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Oral priming with *Salmonella* Typhi vaccine strain CVD 909 followed by parenteral boost with the *S. Typhi* Vi capsular polysaccharide vaccine induces CD27⁺ IgD⁺ *S. Typhi* specific IgA and IgG B memory cells in humans

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Abstract

Attenuated live oral typhoid vaccine candidate CVD 909 constitutively expresses *Salmonella* Typhi capsular polysaccharide antigen (Vi). A randomized, double-blind, heterologous prime-boost clinical study was conducted to determine whether immunity to licensed parenteral Vi vaccine could be enhanced by priming with CVD 909. Priming with CVD 909 elicited higher and persistent, albeit not significant, anti-Vi IgG and IgA following immunization with Vi, than placebo-primed recipients. Vi-specific IgA B memory (B_M) cells were significantly increased in CVD 909-primed subjects. *S. Typhi*-specific LPS and flagella IgA B_M cells were observed in subjects immunized with CVD 909 or with the licensed Vi-negative oral typhoid vaccine Ty21a. CVD 909-induced B_M cells exhibited a classical B_M phenotype (i.e., CD3⁺CD19⁺IgD⁺CD27⁺). This is the first demonstration of classical B_M cells specific for bacterial polysaccharide or protein antigens following typhoid immunization. The persistent IgA B_M responses demonstrate the capacity of oral typhoid vaccines to prime mucosally relevant immune memory.

Keywords

S. Typhi; prime-boost immunization; Vi capsular polysaccharide; CVD 909; B memory; vaccine

1.0 Introduction

Typhoid fever caused by *Salmonella enterica* serovar Typhi (*S. Typhi*) remains a major public health concern globally, but especially in developing countries [1,2]. At present, two commercial vaccines are available in the US to prevent typhoid fever; attenuated Ty21a

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strain live oral vaccine Vivotif® (Berna Biotech), henceforth called Ty21a, and parenteral Vi polysaccharide vaccine Typhim®Vi (Sanofi Pasteur), henceforth referred to as Vi. Ty21a, formulated in enteric coated capsules is administered orally in 4 consecutive doses, every other day, while Vi is given parenterally as a single dose. Although the cumulative efficacy at 3 years for the polysaccharide Vi and Ty21a vaccines were comparable: 55% (95% confidence interval [CI] 30%, 71%) [3] and 67% (95%CI 47%, 79%)[4], respectively, the protection elicited by Ty21a persisted for up to 7 years (cumulative efficacy 62%, 95%CI 48%, 73%)[4]. The Vi polysaccharide vaccine induces a robust serum antibody response, but this response wanes over time and cannot be boosted with subsequent immunizations [5], affording relatively short term protection [6]. Similar to other purified unconjugated polysaccharide vaccines, it is poorly immunogenic in young children [7] and is not expected to induce mucosal or T cell-mediated immune responses (CMI). In contrast, Ty21a generates a wide array of immune responses that include mucosal and systemic antibodies and CMI against *S. Typhi* lipopolysaccharide (LPS), flagella and other relevant bacterial antigens [8–11]. Notably Ty21a, does not express the Vi capsular polysaccharide, a well established virulence factor, and therefore does not induce antibodies to Vi, known to mediate protection against infection. Additionally, the requirement for multiple doses limits its use as a public health tool for large scale immunization.

A new generation of typhoid live vector vaccines has emerged in recent years and several of these candidates have been successfully tested in clinical trials. These vaccine strains include CVD 908-*htrA*, Ty800 and MO1ZH09 [12,13]. CVD 908-*htrA* was shown to induce vigorous antibody and CMI responses in systemic and mucosal tissues, following a single oral immunization. These responses proved similar or even higher in magnitude than those elicited by multiple doses of Ty21a [14–16]. Similar to Ty21a, none of these vaccines reliably stimulates serum Vi antibodies that might contribute to protection [16]. In an attempt to improve the efficacy of the live typhoid vaccine approach, CVD 908-*htrA* was engineered to constitutively express *S. Typhi* Vi, resulting in a new vaccine strain CVD 909 [17]. The expectation was that oral immunization with CVD 909 would elicit serum Vi antibodies in addition to other various responses induced by the parent strain, CVD 908-*htrA*. CVD 909 was found to be safe and immunogenic in a Phase 1 clinical trial [18]. Although it failed to raise significant levels of serum Vi antibodies, the majority of the vaccine recipients developed mucosally-primed Vi-specific IgA antibody secreting cells (ASC) detected among peripheral blood mononuclear cells (PBMC), one week after oral immunization [18]. These volunteers also developed robust CMI, which included memory T cell responses similar to those induced by the parent strain CVD 908-*htrA* [18,19].

Immunological memory is a hallmark of vaccine-induced long-term protection [20]. It is widely accepted that classical B_M cells are generated exclusively in response to T-dependent (T-D) antigens. However, this concept has been challenged in a number of recent publications that report the detection of classical B_M responses to T-independent (T-I) antigens both in mice and humans [21–24]. Despite the role that antibody responses to both T-D and T-I antigens are likely to play in protection from *S. Typhi* disease, no information is available concerning the induction and persistence of B_M cells specific for *Salmonella* antigens in response to the live oral or the parenteral Vi typhoid vaccines. Thus, in this study we characterized the antibody and B_M cell responses in a clinical trial in which oral priming with *S. Typhi* vaccine CVD 909 or placebo preceded a single immunization with the typhoid Vi polysaccharide vaccine. Our goals were: 1) to ascertain whether priming with a live *S. Typhi* strain constitutively expressing Vi could influence the humoral responses to parenterally administered Vi, and 2) to study the generation and persistence of IgG and IgA B_M responses against both T-I (Vi, LPS) and T-D (flagella) antigens from *S. Typhi*.

2.0 Materials and Methods

2.1 Subjects and vaccination protocols

Twenty healthy adult volunteers (9 male, 11 female, 18 to 45 years of age) were enrolled in the CVD 909 study and 10 healthy adult volunteers (3 male, 7 women, 28 to 53 years) participated in the Ty21a study. Subjects were recruited from the Baltimore-Washington, DC area and University of Maryland Baltimore community. Their medical history was reviewed and physical and laboratory examinations were performed to ensure that they were in good health. Any volunteer who had a past history of typhoid fever or immunization against typhoid fever was excluded from participating in this study. Prior to enrollment, the purpose of the study was explained to the subjects and they passed a written test containing questions regarding the rationale for the study, risks and procedures. Informed consent was obtained from all participants and the study was approved by the UMD Institutional Review Board.

2.2 Immunization and sample collection protocol

2.2.1 CVD 909 study—Subjects were randomized to receive oral priming with either 5×10^9 CFU of vaccine CVD 909 administered with sodium bicarbonate buffer (n=11) or placebo (bicarbonate buffer only, n=9), as previously described [19]. Three weeks later, all volunteers were administered the Vi polysaccharide vaccine (Typhim®Vi; Sanofi Pasteur) containing 25 µg of Vi in 0.5 ml by the intramuscular route. Blood was collected before immunization (day 0) and on days 10, 14±2, 21±2, 28±2, 35±2, 42±2 and 84±7, and week 29±2 and 55±4 post vaccination; serum and peripheral blood mononuclear cells (PBMC) were obtained and appropriately frozen (sera) or cryopreserved (PBMC) until used as described previously [19]. Details for this study can be found at ClinicalTrials.gov Identifier: NCT00326443 <http://clinicaltrials.gov/ct2/show/NCT00326443>

2.2.2 Ty21a study—Volunteers were vaccinated following the routine U.S. immunization schedule for the Ty21a typhoid vaccine, i.e., four spaced doses of 2×10^9 to 6×10^9 CFU of Ty21a at an interval of 48 h between doses. Blood was collected before immunization (day 0) and on day 70, post vaccination to obtain PBMC samples.

The experimentation guidelines of the US Department of Health and Human Services and those of the University of Maryland, Baltimore, were followed in the conduct of the present clinical research.

2.3 Antibody assays

Serum IgG, IgA and IgM antibody titers to *S. Typhi* LPS (Difco), and H:d flagella antigen [25,26] were measured by ELISA as previously described [18]. Antibodies to Vi were also measured as previously described [18], with the following modifications: wells were pre-treated with 100 µl of poly-L-lysine hydrobromide (3.0 µg/ml) for 30 minutes at room temperature and then coated with 2.0 µg/ml of Vi polysaccharide (from *C. freundii*, kindly provided by Dr. John Robbins, NIH) for 3 h 37°C. End-point titers were calculated through linear regression parameters as the inverse of the serum dilution that produces an OD of 0.2 above the blank (Elisa Units per ml).

2.4 B cell memory (B_M) ELISpot

The method used was adapted from that originally reported by Crotty et al. [27]. Briefly, frozen PBMCs were thawed and expanded with B cell-expansion media consisting of 5 µM 2β-ME (Biorad), 1:100,000 pokeweed mitogen (kindly provided by Dr. S. Crotty), 6 µg/mL CpG-2006 (Qiagen/Operon, Huntsville, AL), 1:10,000 *Staphylococcus aureus* Cowan (SAC,

Sigma–Aldrich, St. Louis, MO), and 50 ng/ml human IL-15 (PeproTech Inc. Rocky Hill, NJ) in RPMI-1640 (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (CellGro, Manassas, VA), 50 µg/ml gentamicin (HyClone, Logan, UT), 2 mM L-glutamine, 2.5 mM sodium pyruvate, 10 mM HEPES and 10 % heat inactivated fetal bovine serum (BioWhittaker, Walkersville, MD) (complete RPMI). Cells were expanded for 5 days (1×10^6 cells/well in 6-well plates) and fed with complete RPMI after 3 to 4 days of incubation. The B_M ELISpot was performed as follows: Nitrocellulose plates (MAHAN, Millipore, Billerica, MA) plates were coated with Vi (5 µg/ml), LPS (10 µg/mL), flagella (5 µg/ml), total goat anti-human IgG (Jackson Immuno Research lab; 5 µg/mL) or total goat anti-human IgA (Jackson Immuno Research lab; 5 µg/mL) diluted in PBS. Plates were incubated overnight at 4 °C and blocked with 10% FBS in RPMI-1640 for 2 h at 37°C. For *S. Typhi* specific antigens, expanded cells were added at 1.5×10^5 cells/well in triplicates and incubated for 6 h at 37 °C, 5% CO₂. For total IgG and IgA, expanded cells were added at 5×10^3 cells/well in triplicate and serially diluted 2-fold for a total of 7 times. Plates were then washed with PBS-T followed by PBS and incubated with mouse anti-human biotin conjugated Pan-IgG antibody (Hybridoma Reagent Laboratory, Baltimore, MD) or anti-human Pan-IgA (Jackson Immuno Research Labs.) overnight at 4°C followed by HRP-conjugated Avidin D (Vector Laboratories, Burlingame, CA) for 1h at room temperature. Spots were detected by adding 100 µl/well of 3-Amino-9 etheylcarbazole C (AEC substrate; Calbiochem, La Jolla, CA, USA) at room temperature in the dark. The reaction was stopped after 8–10 minutes by washing with distilled water. Spots were counted in an automated ELISpot reader (Immunospot®, Cellular Technology Ltd., Cleveland, OH, USA) and analyzed using the Immunospot® version 5.0 software. Results are expressed as percent of antigen-specific spot forming cells (SFC) per total IgA or IgG or per 10^6 expanded cells.

Adequate expansion of B_M cells, assessed by the frequency of total IgG or IgA detected by ELISpot, is critical to the sensitivity and consistency of this method. Thus, specimens which did not reach a minimum cut-off level following expansion (arbitrarily defined as the 10th percentile of the levels reached by all volunteers at any time point: i.e., more than 13,000/ 10^6 total IgG or more than 5,000/ 10^6 total IgA) were excluded from further analysis. Based on this criterion, one subject whose expanded cells had a meager response at all time points tested, was excluded from analysis. The level of detection (LOD) for the B_M assay was defined as 2 antigen-specific SFC per well, which corresponds to 13 SFC per 10^6 mononuclear cells when seeding 1.5×10^5 cells per well. Positive responses were defined as those that exceeded a threshold level of 0.05%; this cut off was defined by taking into account the LOD (13 antigen-specific SFC/ 10^6 PBMC) and the median total IgG SFC (26,000/ 10^6 PBMC) obtained during assay validation with study specimens. A cut-off for post vaccination responders was defined as any subject that exhibited an increase in antigen specific B_M per total IgG or IgA that was equal or greater than 0.05% above baseline (day 0).

2.5 Flow cytometric sorting of B_M cell subsets

Cryopreserved PBMC were thawed, rested overnight in complete media at 37°C, 5% CO₂, washed and stained with monoclonal antibodies (mAbs) to identify and sort naïve B cells and B_M subpopulations following standard procedures. Cells were surface stained with mAbs to IgD-FITC (clone IA6-2, BD Pharmingen), CD27-PE (clone M-T271, BD Pharmingen), CD 19-ECD (clone J4.119, Coulter), and CD3-PE-Cy7 (clone UCHT1, Coulter) and sorted using a 4-way MoFlow flow cytometer/cell sorter system (Coulter, FL, USA). The gating strategy is shown in Figure 4; four populations were sorted as follows: 1. B_M (CD3⁺ CD19⁺ IgD⁺ CD27⁺); 2. Naïve B (CD3⁺ CD19⁺ IgD⁺); 3. CD27⁺ B_M (CD3⁺ CD19⁺ IgD⁺ CD27⁺) and 4. non-B (CD19⁺) cells. The latter cells were used as feeders in the experiments.

2.6 Measurement of Antibodies in Lymphocyte Supernatant (ALS) of B_M cultures

Unsorted PBMC or sorted B cell subsets were cultured in expansion media at $1.5 \times 10^6/\text{ml}$ or at $5 \times 10^4/\text{ml}$, respectively. Preliminary experiments showed poor recovery and viability of sorted B cell subsets following expansion in the absence of feeders (data not shown). Thus, in subsequent experiments, sorted cells were co-cultured during expansion with non-B feeder cells (CD19⁻) at a B cell:feeder ratio of 1:5. Cultures were maintained for 6 days and supernatants collected and stored at -70°C until evaluated. Antibody titers were measured by ELISA as described above.

2.7 Statistical analysis

Statistical analyses were performed using Prism 5.0 (GraphPad) software. One tail Mann-Whitney U or Chi² tests were used to evaluate the statistical differences. Significance of correlation coefficients (non-parametric) was calculated using Spearman tests. Seroconversion was defined as ≥ 4 -fold rise in antibody titers compared to pre-vaccination levels at any time point post-vaccination. Fold increases were calculated as post-vaccination titers at each time point vs. pre-vaccination titers. For all tests, p-values <0.05 were considered significant.

3.0 Results

3.1 CVD 909 heterologous prime-boost study

Subjects were randomly allocated to receive a single oral dose of CVD 909 or placebo on day 0, and all received one dose of the parenteral Vi vaccine on day 21. We measured the serum antibody titers to *S. Typhi* Vi polysaccharide, LPS and flagella at different time points of the study to evaluate the ability of CVD 909 to elicit immune responses as well as whether it can boost the responses of subsequent parenteral Vi immunization. The kinetics of serum IgM, IgG and IgA responses to these antigens are shown in Figure 1.

3.1.1. anti-Vi polysaccharide—No increases in geometric mean titers (GMT) of Vi IgM, IgG or IgA serum antibodies were observed during the first 21 days following priming with CVD 909 or in subjects who received placebo (Fig 1A-C)

Following Vi vaccination on day 21, seroconversion was observed for anti-Vi IgM, IgG and IgA in both CVD 909 and placebo recipients (Fig 1A-C). For IgM, seroconversion was observed in 27% (3/11) of subjects in the CVD 909-primed group and in 44.4% (4/9) in subjects “primed” with placebo. IgM responses were detected as early as one week after parenteral vaccination (day 28), peaking on day 35 and remaining elevated for up to 3 months (day 84) before returning to baseline levels within a year. No significant differences in IgM anti-Vi GMT (Fig. 1A), fold increases or seroconversion rates were observed between the groups at any time point.

The seroconversion rates for IgG anti-Vi following parenteral Vi vaccination were 82% (9/11) in the CVD 909-primed subjects and 66% (6/9) subjects of the placebo-primed groups, respectively. This difference in seroconversion rates, however, was not statistically significant. Significant rises in IgG anti-Vi antibody titers from their respective base line levels were detected in both groups by day 35 (i.e., 14 days after parenteral Vi). Levels peaked at day 84 before declining, but remaining elevated up to day 365 in both groups (Fig. 1B). The seroconversion rates for anti-Vi IgA was 46% in the CVD 909-primed and 56% in the placebo-primed subjects. The kinetics of IgA anti-Vi showed a rapid increase (day 28) which peaked at day 35, followed by a gradual decline, remaining significantly elevated for up to 1 year (Fig. 1C). Of note, anti-Vi IgA responses were faster than those of IgG (Fig. 1B), reaching peak levels 2 months earlier (Fig. 1C)

Although there was a trend towards higher serum anti-Vi IgG and IgA responses in the CVD 909-primed group compared to the placebo-primed subjects following parenteral Vi, no significant differences could be demonstrated between the two groups, in GMT titers or average fold-increase responses at any time point (Figs. 1A-1C).

3.1.2 anti-LPS—Priming with CVD 909 resulted in a rapid increase in anti-LPS IgM, IgG and IgA serum antibody titers that significantly ($p<0.001$) surpassed those in the placebo-primed subjects during the first 3 weeks following immunization, i.e., before parenteral vaccination with Vi on day 21 (Fig 1D-F). The seroconversion rates for anti-LPS IgM were 36.4% (4/11) and 9% (1/9) subjects primed with CVD 909 or placebo, respectively. In the CVD 909-primed group all four subjects who seroconverted did so by day 10, while the single seroconverter in the placebo-primed group became positive at day 28, one week after receiving parenteral Vi. Although in the placebo-primed group there were no changes in anti-LPS IgM levels from day 0 to day 21, following administration of the Vi vaccine, placebo recipients exhibited a modest rise (~2 fold) from their respective baseline level ($p<0.09$, days 28–42) (Fig. 1D)

CVD 909-primed subjects also showed a rapid and significantly higher anti-LPS IgG response with a seroconversion rate of 63.6% (7/11) within the first 21 days, while no seroconversions were noted in the placebo-primed recipients. The GMT of anti-LPS IgG in CVD 909 group was significantly higher by day 10, peaking at day 14 and remaining elevated until day 42 before gradually returning to baseline by day 212 (Fig 1. E). Similar to anti-LPS IgM, a very modest increase in anti-LPS IgG was also seen in placebo recipients at day 28 ($p=0.056$), returning to baseline by day 212 (Fig 1. E).

The CVD 909-primed subjects also had significantly elevated, although transient, anti-LPS IgA responses. The kinetics showed a sharp increase on day 10, followed by a rapid decline, returning to baseline by day 35 (Fig 1F). Before the administration of parenteral vaccine, the seroconversion rate for anti-LPS IgA was 63.6 % (7/11) in the CVD 909-primed subjects, while none seroconverted in the in placebo-primed group. However, one week following parenteral Vi (day 28), 33% (3/9) of placebo-primed subjects seroconverted along with modest increases in GMT above the baseline (Fig. 1F). Results were similar when these antibody responses were examined as fold-increase instead of GMT (data not shown).

3.1.3 anti-Flagella—Priming with CVD 909 also resulted in a rapid, albeit low magnitude, increase in anti-flagella IgM, IgG and IgA serum antibody titers during the first 3 weeks following immunization, i.e., before parenteral vaccination with Vi on day 21 (Fig 1G-I). Increases were modest with lower percentages of seroconversion rates (only one subject seroconverted in the CVD 909-primed group, while none did so in the placebo-primed control). Despite the low seroconversion rate, significant ($p=0.04$) increases were observed in anti-flagella IgM GMT levels in subjects who were primed with oral CVD 909 which surpassed those of the placebo-primed recipients on days 21–35, reaching peak levels on day 21. Titers remained elevated until day 35, returning to pre-vaccination levels on day 212 (Fig 1G).

Similarly, only 18% (2/11) of CVD 909-primed subjects but none of the placebo-primed subjects seroconverted for anti-flagella IgG. Nevertheless, significant increases ($p<0.05$) were observed in anti-flagella IgG fold-increases in CVD 909-primed subjects when compared with the placebo-primed group at days 21 to 84. The rise of IgG titers peaked at day 10 and declined thereafter (Fig 1H).

Anti-flagella IgA responses were also induced following immunization with CVD 909. A 27.3% (3/11) seroconversion rate was observed in the CVD 909-primed subjects while none

was observed in the placebo-primed group. As described above with anti-flagella IgG responses, the CVD 909-primed group showed 4 times significantly ($p<0.03$) higher levels of anti-flagella IgA responses than those observed in the placebo-primed group on days 10 to 84. As with IgG and IgM, the anti-flagella IgA levels raised significantly from their baseline level in the CVD 909-primed subjects, peaking on day 10 and gradually returning to pre-vaccination levels by day 212 (Fig 1I).

3.2 IgG and IgA B_M memory (B_M) cells specific for *S. Typhi* Vi and flagella antigens after oral CVD 909 prime - parenteral Vi boost immunizations

Given the importance of antibodies in protection against typhoid infection, particularly antibodies to Vi, we investigated the induction of IgG and IgA B_M cells in CVD 909-primed and placebo-primed subjects before and after parenteral immunization with Vi polysaccharide. The frequency of antigen-specific B_M cells was measured in circulating PBMC following an in-vitro polyclonal expansion, as described by Crotty et al. [27,28] with some modifications. This method enables the measurement of antigen specific B_M in PBMC (see section 2.4 for details).

A summary of IgG and IgA B_M responses to *S. Typhi* Vi and flagella are shown in Figure 2. No Vi IgG B_M cells were detected in CVD 909-primed or placebo-primed subjects at any of the time points examined (Fig. 2A,E). However, IgG B_M responses to flagella were observed predominantly in CVD 909-primed individuals (Fig 2B). The mean percentage increases in the frequency of flagella specific IgG B_M after vaccination were significantly higher in CVD 909-primed subjects compared with those who received placebo at days 212 ($p=0.03$) and 365 ($p=0.012$), respectively. The proportion of IgG B_M responders to flagella was also higher in the CVD 909 recipients than those who received placebo; 45.5% (5/11) and 12.5% (1/8), respectively ($p=0.06$).

In contrast to what was observed for IgG B_M cells, strong and consistent IgA anti-Vi IgA B_M responses were observed in CVD 909-primed subjects. The average percentage increase in anti-Vi IgA B_M levels at day 84 in CVD 909-primed subjects was significantly higher ($p=0.05$) than that of placebo recipients (Fig. 2C). Interestingly, one subject in the placebo group had a positive response on day 84, with a very high post-vaccination B_M percent increase (0.636%), while the remaining 7 volunteers showed no responses ($<0.045\%$). The percentage of B_M responders at day 84 in the CVD 909 group (55%; 6/11) was significantly higher ($p=0.02$) than those in the placebo group (12.5%; 1/8). Furthermore, the proportion of individuals with a positive anti-Vi IgA B_M response at any time post-vaccination in the CVD 909-primed subjects (64%; 7/11) significantly exceeded ($p=0.048$) that in the placebo-primed control individuals (25%; 2 out of 8) (Fig. 2E). Significant increases in flagella specific IgA B_M responses were also observed on day 212 in CVD 909 recipients as compared with those that received placebo ($p=0.012$), respectively. At this same time point, significantly higher proportions ($p=0.01$) of subjects in the CVD 909-primed subjects had positive post-vaccination responses compared to the placebo group (45%; 5 out of 11 vs 0%; 0 out of 8, respectively) (Fig. 2D). The same trend towards increases in anti-flagella IgA B_M response frequency, albeit not reaching statistical significance, was observed on days 84 ($p=0.12$) and 365 ($p=0.09$). Overall, IgA B_M responses to flagella were demonstrated in a total of 8 out of 11 (73%) CVD 909 recipients and 4 out of 8 (50%) placebo controls ($p=0.14$). Representative B_M Elispot data are shown in Fig. 2F.

3.3 IgG and IgA B_M cells specific for *S. Typhi* antigens in Ty21a vaccinees

We then assessed whether the B_M responses to *S. Typhi* antigens were also elicited in subjects who were immunized with the licensed live oral typhoid vaccine Ty21a, which confers protection without inducing a Vi response. To this end, anti-LPS and flagella-

specific B_M cells were measured in volunteers who received the recommended four doses of Ty21a in PBMC obtained before and 70 days after vaccination (Fig. 3).

We found a significant increase in mean anti-LPS IgG B_M responses reported as percent of total IgG SFC at day 70 (0.051 ± 0.032) compared to the day 0 baseline levels (0.009 ± 0.005 , $p=0.05$). However, the individual responses were of low magnitude and only one subject exceeded the detection threshold (0.05%) (Fig. 3A). In contrast, strong anti-LPS IgA B_M responses were observed at day 70 (0.10 ± 0.023), which significantly exceeded pre-vaccination levels (0.055 ± 0.023 , $p=0.033$). Half of the vaccinees (5/10) had post-vaccination increases in IgA B_M frequencies at day 70 (Fig 3C). Of note, 2 volunteers had very strong responses before immunization without further increases after receiving Ty21a (Fig. 3C).

The mean percentage of flagella-specific IgG B_M at day 70 (0.042 ± 0.019) was higher, albeit not statistically significant, than that observed at day 0 (0.019 ± 0.0085 , $p=0.20$). Post-vaccination increases were observed in 30% (3 out of 10) of the subjects (Fig 3B). IgA B_M cells specific for flagella were also detected; higher mean frequencies were detected on day 70 (0.092 ± 0.048) compared to baseline (0.019 ± 0.007 , $p=0.05$). Post vaccination increases were observed in 40% (4/10) of the subjects (Fig. 3D).

3.4 Correlation between the B_M frequency and antibody levels in culture supernatants during expansion (ALS)

To evaluate whether the induction of B_M cells is also accompanied by the release of specific antibodies in the supernatants during B_M expansion, culture supernatants were collected and the accumulated total and antigen specific antibodies secreted during the 5 to 6-day expansion period measured using an adapted Antibody in Lymphocyte Supernatant (ALS) assay. The levels of antigen-specific antibodies produced by the expanded B_M cells from Ty21a recipients were compared with the frequency of antigen-specific B_M cells (Fig. 4). A close correlation was observed between the two measurements. ALS results were strongly correlated with the frequency of total IgG and IgA SFC measured in the B_M ELISpot assays (Fig. 4A, B). Similarly, significant correlations were observed between LPS (Fig. 4C,D) and flagella (Fig. 4E,F) specific ALS and the corresponding frequencies of LPS and flagella B_M (expressed as percent of total IgG or IgA SFC). These data further confirm that ALS can be used as an alternative measurement of antigen specific B_M by ELISpot [29], particularly when limited number of cells are available, a situation frequently encountered with specimens obtained from clinical vaccine trials or when using sorted cell subsets. Given the strong correlations observed in these studies between ALS and B_M by ELISpot assays, subsequent investigations directed to identify the effector B cell subsets using sorted populations were performed using the ALS methodology.

3.5 Characterization of the antibody secreting cell subsets in B_M expanded populations

We next characterized the phenotype of the B_M cell subsets induced by oral immunization with CVD 909, based on cell surface marker expression. Previous studies reported that B_M cells specific for protein antigens express CD27 but lack IgD [30,31]. However, no information is available on the phenotype of B_M cells specific for polysaccharide antigens. To this end, B cells (CD19⁺ CD3⁻) present in PBMC preparations were sorted simultaneously into three different subpopulations based on the surface expression of IgD and/or CD27; the gating strategy is shown in Fig. 5. The purity of the sorted cells was typically 85–95%. CD19 negative PBMC were simultaneously sorted and used as feeder cells during the expansion of purified B cells. Antibodies produced by these cells were measured by ELISA in culture supernatants. Results indicated that total and anti-*S. Typhi* LPS, -Vi and -flagella specific antibodies were produced almost exclusively by IgD⁺CD27⁺

cells (Fig. 6). Of note, small amounts of total IgA were also detected in supernatants of IgD⁺CD27⁻ cells (Fig. 6D). However, this might be due to small numbers of IgD⁺CD27⁺ cells that remained after sorting. These results indicate that total, as well as protein-specific and polysaccharide-specific IgG and IgA antibodies, are predominantly secreted by classical B_M cells (CD3⁺CD19⁺IgD⁺CD27⁺).

4.0 Discussion

We have previously shown in volunteers that oral immunization with CVD 909, an attenuated typhoid vaccine strain that constitutively expresses the Vi polysaccharide, induced not only robust CMI to *S. Typhi* antigens, but also Vi-specific IgA ASC [18,19,32]. In this study we investigated the capacity of CVD 909 to elicit antibodies and antigen-specific B_M cells as immunological responses usually associated with long-term vaccine-induced protection. Specifically, we examined whether oral immunization with CVD 909 could prime the human immune system so that anamnestic responses could be elicited following a subsequent encounter with Vi (i.e., following administration of the typhoid Vi vaccine). Antibody levels and frequency of B_M responses to *S. Typhi* antigens were measured up to one year in human subjects orally primed with CVD 909 (or placebo) and boosted parenterally with the licensed Vi polysaccharide vaccine.

No rises in serum Vi antibodies were detected during the 21-day period that followed the CVD 909 priming, prior to the parenteral Vi boost. This observation was consistent with data reported previously [18]. Likewise, the overall seroconversion rate for anti-Vi IgG after parenteral Vi in the present study (75%) is in agreement with those previously reported (~74–98%) in vaccinated subjects from both non-endemic and endemic areas [5]. Despite the fact that serum Vi antibodies were undetectable following CVD 909 priming, there was a trend at several time points after vaccination towards increased IgG and IgA Vi responses in CVD 909-primed subjects following administration of the Vi vaccine. The relatively small number of volunteers who participated in this study may have limited our capacity to detect significant differences when comparing responses between groups. Interestingly, IgA antibodies to Vi had a faster rise compared with Vi-specific IgG (peak response was attained 7 weeks earlier). While the reason for this observation is unclear, it is reasonable to speculate that this accelerated IgA response is the result of existing Vi-specific IgA-secreting plasma cells, presumably primed by the live vector in the gut mucosa. We observed that IgG and IgA anti-Vi antibodies remained elevated above baseline for up to a year after immunization. These data, which are in agreement with those previously reported [3], is suggestive of the presence of long-lived plasma and/or B_M cells. To assess this possibility, we studied whether CVD 909 or Vi immunization resulted in the presence of anti-Vi specific B_M cells.

Antibodies to LPS and other *S. Typhi* antigens are likewise important elements contributing to the host's immune defense against this pathogen [11,13,18,33–36], although they may not represent the ultimate operative mechanism of protection [11,37] in *S. Typhi* infection. Thus, persistence of antibody responses is an important consideration in evaluating the potential effectiveness of novel *S. Typhi* vaccines. The original CVD 909 studies described IgG and IgA responses against *S. Typhi* LPS and flagella, but the kinetics of antibody production and persistence were not examined [18]. One striking observation in our study was the difference in the kinetics of appearance and the persistence of antibodies induced against the individual antigens. LPS antibodies of all three Ig classes increased sharply after vaccination (reaching peak responses within 2 weeks) but rapidly declined, with anti-LPS IgG remaining elevated the longest (day 212). In contrast, antibodies to Vi also reached peak levels by day 14–21 after parenteral immunization, but remained elevated longer (days 84–212). Interestingly, placebo recipients had positive LPS responses after receiving the Vi

vaccine, which could be attributed to the presence of low levels of LPS in the commercial Vi preparation [35]. This modest rise in antibodies most likely also occurred in the CVD 909 recipients following parenteral Vi vaccination, but it was probably masked by the overwhelming LPS responses induced by the live vaccine. IgM, IgG and IgA responses to flagella also developed in CVD 909 vaccinees, albeit at lower levels, lasting for 3 to 7 months. We have observed similar kinetics of LPS responses in subjects immunized with the Ty21a typhoid vaccine (unpublished data).

Vaccine-induced long term protection requires additional immunological effectors such as memory B and T cells that are capable of recalling an immediate protective response. Typically, protein antigens generate memory B cells (B_M) in germinal centers (GC) with cognate help from the helper T cells (T-D) [38,39]. The antibodies produced exhibit high levels of somatic mutation in Ig loci and isotype switching. The B cell responses to T-I antigens, which can be divided into type I and type II antigens, originate differently [40]. Type I antigens such as LPS and poly-IC stimulate B cells and induce polyclonal B cell activation via Toll like receptor (TLR) interactions, whereas Type II antigens (e.g., polysaccharides such as Vi) activate B cells through cross linking of the B-cell receptor and these cells differentiate into antibody secreting short-lived plasma cells [41]. As with other T-I polysaccharide-based vaccines, the typhoid Vi capsular polysaccharide induces a relatively short-lived antibody response in humans and fails to elicit recall responses [42]. This has also been the experience with several other polysaccharide vaccines, supporting the long-held notion that classical B_M cells are generated exclusively in response to T-D antigens. This concept, however, has been challenged in a number of recent publications reporting classical B_M responses to T-I antigens both in mice and humans. For example, CpG-adjuvanted *S. pneumoniae* capsular polysaccharides (PS) were shown to generate a PS-specific B_M pool of long-lived plasma cells that conferred full protection against *S. pneumoniae* infection [43]. There is also evidence that polysaccharide antigens can elicit responses by phenotypically distinct B_M cell subsets in mice [44]. We have reported that a single oral immunization with attenuated *S. flexneri* 2a vaccine strains CVD 1204 and CVD 1208 generated LPS-specific IgG B_M cells that can be detected in circulation, 28 days after vaccination [23]. Similarly, a recent paper described the presence of IgG and IgA B_M cells specific for LPS in subjects naturally infected with *V. cholerae* 01[24]. In contrast, to our knowledge, the generation of B_M specific for Vi following parenteral immunization with the Vi typhoid vaccine, or B_M responses to Vi, LPS or flagella following oral immunization by attenuated *S. Typhi* oral vaccines (e.g., CVD 909 or Ty21a) have not been reported. In the present study we did not observe the induction of detectable IgG B_M against the Vi antigen either following parenteral Vi or after oral CVD 909 immunization. In contrast, strong IgA B_M responses to Vi were observed predominantly in the CVD 909-primed group as early as day 84 and persisted in some volunteers up to 1 year after immunization. These results suggest that immunization with CVD 909 was indeed capable of mucosally priming the human immune system to respond with robust and sustained Vi B_M responses to a subsequent exposure to parenterally administered Vi. The observations that oral immunization with the Ty21a typhoid vaccine, unlike systemic immunization with other antigens, elicits IgA ASC cells that preferentially express gut homing receptors [45], the major role that the GALT (Gut Associated Lymphoid Tissue) has in the production of secretory IgA [46] and that mucosal immune responses play a key role in protection from *S. Typhi*, as well as other enteric pathogens [47] support this contention. The trend of higher anti-Vi serum antibody levels in primed individuals in our study, also supports this possibility. However, we can not exclude the possibility that the appearance of anti-Vi B_M responses in the CVD 909-primed group could have been the result, at least in part, of a delayed kinetic of appearance which was independent of the parenteral administration of Vi.

B_M responses to LPS were also observed in CVD 909-primed vaccinees and placebo-primed controls, but were modest compared to those against Vi (data not shown). Of note, IgA LPS-specific B_M cells predominated over IgG LPS-specific B_M responses. To study in further detail the induction of B_M to LPS following oral immunization with attenuated Typhi vaccines, we measured anti-LPS B_M responses in volunteers immunized with Ty21a. While most of the vaccinees developed IgA B_M responses to LPS by day 70 after vaccination, only one volunteer exhibited an IgG B_M response to LPS. Immunization with CVD 909 and Ty21a also induced significant flagella IgG and IgA B_M responses. Interestingly, a few volunteers exhibited elevated frequencies of IgG and IgA B_M cells prior to vaccination, which likely resulted from natural exposure to organisms that express flagella with a high degree of homology to that expressed by *S. Typhi*. For example, *S. Typhi* expresses the phase 1 H (flagellar) d antigen (H(d)) [48] which is also expressed by some other *Salmonella enterica* serovars that cause enteric infections in humans (e.g., *S. Stanley*, *S. Livingstone*, *S. Muenchen*) [49,50]. Moreover, considerable homology (50–80%) is present between the amino acid sequences of flagellin from *S. Typhi* and other *Salmonella enterica* organisms which express different Phase 1 H antigens (e.g., *S. Typhimurium*, H(i); *S. Enteritidis*, H(g,m)) which are major causative agents of gastroenteritis. Notably, flagella-specific B_M cells were also elicited in a few volunteers that received placebo.

An important finding was the strong association between the frequency of antigen-specific B_M cells and the level of antibody produced by these cells. In our hands, the ALS was found to be a useful surrogate for the measurement of antigen-specific B_M cells by Elispot, as reported by others [29,51]. This is particularly advantageous in situations where the availability of specimens and resources is a limiting factor to perform cell-based assays.

It is generally accepted that B_M cells express high levels of CD27 since this B cell subset contains the majority of hypermutated Ig heavy and light chain cells [30,52]. An original contribution of this study is the demonstration of B_M cells specific for both T-I (i.e., Vi and LPS) and T-D (i.e., flagella) *S. Typhi* antigens following oral immunization with live typhoid vaccines. Thus, it was of great interest to characterize the phenotype of these B_M cells. To this end, we sorted the B cells (CD19⁺) obtained from CVD 909-immunized volunteers based on their expression of IgD and CD27. Our results show that both IgG and IgA B_M responses to *S. Typhi* flagella were mediated by CD19⁺ IgD⁻ CD27⁺ cells. This observation is in agreement with previous studies describing B_M cells specific for bacterial proteins. Additionally, we provided the first evidence that IgA B_M responses to T-I LPS and Vi polysaccharides are also mediated by CD19⁺ IgD⁻ CD27⁺ “classical” B_M cells. Studies are ongoing to further characterize these B_M cell subsets in terms of their homing potential and the expression of activation molecules.

It is important to emphasize that protection from immunization with the Ty21a vaccine lasts for up to 7 years [4], while the anti-*S. Typhi* antibody levels persist for just a few months adds further support to the notion that serum antibodies to *S. Typhi* antigens may have some role in protection against *S. Typhi*, but are unlikely to represent the dominant long-term protective immune responses induced by immunization with Ty21a [11]. In contrast, the presence of robust CMI elicited by immunization with Ty21a and new attenuated *S. Typhi* vaccine candidates which has been shown to sometimes persist for years [8,9] lead to the hypothesis that CMI might play a key role in protection from *S. Typhi* infection [11,37]. The data presented in this manuscript raises the interesting possibility that in addition to the “classical” effector CMI responses likely to contribute to protection (e.g., production of IFN- γ and other cytokines, CTL responses, etc), CMI might contribute to protection by providing help for the expansion and persistence of a sizable B_M pool.

In sum, this is the first demonstration that a single oral vaccination with an attenuated *S. Typhi* vaccine that constitutively expresses the Vi polysaccharide (i.e., CVD 909) elicits long-term B_M responses against this T-I antigen. We also report that oral immunization with Ty21a and CVD 909 generates long lasting B_M responses against LPS and flagella. These observations support the notion that immunization with oral typhoid vaccines elicits a broad range of persistent effector immune responses that might play critical roles in long term protection.

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M.B.S. and M.M.L. are co-inventors in a patent for attenuated *Salmonella enterica* serovar Typhi strains that constitutively express Vi capsular polysaccharide antigen. However, no company has licensed this technology.

Abbreviations used in this paper

ALS	antibody in lymphocyte supernatant
ASC	Antibody secreting cell
B_M	memory B cells
CMI	cell-mediated immunity
CI	confidence interval
GMT	geometric mean titer
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells
SFC	Spot forming cells
T-D	T cell-dependent
T-I	T cell-independent

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Figure 1. IgM, IgG and IgA serum antibody titers to *S. Typhi* Vi, LPS and flagella in CVD 909-primed and placebo-primed subjects

Shown are geometric means of IgM, IgG and IgA antibody titers in CVD 909-primed (closed circles) and placebo-primed (open squares) subjects at pre-vaccination (day 0) and days 10, 14, 21, 28, 35, 42, 84, 212 and 365 post-vaccination against Vi (panels A, B, C), LPS (panels D, E, F) and flagella (panels G, H, I). The broken vertical line represents day 21, the time at which all volunteers received the parental Vi vaccine. * $p < .05$ compared to the corresponding levels in the placebo-primed group.

Figure 2. Kinetics of post-vaccination increases in specific B_M against *S. Typhi* Vi and flagella in CVD 909-primed and placebo-primed subjects

Shown are Vi-specific (panels A, C) and flagella-specific (panels B, D) IgG (panels A, B) and IgA (panels C, D) B_M cell in CDV 909-primed subjects (closed circles, n=11) and placebo-primed subjects (open circles, n=8). The data are presented as the percentage of antigen specific B_M per total IgG or IgA at the indicated days after subtracting day 0 values. The broken horizontal line represents the cut-off for post vaccination responders defined as described in the Methods section. The solid horizontal lines represent the median in each group. The percentages of volunteers who responded to immunization with increased Vi-specific B_M in placebo (open bars) and CVD 909 (solid bars) groups are shown in panel E. Panel F shows representative pictures of B ELISpots rendered by the automated counter. * p < 0.05 as compared to the corresponding placebo-primed group.

Figure 3. *S. Typhi* antigen-specific IgG and IgA B_M in volunteers orally vaccinated with Ty21a Shown are LPS-specific (panels A, C) and flagella-specific (panels B, D); IgG (panels A, B) and IgA (panels C, D) B_M cells before (Day 0) and after (Day 70) vaccination with Ty21a. Connecting lines show pre-vaccination (day 0, open circles) and post-vaccination (day 70, solid triangles) levels for each volunteer. The horizontal broken line represents the cut-off for positive B_M frequency as described in Materials and Methods.

Figure 4. Correlation of antibody titers in culture supernatants of expanded cells and corresponding B ELISpot assays

PBMC samples ($n=12$) were collected at 3 different time points from each of 4 randomly selected volunteers immunized with Ty21a before and after vaccination. Antibody titers are presented as net OD₄₅₀ units calculated as the sample OD₄₅₀ - mean+3SE of the blanks. Total IgG (panel A) and IgA (panel B) antibodies in supernatants were measured at 1:2,000 dilutions, while *S. Typhi* antigen specific IgG (panels C,E) and IgA (panels D,F) were measured at 1:2 dilutions. Total isotype B_M spots (A,B) are presented as SFC/10⁶ expanded cells while antigen specific B_M (C,D,E,F) are presented as percentage of the respective total IgG or IgA. The correlation factor r (Spearman) and corresponding P values are shown in each panel.

Figure 5. Representative sequential gating strategy for isolating B_M and B naïve subsets by flow cytometry

Shown is a representative gating strategy for the isolation of B cell populations from a volunteer primed with CVD 909 followed by parenteral Vi. Histograms show the phenotype of cells before (panels A, B, C) and after (panels D, E, F and G) sorting. The percentages of cells within the indicated regions are shown in the corresponding histograms.

Figure 6. Measurement of specific antibodies in culture supernatants of sorted B_M and naïve subpopulations expanded with B cell mitogens

Antibody titers are shown as net OD₄₅₀ units (sample OD₄₅₀ – mean+3 SE of blanks).

Shown are results for total IgG (panel A) and specific IgG to LPS (panel B) and *S. Typhi* flagella (panel C) and total IgA (panel D) and specific IgA to Vi (panel E), LPS (panel F) and *S. Typhi* flagella (panel G). Total IgG and IgA antibodies in supernatants were measured at 1:1,000 dilutions, while *S. Typhi* antigen specific IgG and IgA were measured at 1:2 dilutions.