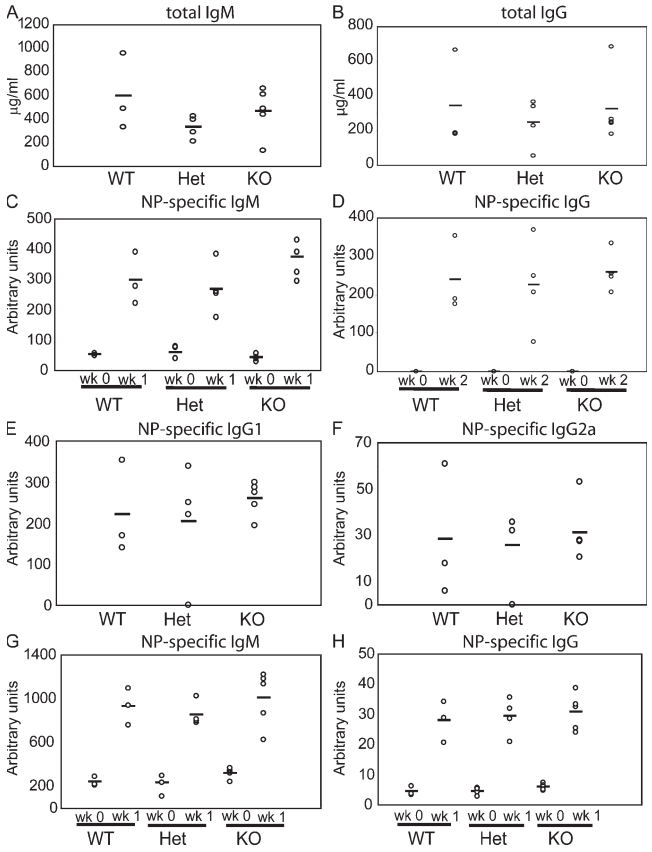

Figure 3. B cell–intrinsic MyD88 signals are not required for primary or memory responses. Splenic B cells from MyD88 WT, Het, and KO littermates were transferred into µMT mice. Mice were immunized with NP-CGG in alum, and IgM and IgG levels were determined at 1 and 2 wk after immunization, respectively. (A and B) Preimmunization IgM and IgG, (C and D) NP-specific IgM and IgG before and after immunization, and (E and F) NP-specific IgG2a and IgG1 2 wk after immunization. (G and H) 4 mo after secondary immunization, mice were challenged with NP-CGG in PBS, and antibody levels for NP-specific IgM and IgG were determined. Data from one of three independent experiments are shown. Total number of animals: WT, 12; Het, 8; KO,16.

To directly test memory responses, we also injected NP-CGG in PBS at >3 mo after the initial antigenic challenge. As expected, both IgM and IgG NP-specific antibody titers increased markedly within 1 wk of antigen challenge. However, we observed no significant differences in this response in mice that received MyD88 WT, Het, or KO B cells (Fig. 3, G and H, wk 1). We also failed to detect any significant differences in the relative affinity of anti-NP–specific IgG antibodies (not depicted). Additionally, we observed no difference in antibody titers >8 mo after primary and secondary immunization (see below).

These data strongly suggest that the number of plasma cells and/or the rate of antibody production were comparable among all animal cohorts, and that neither the magnitude of the recall response nor the affinity of the antibodies produced is dependent on endogenous TLR signals. Our combined observations suggest that B cell–intrinsic TLR signals are not required for generation or maintenance of B cell memory in mice.

B cell–intrinsic MyD88 signals partially enhance antibody responses

It has long been recognized, even before the discovery of TLRs, that microbial products, including LPS, enhance TD immune responses. What role B cells play in this augmented response is not fully understood. Although our results indicate that B cell–intrinsic TLR signals are not required for TD B cell responses, we sought to determine whether such signals could contribute to enhanced TD immune responses.

In WT mice, the immune response to NP-CGG is greatly increased by the addition of LPS, and this effect is completely abolished in MyD88 KO mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071250/DC1). Interestingly, the TRIF adaptor pathway does not appear to be required for these events, as TRIF KO and WT mice exhibit a similar amplification in antigen-specific responses (not depicted). To investigate the contribution of B cell–intrinsic MyD88 signals, we transferred B cells from MyD88 WT or KO mice into µMt mice and immunized recipient animals with NP-CGG in alum with and without the addition of LPS. Total and NP-specific IgM levels were significantly amplified only in mice receiving both adoptively transferred WT B cells and LPS (Fig. 4, A and B). Most notably, despite the presence of other hematopoietic derived lineages expressing MyD88, recipients of MyD88 KO B cells exhibited no amplification in IgM antibody titers with LPS.

Total IgG levels 1 wk after immunization were similarly affected. Only WT recipients receiving LPS in association with the immunogen showed amplified IgG levels (Fig. 4 C). In contrast, NP-specific IgG levels increased equivalently in all cohorts receiving LPS, including mice reconstituted with MyD88 KO B cells (Fig. 4 D). 2 wk after primary immunization, both total and NP-specific IgG levels were higher in mice that received LPS, and this change was independent of the type of B cells transferred (Fig. 4, E and F). NP-specific IgG2a levels, however, were significantly lower in mice that received MyD88 KO versus WT B cells in associations with LPS (Fig. 4, G and H).

Finally, we evaluated the role for exogenous TLR signals in activation of memory B cells. After MyD88 WT, Het, or KO B cells were adoptively transferred into µMT mice, recipients were immunized twice with NP-CGG in alum and subsequently challenged with NP-CGG in PBS (Fig. 5 A). 5 mo later, all animals were injected with 5 µg LPS. NP-specific IgM and IgG titers increased significantly in recipients of adoptively transferred MyD88 WT or Het B cells, but not in recipients of MyD88 KO B cells (Fig. 5, B and C). In addition, total Ig levels also increased slightly both in recipients of WT and Het B cells, but not in recipients of KO cells (not depicted). These increases in specific and polyclonal antibody levels were transient and declined to nearly prestimulation levels within 4 wk after LPS injection (not depicted).

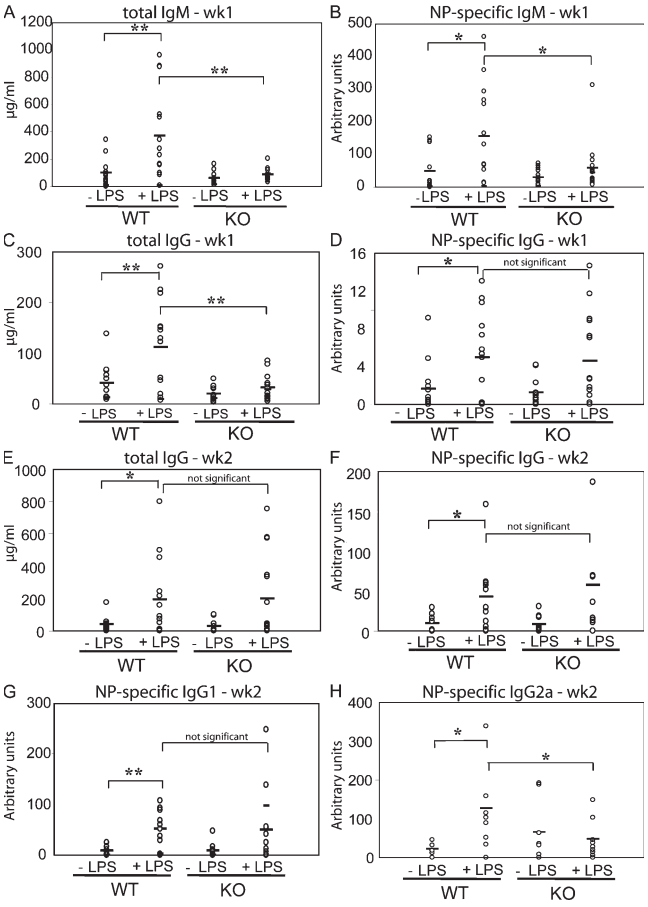

Figure 4. The addition of LPS promotes B cell–intrinsic MyD88-dependent IgM responses. WT or MyD88 KO B cells were transferred into µMT mice, and recipients were immunized with NP-CGG in alum with or without LPS (5 µg/mouse). (A–D) Total and NP-specific IgM and IgG 1 wk after immunization. (E–H) Total and NP-specific IgG, IgG1, and IgG2a 2 wk after immunization. Data are a summary of four independent experiments with a total of 14 mice per group. *, $P < 0.05$; **, $P < 0.01$.

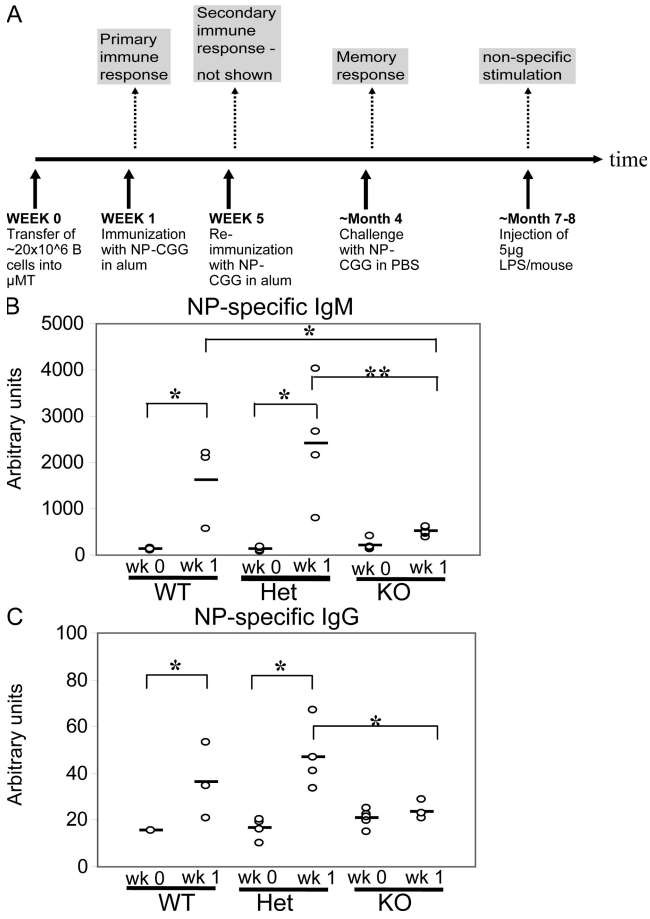

Figure 5. LPS promotes a B cell–intrinsic MyD88-dependent increase in specific antibody titers. (A) Schema for cell transfer and immunization. (B and C) MyD88 WT, Het, or KO B cells were transferred into µMT mice, and recipients were immunized as in A. 8 mo after transfer, 5 µg LPS was injected. (B) NP-specific IgM and (C) IgG before and 1 wk after LPS injection. *, $P < 0.05$; **, $P < 0.01$.

Collectively, our data indicate that B cell–intrinsic MyD88-dependent signals can significantly amplify early immune responses including, most notably, IgM production. These findings suggest that LPS can directly activate GC B cells leading to enhanced early antibody production. Although this rapid rise in both antigen-specific and nonspecific IgM likely represents a low-affinity response, it may play an important role during acute infection. Circulating IgM is comprised of both "natural" IgM produced by B1 cells (a cell population not present in our chimeric model) and antigen-induced IgM generated principally by conventional B2 cells (23). IgM from both sources is important during infection and can limit early viral and bacterial distribution (24). Recent studies have also suggested that patients with bacterial sepsis may benefit from polyclonal IgM-enriched Ig therapy (25). Thus, this rapid B cell–intrinsic MyD88-dependent increase in specific IgM may promote pathogen clearance via high-avidity pentameric IgM. Loss of the early B cell–intrinsic TLR-dependent IgM response described here may contribute to the unique susceptibility of IRAK-4–deficient humans to encapsulated bacteria (26). Additional studies using this chimeric model are needed to define the importance for this response during specific infectious challenges.

The addition of LPS to the immunogen also enhanced antigen-specific IgG responses. However, this effect occurred independently of TLR signaling in B cells. Consistent with this, several reports have demonstrated that TLR signals are critically involved in the initiation and enhancement of adaptive immune responses (4). The only exception to this paradigm was IgG2a production, which required B cell–intrinsic TLR signaling. This latter result is in accordance with work showing that TLR9 ligands can directly stimulate B cells to undergo isotype switching to IgG2a (27).

Although B cell–intrinsic TLR signals are not required for maintenance of humoral memory, our data demonstrate that such signals promote a marked B cell–intrinsic increase in both nonspecific and specific Ig production. This supports the idea that memory B cells can directly respond to TLR ligands as suggested previously (10). However, because this

effect is short-lived (at least in our model system), it may have only a limited impact on long-term antibody titers.

Overall, our data provide a better understanding of how TLR signals contribute to enhanced immune responses. TLR responsiveness is increased during the GC reaction, and changes in the expression of key molecules within the TLR pathway likely control this process. Although not required for TD immune responses, TLR signals can lead to increased Ig production via at least two different mechanisms. First, such signals can directly activate B cells and promote enhanced IgM antibody production. Second, LPS-activated non–B cells enhance activation of T cells, and this ultimately leads to increased costimulation of B cells presumably within the GC microenvironment. Consistent with this, early IgM responses are largely T cell independent, whereas IgG isotype production is dependent on T cells (28). In addition, class switching to IgG2a is enhanced by B cell–intrinsic TLR signals. Finally, TLR signals can directly activate memory B cells leading to a transient rise in both antigen-specific and nonspecific Ig production via a B cell–intrinsic MyD88-dependent signaling cascade.

This knowledge is important for the design of new or improved vaccine strategies. In addition, TLR signals clearly play a role in human autoimmune disease. Understanding the circumstances that promote B cell activation via TLRs should help to better understand the pathogenesis and course of specific autoimmune disorders, and may also guide treatments targeting this pathway within B cells.

MATERIALS AND METHODS

Mouse strains. C57BL/6, MyD88 WT, Het, KO, and μMT mice (on a C57BL/6 background) were bred and maintained in the specific pathogen-free animal facility of the Children's Hospital and Regional Medical Center (Seattle, WA) and handled according to the Institutional and Animal Care Use Committee guidelines.

Antibodies and reagents. The following antibodies were used: CD24 and CD21 (BD Biosciences), CD23 (Invitrogen), PNA (Vector Laboratories), and TLR4/MD2 and RP105 (eBiosciences). Anti-IgM, anti-IgG, anti-IgG1, and anti-IgG2a for ELISA were purchased from SouthernBiotech; LPS, HSA, and alum were from Sigma-Aldrich; polyclonal goat F(ab')2 fragment anti–mouse IgM were from Jackson ImmunoResearch Laboratories; and NP-CGG and NP-BSA were from Biosearch Technologies. Anti-CD40 was provided by G. Cheng (University of California, Los Angeles, Los Angeles, CA).

Immunization. For induction of splenic GCs, mice were injected once i.p. with 0.2 ml of 10% vol/vol fresh sheep red blood cells (Colorado Serum Company) as described previously (29) and analyzed or sort-purified 6–8 d later.

Flow cytometry and cell sorting. FM and MZ B cells were sorted from CD43-depleted splenocytes based on CD24, CD21, and CD23 expression, and GC B cells according to B220 and PNA expression. Postsort analysis showed purities of >90% for all subsets. All FACS data were collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cell culture. Splenocytes were cultured in RPMI 1640 with 10% FCS, 55 μM 2-ME, 10 mM Hepes, penicillin, and streptomycin at 37°C. Cells were stimulated with polyclonal goat anti–mouse IgM F(ab')2 fragment in concentrations as indicated, 10 μg/ml anti–mouse CD40, 10 μg/ml LPS, 50 μM polyIC, or 1 μg/ml Pam3 .

[^{3}H]Thymidine uptake proliferation assay. Purified cells were incubated at 5×10^4 cells per well in complete media. Cells were pulsed with 1 μCi [^{3}H]thymidine 8 h before harvesting. [^{3}H]Thymidine incorporation was measured in triplicate wells using a TopCount Scintillation Counter (PerkinElmer).

Real-time PCR. RNA was isolated using the RNeasy Micro kit (QIAGEN) and converted into cDNA by Superscript II (Invitrogen), and quantitative real-time PCR was performed using SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. Ratios of target expression with mouse β-2 microglobulin (a housekeeping control) were calculated using Pfaffl's mathematical model for relative quantification (30). All real-time PCR analysis includes at least three independent experiments. Primer sequences used include: IRAK-M: forward primer, GCGGTGGCAGGAAACATCTG, reverse primer, CGTGTTTCGGGTCATCCAGC; MyD88: forward primer, AGAGCTGCTGGCCTTGTTAGACC, reverse primer, AGGCTTCTCGGACTCCTGGTT; Mal: forward primer, CGAGTCCTACCAAGCCACTTTTCA, reverse primer, AGCCGATTCCAGGTAGATTGCAT; and TRIF: forward primer, GGTTCACGATCCTGCTCCTGAC, reverse primer, GCTGGGCCTGAGAACACTCAAG. Other primers are available upon request.

Adoptive cell transfer. 15-20 × 10^6 CD43-depleted splenic B cells (purity >97%) from MyD88 WT, Het, or KO littermates were injected i.v. into μMT mice. Recipients were immunized i.p. with 100 μg NP_{36}-CGG in 200 μl alum 5–7 d after cell transfer. Animals were reimmunized with NP_{36}-CGG in alum and challenged with 20 μg NP_{36}-CGG in PBS. 5 μg LPS was injected in addition to the immunogen as indicated or in PBS.

ELISA. Plates were coated with NP30-BSA or NP3-BSA at 10 μg/ml, blocked with 2% BSA in PBS, and incubated with serial dilutions of the sera. This was followed by incubation with horseradish peroxidase–conjugated secondary antibodies. The ELISA was developed using the TMB ELISA kit from BD Biosciences, and absorbance was measured at 450 nm on a Victor3. Ig levels were quantified by comparison with titrated Ig standards.

Western blot analysis. For analysis of MyD88 protein expression, stimulated B cells were lysed in RIPA buffer (0.1% SDS, 0.1% sodium deoxycholate, 1% NP-40, 250 mM NaCl, 50 mM TrisCl, pH 7.5) at the indicated time points. Whole cell lysates were run on a 10% SDS gel and transferred to a nitrocellulose membrane, and blots were probed with anti-MyD88 antibody (1:1,000; Stessgen).

Online supplemental material. Fig. S1 shows TD immune responses after immunization with HSA of μMT mice after transfer of B cells from WT versus MyD88 KO mice. Fig. S2 shows antibody responses in C57BL/6 versus MyD88 KO mice after immunization with NP-CGG in alum with or without the addition of LPS. Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20071250/DC1.

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