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The Capsular Polysaccharide Vi from \textit{Salmonella} Typhi Is a B1b Antigen

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Vaccination with purified capsular polysaccharide Vi Ag from \textit{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 \textit{Salmonella} that express Vi Ag. Expression of Vi by \textit{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 \textit{Salmonella}, which induce Ab–mediated protection, was reduced postinfection with Vi-expressing \textit{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. \textit{The Journal of Immunology}, 2012, 189: 5527–5532.

Typhoid infections, caused by \textit{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 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-acety galacturonic acid, 60–90% O-acetylated at the C-3 position and is encoded within the \textit{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.
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 IgM<sup>+</sup> IgD<sup>+</sup> CD21<sup>+</sup> CD23<sup>+</sup> CD19<sup>−</sup> and are CD11b 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 pneumococcus, *Borrelia*, and *Salmonella enterica* serobar 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<sup>+</sup>). Lastly, we show that Vi expression by *Salmonella* 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/6 (wild type [WT]) were obtained from Harlan Laboratories (Bicester, U.K.), and TCR<sup>β</sup><sup>−/−</sup> mice from Jax. Mouse colonies were maintained in the Biomedical Services Unit, University of Birmingham, under specific pathogen-free conditions. Mice were immunized with 10 µg Typhim Vi (Sanoﬁ Pasteur MSD, Maidenhead U.K.), 20 µg porins (21), or 5 × 10<sup>5</sup> STm AroC-deﬁcient bacteria or 5 × 10<sup>5</sup> STmVi<sup>−</sup> 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 ∼30 kb of S. Typhi genomic sequence, including the SPI-1 island encoding the *via B* locus) and SGB1 (C5.507 ΔtviB::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 pSiM18 using a PCR product amplified from pKD3 using primers 5′-ggctctagcaaaacctgcaagcagcgcaatcggttatttgcattcTGTAGGCGCTGACCTTGCGC-3′ and 5′-attattaatgtagattaacagcgacgctataggactattgCATATGATTATCCTCTC-3′ (23, 24). These primers were designed to precisely delete the *aroC* gene and replace it with the *cat* gene. The *aroC* deletion was transferred by P22-mediated transduction by selecting for the *aroC-cat* using chloramphenicol in the recipient strain. Transduction into recipient strain C5.507 gave rise to strain RAK109, and transduction into a strain derived from strain SGB1 by removal of the *cat* gene from the *tviB* locus (maintaining the deletion of *tviB*) by expression of FLP recombinase from plasmid pCP20 gave rise to strain RAK112. At the end of the experiment, peritoneal exudate cells, spleens, and serum were extracted for further analysis. TLR grade STm LPS was purchased from Axxora, and Ficoll was generated, as described elsewhere (25). To generate STm OmpA, the transmembrane domain (residues 22−211) plus an N-terminal (MHHHHHHH SSC-) purification tag was synthesized and cloned into pET8c (Integrated DNA Technologies), as described (26), to create plasmid pStOmpA. The StOmpA protein was expressed from plasmid pStOmpA as inclusion bodies in Escherichia coli, purified, and refolded, as described previously (27).

For immunization experiments, a single serum was used per mouse and all sera were heat inactivated at 56 C for 0.5 h. Bacteria (2.5 × 10<sup>9</sup>/ml) and sera (1.1−200) were mixed for 0.5 h before infection and bacterial viability and lack of agglutination confirmed.

**Flow cytometry**

For flow cytometric analysis, single-cell suspensions of spleen or peritoneal exudate cells were blocked using CD16/32 Ab (eBiosciences, Hatﬁeld, U.K.) and were subsequently stained using combinations of the following: CD23 PE, B220 PE Texas Red, CD5 PE Cy5, CD11b Pacific Blue, CD21 allophycocyanin, CD19 allophycocyanin Cy7 (all BD Biosciences, Oxford, U.K.), and IgM PE Cy7 (eBiosciences). When Ki67 Ab staining was required, cell suspensions were ﬁxed and stained intracellularly using the Fix/Perm kit from BD Biosciences, according to the manufacturer’s instructions, and then stained using the FITC Mouse Anti-Human Ki-67 Set, which cross-reacts with mouse (BD Biosciences). Samples were acquired using a CyAn flow cytometer (Beckman Coulter, High Wycombe, U.K.) and analyzed using FlowJo software (Tree Star).

**Immunohistology**

The 6-µm spleen sections were stained using the following anti-mouse Abs: IgD (Abcam, Cambridge, U.K.), IgG3 and CD138 (BD Biosciences), and IgM (Abd Serotec, Kidlington, U.K.) for 1 h in Tris buffer, as described elsewhere (28). Secondary reagents were HRP-labeled donkey anti-sheep (The Binding Site, Birmingham, U.K.) and biotin-labeled rabbit anti-rat Ig (Dako, Cambridge, U.K.) for 45 min, followed by streptavidin-conjugated alkaline phosphatase (Dako) for 30 min. Colors were developed using 3,3′-diaminobenzidine tetrahydrochloride and Fast blue substates. CD138<sup>+</sup> cells were evaluated by point-counting technique.

**ELISA and 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 1/50 dilution and was titrated in 5-fold steps. 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.

ELISPOT was carried on prewetted MultiScreen HTS 96-well plates (Millipore) coated with 5 µg/ml Typhim Vi. Plates were blocked with 1% BSA PBS. A total of 10<sup>5</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) was added in 1% BSA PBS overnight at 4°C. Final detection was carried out using 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich). Spots were counted using an AID ELISPOT reader system (Autoimmun Diagnostika, Strassburg, Germany) with Eli4 software (Autoimmun Diagnostika).

**Cell transfer**

Peritoneal B cells were harvested from TCR<sup>β</sup><sup>−/−</sup> or WT mice. A total of 10<sup>6</sup> peritoneal B cells was transferred into recipient C57BL/6 Rag1<sup>−/−</sup> mice i.p. 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 × 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 (TCR<sup>β</sup><sup>−/−</sup>) mice, which lack all T cells, induced a similar response (Fig. 1A). Thus, purified Vi induces a classical TI response.
These splenic B cell subsets in cell cycle as determined by Ki67 expression using flow cytometry (Fig. 3A). This was the case independent of whether Vi Ag was given i.p. or i.v. (data not shown). In contrast, examination of the response in peritoneal B1a, B1b, and CD23+ cell subsets revealed there was a significant increase in the proportion of B1b cells after i.p. immunization, 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 Ag, 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 examining 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 ArOC-derivative of STm strain C5.507 engineered to express Vi on its surface [called STmVi⁺ 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 STmVi⁺. 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 × 105 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

FIGURE 4. Peritoneal B cells are sufficient to produce anti-Vi Ab. (A) Peritoneal B cell chimeras were generated by transferring 10^6 peritoneal cells from TCRβ−/− mice into B6 Rag−/− mice and allowing these cells to reconstitute for 14 d. After this, chimeras were either nonimmunized (NI) or immunized i.p. with 10 μg Vi Ag, and 7 d later Vi-specific serum IgM Ab titres were assessed by ELISA. (B) B cell subset chimeras were generated by transferring sorted B1a, B1b, or CD23+ B cells into Rag−/− mice and allowing these cells to reconstitute for 14 d. Mice were immunized i.p. with 10 μg Vi Ag, and 11 d later Vi-specific serum IgM Ab titres were assessed by ELISA. Graph shows mean ± 1 SD. *p ≤ 0.05, **p ≤ 0.01, two-tailed Mann–Whitney U test. ND, Not detected.

FIGURE 5. Ab derived from B1 cells is sufficient to protect against infection with Vi-expressing Salmonella. (A) TCRβ−/− mice were either immunized with 10 μg Vi Ag (Vi) i.p. injection or remained nonimmunized (NI). After 14 d, mice were challenged with 10^8 STmVi+ and splenic bacterial burdens assessed 4 d postinfection. Data from two experiments combined. (B) Left graph shows bacterial burdens in NI peritoneal B cell chimeras (generated as in Fig. 4) and peritoneal B cell chimeras immunized for 14 d with Vi Ag before challenge with 10^8 STmVi+ for 4 d. Right graph shows splenic bacterial burdens from mice that had been infected with STmVi+ opsonized with sera from naïve (NI) or from Vi Ag-challenged peritoneal B cell chimeras. Graphs show mean and SD from two independent experiments combined. **p ≤ 0.01, Mann–Whitney two-tailed U test.
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 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 Ig 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 Flic. 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.