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B1b Lymphocytes Confer T Cell-Independent Long-Lasting Immunity

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Summary

Many microbial pathogens employ antigenic variation as a strategy to evade the immune system, posing a challenge in vaccine development. To understand the requirements for immunity against such pathogens, we studied *Borrelia hermsii*, a relapsing fever bacterium. We found that mice deficient in T, follicular B, marginal zone B, or B1a lymphocytes resolved *B. hermsii* bacteremia and became resistant to reinfection. The resolution of bacteremia coincided with an expansion and persistence of B1b lymphocytes, and purified B1b lymphocytes from convalescent wild-type or TCR-βx$\delta^{-/-}$ mice conferred immunity to Rag1$^{-/-}$ mice. The B1b lymphocytes in the reconstituted Rag1$^{-/-}$ mice provided long-lasting immunity by rapidly generating *B. hermsii*-specific IgM but not IgG upon bacterial challenge. Unmutated IgM is sufficient to eliminate *B. hermsii*, because AID$^{-/-}$ mice deficient in somatic hypermutation and class switch recombination efficiently resolved all bacteremic episodes. These data demonstrate that B1b lymphocytes can provide long-lasting T cell-independent IgM memory.

Introduction

A hallmark of the adaptive immune system is immunological memory, which generates long-lasting immunity and is central to the concept of vaccination (MacLennan et al., 2000). Conventional B cell memory is dependent upon T cells and develops during the initial exposure to antigens, during which a few antigen-specific B cells proliferate and differentiate into short- and long-lived antibody-secreting plasma cells and long-lived memory B cells. When the immune system is rechallenged with the same antigen, memory B cells mount a very rapid antibody response, providing resistance to reinfection. A crucial event in the generation of B cell memory is the germinal center reaction, during which affinity maturation of antibody by somatic hypermutation (SHM) of Ig variable regions and a change in Ig isotype by class switch recombination (CSR) occur. T cells are essential for the formation of germinal center reactions, as they provide crucial interactions necessary for the B cell responses (McHeyzer-Williams, 2003). This type of response is referred to as a T cell-dependent (TD) antibody response. TD antigens are proteins such as tetanus, diphtheria, and pertussis toxins and can be effective vaccines that generate memory.

Unlike the TD antigens, the T cell-independent (TI) antigens do not typically generate B cell memory, and the antibody responses therefore are short-lived (Lesinski and Westerink, 2001). TI antigens are classified as type-1 (TI-1) and type-2 (TI-2). TI-1 antigens (e.g., lipopolysaccharide [LPS]) can stimulate antigen-specific B cells. In high concentration, TI-1 antigens will also stimulate nonspecific B cells. TI-2 antigens are multivalent polysaccharides or other antigens with repetitive structures that can extensively crosslink specific B cell antigen receptor (BCR) and thereby induce a specific antibody response (Vos et al., 2000). Clinically important TI-2 antigens include those present in *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, and these pathogens are leading causes of morbidity and mortality in children (Lesinski and Westerink, 2001). Although certain TI-2 antigens provide short-term protection in adults, they are not effective vaccines in children or the elderly, and the cellular and molecular basis for this impairment in immunologic memory is not completely understood (Lesinski and Westerink, 2001).

Distinct subsets of B cells respond to TD and TI antigens. Mature B cells can be divided into follicular (FO), marginal zone (MZ), B1a, and B1b cell subsets and each has a distinct function in the immune system (Martin and Kearney, 2001). FO B cells are the major subset in the body, recirculate among the B cell-rich lymphoid follicles, and participate in TD antigen responses. MZ B cells, a minor subset, are localized to the marginal sinus of the spleen. The FO B and MZ B subsets are commonly referred to as B2 cells. B1 cells are most abundant in coelomic cavities and can be subdivided into B1a and B1b subsets (Stall et al., 1992). The B1a and MZ B cells participate in TI antigen responses (Martin et al., 2001). Among the B cell subsets, a direct role for B1b cells in immunity to infection has not been established.

A number of microbial pathogens employ antigenic variation to evade the host immune system and pose a challenge for vaccine development. Relapsing fever is an infection caused by several species of Borreliae, including *Borrelia hermsii* (Burgdorfer, 1976). Relapsing fever bacteria are predominantly localized in the blood, causing recurring episodes of bacteremia. The periodic recurrence in bacteremia is associated with alterations in the expression of surface antigens by DNA rearrangements, resulting in escape from the host adaptive immune system (Barbour, 1990).

Relapsing fever has been utilized as a model for investigating the immune responses to pathogens that undergo antigenic variation. B cells and IgM, but not T cells, are essential for the elimination of relapsing fever Borreliae (Alugupalli et al., 2003a; Barbour and Bundoc,

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2001; Connolly and Benach, 2001; Newman and Johnson, 1984). We recently showed that FO B cells are dispensable for the clearance of *B. hermsii* and that MZ B cells appear to play a role in protection only when *B. hermsii* load is high (Alugupalli et al., 2003a). In contrast, *xid* mice, which are severely deficient in both B1a and B1b subsets, suffer particularly severe episodes of bacteremia (Alugupalli et al., 2003a). B1b cells expanded concurrently with the resolution of bacteremia in wt mice and even more markedly in *xid* mice, suggesting that B1b cells are involved in clearance of *B. hermsii* (Alugupalli et al., 2003a).

In the present study, we utilized adoptive transfer to show that B1b cells from convalescent mice mount a *B. hermsii*-specific antibody response and confer long-lasting T cell-independent immunity. Unmutated IgM was the sufficient component of this immune response, as neither affinity maturation nor isotype switching of Ig were required to eliminate *B. hermsii*.

Results

Mice Deficient in T, FO B, MZ B, or B1a Cells Are Resistant to *B. hermsii* Reinfection

Wild-type mice and mice deficient in T (TCR-βx$\delta^{-/-}$), FO B (IL-7$^{-/-}$), and MZ B (splenectomized) cells resolve *B. hermsii* DAH-p1 bacteremia, suggesting that B1 cells control *B. hermsii* infection (Alugupalli et al., 2003a). Consistent with such a possibility, *xid* mice that have very low numbers of B1a and B1b cells suffer more severe bacteremia than wt mice. However, the resolution of bacteremia in *xid* mice coincided with a remarkable expansion of B1b but not B1a cells (Alugupalli et al., 2003a). We hypothesized that B1b cells could generate protective responses to reinfection in convalescent mice. To test this, we challenged wt, TCR-βx$\delta^{-/-}$, IL-7$^{-/-}$, splenectomized, and *xid* mice with DAH-p1 60 or 230 days after the primary infection. Indeed, bacteremia in these mice was undetectable, indicating that the primary infection generated protective immunity and that T, FO B, MZ B, or B1a cells are not required for this protection (Supplemental Table S1 at http://www.immunity.com/cgi/content/full/21/3/379/DC1). Furthermore, these results suggest that B1b cells might confer long-term immunity.

B1b Lymphocytes of Convalescent Mice Confer Protection against *B. hermsii*

To directly determine whether B1b cells are capable of providing immunity, we transferred the B1b cells of convalescent mice into Rag1$^{-/-}$ mice that are otherwise completely incapable of eliminating *B. hermsii* (Figure 2B; Alugupalli et al., 2003a). T, B1a, and B2 cells in the PerC cell preparation were depleted by MACS ($\leq$2% after depletion), thereby enriching B1b cells by $\sim$5-fold, such that $\sim$27% of the remaining cells were B1b cells (Figure 1). Rag1$^{-/-}$ mice reconstituted with a MACS-enriched PerC cell preparation containing 2×10^5 B1b cells, when challenged with the virulent *B. hermsii* strain DAH-p1 1 day after reconstitution, resolved bacteremia after 2 to 3 days and did not exhibit detectable relapses at later time points (Figure 2C). Moreover, transferred B1b cells were retained among the PerC cells of these reconstituted Rag1$^{-/-}$ mice after resolution of infection (data not shown), and the mice were completely resistant to reinfection when rechallenged 37 days later (Figure 2C).

To address the possibility that other cells (i.e., IgM-negative cells in Figure 1) in the MACS-enriched preparation were responsible for this protection, we further purified the B1b cells from this preparation by FACS using IgM and Mac1 (Figure 1). In contrast to unreconstituted Rag1$^{-/-}$ mice, Rag1$^{-/-}$ mice reconstituted with 2×10^5 FACS-purified B1b cells (IgM^{high} Mac1^{+}) were capable of clearing the first episode of bacteremia after challenge with *B. hermsii* DAH-p1 a day after reconstitution (compare Figures 2B and 2D, panel I). Although these mice exhibited a low to moderate bacteremic relapse 1–3 weeks after challenge (Figure 2D, panel I), they remained subsequently bacteremia-free for an additional 60 days (data not shown). In addition, reconstitution for longer periods before challenge improved the protection of FACS-purified B1b cells. Mice reconstituted for 21 days exhibited a delay in the onset of bacteremia by 2–4 days, and one out of three mice experienced only a single episode of bacteremia (Figure 2D, panel II versus panel I). These mice were bacteremia-free when followed for 4 months and exhibited complete resistance to DAH-p1 rechallenge (data not shown). Finally, when Rag1$^{-/-}$ mice were reconstituted with FACS-purified B1b cells for 60 days, only one of three mice exhibited detectable bacteremia upon *B. hermsii* challenge (Figure 2D, panel III). B1b cells were required for the observed protection, because all the mice reconstituted for 21 days with the "B1b-depleted" preparation of cells (Figure 1) suffered multiple episodes followed by persistent bacteremia and became moribund (Figure 2E). The initial control of bacteremia observed in this group of mice could be due to the presence of residual $\sim$4% B1b cells in the inoculum (see Figure 1). These results demonstrate that highly purified B1b cells from convalescent mice can provide resistance to *B. hermsii* infection.

Naive B1b Lymphocytes Offer Partial Control of *B. hermsii* Bacteremia

Rag1$^{-/-}$ mice reconstituted with 2×10^5 of B1b cells from convalescent mice experienced fewer episodes of bacteremia following challenge with strain DAH-p1 than did naive mice (Figures 2A versus 2D), suggesting that B1b cells from naive mice have less protective activity than those from convalescent mice. To test the extent to which naive B1b cells protect against *B. hermsii* infection, we reconstituted Rag1$^{-/-}$ mice either with 2×10^5 FACS-purified naive B1b cells for 1 day or with MACS-enriched preparation containing 2×10^5 naive B1b cells for 21 days and then challenged with either a high- (DAH-p1) or moderate-virulence (DAH-p19) strain of *B. hermsii*. The reconstituted mice suffered lower levels of bacteremia compared to unreconstituted Rag1$^{-/-}$ mice, a difference that was particularly apparent following infection with DAH-p19 (compare Figure 3A, panels II versus III, or Figure 3B, panel II). Nevertheless, the naive B1b cell-reconstituted Rag1$^{-/-}$ suffered more severe infec-

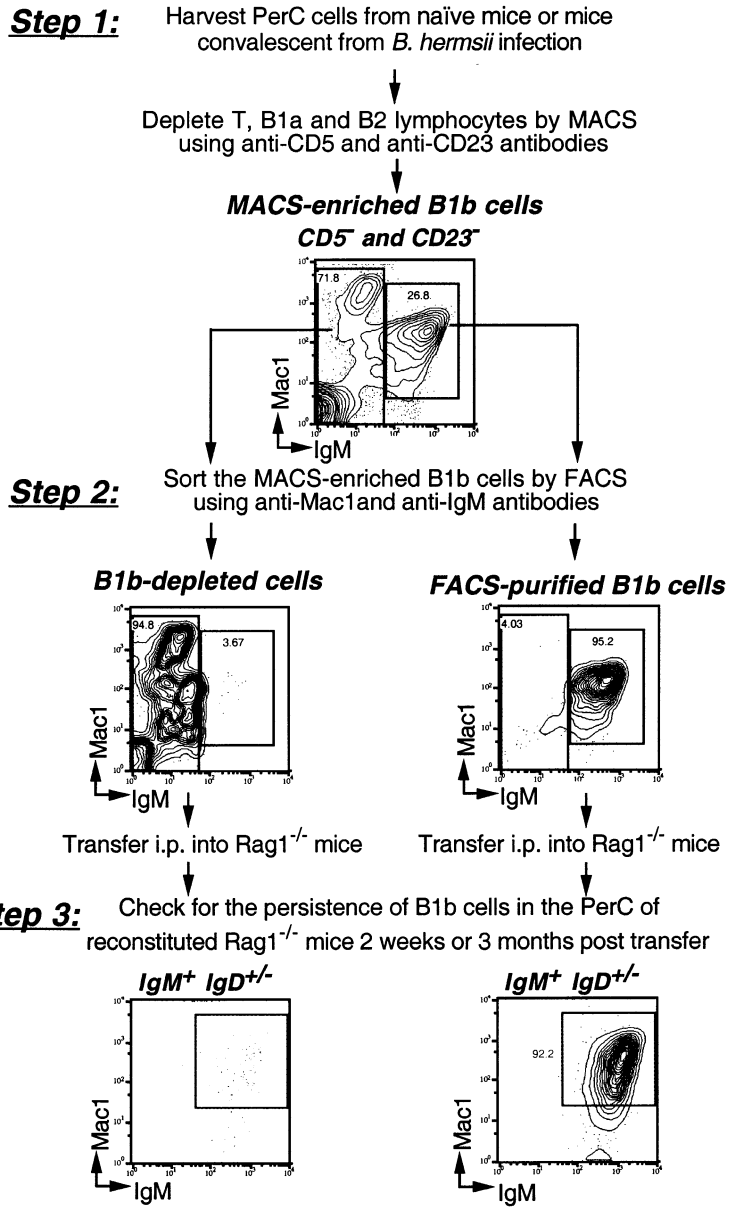

Figure 1. Purification of B1b Lymphocytes and Their Persistence in Rag1$^{-/-}$ Recipients

Step 1: T and B1a cells (using anti-CD5) and B2 cells (using anti-CD23) in the PerC cell preparation of C57BL/6 naive or convalescent mice from *B. hermsii* strain DAH-p1 infection (i.e., >2 months postinfected mice) were depleted by MACS. After confirming the depletion of T, B1a, and B2 cells by FACS (plots not shown), the negatively selected PerC cell preparation was stained with anti-Mac1-PE and anti-IgM-FITC. Approximately 27% of this preparation contained B1b cells, and at this stage, they are referred to as "MACS-enriched B1b cells."

Step 2: The B1b cells, in the MACS-enriched B1b cell preparation, were purified by FACS sorting (IgM and Mac-1 double-positive cells); hence the term "FACS-purified B1b cells." After checking the purity (>95%), 2×10^5 B1b cells were transferred intraperitoneally into C57BL/6-Rag1$^{-/-}$ mice. A total number of 8×10^5 B1b-depleted cells obtained from the FACS were also transferred in parallel to Rag1$^{-/-}$ mice.

Step 3: The reconstitution of B1b cells in the PerC of the recipient Rag1$^{-/-}$ mice was confirmed 2 weeks or 3 months posttransfer. The plots shown are after gating on IgM^{+} and IgD^{low} cells (plots not shown), followed by the identification of B1b cells using IgM^{high} Mac1^{+}. These cells were also confirmed to be CD5^{-} (plots not shown).

tion than did wt mice (compare Figure 3A, panels I versus III, or Figure 3B, panel II) or Rag1$^{-/-}$ reconstituted with B1b cells from convalescent mice (compare Figure 3B, panel II, versus Figure 2D, panel I). These results suggest that naive B1b cells can partially control *B. hermsii* bacteremia.

B1a Cells Are Not Essential for Clearing *B. hermsii*

The observation that Rag1$^{-/-}$ reconstituted with naive B1b cells suffered more severe bacteremia than did wt mice raised the possibility that other B cell subsets may also contribute to the clearance of *B. hermsii*. We previously showed that while MZ B cells might play a role in resolving high-level bacteremia by DAH-p1, they are not essential for eliminating low-level bacteremia by DAH-p19 (Alugupalli et al., 2003a). Although B1a cells express CD5, they resemble B1b cells with respect to other phenotypic characteristics, such as tissue distribution and development (Stall et al., 1992) and are known to mount immune responses to blood-borne TI antigens (Martin et al., 2001). To examine the possible contribution of B1a cells to protection, we used BM chimeras that efficiently reconstitute FO B, MZ B, and B1b cells, but not B1a cells (Kantor et al., 1992). These B1a-deficient BM chimeras controlled *B. hermsii* DAH-p1 and p19 bacteremia as efficiently as the wt mice (Figure 3C, compare panels I and II), and supplementation of B1a cells to these BM chimeras, by the addition of PerC cells, did not accelerate the clearance of bacteremia (Figure 3C, compare panels II and III). Finally, to determine the extent to which naive B1a cells could provide protection, we transferred 2×10^5 FACS-purified B1a cells into Rag1$^{-/-}$ mice and challenged these mice

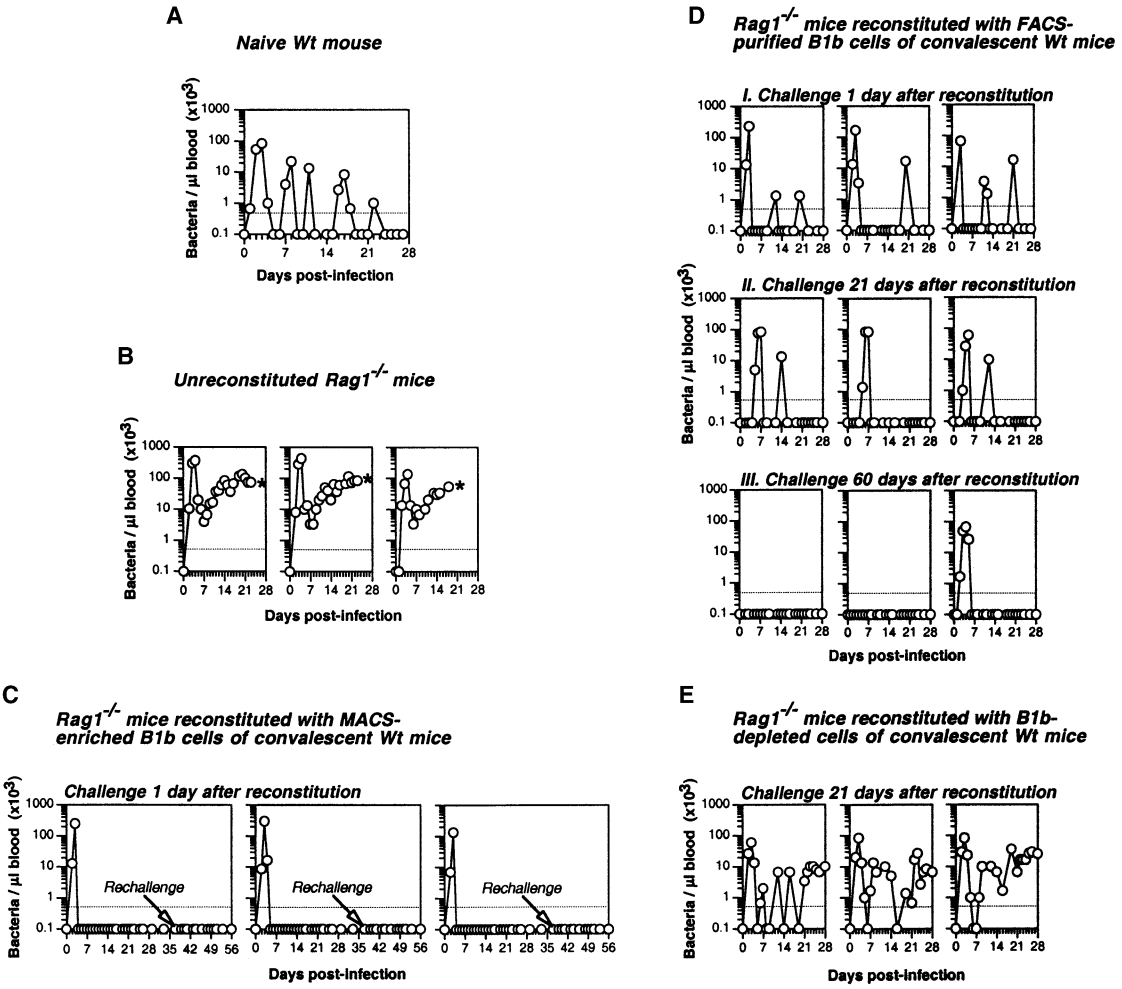

Figure 2. B1b Lymphocytes from Convalescent Mice Confer Immunity

A naive wt mouse (A); unreconstituted Rag1$^{-/-}$ mice (B); Rag1$^{-/-}$ mice reconstituted with 2×10^5 MACS-enriched B1b cells (C; mice in this panel were rechallenged with 5×10^4 DAH-p1, 37 days after the first challenge and indicated by arrow); Rag1$^{-/-}$ mice reconstituted with 2×10^5 FACS-purified B1b cells (D); or Rag1$^{-/-}$ mice reconstituted with 8×10^5 B1b-depleted cells (E) were challenged with 5×10^4 DAH-p1 either 1 day or 21 or 60 days after reconstitution, and the bacteremia was measured by microscopic counting. Each plot represents bacteremia in an individual mouse. The broken line in each plot indicates the detection limit for bacteremia. Mice that died during this bacteremia monitoring period were indicated by an asterisk.

with DAH-p1 or -p19 1 day later. In contrast to naive B1b cells (Figure 3B, panel II), naive B1a cells did not confer any detectable protection, as bacteremia in these mice was comparable to that of bacteremia in unreconstituted-Rag1$^{-/-}$ mice (Figure 3B, panel III). These results, as well as the previously documented lack of an expansion of B1a cells during *B. hermsii* infection (Alugupalli et al., 2003a), suggest that B1a cells do not play a central role the resolution of *B. hermsii* infection.

T Cells Are Dispensable for Both the Expansion of B1b Cells and the Generation of Immunity by B1b Cells

The $\gamma\delta$ T cell subset can facilitate B1 cell migration from PerC to mesenteric lymph nodes, where they differentiate into antibody-secreting cells, and may also induce B1 cell differentiation in vivo (Watanabe et al., 2000). Likewise, the $\gamma\delta$ T cells (Watanabe et al., 2000) could ontribute to the expansion of B1b cell numbers seen in convalescent wt mice (Alugupalli et al., 2003a). To examine whether T cells play a role in the expansion of B1b cells during *B. hermsii* infection or their function in conferring immunity, we infected mice that completely lack mature T cells (TCR $\beta x \delta^{-/-}$) with DAH-p1. We found a 2-fold increase in PerC B1b cell frequency (Figure 4A) and 3-fold increase in their absolute numbers (Figure 4B, Supplemental Table S1) in convalescent mice compared to naive mice. In contrast, the numbers of B1a and B2 cells remained unaltered by infection (Figure 4B). Consistent with this, when tested for the ability to confer control of *B. hermsii* bacteremia upon reconstituted Rag1$^{-/-}$ mice, the expanded B1b cells from convalescent TCR $\beta x \delta^{-/-}$ mice were indistinguishable from

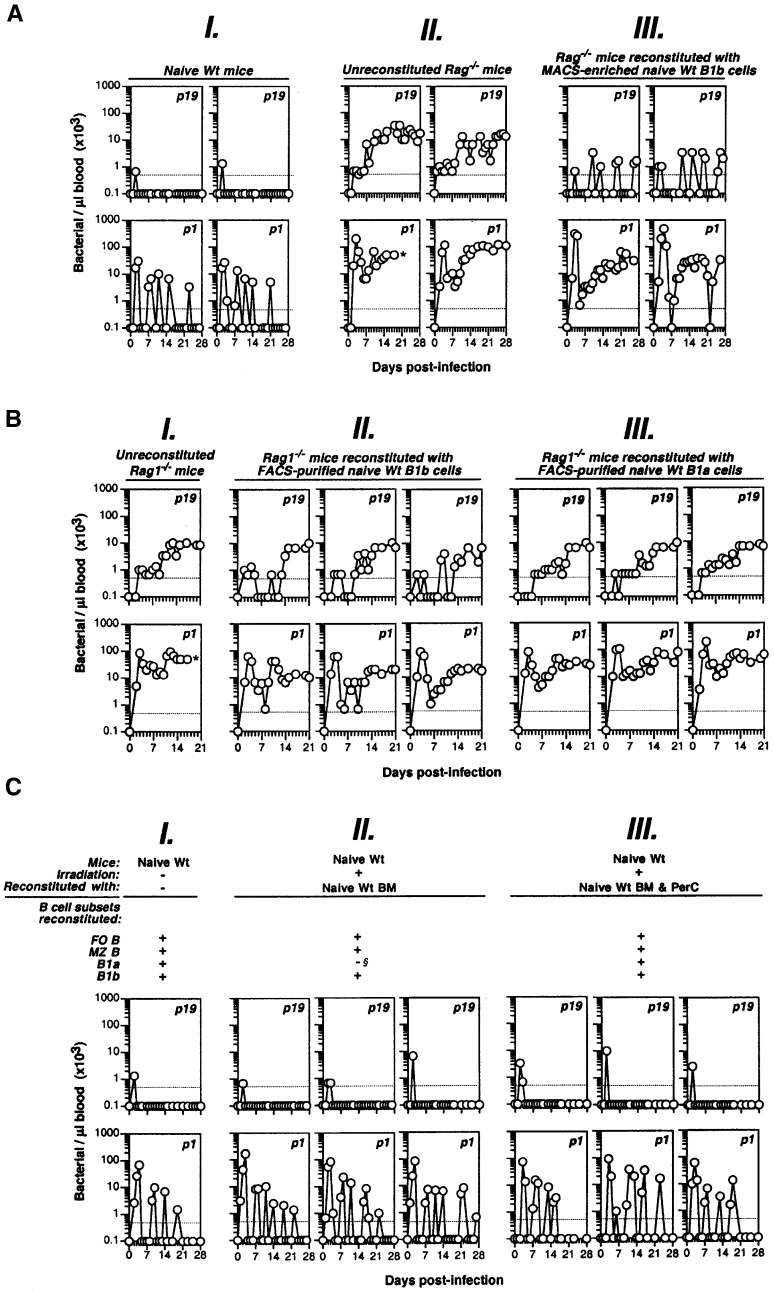

Figure 3. Naive B1b but Not B1a Lymphocytes Provide Partial Control of *B. hermsii* Bacteremia

(A) Naive wt mice (I); unreconstituted Rag1$^{-/-}$ (II); or Rag1$^{-/-}$ mice reconstituted for 21 days with MACS-enriched B1b cells of naive wt mice (III).

(B) Unreconstituted Rag1$^{-/-}$ (I) and Rag1$^{-/-}$ mice reconstituted for a day either with FACS-purified naive B1b cells (II) or with FACS-purified naive B1a cells (III).

(C) Unirradiated wt (I); irradiated wt mice reconstituted with wt BM (II) or irradiated wt mice reconstituted with wt BM and PerC cells (III). All the mice were infected with 5×10^4 DAH-p19 or DAH-p1 and the bacteremia was measured. Mice that died during this bacteremia monitoring period were indicated by an asterisk; § indicates <5% of normal levels of B1a cells; plus sign indicates reconstitution of respective B cell subset to normal levels.

B1b cells from convalescent wt mice (compare Figures 4C and 4D and Figures 2C and 2D, panel I). These results demonstrate that T cells are required neither for the B1b expansion nor for the resolution of *B. hermsii* bacteremia (Alugupalli et al., 2003a) and suggest that B1b cells generate immunity independent of T cell help.

IgM Is Sufficient and Affinity Maturation Is Not Required for Resolving *B. hermsii* Infection

IgM is a major immunoglobulin secreted by B1b cells, and we previously showed that IgM was essential for the resolution of *B. hermsii* bacteremia (Alugupalli et al., 2003a). Nevertheless, it is possible that other isotypes play a role in the clearance of *B. hermsii*, because IgG3 and IgG2b from convalescent mice were found to provide passive protection in a similar relapsing fever bacteremia model (Yokota et al., 1997). To examine whether IgM is sufficient for the resolution of bacteremia, we infected AID$^{-/-}$ mice, which are defective in CSR and cannot generate isotypes other than IgM. Moreover, AID$^{-/-}$ mice are defective in SHM and are thus incapable of generating mutated, affinity-matured antibodies. In spite of these defects, AID$^{-/-}$ mice cleared the first bacteremic episode as rapidly as did the wt mice, and the duration and peak bacterial density of the relapses were indistinguishable (Figure 5A). For both wt and AID$^{-/-}$ mice, the induction of *B. hermsii*-specific IgM occurred with identical kinetics and coincided with the clearance

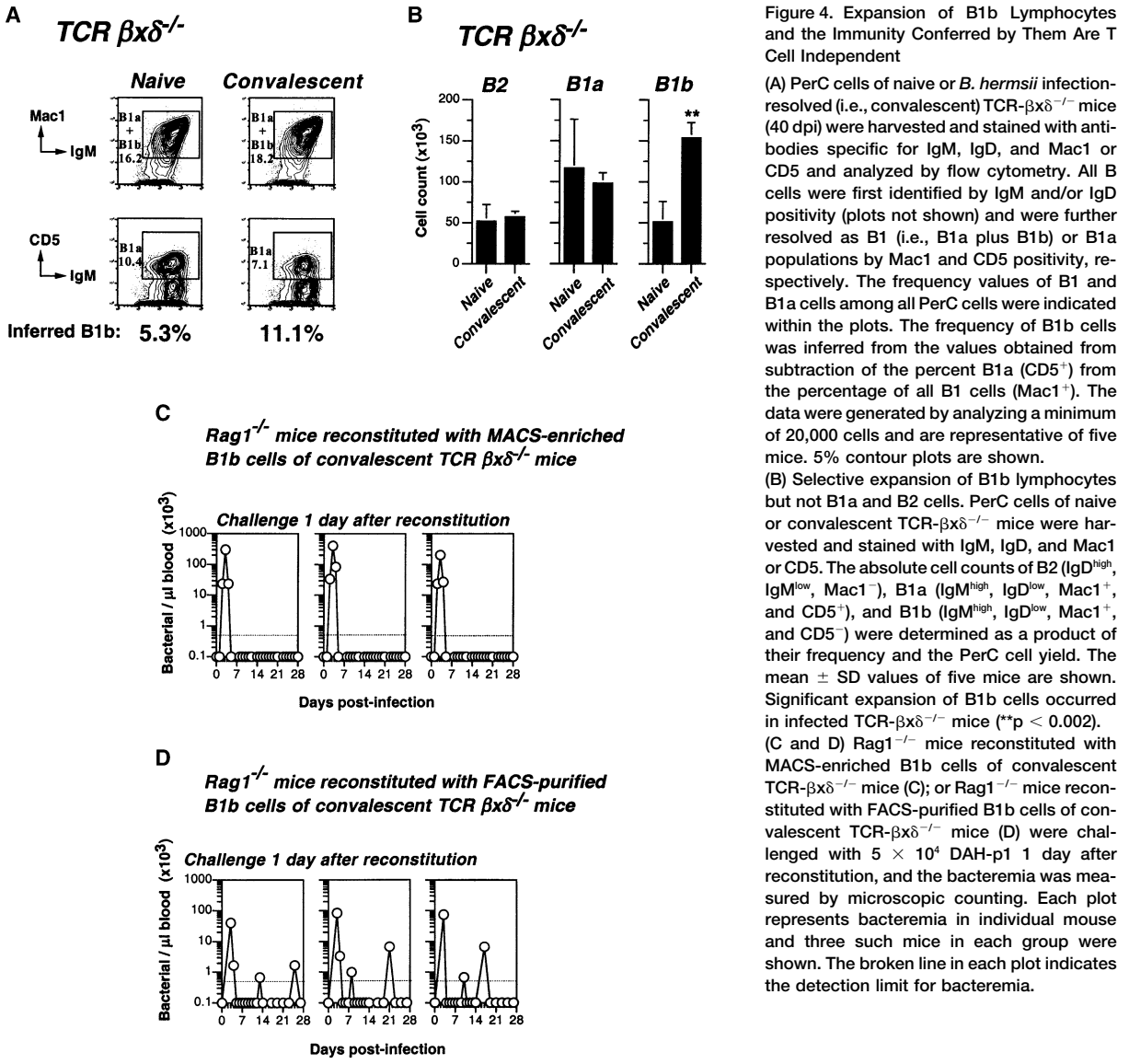

Figure 4. Expansion of B1b Lymphocytes and the Immunity Conferred by Them Are T Cell Independent

(A) PerC cells of naive or *B. hermsii* infection-resolved (i.e., convalescent) TCR-$\beta x\delta^{-/-}$ mice (40 dpi) were harvested and stained with antibodies 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 (i.e., B1a plus B1b) or B1a populations by Mac1 and CD5 positivity, respectively. The frequency values of B1 and B1a cells among all PerC cells were indicated within the plots. The frequency of B1b cells was inferred from the values obtained from subtraction of the percent B1a ($CD5^+$) from the percentage of all B1 cells ($Mac1^+$). The data were generated by analyzing a minimum of 20,000 cells and are representative of five mice. 5% contour plots are shown.

(B) Selective expansion of B1b lymphocytes but not B1a and B2 cells. PerC cells of naive or convalescent TCR-$\beta x\delta^{-/-}$ mice were harvested and stained with IgM, IgD, and Mac1 or CD5. The absolute cell counts of B2 (IgD^{high}, IgM^{low}, $Mac1^-$), B1a (IgM^{high}, IgD^{low}, $Mac1^+$, and $CD5^+$), and B1b (IgM^{high}, IgD^{low}, $Mac1^+$, and $CD5^-$) were determined as a product of their frequency and the PerC cell yield. The mean ± SD values of five mice are shown. Significant expansion of B1b cells occurred in infected TCR-$\beta x\delta^{-/-}$ mice (**$p < 0.002$).

(C and D) $Rag1^{-/-}$ mice reconstituted with MACS-enriched B1b cells of convalescent TCR-$\beta x\delta^{-/-}$ mice (C); or $Rag1^{-/-}$ mice reconstituted with FACS-purified B1b cells of convalescent TCR-$\beta x\delta^{-/-}$ mice (D) were challenged with 5×10^4 DAH-p1 1 day after reconstitution, and the bacteremia was measured by microscopic counting. Each plot represents bacteremia in individual mouse and three such mice in each group were shown. The broken line in each plot indicates the detection limit for bacteremia.

of bacteremia (Figure 5B). These results demonstrate that unmutated IgM is sufficient and all other Ig isotypes are dispensable for the elimination of each bacteremic episode of *B. hermsii*.

B1b Lymphocytes from Convalescent Mice Generate *B. hermsii*-Specific IgM but Not IgG

Given that clearance of bacteremia in $AID^{-/-}$ mice indicated that IgM is sufficient for the resolution of *B. hermsii*, we postulated that the transferred B1b cells in $Rag1^{-/-}$ mice confer protection by mounting a specific IgM response against *B. hermsii*. To test this, we analyzed the specific IgM as well as IgG levels in $Rag1^{-/-}$ mice reconstituted for 1 day with FACS-purified B1b cells of convalescent wt mice. Indeed, these mice generated *B. hermsii*-specific IgM by 4–5 days after the infection, and the rise of this specific IgM response coincided with the resolution of bacteremia (Figure 5C). No *B. hermsii*-specific IgG was detected. These results demonstrate that the specific IgM required and sufficient for the clearance of *B. hermsii* can be generated by B1b cells.

Persistent Expansion of B1b Lymphocytes in *B. hermsii*-Infected Mice

Given the previously shown expansion of the B1b cell population during *B. hermsii* infection (Alugupalli et al., 2003a) and the long-lasting resistance to reinfection (Supplemental Table S1), we examined the kinetics of B1b cell expansion in *B. hermsii*-infected mice. We chose *xid* mice to address this issue, since their basal level of B1b cells is much lower than that of wt mice and any expansion form is easily measured. Indeed, a detectable expansion of *xid* B1b cells is apparent by 7 days postinfection (Figure 6A), a time point coinciding with the resolution of the first bacteremic episode. As shown previously, a dramatic expansion of B1b cells was apparent at 40 days postinfection (Alugupalli et al.,

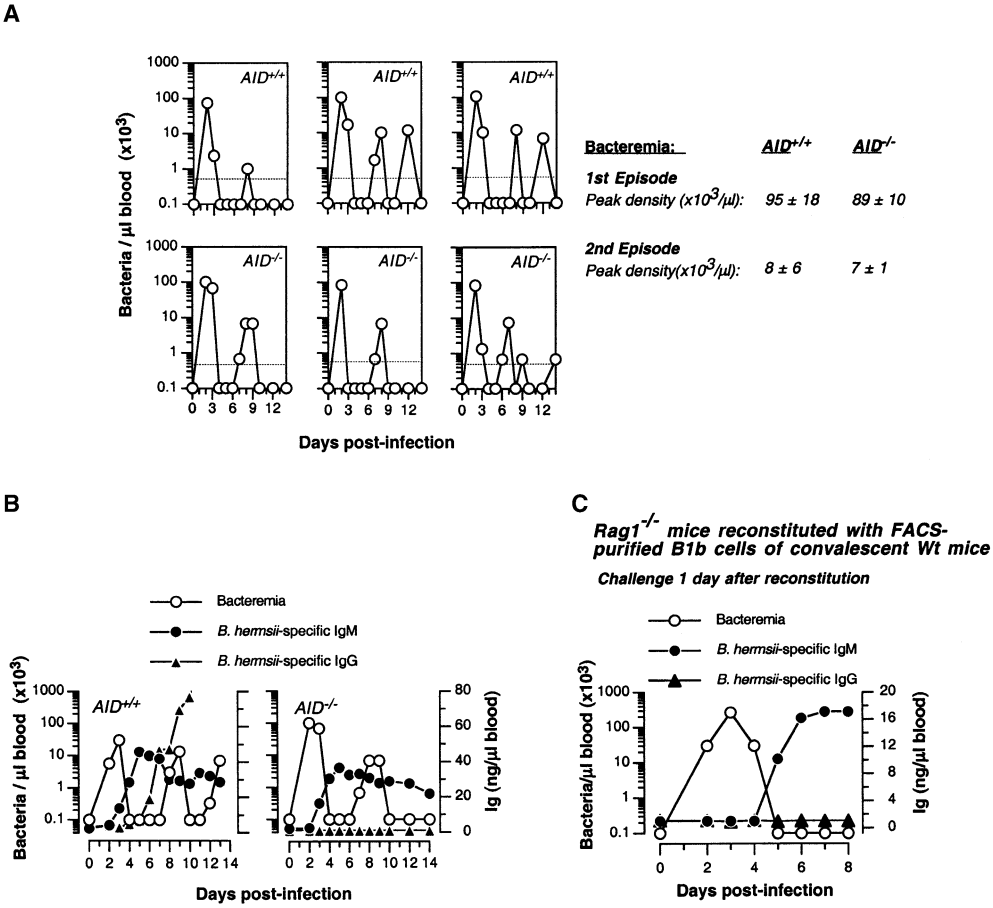

Figure 5. Unmutated IgM Is Sufficient for Controlling *B. hermsii* Bacteremia

(A) The AID$^{+/+}$ (wt; n = 3) and AID$^{-/-}$ (n = 3) mice were infected with 5 × 10^4 DAH-p1, and the bacteremia was measured by microscopic counting. The peak density and duration of the first as well as the second episodes of bacteremia were not statistically significant (by Student's t test), and each plot represents bacteremia in an individual mouse. The broken line indicates the detection limit for bacteremia.

(B) DAH-p1-specific IgM and IgG levels in blood were measured by ELISA using Ig standards (see Experimental Procedures).

(C) Rag1$^{-/-}$ mouse reconstituted for 1 day with FACS-purified B1b cells of convalescent wt mice were infected, and bacteremia, *B. hermsii*-specific IgM, and IgG were measured. The data from one representative mouse is shown.

2003a) and persisted for a remarkably long period, i.e., greater than 230 days postinfection (Figure 6A). This expansion was not detected in B2 or B1a cell subsets (Figure 6B). These results indicate that long-term resistance to reinfection correlates with a sustained B1b expansion.

Generation of *B. hermsii*-Specific IgM by the Expanded B1b Lymphocytes Is Not Constitutive but Is Induced upon Antigenic Stimulation

B cells specific for haptenated-LPS, a model TI-1 antigen, cease generating anti-hapten antibodies within 2 weeks after transfer into donor mice in the absence of antigen restimulation (Colle et al., 1988). To determine whether the *B. hermsii*-specific IgM is produced upon reconstitution or is antigen induced, we followed specific antibody levels in Rag1$^{-/-}$ mice reconstituted with purified B1b cells from convalescent wt mice. During the 60 days following B1b cell transfer, *B. hermsii*-specific IgM was undetectable by ELISA (Figure 7). However, when these mice were challenged with *B. hermsii* DAH-p1 on the 60th day postreconstitution, specific IgM appeared as early as 48 hr after challenge (Figure 7, Mouse #1) and reached maximal levels by 4 days. In fact, detectable bacteremia occurred in only one of three mice, and this episode was cleared concurrent with the appearance of IgM. These results indicate that long-term immunity to *B. hermsii* was not due to long-lived plasma cells; rather, the expanded B1b cells persisted for long time periods and generated specific IgM only upon antigenic stimulus.

Discussion

The ability to rapidly produce large amounts of neutralizing antibodies directed against blood-borne pathogens is often critical in the prevention of infection of vital organs. TI antibody responses develop more quickly than TD responses and therefore can play a crucial role in decreasing the bacterial burden early in infection

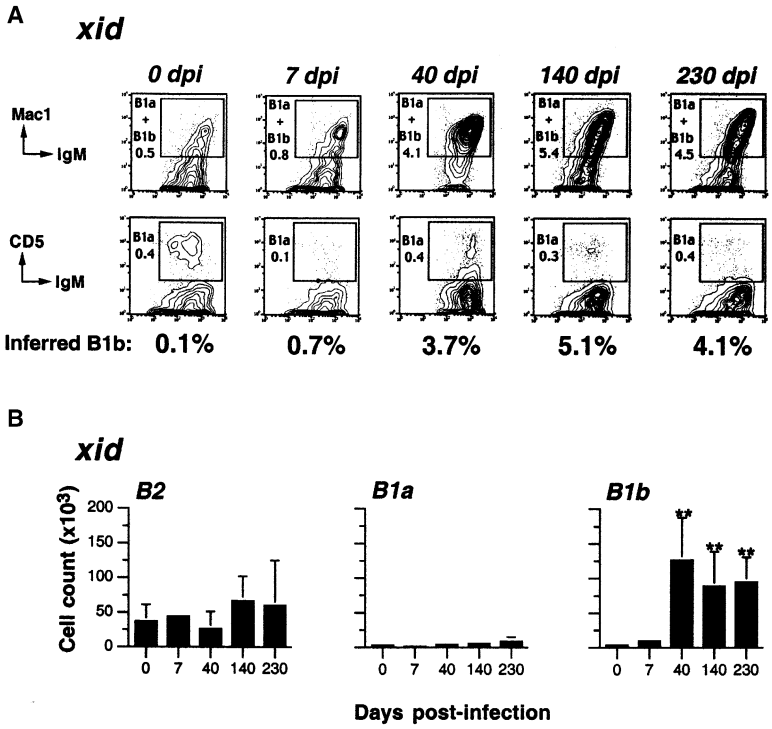

Figure 6. Persistent Expansion of B1b Lymphocytes in *B. hermsii*-Infected Mice

(A) PerC cells of uninfected or *B. hermsii*-infected *xid* (CBA/N) mice at the indicated days postinfection (dpi) were harvested, and B1a + B1b (Mac1$^+$) and B1a (CD5$^+$) frequencies were measured as in Figure 4 legend. The data were generated by analyzing a minimum of 20,000 cells and are representative of 3 mice per time point. 5% contour plots are shown.

(B) Selective expansion of B1b but not B1a and B2 subsets. The absolute cell counts of B2 (IgD^{high}, IgM^{low}, Mac1$^-$), B1a (IgM^{high}, IgD^{low}, Mac1$^+$, and CD5$^+$), and B1b (IgM^{high}, IgD^{low}, Mac1$^+$, and CD5$^-$) were determined as in Figure 4 legend. The mean ± SD values of respective subsets of three mice at various dpi are given. Significant expansion of B1b cells occurred in infected *xid* mice (**$p < 0.002$).

(Martin and Kearney, 2001). However, TI responses have not previously been thought to be capable of generating long-term B cell memory. In the present study, we show that TI responses generated by *B. hermsii* infection result in the expansion of a population of B1b cells that is sufficient to conferring long-lasting immunity upon otherwise immunodeficient mice. These B1b cells produce antigen-specific IgM, and immune mice produce this antibody only after *B. hermsii* challenge, indicating that immunity is not mediated by long-lived plasma cells (Slifka et al., 1998). Rather, antigenic stimulation is required to promote differentiation of these lymphocytes to plasma cells, suggesting that B1b cells produced by a TI response are capable of functioning as long-lived IgM memory B cells.

A TI IgM response is necessary for the control of relapsing fever infection (Alugupalli et al., 2003a; Barbour and Bundoc, 2001; Connolly and Benach, 2001; Newman and Johnson, 1984), suggesting that MZ, B1a, and/or B1b cells may play a role in *B. hermsii* clearance (Martin et al., 2001). MZ B cells have been shown to mount rapid responses to blood-borne TI antigens, but splenectomized mice, which lack these cells, showed a defect in *B. hermsii* clearance only during very high bacterial loads (Alugupalli et al., 2003a, 2003b). Given that splenectomized mice also display a 4-fold defect in the number of B1a cells (Wardemann et al., 2002), these results additionally suggest that this B cell subset plays at most a subsidiary role in the control of relapsing fever. Indeed, we showed here that B1a-deficient chimeric mice clear *B. hermsii* as efficiently as do normal mice and that B1a cells purified from naive mice are incapable of conferring any degree of control of *B. hermsii* bacteremia in otherwise immunodeficient (Rag1$^{-/-}$) mice. In contrast, B1b cells purified from naive mice, when transferred to Rag1$^{-/-}$ mice, provide a level of protection, albeit incomplete, against both high and moderate virulence *B. hermsii*.

We previously showed that clearance of *B. hermsii* was accompanied by an expansion of B1b cells (Alugupalli et al., 2003a). In the current study, expansion was apparent at the first time point tested, 7 days postinfection, just after clearance of the first episode of bacteremia. The cellular mechanisms behind this dramatic expansion are not known. High concentrations (e.g., 1000 ng/ml) of IL-9 promote expansion of B1b cell numbers in vivo (Vink et al., 1999), but we found no evidence for a role for this cytokine in B1b cell expansion following *B. hermsii* infection. First, the major cellular source of IL-9 is the T_H2 lymphocyte (Renauld et al., 1995), and we found that the B1b cell expansion is T cell independent. In addition, IL-9 levels in the serum of *B. hermsii*-infected wt or T cell-deficient mice remained below detectable limits (i.e., <0.3 ng/ml; K.R.A., unpublished observation).

Remarkably, expansion of B1b cells occurred during *B. hermsii* infection in *xid* mice (Figure 6; Alugupalli et al., 2003a), which are defective in BCR signaling, deficient in B1 cells, and defective in generating TI-2 responses (Berland and Wortis, 2002; Khan et al., 1995; Thomas et al., 1993). *xid* mice can respond to TI-2 antigen-specific BCR-mediated activation when costimulatory signals are provided (Couderc et al., 1987; Vinuesa et al., 2001), suggesting that *B. hermsii* generates additional signals to induce B cell activation and B1b expansion. Given that the expanded B1b cells generate antibody specific for *B. hermsii* (see below), the expansion is likely to be antigen driven. Neonatal B cells, like B cells from *xid* mice, do not respond to TI-2 antigens (Chelvarajan et al., 1998), so identification of costimulatory pathways

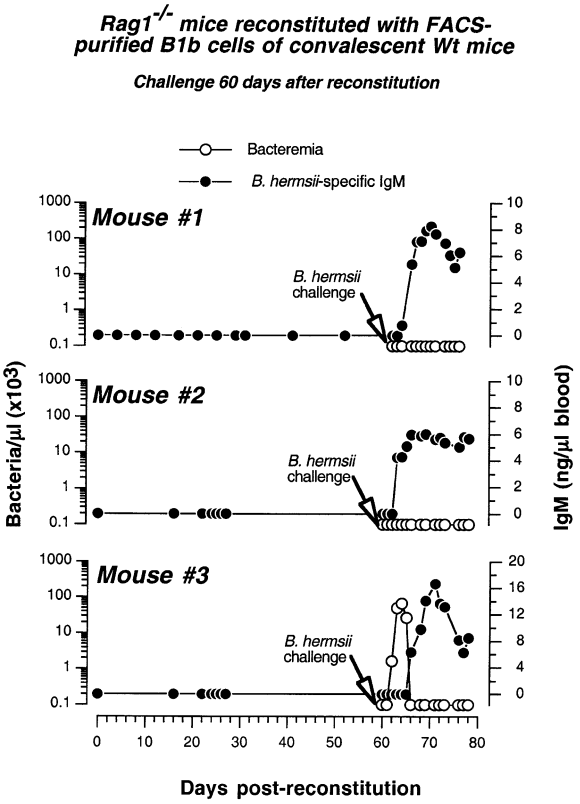

Figure 7. Generation of *B. hermsii*-Specific IgM Response by B1b Lymphocytes Is Not Constitutive but Induced upon Antigen Stimulation

Rag1$^{-/-}$ mice were reconstituted with FACS-purified B1b cells of convalescent wt mice. *B. hermsii*-specific IgM was measured by ELISA. On day 60 postreconstitution, mice were challenged with 5×10^4 DAH-p1, indicated by arrow, and bacteremia was measured. Each plot represents data from individual mouse.

induced by *B. hermsii* could provide strategies to enhance immune responses to TI-2 antigens in children.

The expansion of B1b cells during *B. hermsii* infection was associated not only with a greater number of these cells, but also with enhancement of their protective activity. B1b cells continued to expand over approximately the first 40 days postinfection, and this progressive expansion correlated with episodes of bacteremia of diminishing severity. Indeed, maximal expansion correlated with the complete resolution of infection (Alugupalli et al., 2003a). More importantly, in contrast to a preparation of MACS-enriched B1b cells from naive mice, an analogous preparation of B1b cells from convalescent mice provided a high level of protection to Rag1$^{-/-}$ mice after only 1 day of reconstitution. FACS-purified B1b cells from convalescent mice also provided almost complete protection, although a longer period of reconstitution was required. The requirement for longer reconstitution by FACS-purified compared to MACS-enriched B1b cells did not appear to be due to the IgM and Mac1 labeling procedure used for the FACS selection of B1b cells, since Rag1$^{-/-}$ mice reconstituted for 1 day with a MACS-enriched B1b cell preparation that had been labeled with anti-IgM and Mac1 still exhibited only one episode of bacteremia (K.R.A., unpublished observation). It is possible that accessory cells in the MACS-enriched B1b cell preparation synergized with B1b cells to promote clearance. Indeed, bacteria-primed peritoneal macrophages promote the differentiation of B1a cells into plasmablasts (Balasz et al., 2002), suggesting the possible involvement of primed accessory cells of convalescent mice. Regardless of the differences in protective activity of MACS-enriched versus FACS-purified B1b cells, the observation that B1b cells from convalescent mice are more protective than those from naive mice suggests that a subpopulation of *B. hermsii*-stimulated B1b cells that are highly protective undergo expansion during infection.

The protective activity produced by B1b cells is clearly IgM. First, this isotype is one of the major isotypes produced by these cells, and mice defective for IgM production or secretion are severely defective for control of relapsing fever (Alugupalli et al., 2003a; Connolly and Benach, 2001). Second, the appearance of spirochete-specific IgM, but not IgG, coincided with clearance of spirochetes (Figure 5; Connolly and Benach, 2001). Third, antibodies that protect against relapsing fever spirochetes are of the IgM isotype (Barbour and Bundoc, 2001; Connolly et al., 2004; Yokota et al., 1997). Finally, AID$^{-/-}$ mice, which produce only IgM, were entirely competent for clearance of *B. hermsii*.

While IgM produced by B1b cells is clearly an essential effector, the nature of the antigen that is recognized by this IgM has not yet been determined. Interestingly, the ability of AID$^{-/-}$ mice, which are incapable of SHM, to clear *B. hermsii* infection indistinguishably from wt mice demonstrates that unmutated IgM is sufficient to eliminate *B. hermsii*. The present study is the first demonstration that CSR and SHM can be dispensable for clearance of an active infection. Two monoclonal antibodies that have been shown to protect against relapsing fever spirochetes have both been directed against a variable major protein (Vmp) (Barbour and Bundoc, 2001; Connolly et al., 2004), the surface protein that determines serotype and that undergoes high-frequency antigenic variation (Barbour, 1990). These antibodies have been shown to be directly bactericidal, even in the absence of complement (Barbour and Bundoc, 2001; Connolly et al., 2004). However, both of these antibodies were produced by hybridomas generated from splenic B cells, and whether the parental B cells were of the B1b subclass is not known. It is curious that the repertoire of unmutated IgM would allow B1b cells to comprehensively respond to hypervariable antigens such as Vmps. The pool of natural antibody, to which B1b cells contribute, include antibodies directed against highly conserved antigens (Casali and Schettino, 1996), and it is possible that an element of protection may involve targeting of conserved, non-Vmp antigens on the surface of *B. hermsii*. An important future goal is to identify the target(s) of IgM produced by protective B1b cells.

Remarkably, and unlike any previously characterized TI responses, the TI B1b cell response to *B. hermsii* infection confers long-lasting immunity. This TI response does not closely resemble a TI-2 response, because not only have TI-2 antigens failed to elicit long-lasting B cell memory (Lesinski and Westerink, 2001), but in the current study, *xid* mice, which are deficient in TI-2 responses (Thomas et al., 1993), were found to

be resistant to reinfection (Supplemental Table S1). The model TI-1 antigen haptenated-LPS generates antigen-specific B cells that persist for less than 2 weeks in recipient mice (Colle et al., 1988), whereas we showed that B1b cells from convalescent mice survived and provided protective immunity to *B. hermsii* infection for more than 80 days in recipient Rag1$^{-/-}$ mice. Long-term responses to TI-1 antigens require continuous B cell renewal from BM and perhaps antigen persistence as well (Burlen et al., 1988; Colle et al., 1988; Zhang et al., 1988). In contrast, B1b cells generated by the TI response to *B. hermsii* infection do not require B cell influx from the BM: 12-week-old IL-7$^{-/-}$ mice, which are arrested in B lymphopoiesis (Carvalho et al., 2001), resolve *B. hermsii* bacteremia as efficiently as wt mice (Alugupalli et al., 2003a) and are resistant to reinfection (Supplemental Table S1). These B1b cells persist for long periods (Figure 1) and mount antigen-specific IgM response only upon antigen stimulation. Thus, the long-term immunity provided by B1b cells appears similar to the immunity conferred by conventional memory B cells (Schittek and Rajewsky, 1990) but is generated and maintained in the complete absence of T cells. Elucidation of the pathways that result in B1b expansion and the acquisition of long-lasting immunity in the absence of T cells may provide approaches to enhance the efficacy of TI responses in humans.

Generation of unmutated IgM memory B cells specific to the immunizing antigen can occur independent of germinal center formation (Toyama et al., 2002). The present study demonstrates that unmutated yet *B. hermsii*-specific IgM can be generated during an infection and are sufficient to resolve bacteremia. Ideal approach for vaccine development against pathogens using antigenic variation requires the identification of protective and conserved epitopes. The sufficiency of unmutated IgM for eliminating *B. hermsii* suggests that B1b cells selected naturally during an infection may recognize conserved epitopes and provide immunity. Therefore, screening for B1b cells with a BCR repertoire recognizing protective antigenic determinants will be of tremendous value in vaccine development against pathogens that undergo antigenic variation.

Experimental Procedures

Mice and Infections

Mice housed in micro-isolator cages with free access to food and water were maintained in a specific pathogen-free facility in the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS). C57BL/6J, C57BL/6J-Rag1$^{tm1\,Mom}$ (Rag1$^{-/-}$), and C57BL/6J-TCR-$\beta^{tm1\,Mom}$ TCR-$\delta^{tm1\,Mom}$ (TCR-βx$\delta^{-/-}$) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). C57BL/6J mice splenectomized at the age of 4 weeks were also purchased from Jackson Laboratories and were infected with *B. hermsii* 2 or 6 weeks after splenectomy. IL-7$^{-/-}$ mice on the 129SvC57BL/6 background were obtained from Dr. Richard Murray, DNAX Research Institute, Palo Alto, CA (von Freeden-Jeffry et al., 1995). CBA/Ca and CBA/N (*xid*; X-linked immunodeficient) mice were purchased from National Cancer Institute (Fredrick, MD). The AID$^{-/-}$ mice on C57BL/6 background (Muramatsu et al., 2000) were bred and maintained at UMMS.

Mice were infected intravenously via tail vein with 5×10^4 bacteria of a fully virulent *B. hermsii* strain DAH-p1 (Schwan and Hinnebusch, 1998) or a partially attenuated strain DAH-p19, generated by serial passage of DAH-p1 in vitro 18 times (Alugupalli et al., 2001), and the bacteremia was monitored by dark-field microscopy (Alugupalli et al., 2001).

Flow Cytometry

Peritoneal cavity (PerC) cells were harvested from individual mice and the cell density was adjusted to 2.5×10^7/ml in staining medium (deficient RPMI medium 1640 [Irvine Scientific, Santa Ana, CA] with 3% new calf serum, 1 mM EDTA). After blocking the Fc receptors with 2.4G2 antibody (1 μg per 10^6 cells), an aliquot of 25 μl of PerC cells was incubated in a microtiter plate with appropriately diluted antibody. The antibodies, 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 (San Diego, CA), and streptavidin-PE-Cy5 was from PharMingen (San Diego, CA). After staining, cells were washed twice with staining medium and the preparations were run on a FACScalibur (Becton Dickinson, Mountain View, CA) using CELLQuest software for acquisition of the data (Becton Dickinson). Data was analyzed using FlowJo software program (Treestar, San Carlos, CA).

Irradiation Chimeras

Irradiation chimeras were constructed using 2-month-old lethally irradiated (1000 rads) C57BL/6J (Ly5.2) mice as recipients. A volume of 500 μl (deficient RPMI medium 1640 [Irvine Scientific] with 1 mM EDTA) containing 3×10^6 bone marrow (BM) cells with or without 5×10^6 PerC cells from donor C57BL/6J (Ly5.1) mice were injected intravenously into the irradiated recipient mice via tail vein 1 day after irradiation. Chimeras were analyzed for reconstitution 2–3 months after cell transfers. As reported previously (Kantor et al., 1992), BM cell transfer efficiently reconstituted FO, MZ, and B1b cell subsets but not the B1a subset (B1a $<$ 5% of normal levels, data not shown). The donor-derived B cell reconstitution was found to be 90%–97% (Ly 5.1 positive).

Reconstitution of Rag1$^{-/-}$ Mice with B1b Lymphocytes

We chose Rag1$^{-/-}$ mice as recipients because these mice are incapable of clearing *B. hermsii* (Alugupalli et al., 2003a) and lack not only B cells but also T cells. A lack of competition by host lymphocytes in Rag1$^{-/-}$ mice facilitates efficient reconstitution of donor B1b cells without any requirement for a marker for chimerism or irradiation prior to donor cell transfer. Purification of B1b cells is outlined in Figure 1. In brief, PerC cells (an anatomical compartment rich in B1b cells) were harvested from naive mice or mice convalescent from DAH-p1 infection ($>$2 months postinfection). B2, B1a, and T cells were depleted from the PerC cell preparation by magnetic activated cell-sorting (MACS) (AutoMACS, Miltenyi Biotec, Auburn, CA) using PE-conjugated anti-CD23 (to label B2 cells) and anti-CD5 (to label B1a and T cells) followed by anti-PE micro beads (Miltenyi Biotec). The depletion of B2 ($>$97%), B1a ($>$98%), and T ($>$97%) cells was confirmed by flow cytometry (data not shown). Thus, the lymphocyte population enriched in this negatively selected preparation is B1b cells, also referred as "MACS-enriched B1b cells" (Figure 1). The B1b cells from the MACS-enriched preparation were sorted by FACSVantage SE (Becton Dickinson) using anti-IgM-FITC and anti-Mac1-PE antibodies to $>$95% purity, and this preparation of B1b cells is referred as "FACS-purified B1b cells" (Figure 1). A volume of 500 μl deficient RPMI 1640 with 1 mM EDTA containing 2×10^5 B1b cells (comparable to the numbers of B1b cells in a naive murine peritoneal cavity) was injected i.p. into Rag1$^{-/-}$ mice and the mice were infected 1, 21, or 60 days postreconstitution. A B1b cell-depleted preparation obtained from a FACS sort containing 8×10^5 cells was also transferred into Rag1$^{-/-}$ mice for reconstitution. The transferred B1b cells of naive or of convalescent mice maintained their numbers in the PerC of Rag1$^{-/-}$ mice, when analyzed 2 (Figure 1) or 12 (data not shown) weeks postreconstitution, consistent with the self-replenishing characteristic of B1b cells (Kantor et al., 1995).

Reconstitution of Rag1$^{-/-}$ Mice with Naive B1b or B1a Lymphocytes

IgM$^+$ cells were isolated from the PerC cell preparation by MACS (Miltenyi Biotec). The B1b cells (IgM^{high}, Mac1$^+$, CD5$^-$) or B1a cells (IgM^{high}, Mac1$^+$, CD5$^+$) from the MACS-isolated IgM$^+$ cells were

FACS sorted to >95% purity. A volume of 500 μl deficient RPMI 1640 with 1 mM EDTA containing 2×10^5 B1b or B1a cells were injected i.p. into Rag1$^{-/-}$ mice and the mice were infected 1 day postreconstitution.

Enzyme-Linked Immunosorbent Assay

IgM and IgG levels in blood were measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). The *B. hermsii*-specific IgM and IgG were determined by coating 96-well plates (ICN Biomedicals, Aurora, OH) with in vivo grown *B. hermsii* DAH-p1 (10^5 wet spirochetes), and the specific antibody levels were interpreted as ng/μl using IgM and IgG standards.

To determine the IL-9 levels in the serum of *B. hermsii*-infected mice (0, 2, 3, 4, or 6 day postinfected serum), ELISA was utilized in a sandwich format with rat anti-mouse IL-9 (clone: D8402E8) as a capturing mAb and biotinylated hamster anti-mouse IL-9 (clone: D9302C12) as detecting mAb. Recombinant mouse IL-9 was used as a positive control and standard. All the reagents for the IL-9 detection except streptavidin-horseradish peroxidase conjugate (Zymed Laboratories, San Francisco, CA) were purchased from Becton Dickinson and the ELISA was performed according to manufacturer's protocol.

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