MyD88- and Bruton's Tyrosine Kinase-Mediated Signals Are Essential for T Cell-Independent Pathogen-Specific IgM Responses

Kishore R. Alugupalli, Shizuo Akira, Egil Lien and John M. Leong

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Bacteremia is one of the leading causes of death by infectious disease. To understand the immune mechanisms required for the rapid control of bacteremia, we studied *Borrelia hermsii*, a bacterial pathogen that colonizes the blood stream of rodents to an extremely high density. A T cell-independent IgM response is essential and sufficient for controlling *B. hermsii* bacteremia. Mice deficient in Bruton’s tyrosine kinase (Btk), despite their known defect in BCR signaling, generated *B. hermsii*-specific IgM and resolved bacteremia, suggesting that an alternative activation or costimulatory pathway remained functional for T cell-independent B cells in Btk<sup>−/−</sup> mice. *B. hermsii* contains putative ligands for TLRs, and we found that mice deficient in TLR1, TLR2, or the TLR adaptor MyD88 generated anti-*B. hermsii* IgM with delayed kinetics and suffered more severe episodes of bacteremia. In striking contrast to the anti-*B. hermsii* IgM response in mice deficient only in Btk, mice deficient in both Btk and MyD88 were entirely incapable of generating *B. hermsii*-specific Ab or resolving bacteremia. The response to a T cell-dependent model Ag was unaffected in Btk<sup>−/−</sup> x MyD88<sup>−/−</sup> mice. These results suggest that MyD88 specifically promotes T cell-independent BCR signaling and that, in the absence of Btk, this TLR-mediated stimulation is a required component of this signal. *The Journal of Immunology*, 2007, 178: 3740–3749.

MyD88- and Bruton’s Tyrosine Kinase-Mediated Signals Are Essential for T Cell-Independent Pathogen-Specific IgM Responses<sup>1</sup>

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Bacteremia is one of the leading causes of death by infectious disease. To understand the immune mechanisms required for the rapid control of bacteremia, we studied *Borrelia hermsii*, a bacterial pathogen that colonizes the blood stream of rodents to an extremely high density. A T cell-independent (TI)<sup>4</sup> Ab response can be highly protective and develop much faster than T cell-dependent (TD) Ab responses (2), suggesting a potential utility of TI responses as preventive and therapeutic interventions against a broad range of clinically important bacteria, including bioterror agents.

One approach to identify mechanisms that rapidly clear microbial pathogens from the bloodstream is to investigate bacteria that are uniquely adapted to growth in the vascular compartment. *Borrelia hermsii*, a bacterium that causes relapsing fever, is extremely virulent in mice. This article must therefore be hereby marked in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 E.L. and J.M.L. contributed equally to this study.

4 Abbreviations used in this paper: TI, T cell independent; TD, T cell dependent; FO, follicular; MZ, marginal zone; DKO, double knockout; NP-CGG, nitrophenyl-conjugated chicken gammaglobulin; wt, wild type.

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by stimulating mitogenic receptors (2). As a result, and in contrast to the Ab response to B. hermsii, TI-I responses are not necessarily Ag-specific, particularly in the context of relatively large amounts of Ag, such as would be the case for B. hermsii bacteremia. TI-2 Ags, such as the polymeric capsules of the anthrax agent, *Bacillus anthracis* (13) and *S. pneumoniae* (14), contain repetitious epitopes that can efficiently stimulate B cells in the absence of T cell help primarily by cross-linking BCRs. These Ags do not induce protective Ab-responses in X-linked immunodeficient (sid) mice, which carry a mutation in Bruton’s tyrosine kinase (Btk), a cytoplasmic kinase crucial for BCR-mediated activation (15, 16). In contrast, *B. hermsii* triggers an expansion of B1b cells in sid mice, inducing an Ab response capable of controlling infection, albeit with somewhat delayed kinetics (7). These results indicate that, remarkably, even in the absence of a normal BCR signaling, *B. hermsii* is capable of rapidly activating Ag-specific B cells, presumably by stimulating other signaling pathways.

TLRs play important roles in activation of the immune system (17). TLRs are germline-encoded receptors expressed on B cells as well as a variety of innate immune cells, and recognize a wide range of evolutionarily conserved microbial products from bacteria, viruses and fungi, in addition to certain host-derived molecules. TLRs are type-I transmembrane glycoproteins with an extracellular domain that features a remarkable plasticity in terms of ligand recognition, and at least 11 TLRs (TLR1–11) have been described in mammals. The diversity and specificity of the TLR response is also determined by the selective use of four intracellular adaptor molecules; MyD88, Mal, Trif and Tram. These adaptors mediate the proximal interactions with the intracellular domains of the TLRs and create a platform to a cascade of kinases and transacting factors, events that ultimately result in activation of various components of the immune system.

In the present study, we have investigated mice deficient in specific TLRs, their adaptors and coreceptors and found that TLR signaling pathways, particularly the TLR2-mediated pathway, play a crucial role in protective immunity to *B. hermsii*. Simultaneous disruption of Btk and MyD88 severely impaired the immune response essential for controlling the bacteremia, demonstrating that coordinated stimulation of TLR and BCR signaling activates B cells and triggers the rapid induction of Ag-specific IgM responses.

### Materials and Methods

**Mice and infections**

Mice housed in microisolator cages with free access to food and water, were maintained in a specific pathogen-free facility of Thomas Jefferson University and University of Massachusetts Medical School. The studies have been reviewed and approved by Institutional Animal Care and Use Committees. C57BL/6J, 129Sv/J, B6129SF1/J, B6129SF2/J, C57BL6/J- TCR-β<sup>H</sup>tm1 Mon TCR-β<sup>dx</sup>tm1 Mon (TCR-β<sup>dx</sup>−/−), and IL-1R<sup>α</sup>tm1Rom1 (IL-1R<sup>α</sup>−/−) mice were purchased from The Jackson Laboratory. CBA/Ca and CBA/N (sid) mice were purchased from National Cancer Institute. Btk−/− mice (16) were provided by Dr. W. Khan (Vanderbilt University, Memphis, TN). CD14<sup>−/−</sup> mice (18) were provided by Drs. M. Freeman and K. Moore (Harvard Medical School, Boston MA), and MD2<sup>−/−</sup> mice (19) were provided by Dr. K. Miyake (University of Tokyo and Japan Science and Technology, Tokyo, Japan). TLR1<sup>−/−</sup> (N5); TLR2<sup>−/−</sup> (21) (N10); TLR3<sup>−/−</sup> (21) (N3); TLR4<sup>−/−</sup> (23) (N10); TRLE<sup>−/−</sup> (24) (N6); TLR9<sup>−/−</sup> (25) (N10); MyD88<sup>−/−</sup> (26) (N3); Mal<sup>−/−</sup> (27) (N6); Trif<sup>−/−</sup> (25) (N3); Tram<sup>−/−</sup> (28) (N3); CD14<sup>−/−</sup> (18) (N10); and MD2<sup>−/−</sup> (19) (N6) mice were bred on C57BL6 background and the generation numbers were indicated in parentheses.

### Table I. *B. hermsii* bacteremia in various wt mouse strains and Btk<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Peak density (×1000/μl)</th>
<th>Duration (days)</th>
<th>Significance&lt;sup&gt;a&lt;/sup&gt; (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First episode</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>5</td>
<td>55 ± 17</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>129Sv/J</td>
<td>4</td>
<td>36 ± 9</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>B6129SF1/J</td>
<td>5</td>
<td>40 ± 23</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>B6129SF2/J</td>
<td>5</td>
<td>39 ± 11</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>B6129s-Btk&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6</td>
<td>215 ± 118</td>
<td>4.8 ± 1.2 &lt;0.02 (&lt;0.005)</td>
</tr>
</tbody>
</table>

#### Notes

* Mice were infected i.v. with 4 × 10<sup>8</sup> *B. hermsii* DAH-p1. Bacteremia was measured by microscopic counting.

* The difference in the peak density or the duration of bacteremia in C57BL6J mice was compared with that in various other wt mouse strains or to Btk<sup>−/−</sup> mice by Student’s t-test. Two-tailed p values were given. Values of p for duration were given in parenthesis. N.S. denotes not statistically significant.

#### Significance<sup>a</sup>

The significance level of the difference in peak density or the duration of bacteremia between wt mice and Btk<sup>−/−</sup> mice was determined by Student’s t-test. Two-tailed p values were given. Values of p for duration were given in parenthesis. N.S. denotes not statistically significant.

#### Generation of Btk and MyD88 double knockout (DKO) mice

MyD88<sup>−/−</sup> mice (26) were crossed to Btk<sup>−/−</sup> mice (16) to generate Btk/ MyD88 DKO mice and control mice for these DKO mice were heterozygous for both mutations. MyD88 genotype was determined by PCR using the following primers: Myd88<sup>f<sub>5</sub>-3</sup> (−TGG CAT GCC TCC ATC ATA GTT AAC C-3<sup>f</sup>), and Myd88<sup>r<sub>5</sub>-3</sup> (−AGC CCA AAC GAC CTT CCA AA-3<sup>r</sup>). These DKO mice were generated by crossing Btk<sup>−/−</sup> and Myd88<sup>−/−</sup> mice, and the generation numbers were indicated in parentheses.

#### Flow cytometry

To determine the frequency of B1b cells, peritoneal cavity cells were harvested from individual mice and the cell density was adjusted to 2.5 × 10<sup>6</sup>/ml in staining medium (deficient RPMI 1640 medium (Irvine...
FIGURE 1. Btk contributes to, but not required for the control of B. hermsii. A. CBA/Ca (wt) or CBA/N (xid) mice were infected i.v. with 5 x 10^6 B. hermsii DAH-p1. Blood was sampled daily and B. hermsii-specific IgM was quantitated by ELISA. Each time point represents five mice in each group. Mean ± SD values were shown. B, wt (C57BL6; n = 5) or T cell-deficient (TCR-β−/−; n = 3) mice or Btk−/− mice (n = 3) were infected, and kinetics of B. hermsii-specific IgM responses was measured by ELISA. Mean ± SD values were shown. C, Blood was sampled from 6 days postinfected mice, and specificity of the IgM response was determined using microtiter plates coated with indicated bacterial species. Mean ± SD values were of three mice from each genotype were shown. D, wt (B6129Sv) or Btk−/− mice were infected and bacteremia was measured by dark-field microscopic counting. Each plot represents bacteremia in an individual mouse. The broken line indicates the detection limit for bacteremia. Statistically significant differences between wt and mutant mice were indicated with p values <0.05 (*), <0.005 (**), and <0.001 (***).

Scientifically with 3% calf serum, 1 mM EDTA). After blocking the FcRs with 2.4G2 Ab (1 µg per 10^6 cells), an aliquot of 25 µl of peritoneal cavity cells was incubated in a microtiter plate with appropriately diluted Ab. To determine the frequency of MZ B cells, 25 µl of spleen cells was stained with appropriate Abs. The Abs, anti-IgM-FITC (clone 1B4B1), anti-IgD-biotin (clone 11-26), anti-Mac1-PE (clone M1/70) and anti-CD5 PE (clone 53-7.3) were purchased from eBioscience; streptavidin-PE-Cy5, CD23-PE (clone B3B4), CD21-FITC (clone 7G6) were from BD Pharmingen and with B220-TC (clone RA3-6B2) was purchased from Caltag Laboratories. After staining, cells were washed twice with staining medium and the preparations were run on a FACSCalibur (BD Biosciences) using CellQuest software for acquisition of the data (BD Biosciences). Data was analyzed using FlowJo software program (Tree Star).

**Immunization**

To measure the TD immune responses, wt mice or mice deficient in both btk and MyD88 (i.e., DKO) were immunized with 50 µg of nitrophenyl-conjugated-chicken gammaglobulins (NP-CGGs; Research Technologies) precipitated in alum and injected i.p. Blood samples obtained on 0, 4, 7, 10, and 14 days postimmunization were diluted and NP-specific response was determined by ELISA using NP-conjugated BSA (NP-BSA; Research Technologies) (5 µg/ml)-coated microtiter plates.

**Statistical analysis**

Statistics were performed using InStat 2.01 software program. To determine statistically significant differences, Student’s unpaired t test was performed and two-tailed p values were given.

**Results**

**Mice deficient in Btk can control B. hermsii bacteremia**

Mutations affecting the BCR signaling pathway result in a deficiency of B1 cell subsets, which are the major producers of IgM (29), and xid mice (CBA/N), harboring a point mutation in btk that diminishes BCR signaling, display a deficiency of B1 cells and basal IgM levels (7). Nevertheless, xid mice, after initially suffering more severe bacteremic episodes than wt mice, can control the B. hermsii bacteremia (7). Because IgM is essential for controlling this infection, we predicted that xid mice are capable of mounting an anti-B. hermsii IgM response, despite their inherent deficiency in preimmune IgM levels. As predicted, with some delay, xid mice generated a specific IgM response, one which was of somewhat lower magnitude than the wt response but coincident with the resolution of bacteremia by day 4 postinfection (Fig. 1A) (7).

The Btk protein in xid mice has a substitution (R28C) in the pleckstrin homology (PH) domain that disrupts Btk recruitment to the BCR signalosome, and is expressed at 70% of wt levels (15). This substitution is not likely to disrupt the kinase activity of Btk, because Btk deleted for the PH domain has wt basal levels of catalytic activity (30). To unequivocally determine whether the ability of xid mice to generate B. hermsii-specific IgM and to control bacteremia was due to residual Btk activity, we examined mice that have a targeted deletion in the kinase domain of Btk and do not express any detectable Btk protein (16). Despite the inherent deficiency of Btk−/− mice in serum IgM as well as BCR-mediated signaling (16), infected mice mounted a robust IgM response to B. hermsii (Fig. 1B). A TI response is sufficient for the control of B. hermsii infection (7), and consistent with this T cell-deficient mice rapidly generated anti-B. hermsii IgM (Fig. 1B). The infection-induced IgM did not bind efficiently to a closely related pathogen, B. burgdorferi, to an unrelated Gram-negative enteric pathogen, Escherichia coli, or to a Gram-positive oral pathogen, Streptococcus sanguis, demonstrating the involvement of Ag-specific BCR repertoire in this response (Fig. 1C).

Consistent with the somewhat delayed IgM response in Btk−/− mice (Fig. 1B), the Btk−/− mice suffered more severe episodes of bacteremia than wt mice (Fig. 1D). For example, the peak bacterial
density (215,000 ± 118,000 vs 55,000 ± 17,000 per μl blood; p < 0.02) and duration (4.8 ± 1.2 vs 2.4 ± 0.6 days; p < 0.005) of the first episode of bacteremia was significantly higher and prolonged in the Btk−/− mice compared with wt mice (Table I). Nevertheless, Btk−/− mice were capable of controlling bacteremia with delayed kinetics (Fig. 1D), similar to that observed for xid mice (7). These results indicated that immunostimulatory pathways independent of Btk and T cells contribute to the development of an Ag-specific IgM response during bacteremia.

MyD88-mediated signaling plays an important role in controlling B. hermsii bacteremia

The TLR signaling system has been shown to play an important role in controlling adaptive immune responses (17). B. hermsii bacteremia

### Table II. B. hermsii bacteremia in mice deficient in adaptors required for TLR signaling

<table>
<thead>
<tr>
<th>Genotype</th>
<th>First episode</th>
<th>Second episode</th>
<th>Third episode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak density (×1000/μl)</td>
<td>Duration (days)</td>
<td>Significance (p value)</td>
</tr>
<tr>
<td>wt (9)</td>
<td>37 ± 10</td>
<td>2.3 ± 0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MyD88−/− (7)</td>
<td>1106 ± 148</td>
<td>12.7 ± 3.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mal−/− (8)</td>
<td>155 ± 129</td>
<td>5.0 ± 2.7</td>
<td>&lt;0.02 (&lt;0.02)</td>
</tr>
<tr>
<td>Trif−/− (8)</td>
<td>44 ± 11</td>
<td>2.5 ± 0.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Tram−/− (7)</td>
<td>43 ± 16</td>
<td>2.1 ± 0.4</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* Mice were infected i.v. with 4 × 10⁸ B. hermsii DAH-p1. Bacteremia was measured by microscopic counting.

* The difference in the peak density or the duration of bacteremia in wt (C57BL/6J) mice was compared with that of mice deficient in TLR adaptors by Student’s t test. Two-tailed p values were given. Values of p for duration were given in parenthesis. N.S. denotes not statistically significant.
possesses a number of potential TLR ligands, such as lipoproteins (31) and CpG DNA, that could activate distinct members of TLR family, thereby generating functional redundancy in TLR(s) signaling. MyD88 is a common cytoplasmic adaptor for all TLRs family, thereby generating functional redundancy in TLR(s) signaling. MyD88 is known to play a role in IL-1R signaling in addition to TLR2 signaling (17), the defect in controlling bacteremia in mice deficient in specific TLR signaling pathways except TLR3, so we investigated B. hermsii infection in mice deficient in each of the four known TLR adaptors.

MyD88−/− mice were delayed in the generation of specific IgM (Fig. 2A). The delay in the generation of B. hermsii-specific IgM correlated with more severe and prolonged episodes of bacteremia (Fig. 2B). For instance, the bacterial burden during primary episode in MyD88−/− mice was ~20-fold higher than that of wt mice, and the duration of bacteremia was significantly prolonged (Table II). Consistent with the significant impairment in controlling B. hermsii infection (Table II), a fraction of MyD88−/− mice died during episodes of severe bacteremia (Fig. 2B). Although MyD88 is known to play a role in IL-1R signaling in addition to TLR signaling (17), the defect in controlling B. hermsii bacteremia by MyD88−/− mice was not due to this activity because IL-1R plays no apparent role in limiting B. hermsii bacteremia (Table III). Mice deficient in the adaptor Mal, exhibited a moderate susceptibility compared with MyD88−/− mice (Fig. 2B). The mean bacterial density during the primary episode in Mal−/− mice was significantly higher compared with wt mice (Table II). The duration of bacteremic episodes were significantly prolonged (Table II). Surprisingly the kinetics of anti-B. hermsii IgM response in Mal−/− mice was indistinguishable from that of wt mice (Fig. 2A). Mice deficient in Trif and Tram adaptors (17) controlled B. hermsii comparably to wt mice (Table II), and generated anti-B. hermsii Ab responses as efficiently as wt mice (data not shown). These data demonstrate that of the TLR adaptors, MyD88 plays the most crucial role in the rapid IgM response to B. hermsii infection.

TLR2- and TLR1-mediated signaling contributes to anti-B. hermsii IgM response

TLR2 employs MyD88 and Mal as its proximal adaptors for initiating its signaling cascade (17), and we found severe bacteremia in mice deficient in these two adaptors (Fig. 2A and Table II) suggesting a role for TLR2 in anti-B. hermsii response. Moreover, the outer membrane of B. hermsii is predominantly composed of lipoproteins (31), which can be recognized by TLR2 (32). To examine a role for TLR2, we infected TLR2−/− mice and found that compared with wt mice, the B. hermsii-specific IgM response was significantly delayed and reduced in TLR2−/− mice (Fig. 3). Correspondingly, the mice suffered an order of magnitude higher bacterial burden than wt mice during the first episode (Table III). Unexpectedly, after resolution of bacteremia, a large percentage of TLR2−/− mice succumbed to B. hermsii infection. Although the lethality of B. hermsii infection in these mice is striking, the timing of death, after bacterial clearance, indicates that it was not simply due to high levels of bacteremia, and the mechanism of death in these mice is the subject of a separate study.

Heterodimerization of TLR2 with either TLR1 or TLR6 could facilitate specific recognition of triacylated or diacylated microbial lipoproteins (31), which can be recognized by TLR2 (32).
FIGURE 4. Analysis of mature B cell subsets and Ab responses in Btk and MyD88-deficient mice. A, Peritoneal cavity cells of were harvested and stained with Abs specific for IgM, IgD, and Mac1 or CD5 and analyzed by flow cytometry. All B cells were first identified by IgM and/or IgD positivity (plots not shown) and were further resolved as B1 (e.g., B1a plus B1b) or B1a populations by Mac1 or CD5 positivity, respectively. The frequency values of B1 and B1a cells among the total B cells were shown. Frequency values indicated in the parentheses represent the percentage of these populations among the total peritoneal cavity cells. The frequency of B1b cells was inferred from the values obtained from subtraction of the percentage of B1a (CD5<sup>+</sup>) from the percentage of all B1 cells (Mac1<sup>+</sup>). The data were generated by analyzing a minimum of 20,000 cells and are representative of 3–5 mice. Five percent contour plots are shown. B, Spleen cells were stained with Abs specific for B220, CD21, and CD23 and analyzed by flow cytometry. All B cells were first identified by B220 positivity (plots not shown) and were further resolved as MZ B (CD23<sub>low</sub> and CD21<sub>high</sub>) and FO B cells (CD23<sub>high</sub> and CD21<sub>low</sub>) and their frequencies were indicated within the plots. C, Total IgM (circles) and IgG (triangles) levels in the blood were determined by ELISA. Wt (<i>n</i> = 8), Btk<sup>−/−</sup> or y and MyD88<sup>−/−</sup> (<i>n</i> = 6), MyD88<sup>−/−</sup> (<i>n</i> = 8), Btk<sup>−/−</sup> or y (<i>n</i> = 10) and DKO (<i>n</i> = 15). Each symbol represented a mouse and the mean Ab levels were indicated with a solid line. The differences in the IgG levels in all these groups of mice were not statistically significant. The IgM levels in Btk<sup>−/−</sup> or y and DKO mice were indistinguishable but significantly different from that of wt or MyD88<sup>−/−</sup> mice (<i>p</i> < 0.0001). D, wt (<i>n</i> = 4) or DKO (<i>n</i> = 4) mice were immunized with alum precipitated NP-CGG.
lipoproteins, respectively (17). We found that, similar to TLR2−/− mice, TLR1−/− mice exhibit an IgM response that was both diminished and delayed relative to wt (Fig. 3). This muted response corresponded to significantly prolonged episodes of bacteremia (Table III). In contrast, TLR6−/− mice generated anti-B. hermsii IgM and cleared bacteria indistinguishably from wt mice (Table III and data not shown). These results suggest that triacylated but not diacylated lipoproteins drive TLR2-mediated stimulation (Fig. 3). Consistent with a role for CD14 in augmenting the TLR2 response, we also found that CD14−/− mice generated a delayed IgM response (Fig. 3) and suffered more severe bacteremia than wt mice (Table III).

**TLR9-mediated signaling is not required for controlling B. hermsii**

The generation of B. hermsii-specific IgM responses and the control of B. hermsii bacteremia in TLR2−/− mice, albeit with delayed kinetics, indicated that other TLRs might also play a role eliminating B. hermsii. By virtue of being a prokaryote, B. hermsii is expected to contain unmethylated CpG DNA in its genome, which is recognized by TLR9 (25). More importantly, TLR9 has been shown to activate autoreactive B cells (33) and memory B cells (34), indicating a possible role for TLR9 in the development of rapid as well as long-lasting Ab responses to B. hermsii. Despite these expectations, we found that TLR9−/− mice generate rapid IgM responses comparable to those of wt mice (Fig. 3) and do not suffer more severe bacteremia than the wt mice (Table III). These results demonstrate that TLR9 is not essential for controlling B. hermsii bacteremia.

**TLR4-mediated signaling plays a minor role in controlling B. hermsii infection**

Lack of a role for TLR9 prompted us to examine the potential involvement of other members of the TLR family. CD14 is not only involved in TLR2 signaling but also in TLR4-mediated signaling, and although B. hermsii does not contain LPS, it was shown that other sialoglycoproteins could be sensed by TLR4 (35). Thus, we infected TLR4−/− mice and found that the severity of peak B. hermsii bacteremia was on average 3- to 6-fold higher than for infection of wt mice, but reached statistical significance only for the third peak (Table III). The magnitude of the specific IgM response was also somewhat lower than wt, but the differences were not statistically significant (Fig. 3). As expected, this potential TLR4-mediated response did not resemble an LPS-induced response, because mice deficient in MD2, a protein absolutely required for the LPS response (19), did not suffer more severe bacteremia than wt mice (Table III). These results indicate that B. hermsii may also signal through TLR4 in addition to TLR2, by a mechanism distinct from that of LPS-induced stimulation. As expected, given that TLR3 is a receptor for dsRNA, which is not predicted to be present in B. hermsii, IgM response (data not shown) and the magnitude of bacteremia (Table III) in TLR3−/− mice were indistinguishable compared with wt mice, emphasizing TLR1, TLR2 and to a lesser extent TLR4, serve a specific function during B. hermsii infection (Table III).

Severely impaired IgM responses and persistent high-level bacteremia in mice deficient in MyD88 and Btk-mediated signaling

MyD88−/− and Btk−/− mice each are capable of generating a specific IgM response to B. hermsii, albeit in a delayed fashion (Figs. 1B and 2A). We reasoned that MyD88 and Btk might generate signals that play a synergistic or partially redundant role in the specific IgM response, in which case mice defective in both signaling pathways might be severely impaired in their ability to control B. hermsii bacteremia. To test this, we generated Btk and MyD88 DKO mice, here after referred to as DKO, by intercrossing Btk−/− and MyD88−/− mice (data not shown).

We first analyzed DKO mice for immunologic parameters that could be related to the ability to resist B. hermsii infection. DKO mice demonstrated a B1a and B1b cell deficiency comparable to Btk−/− (Fig. 4A; Table IV) or xid mice (7). In addition to B1b cells, MZ B cells, which are strategically located in the spleen and can mount a rapid IgM response to blood-borne particulate Ags (36), contribute to the control of high-level B. hermsii bacteremia, particularly early in infection (11). Analysis of spleen cells by flow

Blood was sampled on indicated days and NP-specific IgM and IgG response in 1/500 diluted-blood was measured by ELISA. Kinetics of specific Ab responses was expressed as OD at 450 nm using NP-BSA as capture reagent. E, The magnitude of NP-specific Ab response in wt and DKO mice was measured for 7-day postimmunized mice. The specific IgG titer was significantly less in DKO mouse (p < 0.05).
cytometry revealed that, similar to Btk<sup>−/−</sup> mice (16), the frequency of MZ B cells in DKO mice was comparable to that of wt mice (Fig. 4B; Table IV). As expected the absolute numbers of MZ mice (Fig. 4). In contrast, littermate controls heterozygous for both mutations showed IgM levels indistinguishable from either wt or MyD88<sup>−/−</sup> mice. The overall IgG levels of DKO mice were comparable to wt, Btk<sup>−/−</sup>, or MyD88<sup>−/−</sup> mice (Fig. 4C). Although it is known that the relative concentrations of different IgG subclasses in MyD88<sup>−/−</sup> and Btk<sup>−/−</sup> mice are distinct (37, 38), we did not analyze the subclasses because IgG is dispensable for the rapid clearance of <i>B. hermsii</i> (10).

Neither Btk nor MyD88 are required for an efficient response to alu-precipitated TD Ags (16, 39), and to test whether DKO mice respond normally to such Ags, they were immunized with alu-precipitated NP-CGG. These mice generated specific IgM and IgG with kinetics similar to that of wt mice (Fig. 4D). Moreover, the magnitude of the NP-specific IgM response was also comparable between wt and DKO mice (Fig. 4E). Although the magnitude of the IgG response in DKO mice was somewhat less than that of wt mice, it was still quite robust (Fig. 4E) and comparable to that of Btk<sup>−/−</sup> mice (38). These results demonstrated that many of the B cell and IgM parameters for DKO mice are similar to those of Btk<sup>−/−</sup> mice, and that simultaneous disruption of Btk and MyD88 does not compromise their ability to mount a TD Ab response.

Heterozygous littermates were capable of generating <i>B. hermsii</i>-specific IgM (data not shown) and controlling infection as rapidly and efficiently as wt mice (Table V). Compared with wt mice, the Btk<sup>−/−</sup> and the MyD88<sup>−/−</sup> mice were delayed in their IgM response by one and two days, respectively (Fig. 5). We next tested whether MyD88 and Btk provide synergistic or partially redundant functions in triggering an Ab response to <i>B. hermsii</i>. DKO mice were strikingly compromised for IgM production and bacterial clearance (Fig. 5). Indeed, DKO mice were incapable of even transient clearance of bacteria, and suffered persistent bacteremia at levels ~20-fold higher than that found in wt mice or heterozygous littermates (Table V). These results suggest that TLR stimulation facilitates BCR-mediated responses leading to the production of Ag-specific IgM, and in fact, in the absence of normal BCR signaling due to the lack of Btk, this MyD88-mediated response is essential to the control of <i>B. hermsii</i> infection.

**Discussion**

The ability of the immune system to generate a rapid and pathogen-specific TI Ab response during bacterial infection can prevent serious pathological consequences. In the current study, we confirmed our previous finding that Btk plays a role in, but is not entirely essential for, the development of an anti-<i>B. hermsii</i> IgM response, suggesting that an alternative activation or costimulatory signal remained functional for TI B cells in these mice. The only
costimulatory pathway previously shown to promote an Ag-specific response in Btk−/− mice involves engagement of CD40 by CD40L during B cell responses to TD Ags (38). Although artificial stimulation of CD40 by the administration of anti-CD40 Abs to xid mice restores responsiveness to TI-2 Ags such as NP-Ficoll (40), engagement of CD40 by CD40L occurs primarily during cognate interaction of T and B cells (41), indicating that this pathway is unlikely to play a role in the TI response to B. hermsii. The observation that CD40L−/− mice generate a robust TI IgM response to the related bacterium B. burgdorferi supports this notion (42).

To determine what signaling cascades might contribute to the activation of B. hermsii-specific B cells in the absence of Btk, we investigated the effect of TLR signaling on the generation of anti-B. hermsii IgM and the clearance of bacteremia. Mice deficient for TLR2 or TLR1 were defective in their IgM response to B. hermsii, and correspondingly were delayed in clearing bacteria from the bloodstream, indicating a role for TLR signaling in the activation of B. hermsii-specific B cells. The (mild) defect in bacterial clearance displayed by TLR4 mice suggested that multiple TLR’s might provide redundant signaling during B. hermsii infection. Consistent with this, the defect of MyD88-deficient mice in clearing bacteremia was more severe than that observed for mice deficient in any single TLR, and a fraction of infected MyD88-deficient mice died during peak bacteremia. (TLR2-deficient mice also suffered significant mortality, but death occurred by unknown mechanisms several days after peak bacteremia; Fig. 3 and data not shown.) It was recently reported that transfer of convalescent serum but not naive serum from wt mice diminishes the bacterial burden in MyD88−/− mice (43), demonstrating an important role for specific Abs in the protective immunity even in the absence of MyD88. The generation of a specific (albeit delayed) Ab response in MyD88−/− mice detected in the current study (Fig. 2A) suggests a role for both MyD88-dependent and MyD88-independent pathways in protective immunity. Mice deficient in Mal, an adaptor that also participates in TLR2 and TLR4 signaling (17), displayed a milder defect in bacterial clearance than the MyD88−/− mice, raising the possibility that this adaptor might provide partially overlapping function. It is noteworthy, that the phenotype of bacteremia in Mal−/− mice (Table II) was less severe than that in TLR2−/− mice (Table III). For example, the peak bacterial density (155,000 ± 129,000 vs 471,000 ± 121,000 per microliter of blood; p < 0.005) of the first bacteremic episode was significantly higher in TLR2−/− mice than that in Mal−/− mice. Moreover, the kinetics of specific IgM response was delayed in TLR2−/− (Fig. 3) and MyD88−/− (Fig. 2A) but not in Mal−/− mice (Fig. 2A), suggesting that a Mal-independent TLR2 signaling also plays an important role in controlling B. hermsii infection. The involvement of TLR2 and MyD88 in the development of a specific Ab response during B. hermsii infection contrasts with the observation that these molecules are not required for the generation of pathogen-specific Ab during B. burgdorferi infection (44–46).

MyD88 plays a central role in the response to LPS, the prototypic TI-1 Ag. LPS can stimulate B cells via TLR4 regardless of their BCR specificity, resulting in a relatively nonspecific Ab response, particularly in the context of high Ag load. In the present study, MyD88−/− mice generated B. hermsii-specific IgM, albeit with delayed kinetics, indicating that B. hermsii does not behave like a typical TI-1 Ag. Furthermore, given that the bacterium generated a vigorous B. hermsii-specific IgM response in Btk−/− mice (Fig. 1, B and C), B. hermsii does not appear to stimulate a TI-2 response, which are severely impaired in the absence of Btk (16) but not MyD88 (39). Therefore, our results suggest that B. hermsii induces a TI response that does not resemble a typical TI-1 or TI-2 response. A completely impaired IgM response in mice lacking both Btk and MyD88 (DKO) suggests that this TI response might involve a combination of TI-1 and TI-2 components of B. hermsii.

It is likely that multiple components of B. hermsii contribute to the induction of a TI Ab response. The TLR1/2 heterodimer facilitates recognition of triacylated lipoproteins, and although B. hermsii lipoproteins have not been characterized in detail, the major lipoproteins OspA and OspB of B. burgdorferi are triacylated (47), and TLR1- and TLR2-deficient mice are hypersensitive to purified OspA (48), suggesting that the abundant lipoproteins of B. hermsii (31) may be an important trigger of the TLR signal. Interestingly, despite the prediction that spirochetal DNA might be an important ligand for TLR9, TLR9-deficient mice displayed no defect in generating specific Ab or clearing bacteremia.

The most striking result of this study was that while production of Ag-specific IgM, and therefore BCR signaling, by B. hermsii remained relatively robust in the absence of Btk alone, the dual absence of Btk and MyD88 entirely abrogated Ab production. The response to alum-precipitated CGGs, a widely used TD model Ag, was unaffected in DKO mice, indicating that the CD40-CD40L costimulatory pathway is likely intact. These results implicate MyD88 in the promotion of a productive TI BCR signal—indeed, in the absence of Btk, MyD88 is a required component of this signal. The role of TLR-stimulation in BCR signaling could be entirely intrinsic to the Ag-specific B cell. For example, although no role for TLR9 was found in activation of B. hermsii-specific B cells in this study, dual engagement of BCR and TLR9 activates autoreactive B cells (33), and it has been proposed that TLR9 is critical to the maintenance of memory B cells (34). The cytoplasmic domains of several TLRs, as well as other molecules that are involved in the downstream TLR signaling cascade, interact with Btk (49), and Btk phosphorylates Mal during TLR2 or TLR4 signal transduction. Alternatively, TLR2 and TLR4 are highly expressed on monocytes, neutrophils, macrophages and dendritic cells (50, 51), raising the possibility that TLR-stimulated responses generated by innate immune cells in response to B. hermsii infection could provide signals leading to costimulation of Ag-specific B cells (52–54). Identification of the relevant TLR-bearing immune cells and further investigation into the mechanism by which TLRs are capable of driving efficient Ab responses from even a weak BCR signal during B. hermsii infection may provide strategies to induce rapid TI responses to prevent or treat bacteremia.

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