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The Capsular Polysaccharide Vi from Salmonella Typhi Is a B1b Antigen

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Vaccination with purified capsular polysaccharide Vi Ag from Salmonella typhi can protect against typhoid fever, although the mechanism for its efficacy is not clearly established. In this study, we have characterized the B cell response to this vaccine in wild-type and T cell–deficient mice. We show that immunization with typhoid Vi polysaccharide vaccine rapidly induces proliferation in B1b peritoneal cells, but not in B1a cells or marginal zone B cells. This induction of B1b proliferation is concomitant with the detection of splenic Vi-specific Ab-secreting cells and protective Ab in Rag1-deficient B1b cell chimeras generated by adoptive transfer-induced specific Ab after Vi immunization. Furthermore, Ab derived from peritoneal B cells is sufficient to confer protection against Salmonella that express Vi Ag. Expression of Vi by Salmonella during infection did not inhibit the development of early Ab responses to non-Vi Ags. Despite this, the protection conferred by immunization of mice with porin proteins from Salmonella, which induce Ab-mediated protection, was reduced postinfection with Vi-expressing Salmonella, although protection was not totally abrogated. This work therefore suggests that, in mice, B1b cells contribute to the protection induced by Vi Ag, and targeting non-Vi Ags as subunit vaccines may offer an attractive strategy to augment current Vi-based vaccine strategies. The Journal of Immunology, 2012, 189: 5527–5532.

Typhoid infections, caused by Salmonella enterica serovar Typhi (ST), are major killers responsible for >200,000 deaths yearly (1). This organism can infect humans and other higher primates, but no other animal reservoir has been recognized. This suggests that it may be possible to eliminate typhoid through the use of vaccination and other public health measures. The death toll from typhoid would be higher except for the availability of vaccines. The two well-tolerated vaccines in use provide partial and temporally limited protection and are quite distinct (2–4). One is a live attenuated typhoid bacterium, strain Ty21a, and is administered orally. How this attenuated bacterium confers protection is not fully understood, but Ab is likely to be important. This live vaccine lacks galE and so has an impaired capacity to synthesize LPS O-chain, and also lacks the capsular polysaccharide (CP) Vi Ag, both considered major targets of protective Ab (5). The importance of Ab to Vi is evidenced by the use of purified Vi Ag as a stand-alone vaccine. Vi Ag is made of repeating units of (1→4)-2-deoxy-2-N-acetyl galacturonic acid, 60–90% O-acetylated at the C-3 position and is encoded within the viaB locus from ST (6). Immunization provides protection against typhoid at levels comparable to Ty21a in adults and older children in the first 2 y postimmunization (2, 3). The protection conferred by immunization with Vi Ag is likely to be mediated via systemic Ab as it has not been found to induce pronounced mucosal Ab responses, nor have a requirement for T cell involvement (7, 8). Therefore, understanding the nature of Ab responses to Vi Ag and other vaccines based on CP is likely to be important in understanding the basis of immunity to many pathogens and improving vaccines that target them.

Classically, CP are regarded as T cell–independent type-II (TI-2) Ags, with splenic marginal zone (MZ) B cells playing an important role in mediating responses to this class of Ags (9–12). This association became apparent in part because of the poor responses to CP seen in asplenic adults (13, 14), and because B cells with the MZ phenotype and responsiveness are mainly located in the spleen. Also, infants have aberrant responses to TI-2 Ags and appear to lack a mature MZ B cell compartment (15). Although it is clear that MZ B cells contribute to these responses (9), recent studies have shown that CP induces a more complex response than previously thought, with increasing evidence suggesting that, at least in mice, B1 cells contribute to TI-2 responses (11). Two subsets of B1 cells are recognized in mice, B1a and B1b cells (16), and although typically found in areas such as the peritoneal cavity, they can be present in other anatomical sites such as the spleen. The role of B1 cell subsets in immune responses can be complex.

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Abbreviations used in this article: ASC, Ab-secreting cell; CP, capsular polysaccharide; MZ, marginal zone; ST, Salmonella enterica serovar Typhi; STm, Salmonella enterica serovar Typhimurium; TI, T cell–independent; TI-2, T cell–independent type-II; WT, wild type.

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Thus, the phosphorylcholine moiety CP from *Streptococcus pneumoniae* is a natural target of Ab produced by B1a cells, but after immunization with pneumococcal CP specific Ab is produced by B1b cells (17). Furthermore, when B1b cells are recruited into responses against model TI-2 Ags such as NF-Picol, then plasmablast differentiation occurs in the spleen, highlighting the importance of this site for both MZ and B1b responses (18).

B1 cells can be identified by their surface marker phenotype. B1a and B1b cells are IgM1IgD20wCD21++CD233 IgCD220wCD19* and are CD1b variable (16, 19). B1a cells are discriminated from B1b cells by their expression of CD5. Their importance has been identified in responses to many pathogens, including pneumococci, *Borrelia*, and *Salmomella enterica* serovar Typhimurium (STM) (17, 19, 20). Interestingly, B1b responses to Ags from these pathogens can occur in the absence of MZ B cells, indicating that B1b cells can be sufficient for protective immunity (19, 20). In light of this, and in the absence of a clear mechanism for the protection induced by Vi Ag, we studied the mode of action of the typhoid vi polysaccharide vaccine in mice. We show that peritoneal B1b cells, but not other B cell subsets, are selectively induced to proliferate and differentiate to Ab-producing cells in response to immunization with purified Vi Ag in a TI manner. Furthermore, transfer of peritoneal B cells is sufficient to confer protection against infection with a STM strain engineered to express Vi Ag (STMVi*) Lastly, we show that Vi expression by *Salmomella* during infection impairs, but does not ablate, the capacity of Ab to other Ags to control infection. This work further emphasizes the importance of B1b cells in conferring protective responses to bacterial pathogens.

**Materials and Methods**

*Animals, bacteria, Ags, and immunizations*

Animal studies were performed with ethical approval and within Home Office guidelines. Age- and sex-matched C57BL/6J (wild type [WT]) were obtained from Harlan Laboratories (Bicester, U.K.), and TCRβ-/- mice from Jax. Mouse colonies were maintained in the Biomedical Services Unit, University of Birmingham, under specific pathogen-free conditions. Mice were inoculated with 10 μg Typhim Vi (Sanofi Pasteur MSD, Maidenhead U.K.). 20 μg porins (21), or 5 x 10<sup>5</sup> STM AroC-deficient bacteria or 5 x 10<sup>5</sup> STMVi* AroC-deficient bacteria. STM strains containing an aroC deletion and that either express Vi (strain RAK109) or an isogenic strain that is Vi negative due to deletion of the tviB gene (strain RAK112) were constructed by modification of previously described strains, C5.507 (strain C5 containing ~300 kb of S. Typhi genomic sequence, including the SPI-1 island encoding the viaB locus) and SGB1 (C5.507 viaB::cat (22). A strain in which the aroC gene was replaced by the cat gene was constructed using red recombinase-mediated allelic exchange in strain SL1344 containing pSmi18 using a PCR product amplified from pKD3 using primers 5’-gctactgacaaaccatgccagcagcgcaatcgcggttttttt-3’ and 5’-attataaattgacaagc-3’. Titres were calculated after plotting the OD of each well against the serum titre of a positive control.

**Immunohistology**

Immunohistology was performed on prepared Multiscreen plates using 10 μg/ml Typhim Vi. Plates were blocked with 1% BSA PBS. A total of 10<sup>6</sup> splenocytes or peritoneal exudate cells was added to each well, and plates were incubated for 6 h at 37˚C at 5% CO<sub>2</sub>. After incubation, plates were washed and then anti-IgM Ab conjugated to alkaline phosphatase (Southern Biotech), followed by p-nitrophenyl phosphate (Sigma-Aldrich). Plates were read at 405 nm to determine absorbance. Relative Ab titres were calculated after plotting the OD of each well against the serum dilution and were derived from linear portion of the resulting curves.

**ELISPOT**

Serum Ab to Vi or STM and its Ags were assessed by ELISA, as described previously (29), and Vi-specific Ab-producing cells were detected by ELISPOT assay. Briefly, ELISA was performed on Nunc Immunosorb 96-well plates (Nunc) coated with 5 μg/ml Ag in carbonate buffer. Serum was added at a 1:50 dilution in triplicate. Bound Ab was detected using goat anti-IgM, IgG3, or IgG conjugated to alkaline phosphatase (Southern Biotech), followed by p-nitrophenyl phosphate (Sigma-Aldrich). Plates were read at 405 nm to determine absorbance. Relative Ab titres were calculated after plotting the OD of each well against the serum dilution and were derived from linear portion of the resulting curves.

**Cell transfer**

Peritoneal B cells were harvested from TCRβ-/- or WT mice. A total of 10<sup>6</sup> peritoneal B cells was transferred into recipient C57BL/6 Rag1<sup>−/−</sup> mice i.v. Peritoneal cells were sorted using a MoFlow cell sorter (Beckman Coulter) after staining with CD23 PE, CD5 PE-Cy5, and CD3 FITC, and sort purity was assessed using B220 allophycocyanin. A quantity amounting to 2 x 10<sup>5</sup> cells was transferred i.v. into C57BL/6 Rag1<sup>−/−</sup> mice, as above. Recipient mice were left for 2 wk, and reconstitution was confirmed by performing ELISA for IgM Ab from tail bleeds. After reconstitution, mice were immunized, as stated in the text.

**Statistics**

All statistics were calculated using the nonparametric Mann–Whitney U test, with P < 0.05 accepted as significant.

**Results**

Vi antigen induces a TI Ab response

The response to Vi Ag in WT mice was assessed 4 d after i.p. immunization with 10 μg purified Vi. This Ag induced a rapid induction of Vi-specific serum IgM Ab in parallel with a significant increase in CD138<sup>+</sup> splenic Ab-secreting cells (ASC) at this time (Fig. 1A). This response did not require T cells because T cell–deficient (TCRβ<sup>−/−</sup>) mice, which lack all T cells, induced a similar response (Fig. 1A). Thus, purified Vi induces a classical TI response.
To study which B cell populations respond to Vi, changes in the TCRβδ−/− subsets were assessed in TCRβδ−/− mice by immunohistology (right panel) and the density of splenic CD138+ cells determined by immunohistology (right panel). To assess whether the route of immunization affected the TI response to Vi Ag, TCRβδ−/− mice were immunized i.p. or i.v. in the tail vein or s.c. over the shoulder, and responses were assessed 7 d later (Fig. 2). These experiments show that immunization via all routes induced similar IgM and IgG anti-Vi titres, although not in absolute numbers (Fig. 3B). Furthermore, there was a marked increase in the proportion and numbers of B1b cells expressing Ki67, suggesting they had entered cell cycle in response to immunization with Vi Ag (Fig. 3B). This increase in Ki67 expression was not seen in other peritoneal B cell subsets. Therefore, peritoneal B1b cells are induced to proliferate in response to Vi Ag.

**Peritoneal B1b cells are sufficient to generate Ab against Vi Ag**

As the above data suggest that B1b cells could respond to Vi, we assessed whether peritoneal B cells were sufficient to generate protective immunity after Vi immunization. B cell chimeras were generated by transferring 10⁶ peritoneal cells from nonimmunized T cell-deficient mice into Rag1-deficient mice that lack both T and B cells. Mice were left to reconstitute for 14 d, and success of reconstitution was confirmed by tail-vein blood for the presence of total IgM by ELISA. Some chimeras were then immunized with 10 μg Vi and at day 7 Ab titres compared between nonimmunized and immunized chimeras. This showed that whereas anti-Vi serum IgM was undetectable in nonimmunized controls, anti-Vi IgM was detectable after immunization (Fig. 4A). To dissect this response further, B cell subset Rag1−/− chimeras were generated by transferring 2 × 10⁵ cell-sorted B1a or B1b or CD23+ peritoneal B cells. After 14 d, assessment of tail bleeds from the chimeras showed that they all had similar levels of serum IgM, indicating successful B cell reconstitution. In the absence of immunization, chimeras lacked anti-Vi Ab. Chimeras were then immunized with 10 μg Vi, and at day 11 anti-Vi titres were assessed. All B1b chimeras generated anti-Vi Ab in response to Vi, whereas in the other groups responses were either absent or weak (Fig. 4B). Therefore, peritoneal B1b cells are sufficient to respond to Vi Ag.

**Ab from Vi-immunized peritoneal B cell chimeras is sufficient to impair infection**

Next, we tested whether TI Ab against Vi could protect against *Salmonella* infection by challenging mice with an ArOcr-derivative of STm strain C5.507 engineered to express Vi on its surface [called STmVi² and detailed in (22, 30)]. T cell–deficient mice were immunized i.p. with 10 μg Vi for 14 d before i.p. challenge with STmVi². Four days postinfection, bacterial burdens were assessed in the spleen. This showed that median bacterial numbers were >10-fold lower in immunized mice compared with nonimmu-
FIGURE 3. Vi Ag selectively induces peritoneal B1b cells to proliferate. Nonimmunized (NI) TCRβ−/− mice or TCRβ−/− mice were immunized with 10 μg Vi Ag (Vi) i.p., and 4 d later the splenic and peritoneal B cell responses were assessed by flow cytometry. (A) The percentage and number of all B cells in splenic B cell subsets (top). MZ B cells, MZ, were identified as IgM+CD19+B220+CD21low/− and follicular B cells as IgM+CD19+B220− CD23+CD21low B cells; Foll, follicular. The bottom graphs show the numbers and proportion of these subsets that are Ki67+. (B) The number and proportion of peritoneal B1 and recirculating B cells, from the same TCRβ−/− mice as (A) above, that were in the B1a, B1b, and CD23 subset. All B1 cells were identified as IgM+CD19+CD21+CD23−B220− cells and B1a cells by coexpression of CD5. CD23 cells were IgM+CD19+CD23+CD21+. The bottom graphs show the number and proportion of peritoneal B1 and recirculating B cells that express Ki67. For gating protocols, see Supplemental Fig. 1 and Materials and Methods. Pooled data from three experiments. **p ≤ 0.01 as assessed by two-tailed Mann–Whitney U test.

Bacterial Vi expression does not prevent Ab-mediated protection to other Ags

Vi can promote immunoevasion through the inhibition of IL-8–mediated neutrophil recruitment (31, 32). To assess whether Vi expression can also promote immunoevasion through reducing the induction of Ab responses to non-Vi Ags, we examined the early IgM Ab response to LPS, porins (OmpF, C, and D), flagellin, and OmpA 7 d after primary i.p. infection with 5 × 10^2 STm or STmVi+. This shows that, at day 7 postinfection, IgM titres were similar irrespective of Vi expression (Fig. 6A). Therefore, Vi expression does not inhibit the development of the early Ab response to STm. Finally, we assessed whether Vi expression can affect Ab-mediated protection after immunization with non-Vi Ags. To do this, mice were immunized with 20 μg purified porins (OmpC, OmpD, and OmpF), which can provide protection via an Ab-dependent mechanism (19). Fourteen days later, we infected porin-immunized mice alongside nonimmunized controls with STm or STmVi+ (Fig. 6B). Three days postinfection, it was apparent that porin immunization reduced bacterial colonization independently of Vi expression, but that this reduction was more pronounced in mice infected with non-Vi-expressing STm (median 28-fold reduction for STm compared with 6-fold for STmVi+). This suggests that although Vi expression does
not inhibit the development of Ab responses to non-Vi Ags, it can provide some limited protection against non-Vi-targeting Ab.

Discussion

In this study, we show that B1b cells respond to Vi Ag, and that anti-Vi Ab derived from peritoneal cells is sufficient to impair infection with a Vi-expressing strain of STm. Finally, Vi expression was shown not to ablate Ab-mediated protection to heterologous Ags. Vaccines derived from CP have been important tools in controlling a host of infections, including typhoid (2). In recent years, numerous studies in mice have implicated a role for B1 cells, and particularly B1b cells, against CP and nonproteinaceous Ags such as the bacterial polysaccharide α-1,3 dextran and Ficoll (17, 18, 33). In addition, B1b cells are important in responses to proteins from other pathogens such as Borrelia and STm, and the response to these proteins can be T independent (19, 20). One of the reasons B1b cells have received limited attention has been the lack of a clear counterpart in humans, in part because many non-B1 human B cells express CD5. Indirect evidence that the immunological mechanisms are conserved to TI-2 Ags, such as CP, in mice and humans is the similarity in the nature of these responses (34). These include a hyporesponsiveness in infants and a limited longevity of the response and the recognition by human B cells of known murine B1b Ags (35). However, a purported B1 phenotype (CD20⁺CD27⁺CD43⁻CD70⁻) in humans has recently been described, and a B1-like cell population has been identified in the blood of patients with common variable immunodeficiency (36, 37). Future studies will assess whether further phenotyping into multiple subsets is possible. A second concern about the relationship between B1 cells and responses to TI-2 Ags has been the importance of the role of MZ B cells in these responses (9, 11). Nevertheless, evidence suggests that B1b cells in infant mice respond poorly to Ag despite being present in near normal numbers (38), thus resembling the diminished MZ response seen in infants. There is evidence that this is because in infant mice B1b cells lack sensitivity to IL-7 (38). Therefore, for differing reasons, MZ and B1b responses may both be defective in infant mice. In contrast to B1b cells, B1a cells do not appear to be recruited in response to

CP (17). If a similar situation occurs in humans, it may explain the reduced or absent responsiveness to CP in infants <5 y. Lastly, the potential importance of B cell subsets other than MZ B cells, including B1b cells, is highlighted by experiments performed in mice that are MZ cell deficient (9). These mice can mount responses to pneumococcal CP with only marginally impaired IgM responses. This suggests there is some redundancy in responses to CP, perhaps because of a selective advantage conferred by being able to respond to TI-2 Ags. Therefore, it is likely that Ab-producing cells responding to CP can derive from multiple B cell subsets.

It is significant that the genus Salmonella contains multiple B1b Ags, Vi, and the porins OmpC, D, and F (19), and suggests that naturally occurring B1b Ags are likely to be more common than previously thought. The Ags do not share any obvious structural similarities, but porins do share the property of Vi Ag to form oligomers, and thus present the immune system with numerous repeating epitopes (39). Because Ab targeted to the porin OmpD from STm and Vi Ag from ST can protect against infection, it offers the tantalizing possibility that there may be a high frequency of protective Ags that are recognized by B1b cells. Therefore, characterizing B1b responses to bacteria may be a profitable way to identify novel vaccine candidates to a range of pathogens.

We have previously shown that the early, extrafollicular Ab response to STm occurs with unusually rapid kinetics (28), suggesting that there is no limit in Ag availability to drive such an extensive response. In the current study, expression of Vi in STm did not markedly impair the development of the Ab responses to a number of STm Ags such as OmpA, porins, LPS, or FicC. This suggests that Vi Ag does not necessarily act to restrict Ag availability from B cells at any significant level. During primary infection, Ab does not control bacterial clearance (40, 41). This is consistent with the major effect of Vi Ag being to reduce innate stimulation, cell recruitment, and uptake postinfection and the production of cytokines rather than to limit B cell responses (31, 42, 43). This is consistent with a role of Vi expression in supporting the dissemination of the organism through the host.

The failure of Vi expression to impair the development of primary Ab responses led us to assess whether Ab to non-Vi Ags can still moderate infection when mice were challenged with Vi-expressing STm. We did this by assessing whether immunizing mice with porins and subsequently infecting with Vi⁺ and Vi⁻ STm affected the protection afforded. The advantage of using porins in these types of experiments is that porin molecules are integral outer membrane proteins that only have relatively short surface loops exposed from the surface of the organism (39). In this situation, anti-porin Abs were still protective, although the fold reduction in bacterial colonization was not as great as in the absence of Vi. Indeed, Abs to typhoid porins are bactericidal to Vi-expressing ST strains (44). Therefore, at best, Vi expression provides partial protection against non-Vi Ab-mediated immunogen. This is significant and encouraging, because it indicates that targeting Ags other than Vi may also offer protective immunity. Further support is evidenced from humans immunized with Ty21a, a vaccine that is protective despite lacking Vi Ag and full competency for LPS O-chain expression (5). This may be significant for the development of future vaccines against typhoid. However, the current purified Vi vaccine is likely to be superseded by more sophisticated conjugated Vi vaccines (45, 46), which may offer greater, longer lasting protection in all age ranges. Nevertheless, because in some instances the use of conjugated vaccines can be problematic (34), it may necessitate the development of other anti-typhoid vaccines that are not Vi derived. If so, then the current study suggests that other non-Vi Ags will be effective protective targets for Ab.
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Disclosures

The authors have no financial conflicts of interest.

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