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Micro RNA 145 targets the insulin receptor substrate-1 and inhibits the growth of colon cancer cells

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A distinct role for B1b lymphocytes in T cell-independent immunity

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Table of contents

1 Introduction..... 5

2 T cell-dependent and -independent antigens..... 5

3 B cell subsets..... 6

4 B1b cells..... 7

5 Role of B1b cells in T cell-independent responses..... 8

5.1 Immunity to *Borrelia hermsii*..... 8

5.2 Immunity to *Streptococcus pneumoniae*..... 11

5.3 Response to NP-Ficoll, a model TI-2 antigen..... 13

6 Activation of antigen-specific T cell-independent B cells..... 14

6.1 Role of Btk in T cell-independent B cell responses..... 15

6.2 Role of co-stimulatory signals in T cell-independent B cell responses..... 16

6.2.1 CD40 and its ligand CD40L..... 16

6.2.2 Complement component C3 and its receptor CR1/2..... 17

6.2.3 Toll-like receptors..... 18

7 Impaired T cell-independent B cell responses..... 20

8 Memory B1b cells..... 21

9 Concluding remarks..... 23

References..... 26

Abbreviations

| | |
|------|---------------------------------------|
| AID | Activation-induced cytidine deaminase |
| BCR | B cell antigen receptor |
| Btk | Bruton's tyrosine kinase |
| CSR | Class switch-recombination |
| FO | Follicular |
| LPS | Lipopolysaccharide |
| MZ | Marginal zone |
| NP | 4-Hydroxy-3-Nitrophenyl-Acetyl |
| PerC | Peritoneal cavity |
| PS | Polysaccharide |
| Rag1 | Recombination-activating gene 1 |
| SHM | Somatic hypermutation |
| TD | T cell-dependent |
| TI | T cell-independent |
| TI-1 | T cell-independent type 1 |
| TI-2 | T cell-independent type 2 |
| TLR | Toll-like receptor |
| Xid | X-linked immunodeficiency |
| XLA | X-linked agammaglobulinemia |

Abstract

Pathogenesis of infectious disease is not only determined by the virulence of the microbe but also by the immune status of the host. Vaccination is the most effective means to control infectious diseases. A hallmark of the adaptive immune system is the generation of B cell memory, which provides a long-lasting protective antibody response that is central to the concept of vaccination. Recent studies revealed a distinct function for B1b lymphocytes, a minor subset of mature B cells that closely resembles that of memory B cells in a number of aspects. In contrast to the development of conventional B cell memory, which requires the formation of germinal centers and T cells, the development of B1b cell-mediated long-lasting antibody responses occurs independent of T cell-help. T cell-independent (TI) antigens are important virulence factors expressed by a number of bacterial pathogens including those associated with biological threats. TI antigens cannot be processed and presented to T cells and therefore are known to possess restricted T cell-dependent (TD) immunogenicity. Nevertheless, specific recognition of TI antigens by B1b cells and the highly protective antibody responses mounted by them clearly indicate a crucial role for this subset of B cells in the host's ability to overcome the restricted TD antibody response. Understanding the mechanisms of long-term immunity conferred by B1b cells may lead to improving the vaccine efficacy for a variety of TI antigens.

1

Introduction

Infectious diseases are the leading cause of mortality and morbidity worldwide. The acquisition of virulence-encoding genetic elements by horizontal transfer, the high mutation rates of pathogens, and the emergence of antibiotic resistance make it difficult to control infectious diseases by therapeutic means. Vaccination is the most effective way to control infectious diseases as it induces long-lasting immunity. A hallmark of the adaptive immune system is the development of B cell memory, which provides a protective antibody response upon re-exposure to the same antigen and is central to the concept of vaccination (MacLennan et al., 2000). T cells are crucial for the formation of germinal centers, a specialized microenvironment of the secondary lymphoid organs in which proliferation of antigen-specific B cells, affinity maturation of B cell antigen receptor (BCR) by somatic hypermutation (SHM), and alteration of antibody isotype by class-switch recombination (CSR) occur. The ultimate accomplishment of the germinal center reaction is the development of memory B cells and long-lived plasma cells that generate high affinity antibodies of different Ig isotypes (McHeyzer-Williams et al., 2001; McHeyzer-Williams, 2003). T cell-independent (TI) antibody responses are highly protective and develop much faster than T cell-dependent (TD) antibody responses (Maizels and Bothwell, 1985; Vos et al., 2000; Martin and Kearney, 2001). In spite of the potential utility of TI responses as a preventive and therapeutic intervention against a wide range of pathogens, little work has been done in this area. One reason is that, until [recently](#), TI antibody responses have been considered exclusively short-lived and incapable of conferring long-lasting protection.

2

T cell-dependent and -independent antigens

Protein antigens such as tetanus, diphtheria and pertussis toxins are potent immunogens. They induce generation of antigen-specific T cells that help B cells by providing cognate and non-cognate interactions crucial for B cell responses. Such antigens are referred to as TD antigens. Vaccines comprised of this group of antigens generate highly protective and long-lasting antibody responses (Zinkernagel, 2000). Antigens that induce antibody responses without T cell-help are referred to as TI

antigens (Fagarasan and Honjo, 2000; Vos et al., 2000). TI antigens are typically resistant to proteolysis and cannot be processed and presented to T helper cells via MHC class II, and thus are not capable of establishing cognate interaction with T cells, which can account for the restricted immunogenic nature of TI antigens (Lesinski and Westerink, 2001). TI type 1 (TI-1) antigens, the prototype of which is bacterial lipopolysaccharide (LPS), activate B cells primarily by stimulating mitogenic receptors e.g. Toll-like receptors (TLRs). Therefore, the antibodies generated by such stimuli are predominantly polyclonal (Vos et al., 2000). On the other hand, type 2 (TI-2) antigens such as bacterial capsular polysaccharides (PS) and other macromolecules with repetitive antigenic determinants, can induce antibody responses by primarily cross-linking the BCR of specific B cells (Vos et al., 2000).

3

B cell subsets

Mature B cells that generate antibody responses can be divided into four subsets; namely, follicular (FO), marginal zone (MZ), B1a and B1b subsets (Martin and Kearney, 2001; Montecino-Rodriguez and Dorshkind, 2006). Each appears to have a distinct function in the immune system. FO B cells (also known as B2 cells), comprise the majority of B cells in the body, re-circulate among the B-cell rich lymphoid follicles, participate in germinal center reactions, and mount TD antibody responses (McHeyzer-Williams, 2003). MZ B cells localized to the marginal sinus of the spleen, are strategically located to capture blood-borne TI particulate antigens and mount a very rapid response (Martin and Kearney, 2000; Martin and Kearney, 2001). The B1a subset is abundant in coelomic cavities but can also be found in the spleen (Martin and Kearney, 2000; Martin and Kearney, 2001). B1a cells though developmentally distinct from MZ B cells are also efficient in generating TI antibody responses (Berland and Wortis, 2002). B1a and MZ B cell subsets recognize evolutionarily conserved antigens such as phosphorylcholine moieties present on bacteria. When mice are challenged with phosphorylcholine-expressing bacteria such as *Streptococcus pneumoniae*, these two B cell subsets unite in the development of a rapid response (Martin et al., 2001). Aspects contribute to this response include, the ability of these B cell subsets differentiate into plasmablasts rapidly. Primed accessory cells of the myeloid lineage such as dendritic cells and macrophages appear to facilitate this response (Balasz et al., 2002; Martin and Kearney, 2001). Nevertheless, B1a cells constitutively secrete antibodies mainly of the IgM isotype and are referred to as natural antibodies because they occur spontaneously in human cord

blood, in naïve “antigen-free” mice, and in normal individuals in the absence of apparent antigen-stimulation (Boes, 2000). These antibodies have been shown to play an important role in controlling a wide range of pathogens (Boes, 2000). Although the B1b subset was described years ago (Stall et al., 1992), a functional role for these cells in protective immune responses has not been found until recently (Alugupalli et al., 2004; Haas et al., 2005; Hsu et al., 2006).

4

B1b cells

B1b cells share a number of features with B1a cells including the expression of cell-surface markers and anatomical location, e.g. the peritoneal cavity (PerC) (Stall et al., 1992). Both subsets express high levels of membrane IgM and Mac1 (integrin α_{Mac}), low levels of IgD and B220 and lack CD23 expression. The only phenotypic marker to date that distinguishes these two B1 subsets is the expression of CD5 on B1a but not on B1b cells. Additional differences in developmental pathways (Montecino-Rodriguez et al., 2006; Tung et al., 2006) and cytokine responses also exist between these two subsets. Unlike the consistent expression of CD5 on B1a cells found elsewhere, e.g. spleen, the expression of Mac1 is restricted to the B1a and B1b cells localized to the PerC. Thus, identification of the B1b subset in sites other than PerC is limited by the lack of any other B1b-specific phenotypic marker. Interestingly, a recent analysis of PerC cells from chimeric mice revealed that an IgM^{high} , $IgD^{low/-}$, $Mac1^{+}$ B1 cell population emerges over time in $Rag1^{-/-}$ mice reconstituted with wildtype peripheral lymph node cells (Hsu et al., 2006). The majority of their PerC B1 cell population lacked CD5 expression, indicating that lymph node cells predominantly reconstitute the B1b but not the B1a compartment. Thus, the number of B1b cells and/or B1b cell precursors in mice could be far more abundant than those found in coelomic cavities. The small number of B cells transferred compared to the numbers of B1b cells recovered in the PerC of the $Rag1^{-/-}$ chimeras likely reflects proliferation of these cells after transfer, as the B1 cell subsets are known to maintain their numbers in the PerC by their unusual self-renewing capability even after an arrest of bone marrow B lymphopoiesis (Carvalho et al., 2001; Kantor et al., 1995). Although the reasons are not clear, the mechanism for the preferential homing of B1a and B1b cell subsets to the PerC appears to be due to their selective attraction to CXCL13, a chemokine produced by cells in the omentum and by peritoneal

macrophages (Ansel et al., 2002). Mice deficient in CCR7 and CXCR5, the receptor for CXCL13, also exhibit a PerC homing defect of B1 cells (Hopken et al., 2004; Muller et al., 2003). As a consequence of this migration impairment the immunity of the coelomic cavity is significantly compromised in these mice (Ansel et al., 2002). Parabiosis experiments revealed that B1 cell subsets do not actively circulate compared to B2 cells (Ansel et al., 2002). Nevertheless, PerC B1 cells have the capability to leave the PerC upon appropriate stimulation. In a transgenic B1 cell mouse model it was shown that microbial products induce the migration of PerC B1 cells to mesenteric lymph nodes where they rapidly differentiate into antibody-secreting cells (Watanabe et al., 2000). Recently, it was demonstrated that Toll-like receptor (TLR)-mediated stimulation induces a rapid and transient down-regulation of integrins resulting in an efficient movement of cells of PerC B1 cells in a chemokine-dependent fashion (Ha et al., 2006). These data and the presence of B1b cells or their precursors in the lymph node suggest that they can move in a dynamic fashion and are poised to encounter pathogens not only in the PerC but elsewhere with high frequency and thus play an important role in protective immunity.

5

Role of B1b cells in T cell-independent responses

5.1

Immunity to *Borrelia hermsii*

While investigating the immune mechanism required for controlling the relapsing fever bacterium *B. hermsii*, we found for the first time a specific function for B1b cells in protective immunity. Rodents are natural reservoirs for relapsing fever bacteria, and murine infection recapitulates the critical pathophysiological aspects of the human disease (Southern and Sanford, 1969; Cadavid et al., 1994; Garcia-Monco et al., 1997; Gebbia et al., 1999; Alugupalli et al., 2001a; Alugupalli et al., 2001b; Alugupalli et al., 2003b). The hallmark of this infection is recurrent episodes of high-level bacteremia ($\sim 10^8$ bacteria/ml blood), each caused by antigenically distinct populations of bacteria generated by DNA rearrangements of the genes encoding the variable major proteins (Barbour, 1990). Remarkably, each episode is resolved rapidly within one to three days (Barbour and Bundoc, 2001; Connolly and Benach, 2001;

Alugupalli et al., 2003a). TI B cell responses are necessary and sufficient for controlling *B. hermsii* infection (Barbour and Bundoc, 2001; Alugupalli et al., 2003a). IgM is the most dominant isotype in TI responses (Martin and Kearney, 2001). We found that mice deficient only in the secretion of IgM, but not SHM or CSR, suffer persistently high bacteremia and become moribund, indicating that IgM is the essential antibody isotype required for controlling *B. hermsii* (Alugupalli et al., 2003a). Consistent with these results, it was shown that passive transfer of IgM from convalescent mice is sufficient to confer protection (Arimitsu and Akama, 1973; Yokota et al., 1997). Nevertheless, it was demonstrated that other IgG isotypes, e.g. IgG3 and IgG2b are also capable of conferring passive protection (Yokota et al., 1997). Since the control of a bacteremic episode occurs rapidly e.g. within one to three days and IgM, the first isotype to be made during primary immune response to antigen/pathogen exposure (Boes, 2000), we anticipated that neither the generation of other immunoglobulin isotypes by CSR nor affinity maturation of variable regions of the immunoglobulin by SHM which typically takes more than a week, play a critical role in this process. Activation-induced cytidine deaminase (AID) is essential for both CSR and SHM that typically occurs during a TD germinal center reaction (Muramatsu et al., 2000). Indeed, the resolution of the bacteremia and the kinetics of the *B. hermsii*-specific IgM response are indistinguishable between wildtype and AID^{-/-} mice, demonstrating that unmutated IgM is sufficient for controlling *B. hermsii* infection (Alugupalli et al., 2004).

To identify the B cell subsets that are capable of generating this protective IgM response we have infected IL-7^{-/-} mice, which are deficient in FO B cells but not B1a, B1b and MZ B cell subsets (Carvalho et al., 2001) and found that they control bacteremia as efficiently as wildtype mice (Alugupalli et al., 2003a). Using bone marrow chimeric mice deficient in B1a cells, we have ruled out a requirement for B1a cells in the protective response against *B. hermsii* (Alugupalli et al., 2004). Severe bacterial burden in splenectomized mice during the primary bacteremic episode suggested that MZ B cells play a role in controlling *B. hermsii* (Alugupalli et al., 2003a; Alugupalli et al., 2003b). Consistent with this, recently Bockenstedt and colleagues have demonstrated that MZ B cells mount anti-*B. hermsii* antibody responses (Belperron et al., 2005). Nonetheless, the rapid control of bacteremia during secondary episodes of a virulent strain or a moderate bacteremic episode by a partially attenuated strain of *B. hermsii* in

splenectomized mice suggested that MZ B cells are not the only subset that contributes to protection. Having ruled out a role for the other three B cell subsets, we investigated whether B1b cells might play a role in controlling *B. hermsii* bacteremia. Mutations affecting the BCR signaling pathway result in a severe deficiency of B1 cell subsets, which are the major producers of IgM (Berland and Wortis, 2002). Due to the lack of a B1b-deficient mouse model, we examined a potential role for B1b cells in X-linked immunodeficient (*xid*) mice (Khan et al., 1995; Thomas et al., 1993). Bacteremic episodes in *xid* mice are more severe than those of wildtype mice, which suggest that B1b cells may play a role in controlling *B. hermsii* (Alugupalli et al., 2003a). Although *xid* mice were clearly impaired in bacterial clearance, by four weeks post-infection bacteremia was completely resolved, an apparent paradox if B1b cells are indeed critical for controlling bacteremia. One possible explanation is that the markedly reduced B1b subset of *xid* mice expands during this infection. In fact Kearney and colleagues (Martin et al., 2001) have previously shown that antigen-specific TI B cell clones expand in mice immunized with *S. pneumoniae*. To detect a potential expansion of B1b cells we analyzed the frequencies of PerC B cells in *xid* mice. Remarkably, the frequency of B1b cells as defined by the surface markers IgM^{high}, IgD^{low}, Mac1⁺ and CD5⁻ in convalescent *xid* mice is significantly greater than in uninfected *xid* mice (Fig. 1A) and comparable to the frequency of B1 cells in wildtype mice (Alugupalli et al., 2003a). This expansion was selective for B1b cells but not for B1a or B2 cell subsets (Fig. 1B) and occurred regardless of whether the *xid* mice were infected with a highly virulent or a partially attenuated strain of *B. hermsii*. These findings indicate a role for B1b cells in the control of both high and moderate bacteremic episodes (Alugupalli et al., 2003a). Since IgM is essential for controlling this infection and B1b expansion was detected as early as by seven days post-infection (Fig. 1B), we predicted that *xid* mice would be capable of mounting an anti-*B. hermsii* IgM response, despite their inherent deficiency in pre-immune IgM levels. As predicted, with some delay, *xid* mice generated a specific IgM response coincident with the resolution of bacteremia (Alugupalli et al., 2007).

A significant increase in B1b cell numbers also occurs in TCR- β δ ^{-/-} mice, demonstrating that this expansion is a TI process (Fig. 1C). Remarkably, the convalescent TCR- β δ ^{-/-} were as resistant as that of the wildtype mice to re-infection suggesting that a memory-like response can be generated by B1b cells independent of a germinal center reaction (Alugupalli et al., 2004).

Indeed, we eliminated a role for two very important TD germinal center events namely SHM and CSR in controlling *B. hermsii* infection (Alugupalli et al., 2004). Movement of PerC B1 cells is crucial for their protective immune responses (Ansel et al., 2002; Ha et al., 2006; Hopken et al., 2004; Muller et al., 2003). We, therefore, anticipated that B1b cells leave the PerC to differentiate into IgM secreting plasma cells that are critical for the *B. hermsii* immunity. Upon infection with *B. hermsii*, a rapid egress of B1b cells from the wildtype mouse PerC was also observed. The magnitude of the egression was directly proportional to the bacterial burden (Fig. 1D). Interestingly, soon after resolution of bacteremia B1b cell numbers in the PerC were rapidly restored to basal levels, suggesting an active turnover and that these cells are capable of participating in protective immunity in the blood stream (Fig. 1D).

To directly determine whether B1b cells are capable of providing long-lasting immunity, we transferred B1b cells from convalescent mice into Rag1^{-/-} mice that are otherwise completely incapable of eliminating *B. hermsii* (Alugupalli et al., 2003a). Rag1^{-/-} mice reconstituted with B1b cells controlled *B. hermsii* bacteremia, demonstrating for the first time a crucial role for B1b cells in protective immunity (Alugupalli et al., 2004). The fact that naïve mice suffer recurrent bacteremic episodes indicates that naïve B1b cells are functionally less effective than convalescent B1b cells. As expected, transfer of the same number of naïve B1b cells to Rag1^{-/-} mice conferred only partial protection (Alugupalli et al., 2004). Consistent with the requirement for IgM in the clearance of bacteremia, the transferred B1b cells in Rag1^{-/-} mice confer protection by mounting a specific IgM response against *B. hermsii*, and the generation of this IgM is coincident with the resolution of bacteremia. B1b cells do not mount an antibody response in the absence of specific stimulation, indicating that they maintain a quiescent state like conventional memory B cells (Alugupalli et al., 2004). This property of B1b cells is not unique to the *B. hermsii* infection, and recently has been extended to another important human pathogen, *S. pneumoniae*.

5.2

Immunity to *Streptococcus pneumoniae*

A number of clinically important pathogens including *S. pneumoniae*, *Haemophilus influenzae*

and *Neisseria meningitidis* express PS capsules (Lesinski and Westerink, 2001). Some pathogens associated with biological threats such as *Bacillus anthracis*, the etiological agent of anthrax, express capsules made of poly- γ -D-glutamic acid (Wang and Lucas, 2004). Due to the restricted immunogenicity for TD responses, the masking of potentially immunogenic TD protein antigens, and the provision of serum resistance, capsules enable the persistence of these pathogens in the host and eventually cause serious diseases ranging from bacteremia to meningitis (Kelly et al., 2004; Scorpio et al., 2007). A critical factor in preventing these infections from becoming systemic is the ability of B cells to rapidly generate protective antibodies against capsules. As mentioned earlier, bacterial capsules are TI-2 antigens and antibodies to capsules are highly protective. In fact, the introduction of conjugate vaccine against the capsular PS (Hib) had a major impact in reducing the incidence of *H. influenzae* type b disease worldwide (Kelly et al., 2004).

Using a well-established murine model of *S. pneumoniae* infection in mice sufficient or deficient in CD19, Haas and colleagues found that CD19, though an important molecule for B cell development is, surprisingly, not necessary for generating protective IgM or IgG3 responses against the capsular PS of *S. pneumoniae* (Haas et al., 2005). CD19^{-/-} mice have a severe deficiency in B1a cells and MZ B cells but not B1b cells suggesting that this subset is responsible for the observed anti-PS specific antibody response. Conversely, CD19 transgenic (CD19Tg) mice that are severely deficient in B1b cells but not B1a or MZ B cells were incapable of mounting an antibody response to capsular PS (Haas et al., 2005). Immunization of CD19^{-/-} but not CD19Tg mice with capsular PS protects mice from a lethal *S. pneumoniae* challenge. These correlations suggest that B1b cells might be involved in generating long-lasting anti-PS responses. To identify the B cell subsets responsible for the anti-PS antibodies, Haas and colleagues transferred wildtype B1b, B1a or MZ B cells into Rag1^{-/-} mice. None of the reconstituted mice spontaneously generated anti-PS antibodies over a period of four weeks. However, upon PS immunization only B1b-reconstituted mice mounted an exuberant anti-PS IgM and IgG3 response (Haas et al., 2005). Unreconstituted mice died as early as two days post-infection when challenged with as few as 100 colony-forming units of *S. pneumoniae*. In striking contrast greater than 75% of Rag1^{-/-} mice reconstituted with B1b cells survived this lethal dose (Haas et al., 2005). These data demonstrate yet another example of B1b cell-mediated immunity to a clinically important bacterial pathogen. In the pneumococcal infection mouse model B1a cell

subsets were also shown to play an important complementary role in protective immunity by virtue of their natural antibody repertoire. Unlike constitutively secreted natural antibodies, antibodies generated by B1b cells are inducible only by specific antigen stimulation (Haas et al., 2005), as in the case of *B. hermsii*-specific B1b cell response (Alugupalli et al., 2004). To date, two independent studies, using clinically distinct murine infection systems, reveal a long-lasting and antigen-specific B1b cell response (Alugupalli et al., 2004; Haas et al., 2005). In both cases the function of B1b cells was revealed unpresumptuously. Therefore, it is tempting to consider an important role for B1b cells in protection against other infections.

5.3

Response to NP-Ficoll, a model TI-2 antigen

Due to the division of labor within TI B cell subset responses during an infection and the host- or tissue-tropism of bacterial pathogens, it is not easy to dissect the role of B1b cells in other infection systems. Moreover, the lack of a B1b cell-specific marker currently poses a challenge in tracking B1b cell responses in compartments such as the spleen, lymph nodes and bone marrow. Nevertheless, while exploring the identity of B cell populations involved in the response to 4-hydroxy-3-nitrophenyl acetyl (NP), a frequently used hapten for studying antigen-specific antibody responses in mice, MacLennan and colleagues revealed an involvement for B1b cells in anti-NP responses (Hsu et al., 2006). NP-conjugated polysaccharide Ficoll is a widely used model antigen for studying TI-2 responses. It is known that extrafollicular antibody responses induced by immunization of NP-Ficoll persist for long periods of time (de Vinuesa et al., 2000). Adoptive transfer experiments were performed using $Rag1^{-/-}$ mice reconstituted with wildtype mouse PerC cells which were depleted of either B2 or B1a cells. These mice generated normal levels of NP-specific IgM and IgG3 antibodies (Hsu et al., 2006). In contrast, $Rag1^{-/-}$ mice reconstituted with B1a and B1b cell-depleted PerC cells failed to generate NP-specific antibody responses indicating B1b cells are involved in this response (Hsu et al., 2006). In support of the specific induction of B1b cell-derived IgM seen in *B. hermsii* and *S. pneumoniae* infection models (Alugupalli et al., 2004; Haas et al., 2005), NP-specific B1b cells did not generate an anti-hapten response spontaneously (Hsu et al., 2006). The magnitude of the anti-NP response increased dramatically following NP-Ficoll immunization (Hsu et al., 2006), revealing once again a requirement for specific-antigen stimulation in the B1b cell response (Alugupalli et al.,

2004; Haas et al., 2005; Hsu et al., 2006). Surprisingly, Rag1^{-/-} mice reconstituted with lymph node B cells also generated NP-specific IgM and IgG3 responses as efficiently as the PerC cell-reconstituted Rag1^{-/-} mice (Hsu et al., 2006). Analysis of PerC cells isolated from lymph node cell-reconstituted Rag1^{-/-} mice revealed a robust generation of B1b cells but not B1a cells. Approximately 2% of lymph node B220⁺ cells are IgM^{high} and IgD^{low} that are characteristics of B1b cells. These results indicate that B1b cells and/or B1b cell precursors are present in lymphoid compartments and are poised to contribute to TI-2 antigen-specific responses. Recently, it was demonstrated that IgM memory B cells are generated upon NP-Ficoll immunization and such cells can be detected in spleen (Obukhanych and Nussenzweig, 2006). The phenotype of these B cells is distinct from that of MZ B and FO B cells. It is possible that these cells might represent splenic B1b cells (Obukhanych and Nussenzweig, 2006).

6

Activation of antigen-specific T cell-independent B cell responses

Antigens driving B1b cell responses appear to be heterogenous. For example, one *B. hermsii* antigen targeted by B1b cells is a protein implicated in virulence of this pathogen (M. J. Colombo and K. R. Alugupalli, unpublished data). In the case of *S. pneumoniae* it is a carbohydrate (Haas et al., 2005), whose expression is implicated in a variety of immune evasion strategies. Thus, the recognition of biochemically different bacterial products and even synthetic haptens such as NP (Hsu et al., 2006) by B1b cells clearly indicates that the B1b repertoire is capable of responding to a wide spectrum of antigens. Unbiased analysis of the CDR3 regions of VH genes revealed that unlike B1a cells, B1b cells have high junctional diversity that is comparable to B2 cells (Kantor et al., 1997; Tornberg and Holmberg, 1995). Furthermore, immunization with NP-Ficoll can result in low levels of SHM in the VH regions even without T cell help (Toellner et al., 2002). This process presumably occurs by BCR cross-linking induced AID expression without the requirement for CD40-CD40L (Faili et al., 2002; Weller et al., 2001). This could also explain the existence of other antibody isotypes e.g. IgG3 by CSR during TI responses (Martin and Kearney, 2001). However, unlike conventional B2 cells, the affinity and overall Ig repertoire of B1b cell-derived antibodies may be limited due to the lack of an involvement with TD germinal center reactions.

6.1

Role of Btk in T cell-independent B cell responses

Although antigen-specific B1b cells respond to various TI antigens e.g. pneumococcal PS, hapten NP and *B. hermsii* surface protein (Haas et al., 2005; Hsu et al., 2006) (M. J. Colombo and K. R. Alugupalli, unpublished data), the mechanism of activation of this response can vary. *Xid* mice, which carry a point mutation in Bruton's tyrosine kinase (Btk), a cytoplasmic kinase belonging to the Tec family of kinases is crucial for BCR-mediated activation. B cells of these mice cannot respond to anti-BCR cross-linking and are therefore impaired primarily in response to TI-2 antigens such as NP-Ficoll (Amsbaugh et al., 1972; Khan et al., 1995; Scher et al., 1975; Thomas et al., 1993). In fact, these mice cannot generate protective antibody responses to capsules of *S. pneumoniae* or *B. anthracis* (Briles et al., 1981; Wang and Lucas, 2004). Mice that have a targeted deletion in the *Btk* gene recapitulate the *xid* mouse phenotype (Khan et al., 1995). Surprisingly, the phenotypes seen in these mice are not identical to X-linked agammaglobulinemia (XLA) patients, the human equivalent of *xid*. Mice deficient in Tec, a kinase belonging to the same family as Btk, have no major phenotypic alterations of the immune system (Ellmeier et al., 2000). Interestingly, mice deficient in both Btk and Tec exhibit a phenotype that is similar to human XLA (Ellmeier et al., 2000). Although the exact reasons for these differences is not clear, it was demonstrated that retroviral-mediated transfer of the human *Btk* gene corrects a number of functions including TI-2 responses in Btk and Tec double knockout mice, indicating a functionally conserved and crucial role of Btk in both mice and humans (Yu et al., 2004). *Xid* mice respond to LPS, a model TI-1 antigen (Hiernaux et al., 1983; Mosier et al., 1977). The moderate impairment of *xid* mouse B cells to LPS stimulation (Khan et al., 1995) is likely due to a potential role for Btk in TLR-mediated signaling rather than its role in BCR-mediated signal (Jefferies et al., 2003; Jefferies and O'Neill, 2004), as the IgM response induced by LPS is polyclonal and non-specific (Andersson et al., 1972; Mosier et al., 1976). In support of this, it was shown that mice deficient in MyD88 involved in TLR- but not BCR-mediated signaling exhibit impaired LPS-induced B cell activation (Kawai et al., 1999). In fact, these mutant mice generate normal anti-NP-Ficoll antibody responses (Schnare et al., 2001). These data together reveals the critical role for Btk in TI-2 responses.

Although *xid* mice are deficient in B1 cells, as previously mentioned (see Sect. 5.1), the impairment of a specific antibody response to TI-2 antigens is not entirely due to a deficiency of these B cell subsets. Increasing the B cell numbers in *xid* mice by blocking apoptosis of B cells with the transgenic expression of Bcl2, does not restore B cell functions including TI-2 responses in *xid* mice (Woodland et al., 1996). Transgenic expression of IL-9 or injection of this cytokine induces selective expansion of B1b cells in wildtype mice (Vink et al., 1999). Such a transgene can even correct the B1b cell deficit of *xid* mice but does not restore the specific antibody response to pneumococcal capsular PS or NP-Ficoll (Knoops et al., 2004). In contrast, a Btk transgene restores NP-Ficoll and antiviral TI-2 responses of *xid* mice in a dose-dependent fashion (Pinschewer et al., 1999; Satterthwaite et al., 1997). Furthermore, the defective anti-PS response of *xid* mice is not attributed to lack of a specific VH gene family involved in TI-2 responses (Feng and Stein, 1991; Selinka and Bosing-Schneider, 1988). These data suggest that the impaired TI response in *xid* mice is due to a compromised BCR-mediated signal rather than a deficiency in B1b cells or the potential deficiency in the BCR repertoire itself.

6.2

Role of co-stimulatory signals in T cell-independent B cell responses

6.2.1

CD40 and its ligand CD40L

Despite a BCR-mediated signaling defect, *xid* or Btk^{-/-} mice generate near normal responses to TD antigens (Khan et al., 1995). This response is likely mediated by CD40-Cd40L co-stimulation provided by T cells, since mice deficient in both Btk and CD40 are severely compromised for TD antibody responses (Khan et al., 1997). In fact, *in vitro* co-stimulation of *xid* B cells with CD40 compensates the need for Btk in BCR-mediated signal as detected by cell cycle progression and nuclear translocation of NF- κ B, a transcription factor involved in a variety of B cell responses (Mizuno and Rothstein, 2003; Mizuno and Rothstein, 2005). Consistent with this, injection of anti-CD40 antibodies as a surrogate for CD40L restores immune responses to NP-Ficoll in *xid* mice (Dullforce et al., 1998; Vinuesa et al., 2001). In striking contrast to that of typical TI-2 responses induced by capsular PS or NP-Ficoll, the antibody response to *B. hermsii*

is clearly different. For example, unlike pure TI-2 antigens, an active infection is expected to engage several immune activation pathways. Although *xid* mice suffer more severe bacteremia than wildtype mice, they control all episodes of *B. hermsii* bacteremia and the resolution of the infection coincides with an expansion of B1b cells in these mice (see Sect. 5.1) (Alugupalli et al., 2003a). *Xid* mouse B cells are defective in homeostatic proliferation (Cabatingan et al., 2002; Woodland and Schmidt, 2005), hence this B1b cell expansion is likely to be antigen-driven. In support of this, *xid* or *Btk*^{-/-} mice generate an antigen-specific IgM response capable of controlling infection (Alugupalli et al., 2007). The magnitude of the specific IgM response in wildtype mice is comparable to that of *TCR-βxδ*^{-/-} mice, implying an unlikely role for CD40-CD40L-mediated co-stimulation, provided by T cells in this infection (Alugupalli et al., 2007) (Fig. 2A). The observation that *CD40L*^{-/-} mice generate a robust TI IgM response to the related bacterium *B. burgdorferi* supports this notion (Fikrig et al., 1996). Although the physiological relevance of CD40-CD40L interaction in TI responses is not clear, the above data indicate that a role exists for alternate pathways of activation during TI responses.

6.2.2

Complement component C3 and its receptor CR1/2

The high expression of CR1/2, the C3 receptor on MZ B cells (Zandvoort and Timens, 2002), is necessary for specific targeting of C3 conjugated TI-2 antigens such as Pneumococcal capsular PS to MZ B cell (Breukels et al., 2005; Guinamard et al., 2000). Mice deficient in C3 have reduced responses to TI-2 antigens (Guinamard et al., 2000). Some viral pathogens also behave like TI antigens and induce TI-2 like responses *in vivo* (Fehr et al., 1998; Ochsenbein et al., 1999; Szomolanyi-Tsuda et al., 2001; Szomolanyi-Tsuda and Welsh, 1998). Studies of vesicular stomatitis virus have demonstrated a correlation between antigen repetitiveness (TI-2 like arrangement) and the degree to which B-cell activation is independent of T cells. The rigidly structured Poliovirus efficiently induces neutralizing IgM antibodies independent of T cell-help (Fehr et al., 1998). For example, Poliovirus and Vaccinia virus expressing vesicular stomatitis virus glycoproteins do not induce antibody responses in *xid* mice (Pinschewer et al., 1999; Szomolanyi-Tsuda et al., 2001). However, as mentioned earlier, a *Btk* transgene complements these responses in *xid* mice suggesting that BCR-signaling is the limiting component

(Pinschewer et al., 1999). However, impaired TI responses to these viral pathogens, in $C3^{-/-}$ or $CR2^{-/-}$ mice, implied that simultaneous engagement of C3-coupled antigen to CR1/2 and BCR promotes B cell stimulation (Ochsenbein et al., 1999; Szomolanyi-Tsuda et al., 2006). In fact, priming of human B cells with C3 engagement to CR1/2 enhances anti-IgM-mediated B cell activation even to very low levels of BCR crosslinking (Carter and Fearon, 1989; Carter et al., 1988). This co-stimulatory pathway seems to be more appropriate for the activation of MZ B cells than B1b cells. For instance, the B1b but not MZ B cells are crucial for the clearance of *B. hermsii* and $C3^{-/-}$ mice control this infection efficiently (Connolly and Benach, 2001; Connolly et al., 2004). The kinetics of the anti-*B. hermsii* IgM response in $C3^{-/-}$ mice is comparable to that of wildtype mice (Fig. 2A). This result is in agreement with the C3-independent IgM-mediated killing of relapsing fever bacteria (Connolly and Benach, 2001; Connolly et al., 2004).

Xid B cells respond to TI-2 antigens when co-stimulated by IL-1 (Couderc et al., 1987). However, we found that $IL-1R^{-/-}$ mice generate robust IgM response to *B. hermsii* (Fig. 2A) (Alugupalli et al., 2007). Our results on the pathogen-specific IgM response in *xid* or $Btk^{-/-}$ mouse infections suggested that even in the absence of normal BCR-mediated signaling, *B. hermsii* is capable of rapidly activating antigen-specific B cells, presumably by stimulating other signaling pathways such as the TLR-mediated pathway. In fact, when *xid* B cells are co-stimulated with TLR ligands, they respond to BCR cross-linking (Couderc et al., 1987).

6.2.3

Toll-like receptors

TLRs play important roles in activation of the immune system (Takeda and Akira, 2005). Importantly, TLR9 has been shown to activate auto-reactive B cells (Leadbetter et al., 2002) and memory B cells (Bernasconi et al., 2002), indicating a possible role for TLR9 in the development of rapid and long-lasting TI-2 antibody responses. *B. hermsii* being a prokaryote is expected to contain a high frequency of CpG DNA, the ligand for TLR9. Despite these expectations, $TLR9^{-/-}$ mice generate rapid anti-*B. hermsii* IgM responses. *B. hermsii* possess other potential TLR ligands such as lipoproteins, the ligands for TLR2 (Shang et al., 1998) that could redundantly activate distinct members of the TLR family, thereby restoring responses in *xid* mice. Indeed,

mice deficient in MyD88, a common cytoplasmic adaptor for all TLRs except TLR3, exhibited a significantly delayed anti-*B. hermsii* IgM response (Fig. 2B). Analysis of individual TLR knockout mice revealed important roles for TLR1 and TLR2 in anti-*B. hermsii* IgM responses (Alugupalli et al., 2007). CD14 is known to augment TLR2-mediated responses and as expected, CD14^{-/-} mice have impaired antibody response to *B. hermsii* (Alugupalli et al., 2007). CD14 is not only involved in TLR2 signaling but also in TLR4-mediated signaling. Although *B. hermsii* is not a Gram-negative bacterium and hence does not contain LPS, other evolutionarily distant microbial components such as glycolipids of *Treponema* (Schroder et al., 2000), pneumolysin of *S. pneumoniae* (Malley et al., 2003) and fusion protein of respiratory syncytial virus (Kurt-Jones et al., 2000) are recognized by TLR4. This may explain the delayed and reduced *B. hermsii*-specific IgM response in TLR2^{-/-} mice. The magnitude of the specific IgM response in TLR4^{-/-} mice was also somewhat lower than wildtype mice (Alugupalli et al., 2007). As expected, this potential TLR4-mediated response did not resemble an LPS-induced response, since mice deficient in MD2, a protein crucial for the LPS response (Nagai et al., 2002), generated normal antibody responses (Alugupalli et al., 2007). These results indicate that *B. hermsii* may also signal through TLR4 in addition to TLR1 and TLR2, by a mechanism distinct from that of LPS-induced stimulation. Mice deficient in TLR3, which is involved in recognizing double-stranded RNA, a signature for viral rather than bacterial infection, generated IgM responses comparable to wildtype mice (Alugupalli et al., 2007).

Due to the redundancy in TLRs, we tested whether MyD88 provides synergistic or partially redundant functions in triggering an antibody response to *B. hermsii* in *xid* mice. Indeed, mice deficient in both Btk and MyD88 are severely compromised in IgM production and bacterial clearance but not TD antibody responses that utilize CD40-CD40L (Alugupalli et al., 2007). Despite a normal response both in terms of kinetics and magnitude of specific IgM or IgG to the model TD antigen NP-conjugated chicken gammaglobulin, Btk x MyD88^{-/-} mice are unable clear *B. hermsii* even transiently, highlighting the critical role for TI responses in protective immunity (Alugupalli et al., 2007) (Fig. 2B). Although B1b cells are capable of efficiently recognizing chemically distinct antigens, these data suggest the involvement of a co-stimulatory mechanism in B1b cell activation during TI responses. Understanding the source of

TLR signaling and how they regulate B1b cell function may help develop strategies to restore efficient responses in individuals with impaired TI responses.

7

Impaired T cell-independent B cell responses

Children and the elderly respond poorly to TI antigens, and as a consequence suffer severe and recurrent infections by both encapsulated and non-encapsulated bacteria (Kelly et al., 2004; Kelly et al., 2005; Lesinski and Westerink, 2001). A number of possibilities could account for this impairment, some of which are reminiscent of the defects seen in *xid* mice, suggesting that the *xid* mouse is an appropriate model for studying impaired TI responses in children. Similar to the BCR-mediated activation defect of *xid* B cells (Couderc et al., 1987), it has been shown that the B cells of neonatal, very young and aged wildtype mice exhibit an activation defect to PS antigens and multivalent membrane Ig crosslinking, a mimic of TI-2 antigen-induced stimuli (Chelvarajan et al., 1998; Chelvarajan et al., 1999; Snapper et al., 1997). This impairment of the surface IgM cross-linking and NP-Ficoll responses in neonatal B cells can be corrected by a number of above mentioned (see Sect. 6) co-stimulatory signals for *xid* B cells (Couderc et al., 1987; Vinuesa et al., 2001) such as CD40-L, TLR ligands, IL-1 and IL-6 (Chelvarajan et al., 1998; Chelvarajan et al., 1999; Snapper et al., 1997). A defect in accessory cell-mediated co-stimulation for neonatal and aged mouse B cell activation has also been proposed for the poor responses of children and the elderly. These accessory cells are mainly of the myeloid lineage, such as monocytes and macrophages but not T cells (Bondada et al., 2000; Chelvarajan et al., 2004; Chelvarajan et al., 2005; Chelvarajan et al., 2006; Landers et al., 2005; Yan et al., 2004a; Yan et al., 2004b). In fact, compared to adult human monocytes, neonatal monocytes express significantly lower levels of MyD88 protein, which is required for IL-1 and almost all TLR-mediated signalings (Yan et al., 2004b). In agreement with these signaling defects, we have recently demonstrated that mice deficient in both MyD88 and Btk are severely compromised for TI antibody responses (see Sect. 6.2.3).

The lack of appropriate B cell subsets and/or IgM memory B cells could also explain the impaired TI responses in the young (Kruetzmann et al., 2003; Zandvoort et al., 2001). For

example, the lack of MZ B cell development and the low expression of CR1/2 on MZ B cells in the spleens of children under 2 years of age has been suggested as a reason for their impaired responses to PS (Zandvoort and Timens, 2002). Nevertheless, upon immunization with NP-Ficoll, *pyk-2^{-/-}* mice, deficient in MZ B cells but not B1 cells, generate IgM and IgG3 antibody responses more than two orders of magnitude higher than pre-immune levels (Guinamard et al., 2000). This implies that other B cells, likely B1b cells, play an important role in this TI-2 response. In agreement with this possibility two independent studies reviewed here (see Sect. 5.2 & 5.3) provided direct evidence using *Rag1^{-/-}* chimeras that B1b cells generate the majority of the anti-TI-2 IgM and IgG3 responses to NP-Ficoll and PS (Haas et al., 2005; Hsu et al., 2006). It is interesting that the splenic localization of PS conjugates can also be independent of the presence of C3 (Breukels et al., 2005), which could explain the significant NP-Ficoll response in *pyk-2^{-/-}* mice (Guinamard et al., 2000). In agreement with this, mice deficient in CD19, the signal-transducing moiety of the C3 receptor CR1/2, generate B1b cells normally and mount efficient protective antibody responses to capsular PS (Haas et al., 2005). In fact, *C3^{-/-}* mice also generate normal IgM responses to *B. hermsii* (Fig. 2A) (Connolly and Benach, 2001; Connolly et al., 2004). While it is not clear that all distinctions between B cell subsets defined in the mouse hold true for humans, these data also suggest that children lack sufficient protection from functional IgM-secreting (memory) B1b cells. Such a lack in the rapid as well as long-lasting responses to TI antigens by B1b cells may explain recurrent infections by encapsulated bacterial pathogens in children.

8

Memory B1b cells

The functional definition of immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously and reflect the pre-existence of clonally expanded populations of antigen-specific lymphocytes (Janeway et al., 2004). The long-term immunity provided by B1b cells functionally resembles that of canonical B cell memory, however it is generated and maintained in the complete absence of T cells (Alugupalli et al., 2004).

The B1b cell responses to three distinct TI antigens reviewed here (Sect. 5.1, 5.2, and 5.3)

have revealed strikingly common characteristics to memory B cell responses. Such characteristics include i) the generation of B1b cell responses that do not require continuous B lymphopoiesis (Alugupalli et al., 2003a; Carvalho et al., 2001); ii) B1b cells maintain their numbers by self-renewal (Kantor et al., 1995); iii) B1b cells maintain a quiescent state in terms of their differentiation into plasma cells, and their activation requires specific-antigenic stimulation (Alugupalli et al., 2004; Haas et al., 2005; Hsu et al., 2006); and iv) B1b cells generate long-lasting antibody responses (Alugupalli et al., 2004; Haas et al., 2005; Hsu et al., 2006). In the *B. hermsii*-infection system, expanded B1b cells persist for a remarkably long-time in convalescent mice (Fig. 1B) and upon challenge, expanded B1b cells rapidly differentiate into antibody secreting cells (Alugupalli et al., 2004). While naïve B1b cells also generate specific IgM, the magnitude of the response is significantly lower compared to that of expanded B1b cells. The kinetics of the IgM response is also considerably delayed (Alugupalli, unpublished data). These properties indicate that a subset of B1b cells behave like memory B cells. Reasons that account for the evolution of the enhanced protection or antibody responses by such a subset may include an expansion of antigen-specific B1b cells. In addition, antigenic stimulation may have conferred upon the immune or convalescent mouse B1b cells a distinct property that naïve B1b cells do not possess, similar to those that distinguish TD memory B cells from naïve B cells as illustrated in Fig. 3.

The robust binding of B1b cell-derived IgM to *B. hermsii* without SHM (Alugupalli et al., 2004) suggests an increased quantity of antigen-specific B1b cells in convalescent mice rather than an affinity maturation of VH regions of specific B1b clones. Interestingly, the VH sequences of the majority of the Ig repertoire of NP-Ficoll immunized mice have no SHM and are of the IgM isotype (Maizels and Bothwell, 1985; Maizels et al., 1988). This unmutated IgM response is not unique to the carrier, Ficoll. In fact, mice deficient in BCL6, which cannot develop germinal center reaction, are capable of mounting hapten-specific antibody responses when immunized with NP-conjugated to chicken gammaglobulins as a carrier, a frequently used model TD antigen. The VH regions of the NP-specific IgM memory B cells in BCL6^{-/-} mice are also unmutated. These results indicate that generation of unmutated IgM memory occurs without germinal center formation and is specific to the antigen driving such response (Toyama et al., 2002). In fact, such an unmutated IgM response is sufficient for conferring immunity even to a rapidly replicating bacterial pathogen, since TI control of *B. hermsii* occurs in AID^{-/-} mice.

Furthermore, we have also shown that convalescent AID^{-/-} mice are also resistant to re-infection demonstrating that the unmutated IgM memory of B1b cells is functional (Alugupalli et al., 2004).

9

Concluding remarks

To date three distinct model systems have revealed that B1b cell subsets mount the majority of the antigen-specific TI response (Alugupalli et al., 2003a; Alugupalli et al., 2004; Haas et al., 2005; Hsu et al., 2006). This B1b cell subset is also capable of generating a unique response that is functionally but not developmentally similar to the classical memory B cell response. The generation of rapid and long-lasting IgM induction from the B1b cell subset, may not be an unnecessary complexity in the seemingly linear conventional TD B cell memory development as opined recently (Tarlinton, 2006). The existence of B1b cell memory does not appear to be a redundant arm of the memory B cell compartment, as B1b cell memory has already been shown to play an indispensable role in two clinically important bacterial infections (Alugupalli et al., 2004; Haas et al., 2005). In fact, TD responses do not contribute to the protective immunity in these systems. The dichotomy in the development of B cell memory, such as the generation of TI memory B1b cells and canonical TD memory B cells seem to imply the humoral immune system's inherent strategy to cover two major categories of immunogens, the TI and TD antigens, respectively. The division of labor by these two distinct arms of B cell memory could ensure achieving "sterilizing" immunity to a wider range of pathogens in an overlapping and timely fashion. The rapid and antigen-specific IgM response enhances the germinal center reactions by forming antigen-IgM complexes and facilitates an accelerated development of high affinity antibody response to TD antigens, suggesting a role for B1b-derived IgM in the development of canonical B cell memory (Corley et al., 2005). In addition, B1b cell-derived TI responses may also be beneficial in decreasing the incidence of morbidity associated with opportunistic pathogens in individuals with decreased T cell counts and T-cell dysfunction (Chinen and Shearer, 2002). For instance, a selective reduction in CD27⁺ conventional memory B cells in drug-naïve and in highly active anti-retroviral therapy-treated HIV-1-infected individuals compromises their TD responses and increases susceptibility to a number of opportunistic infections (Chong et al., 2004). Elucidation of the developmental pathways

4/11/08

involved in B1b cell generation, activation, expansion and long-term maintenance of antigen-specific B1b cells may provide new approaches to the induction of long-lasting protective antibody responses in individuals impaired in TI and/or TD responses.

4/11/08

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4/11/08

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Figure legends

Figure 1.

A. Expansion of the B1b cell population in *B. hermsii*-infected mice. PerC cells of uninfected or four-week post-infected *xid* (CBA/N) mice were harvested and stained with antibodies specific for IgM, IgD and Mac1 or CD5 and analyzed by flow cytometry (Alugupalli et al., 2003a). All B cells were first identified by IgD and IgM dual positivity (plots not shown) and were further resolved as B1 (i.e. B1a + B1b) and B1a populations by Mac1 and CD5 positivity, respectively. The percent frequency values of B1 and B1a cells, among the all PerC cells are indicated within the plots. The frequency of B1b cells was inferred from values obtained from the subtraction of the % of B1a (CD5⁺) cells from the % of all B1 cells (Mac1⁺). Data were generated by analyzing a minimum of 20,000 cells and are representative of four separate experiments. 5% contour plots are shown.

B. Persistent expansion of B1b lymphocytes in *B. hermsii*-infected mice. PerC cells of uninfected or *B. hermsii* infected *xid* (CBA/N) mice at the indicated days post-infection (dpi) were harvested and frequencies of B1b, B1a and B2 subsets were determined by flow cytometry. The absolute cell counts of B1b (IgM^{high}, IgD^{low}, Mac1⁺ and CD5⁻), B1a (IgM^{high}, IgD^{low}, Mac1⁺ and CD5⁺) and B2 (IgD^{high}, IgM^{low}, Mac1⁻) were calculated as a product of their frequency and the PerC cell yield. The mean ± SD values of respective subsets of three mice at the indicated dpi are given. Significant expansion of B1b cells occurred in infected *xid* mice (**, $p < 0.002$) (Alugupalli et al., 2004).

C. Expansion of B1b cells is T cell-independent. PerC cells of naïve or convalescent (40 dpi) TCR- β δ ^{-/-} mice were harvested and stained with IgM, IgD and Mac1 or CD5 and the absolute cell numbers of B1b, B1a and B2 were determined. The mean ± SD values of five mice are shown. Significant expansion of B1b cells occurred in infected TCR- β δ ^{-/-} mice (**, $p < 0.002$) (Alugupalli et al., 2004).

D. Rapid egress of B1b cells from the peritoneal cavity (PerC) during *B. hermsii* infection. Wildtype mice were infected intravenously with 5×10^4 *B. hermsii* strain DAH-p1. On each of the indicated days post-infection, three mice were sacrificed. Bacteremia and frequency of B1b cells in the PerC were measured by microscopic counting and flow cytometry, respectively.

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Significant reduction of B1b cells occurred during the acute phase of the bacteremia (i.e. 2 and 3 dpi) (*, $p < 0.05$)

Figure 2. Kinetics of the *B. hermsii*-specific IgM response in various knockout mice.

A. Wildtype (n=3), T cell-deficient (TCR- $\beta \times \delta^{-/-}$; n=3), C3 $^{-/-}$ (n=5) or IL=1R $^{-/-}$ (n=5) mice; **B.** Wildtype (n=3) or mice deficient in either Btk (Btk KO; n=3) or MyD88 (MyD88 KO; n=3) or both (Btk x MyD88 KO; n=4) were infected intravenously with 5×10^4 *B. hermsii* strain DAH-p1. *B. hermsii*-specific IgM responses in the blood on the indicated days post-infection were measured by ELISA and mean \pm SD values are shown (Alugupalli et al., 2007).

Figure 3. A model for the efficient protection conferred by B1b cells to pathogens in convalescent mice.

Upon infection or exposure to a specific T cell-independent antigen, B1b cells of varying affinities generate a short-lived IgM response. In this primary response, a division of labor by appropriate MZ B and/or B1a subsets (not shown in the figure) may also contribute to the IgM response. While a significant fraction of this IgM may recognize the pathogens, only some of this response is protective during primary infection. On the other hand, a subset of B1b cells driven by specific antigens expand with the help of a T cell-independent co-stimulatory signal (see Sect. 6), persist in convalescent mice with higher frequency, and maintain a quiescent state in terms of their further differentiation into plasma cells. Such B1b cells acquire a functional memory B1b cell phenotype. Compared to their naïve counterparts, the ability of memory B1b cells to mount a heightened IgM response, upon antigen exposure, results in efficient protection from re-infection. Factors that may contribute to this rapid response include an altered transcriptomic profile and surface expression of other signaling receptors. Since AID can be induced by BCR cross-linking and TLR stimulation, IgM secreting B1b cells could also switch to other isotypes, such as IgG3.