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Antigen-specific IgA B memory cell responses to *Shigella* antigens elicited in volunteers immunized with live attenuated *Shigella flexneri* 2a oral vaccine candidates

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Abstract We studied the induction of antigen-specific IgA memory B cells (B_M) in volunteers who received live attenuated *Shigella flexneri* 2a vaccines. Subjects ingested a single oral dose of 10^7, 10^8 or 10^9 CFU of *S. flexneri* 2a with deletions in *guaBA* (CVD 1204) or in *guaBA*, *set* and *sen* (CVD 1208). Antigen-specific serum and stool antibody responses to LPS and Ipa B were measured on days 0, 7, 14, 28 and 42. IgA B_M cells specific to LPS, Ipa B and total IgA were assessed on days 0 and 28. We show the induction of significant LPS-specific IgA B_M cells in anti-LPS IgA seroresponders. Positive correlations were found between anti-LPS IgA B_M cells and anti-LPS IgA in serum and stool; IgA B_M cell responses to IpaB were also observed. These B_M cell responses are likely play an important role in modulating the magnitude and longevity of the humoral response.

Abbreviations: ASC, antibody secreting cell; B_M, memory B cells; CD, cluster designation; CDC, Center for Disease Control and Prevention; CVD, Center for Vaccine Development; CFU, colony forming units; CMI, cell-mediated immunity; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot; Ig, immunoglobulin; Ipa, Invasion plasmid antigen; IU, international units; LPS, lipopolysaccharide; mAb, monoclonal antibodies; mL, milliliter; mM, millimolar; NIH, National Institutes of Health; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PBST, phosphate buffered saline+0.05% Tween 20; PCR, polymerase chain reaction; PWM, pokeweed mitogen; cRPMI, complete Roswell Park Memorial Institute medium; SAC, *Staphylococcus aureus* Cowan; SFC, spot forming cells; μg, microgram; μM, micromolar; VTEU, Vaccine and Treatment Evaluation Unit; WHO, World Health Organization.

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14. ABSTRACT
We studied the induction of antigen-specific lgA memory B cells (BM) in volunteers who received live attenuated Shigella flexneri 2a vaccines. Subjects ingested a single oral dose of 107 , 108 or 109 CFU of S. flexneri 2a with deletions in guaBA (CVD 1204) or in guaBA, set and sen (CVD 1208). Antigen-specific serum and stool antibody responses to LPS and lpa B were measured on days 0, 7, 14, 28 and 42. lgA BM cells specific to LPS, lpa Band totallgA were assessed on days 0 and 28. We show the induction of significant LPS-specific lgA BM cells in anti-LPS lgA seroresponders. Positive correlations were found between anti-LPS lgA BM cells and anti-LPS lgA in serum and stool; lgA BM cell responses to lpaB were also observed. These BM cell responses are likely play an important role in modulating the magnitude and longevity of the humoral response.

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1. Introduction

Shigella infections continue to be a major cause of morbidity and mortality among children under 5 years old living in the developing world. Every year, there are 165 million cases of shigellosis worldwide and 14,000 cases reported in the United States; it is estimated that because of underreporting, the number of actual cases may be twenty times higher [1,2]. The increasing prevalence of resistance to multiple antimicrobials is of concern [3] and *Shigella* is considered a Category B bioterror agent by the CDC [4]. *Shigella flexneri* is endemic throughout the developing world, and causes more mortality than any other species of *Shigella* [5]. There is a high demand for a safe and effective oral vaccine, and the WHO has prioritized the development of a well-tolerated vaccine that induces durable immunity against shigellosis [1,6].

By engineering rational deletions in the wild-type *S. flexneri 2a* strain 2457 T, two vaccine candidates, designated CVD 1204 and CVD 1208, were constructed at the Center for Vaccine Development (CVD). CVD 1204 contains deletions in *guaA* (encoding a guanosine monophosphate synthase) and *guaB* (encoding an inositol monophosphate dehydrogenase), which impair the biosynthesis of guanine nucleotides; CVD 1208 has additional deletions of *set* and *sen* genes that encode *Shigella* enterotoxins 1 and 2, respectively. In a Phase 1 trial CVD 1204 was shown to be clearly attenuated compared to its wild type parent (based on comparison with data from multiple previous challenge studies), while CVD 1208 appeared fully attenuated yet immunogenic [7]. Clinical adverse reactions (diarrhea, dysentery and/or fever) occurred in 8 of 23 recipients of CVD 1204 but in only 1 of 21 recipients of CVD 1208 [7].

Putative correlates of protection against shigellosis reported in the literature include serum IgG antibodies against lipopolysaccharide (LPS) O antigen and serotype specific O antigen peripheral blood IgA antibody secreting cells (ASC) [2,8,9]. Other antibody and cell-mediated immune responses (CMI) against conserved antigens such as invasion plasmid antigens (Ipa) may also play a role in protective immunity [2,10–13]. An optimal vaccine should not only induce enduring systemic and mucosal antibody responses but also allow the host to mount an anamnestic immune response upon subsequent re-exposure to antigen. This response is faster, stronger, and qualitatively better than primary responses and depends on the presence of B_M cells [14]. Following natural *Shigella* infection, as well as after ingestion of some live attenuated *Shigella* vaccines, relatively long-term humoral and secondary secretory IgA immune responses to LPS in stool have been described [15]. We have previously demonstrated the induction of IgG B_M responses by live attenuated *Shigella* vaccines in human volunteers [16]. However, the presence of IgA B_M responses has not been reported. In this study we examined the hypothesis that volunteers who display mucosal and serum antibody responses to CVD 1204 and CVD 1208 live-attenuated oral *Shigella* vaccines also exhibit IgA B_M cell responses specific to LPS, IpaB and other *Shigella* antigens.

2. Materials and Methods

2.1. Specimens

46 healthy adult volunteers 18–45 years of age from the Baltimore–Washington area received a single oral dose of *S. flexneri 2a ΔguaBA* (CVD 1204) or *S. flexneri 2a ΔguaBA Δsen Δset* (CVD 1208) as previously described [7]. Volunteers received 10^7, 10^8, or 10^9 CFU of each vaccine strain or placebo, and sera and stools were collected on days 0, 7, 14, 28, and 42. In addition, peripheral blood mononuclear cells (PBMC) were obtained on days 0 and 28 after oral vaccination. PBMC specimens were cryopreserved and stored in liquid nitrogen until use as previously described [17]. Seroresponse, measured by ELISA [7], was defined as ≥4-fold rise of antigen-specific IgA antibody in serum (seroresponders) and a ≥4-fold rise of antigen-specific IgA/total IgA in stool (mucosal responders) after oral vaccination as compared to pre-vaccination. Adequate specimens were available to assay 13 seroresponders and 11 non-seroresponders; these included subjects immunized with placebo or 10^7, 10^8, or 10^9 CFU of the *Shigella* strains. For B_M assays, subjects from all three dosage level cohorts were analyzed. 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. Antigen Preparation

LPS was purified by the hot aqueous phenol method of Westphal [18]. IpaB, IpaC, IpaD, MxiH, VirG and *Yersinia pestis* LcrV antigens were purified as recombinant proteins from *Escherichia coli*. A PCR fragment of virG encompassing amino acids 68–774 was copied from the vir plasmid of *S. flexneri* 2457 T by standard PCR. The fragment was ligated into pET22b (Novagen, Madison, WI) and the ligation product used to transform *E. coli* NovaBlue. The resulting plasmid was sequenced and used in the protein expression system. IpaD, VirG and LcrV were purified by standard His tag chromatography and dialyzed into PBS as described previously [19,20]. IpaC and MxiH were solubilized from inclusion bodies with 6 M urea, purified by standard His tag chromatography, and refolded by step dialysis into PBS as previously described [21,22]. IpaB was co-expressed with its cognant chaperone, IpgC, as described previously [23]. The complex was purified by standard His tag chromatography via the His tag fused to the IpgC. IpaB was released from IpgC with 1% *n*-octyl-polyoxyethylene.

2.3. Mucosal and Systemic Antibodies

Serum antibodies specific for *S. flexneri* LPS and Ipa B were measured by ELISA as we previously described [7]. Briefly, plates were coated with LPS (5 μg/mL) or IpaB (0.1 μg/mL) and blocked with 10% dried milk in PBS. Samples were evaluated in serial 2-fold dilutions. HRP–labeled goat anti–human Fc α chain (ICN) was used as conjugate and TMB microwell Peroxidase (Kirkegaard & Perry Laboratories, KPL) as substrate. Titers were calculated from linear regression curves as the reciprocal serum dilution that produced an OD of 0.2 above the blank (EU/mL). Total and LPS-specific fecal IgA were also measured by ELISA as previously described [7]. Plates were coated with either α-chain specific anti-human IgA (1 μg/mL; Jackson ImmunoResearch Laboratories) or LPS (10 μg/mL). Stool supernatants were tested in serial 2-fold dilutions. HRP-labeled goat anti-human IgA (Jackson) was used as conjugate and TMB (KPL) as substrate. IgA concentrations were calculated by interpolation into a standard curve of human IgA (Calbiochem). Data are reported as the ratio of LPS-specific/ total IgA levels.

2.4. Antibody Secreting Cells

IgA and IgG ASCs specific for *S. flexneri* LPS and IpaB were detected by ELISpot as we previously described [7,24]. A positive ASC response was defined as a post-vaccination count at least 3 SD above the mean prevaccination count and at least 8 cells/10^6 PBMC.

2.5. PBMC Expansion

Expansion of PBMC to measure B_M cells was performed as described by Crotty et al. [25]. Briefly, PBMC specimens were thawed, washed with complete cRPMI 1640 containing 100 IU/mL penicillin, 100 μg/mL streptomycin (CellGro, Manassas, VA), 2 mM L-glutamine (Bio-Whittaker, Walkersville, MD), and 10% heat-inactivated fetal bovine

serum (HyClone, Logan, UT), and expanded for 5 days in 6-well plates (1×10^6 cells/well) in the presence of 1/100,000 pokeweed mitogen (PWM, kindly provided by Dr. S. Crotty), 6 μg/mL CpG-2006 (Qiagen/Operon, Huntsville, AL), 50 μM β-Mercapto-ethanol, and 1/10,000 *Staphylococcus aureus* Cowan (SAC, Sigma-Aldrich, St. Louis, MO) in cRPMI (expansion media) in a total volume of 2 mL/well. After 2 days of incubation, cells were fed by replacing 2 mL of cRPMI in each well.

2.6. B Memory Cell Assays

96-Well ELISPOT MAHA (Millipore, Billerica, MA) plates were coated with 5 μg/mL LPS, 0.5 μg/mL of IpaB, IpaC, IpaD, VirG, MxiH or *Y. pestis* LcrV (as a negative control) or 5 μg/mL of mouse anti-human IgA in PBS and incubated overnight at 4 °C. The plates were then blocked with 1% bovine serum albumin (Sigma) in RPMI for 2 h at 37° C and 5% CO_2, and 10^5 expanded PBMC added per well coated with LPS, IpaB, IpaC, IpaD, VirG, MxiH or LcrV. For total IgA measurements, serial 2-fold dilutions were performed starting at 7500 expanded cells/well down to 234 cells/well. Cells were incubated for 5 h at 37° C and 5% CO_2, washed with PBST followed by PBS, and incubated with mouse anti-human pan IgA Biotin Conjugated Antibody (Hybridoma Reagent Laboratory, Baltimore, MD) overnight at 4 °C. Subsequently plates were washed with PBST and PBS and labeled with horseradish peroxidase-conjugated Avidin D (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The substrate 3-Amino-9 etheylcarbazole C (Calbiochem, La Jolla, CA, USA) was added at 100 μL per well for 20 minutes at room temperature and the reaction was stopped with ddH_2O. Final enumeration of specific and total SFC was performed using the Immunospot Series 3B Analyzer ELISPOT reader (Cellular Technologies Ltd, Shaker Heights, OH) with aid of the Immunospot software version 4.0 (Cellular Technologies Ltd).

Adequate expansion of B_M cells, assessed by the frequency of total 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., >8300/10^6 total IgA SFC/10^6 expanded cells) were excluded from further analysis. Statistical analysis was performed on the mean number of SFC in antigen-coated wells minus the mean number of SFC in the negative control wells. The limit of detection of antigen-specific/total expanded cells in our assays was 1 in 100,000 (0.001%). The limit of detection of antigen-specific/total IgA-secreting cells in each volunteer was determined by taking into account the maximum number of total IgA SFC for that subject in anti-IgA coated ELISPOT wells. The latter had an acceptable minimum of 0.005% (i.e., 1 specific SFC in the 8300 total IgA SFC/10^6 expanded cells cut-off).

2.7. Flow Cytometry

Eight volunteers had sufficient PBMC available after expansion pre and post vaccination to enable flow cytometric measurements. Of these subjects, 3 volunteers were responders by LPS IgA in serum and stool by ELISA and 5 were non responders. Of the 3 LPS responders, 2 were also IpaB responders. Expanded PBMC were washed with 1% FBS in PBS and labeled with fluorochrome-labeled mAbs against the following antigens: (1) CD19-ECD (clone J3.119, Beckman-Coulter, Fullerton, CA), (2) CD20-APC-Cy7 (clone L27, BD Biosciences, San Jose, CA), (3) CD27-APC-A700 (clone 1A4CD27, Beckman-Coulter), (4) IgA-Biotin (clone G20-359, BD Biosciences—subsequently labeled with streptavidin-Pacific Orange (InVitrogen, Carlsbad, CA), (5) IgG-PE-Cy5 (clone G18-145, BD Biosciences), (6) integrin α_4/β_7-Alexa 647 (the anti-integrin α_4/β_7 ACT-1 mAb was kindly provided by Dr. W. Newman, PaxVax Inc., San Diego, CA and conjugated to Alexa 647 using an Alexa 647-labeling kit (Molecular probes, Eugene, OR), and (7) CD14-PacBlue (clone TÜK4 InVitrogen), CD3-PacBlue (clone UCHT1 BD Biosciences) and Vivid (InVitrogen), which were used to exclude cells staining positively with these mAbs using a "dump" channel gating strategy. Incubation with the mAbs was performed in volumes of 50 μL per tube for 20–30 minutes at 4 °C, washed with 1% FBS in PBS, and fixed in 300 μL of 1% formaldehyde until run. Events were acquired on a MoFlow flow cytometer/cell sorter system (Beckman-Coulter) and analyzed using WinList 6.0 (Verity Software House, Topsham, ME) software.

2.8. Statistical analysis

Microsoft® Office Excel 2007, GraphPad Prism 5.0, and STATA 9.0 were used for statistical analysis. Our hypotheses were evaluated using non-parametric two-sided tests. Pre- and post-vaccination results were paired. Antigen-specific SFC/10^6 expanded cells were divided by total IgA SFC. The Wilcoxon signed rank test was used to assess continuous pre- to post-vaccination antigen-specific B_M responses. Wilcoxon Rank Sum was used to compare responders to non-responders. Correlations between seroresponse and B_M responses were assessed using Spearman rho test for continuous variables and Fisher's exact test for dichotomous variables. Two-sided p values <0.05 were considered significant.

3. Results

3.1. Evaluation of Total and Specific SFC After Expansion of B_M Cells

Twenty four volunteers who received vaccine had sufficient PBMC pre- and post-vaccination to be included in these studies. Thirteen of the 24 subjects were anti-LPS IgA seroresponders. All 13 seroresponders were also mucosal responders by sIgA anti-LPS measured in stool. Three individuals were mucosal responders only with ≥4 fold increases in anti-LPS/total stool sIgA without a serum response. Cells from one anti-LPS seroresponder had to be excluded from analysis due to inadequate expansion. LPS-specific B_M cells increased from a median of 7 SFC/10^6 expanded cells pre-vaccination to a median of 53 SFC/10^6 expanded cells 28 days post-vaccination ($p=0.005$) (Fig. 1). Increases were observed in LPS-specific IgA B_M in 10 out of 12 IgA anti-LPS (83%) seroresponders with adequate expansion. Of 24 vaccinated volunteers who had sufficient PBMC to perform these studies, 7 also displayed an IgA anti-IpaB seroresponse. Mean IgA anti-IpaB B_M cells increased from 4 to 20 SFC/10^6 expanded cells pre- to post-vaccination ($p=0.062$); 4 out of 7 (57%) IgA IpaB seroresponders manifested an increase in IgA anti-IpaB B_M cells. The median percentages of antigen-specific SFC as a proportion of total IgA SFC showed increases from 0.03% pre-vaccination to 0.20% post-vaccination for LPS (p value=0.005) and 0.03% pre-vaccination to 0.10% post-vaccination for IpaB ($p=0.2$) among seroresponders (Fig. 1). Individuals who were not seroresponders did not exhibit a statistically significant increase in antigen-specific B_M responses pre- to post-vaccination.

Because of the importance of other proteins in *Shigella* pathogenesis, e.g., VirG (IcsA), MxiH, IpaB, IpaC and IpaD [26,27], we studied whether immunization with a live oral *Shigella* vaccine also elicited specific B_M to these antigens. Only sporadic B_M responses were observed to IpaC, IpaD, MxiH, and VirG. No responses were detected against *Y. pestis* LcrV (negative control).

The frequency of B_M can be quantified as the number of anti-LPS or as anti-IpaB specific IgA per 10^6 expanded PBMC or the % of anti-LPS or anti-IpaB specific IgA divided by the total number of IgA producing cells in expanded PBMC. As can be observed in Fig. 2, highly significant correlations were observed between the two methods of quantification.

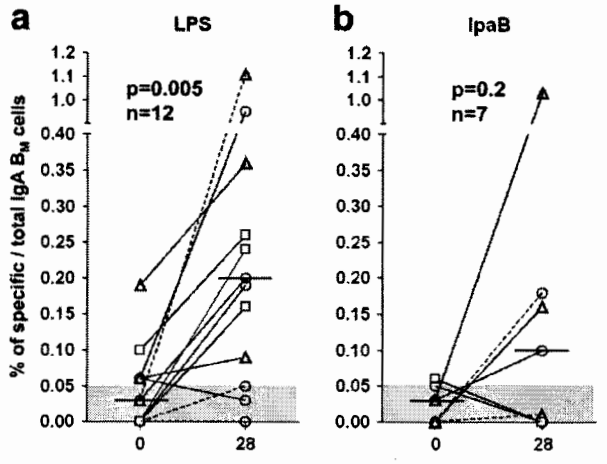

Figure 1 Antigen-specific IgA B_M cell responses. Shown are recipients of 10^7 (triangles), 10^8 (circles) and 10^9 (squares) CFU of CVD 1204 (solid lines) and CVD 1208 (broken lines) who mounted a $\geq$4-fold rise of anti-LPS (n=12) and anti-IpaB (n=7) IgA pre to post vaccination and evidenced appropriate B_M expansion in vitro; LPS (a) and IpaB (b) ELISPOT performed on days 0 and 28; comparisons made by Wilcoxon Signed Rank Test for 1204 and 1208 combined. Results are expressed as the % of specific B_M SFC per total IgA^+ expanded cell populations; horizontal lines represent the median of the corresponding groups.

3.2. Correlation of B_M Responses with Antibody Responses in Serum and Stool

Mucosal responses to vaccination (i.e., the production of secretory IgA in stool/total $\geq$4 fold pre to post oral vaccination) was observed among 16 out of 24 subjects in this study. Among these 16 subjects, strong correlations were observed when comparing their LPS-specific B_M (SFC/10^6 expanded cells) frequencies measured on day 28 with their respective post-vaccination peak seroresponse (Fig. 3A), peak mucosal (stool) IgA response (Fig. 3B), and peak peripheral blood IgA ASC responses (Fig. 3C). Strong correlations were also observed among anti-LPS IgA B_M and both mucosal and peripheral anti-LPS IgA responses among the 12 individuals who were seroresponders (data not shown). Of importance, the correlations remain significant when results were analyzed as percentages of specific anti-LPS IgA B_M divided by total IgA secreting cells in each volunteer. No significant correlations were observed among antibody levels in serum and stool and peripheral B_M cells on day 0 (pre-vaccination, data not shown). Calculations taking into account individual spot sizes (a relative measurement of the amount of antibody produced by each cell) did not change the results. Interestingly, no correlations were observed between the levels of IpaB-specific IgA B_M cells and the serum levels of anti-IpaB IgA antibodies or the levels of anti-IpaB ASC in circulation (stool anti-IpaB antibody levels were not measured)(data not shown). Among the vaccine recipients who exhibited a mucosal response, the frequencies of IgA B_M cells were better correlated with serum LPS IgA titers (Fig. 3) than with serum LPS IgG (Spearman Rho 0.5, p=0.05).

3.3. Expression of Gut Homing Molecules in Plasmablasts and B_M Following Immunization

To evaluate whether immunization with CVD 1204 or CVD 1208 resulted in changes in the proportion of B cell subsets of defined phenotypes,

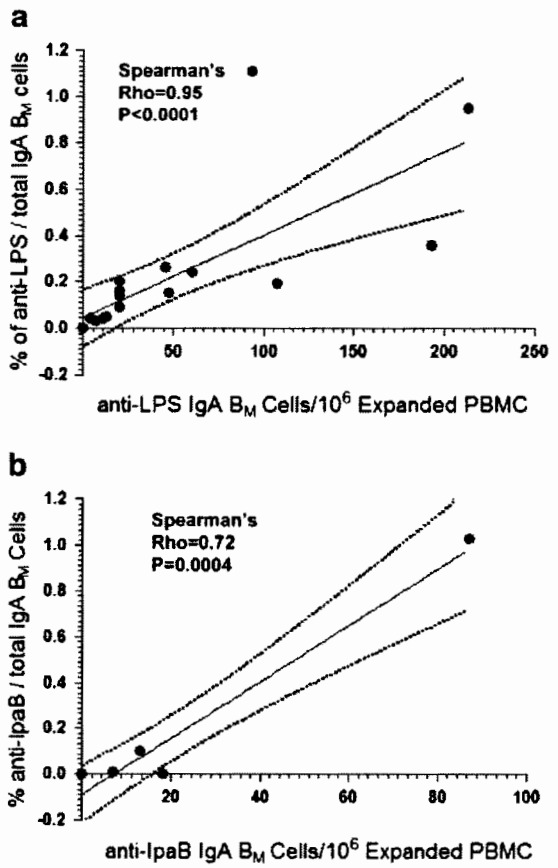

Figure 2 Correlation of antigen-specific B_M cells per 10^6 expanded PBMC with antigen-specific B_M cells/total IgA B_M cells. B_M cells specific for LPS (a) and IpaB (b)/10^6 expanded cells 28 days after immunization were plotted against the % of specific SFC divided by total IgA-secreting expanded cell populations for all volunteers studied; dotted lines represent the 95% confidence interval.

PBMC obtained before and after oral vaccination were expanded and stained to examine B cell subsets and expression of the gut homing integrin α_4/β_7 receptor. Cells were sequentially gated based on forward versus side scatter ("lymph region"), followed by the electronic elimination of doublets, dead cells, $CD3^+$ T cells and $CD14^+$ macrophages. The $CD19^+$ cells co-expressing integrin α_4/β_7 (i.e., with the potential to home to the gut) or not were then gated and analyzed for their expression of IgG or IgA; each of these cell subsets was further gated based on their expression of CD27 and CD20 to define the $CD19^+$ integrin α_4/β_7^+ $CD27^+$ $CD20^+$ [largely B_M] and $CD19^+$ integrin α_4/β_7^+ $CD27^+$ $CD20^{-/low}$ [largely plasmablasts/plasmocytes] subsets [28,29].

The results from these studies demonstrated an increase in IgA secreting cells post-oral vaccination compared to pre-vaccination among the LPS seroresponders. This increase was statistically significant when comparing responders to non-responders by Wilcoxon Rank Sum in the $CD19^+$ integrin α_4/β_7^+ IgA^+ $CD27^+$ $CD20^+$ as well as $CD19^+$ integrin α_4/β_7^+ IgA^+ $CD27^+$ $CD20^{-/low}$ subsets (Fig. 4). No significant differences among responders and non-responders were observed in the percentages of any of the other subsets evaluated (i.e., IgG^+ subsets, $CD19^+$ integrin α_4/β_7^- subsets, $CD19^+$ integrin α_4/β_7^+ $CD27^-$ $CD20^+$ and $CD19^+$ integrin α_4/β_7^+ $CD27^-$ $CD20^-$ subsets).

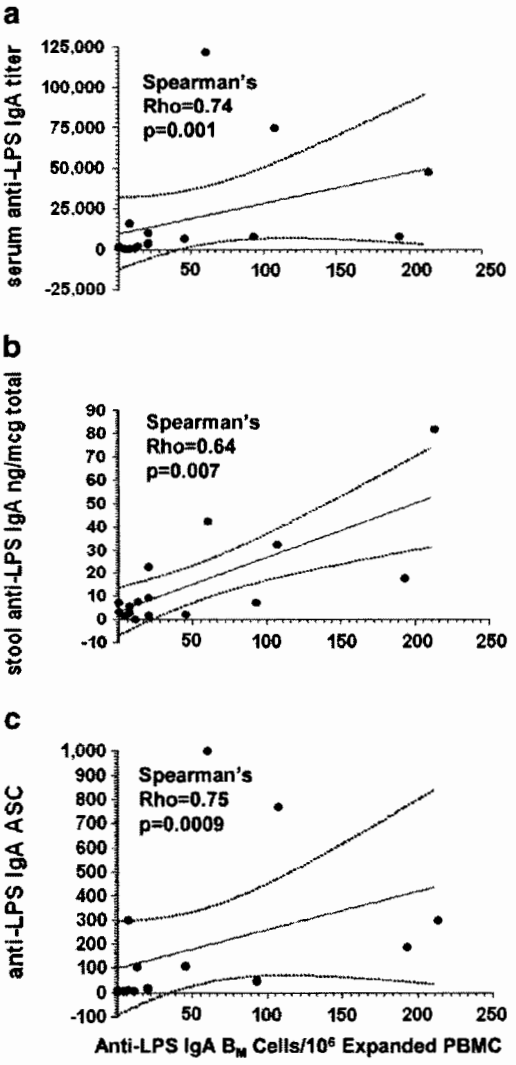

Figure 3 Correlation of anti-LPS B_M cell responses with serum and stool antibodies. Recipients of CVD 1204 and CVD 1208 who mounted a ≥4-fold rise of anti-LPS/total stool sIgA pre to post vaccination (mucosal responders) and evidenced appropriate B_M expansion in vitro ($n=16$) were included in the analysis. Peak serum IgA titers to LPS (a), peak stool IgA specific for LPS divided by total IgA (b), and peak LPS specific IgA ASC (c) were plotted against the number of LPS-specific B_M cells/10^6 expanded cells 28 days after immunization; dotted lines represent the 95% confidence interval.

4. Discussion

Immune responses induced locally provide the first line of defense against the many pathogens that invade the human host via mucosal surfaces. Humans make more IgA than IgG, IgM and IgE combined and allows for active transport of sIgA across mucosal epithelia to facilitate antigen exclusion and neutralization [30]. The normal human colon has a higher proportion of IgA than IgG-producing plasma cells as evidenced by immunohistochemistry [31]. The induction of B_M responses is widely accepted to be a major factor in the ability of vaccines to elicit long lasting, effective immunity. However, the precise role of IgA ASC and B_M cells in primary and secondary [anamnestic] immune responses to infection remains ill defined.

A B_M response can be demonstrated by documenting: (1) an anamnestic secondary antibody response, both stronger and faster than the initial immune response, (2) avidity maturation, and (3) the presence of B_M cells [32]. B_M cells have been described in humans against vaccines known to induce a T-cell dependent response, including live viral vaccines administered parenterally (e.g. smallpox vaccine [33]) and orally (e.g., rotavirus vaccines [34]) and parenteral conjugate vaccines consisting of bacterial polysaccharides (such as pneumococcal [35] and meningococcal [36] capsular polysaccharides) covalently linked to carrier proteins. B_M cells have also been reported following natural infection with bacterial enteropathogens and after the administration of live oral bacterial enteric vaccines [16,37]. We here report the observation that oral immunization with attenuated *S. flexneri 2a* vaccines elicits the generation specific IgA B_M cells.

Although the immunological correlates of protection following *Shigella* infection have not yet been fully elucidated, high numbers of antigen-specific IgA ASC post oral vaccination have been found to be associated with protection from shigellosis after experimental challenge [8]. Interestingly, the volunteers who have high numbers of antigen-specific ASC after vaccination and are asymptomatic after challenge have low levels of antigen-specific IgA ASC in peripheral blood after secondary antigen exposure [38]. In the present study we observed that a single oral immunization with live attenuated *Shigella* vaccine CVD 1204 or CVD 1208 elicited significant increases in antigen-specific IgA anti-LPS B_M responses among subjects who mounted ≥ fourfold specific sIgA or IgA antibody responses as measured by ELISA, respectively, in stool and serum. IpaB seroresponders also exhibited antigen-specific IgA B_M responses to IpaB. Strong correlations were observed between the magnitude of IgA anti-LPS B_M cells and increases in anti-LPS IgA responses, both among individuals who displayed a strong mucosal sIgA stool response and among IgA seroresponders. B_M cells also correlated highly with anti-LPS IgA ASC, both among seroresponders and mucosal responders. It will be important to determine in future experimental challenge studies with wild-type *Shigella* whether antigen-specific IgA and IgG B_M cells are elevated in subjects who had been (or not) previously immunized with oral live attenuated *Shigella* strains and whether B_M cells are associated with protection.

We were surprised that no differences were detected in the magnitude of B_M cell responses among recipients of the slightly reactogenic CVD 1204 or the very well tolerated CVD 1208, or related to the various dosage levels evaluated (10^7, 10^8, or 10^9 CFU). An optimistic interpretation of this observation is that the loss of reactogenicity resulting in a well tolerated further attenuated *Shigella* vaccine strains such as strain CVD 1208 is not accompanied by a diminution of the ability to elicit strong immune responses, including the generation of antigen-specific IgA B_M cells.

In previous studies we determined by flow cytometry the proportions of phenotypically defined total B, B_M and IgG^+ B_M cell populations before and after expansion [16]. This information helped validate the B_M cell ELISPOT assay as well as provide the rationale for limiting the analyses to specimens that showed evidence of appropriate in vitro expansion in the presence of mitogens. By eliminating specimens that exhibited suboptimal expansion of functional cells (defined as ≤8,300 total IgA SFC/10^6 expanded cells) in the current dataset, we observed increased levels of circulating antigen-specific IgA B_M responses elicited by oral vaccination of up to 1.1% antigen-specific B_M SFC/total IgA SFC post-vaccination for T-independent antigen LPS and up to 1.0% for the T-dependent antigen IpaB. These proportions of antigen-specific B_M cells are similar to those previously reported in subjects who received efficacious parenteral vaccines. For example, responses to diphtheria and tetanus toxoids elicited specific B_M cells over total IgG secreting cells in the range of 0.01–1% [39]. Recombinant hepatitis B vaccine showed the induction of 0.07% hepatitis B surface

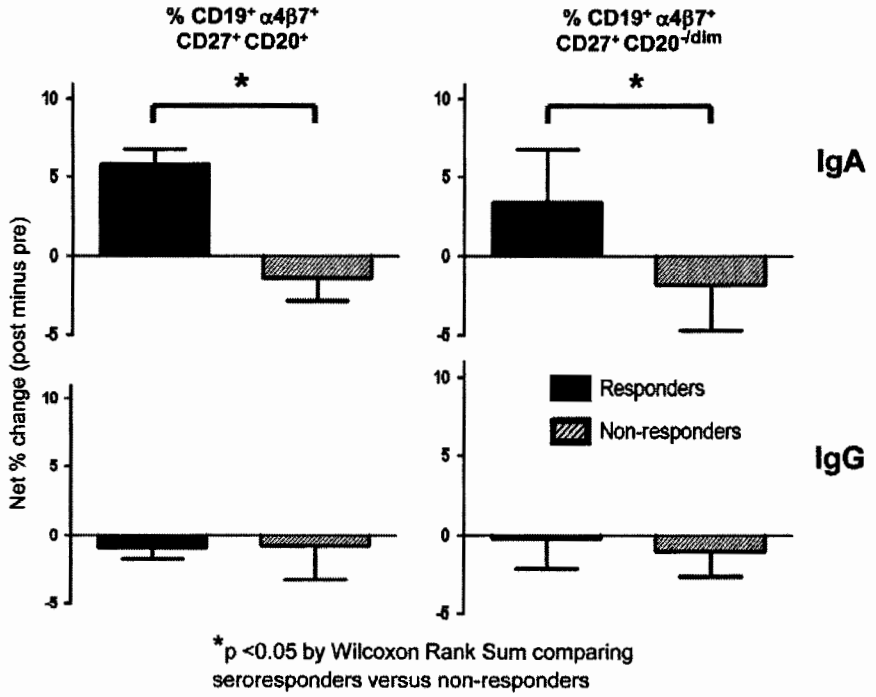

Figure 4 Pre- to post-vaccination assessment of B cell subsets expressing the gut homing receptor integrin α_4/β_7. Shown are the % of $CD19^+$ integrin α_4/β_7^+ cells co-expressing CD27 and CD20 (left panels) and CD27 but not CD20 (right panels) pre- and day 28 post-vaccination for IgA^+ (upper panels) and IgG^+ cells (lower panels) among LPS seroresponders and non-responders. *$p<0.05$ by Wilcoxon Rank Sum comparing net change in % of the indicated subsets post minus pre immunization among LPS seroresponders versus non-responders; LPS seroresponse is defined as $\geq$4-fold rise of antigen-specific antibody in serum (all responders were both IgA and IgG seroresponders).

antigen-specific Ig secreting cells over total Ig secreting cells; 88% of vaccinees had detectable levels of IgG B_M cells and 76% had detectable levels of IgA and IgM B_M cells [40]. In natural cholera infection, anti-LPS IgA B_M cells have been reported to be 0.6% of total IgA B_M cells and proposed to play an important role in the anamnestic mucosal immune response [37]. To our knowledge, the data included in the present manuscript is the first description of the induction of specific IgA B_M cell response to LPS in recipients of an oral live-attenuated bacterial vaccine.

Since *Shigella* enters the host via the gut, it is important to define the local immune responses elicited by immunization. Due to regulatory constraints and other factors, intestinal mucosal biopsies are very difficult to obtain following vaccine administration or challenge with wild-type organisms. Alternative ways to investigate whether antigen-specific IgA and IgG ASC and B_M found to be elevated in the periphery after oral vaccination have the potential to migrate to the gut mucosa involve indirect measurements, such as sIgA in stool and the measurement of gut homing molecule expression, e.g., integrin α_4/β_7, on IgA^+ and IgG^+ ASC and B_M cells. Although in our experience flow cytometry is not as sensitive as ELISPOT in assessing antigen-specific B_M responses, it nevertheless allowed us to characterize the phenotype of the B cell subsets that increased following oral vaccination.

In the present study we found significant increases in the proportion of vaccine-induced $CD19^+$ $CD27^+$ $CD20^+$ integrin α_4/β_7^+ IgA^+ B_M cells with a gut homing pattern in seroresponders as compared to non-seroresponders. Smaller, yet significant, increases have also been observed in $CD19^+$ $CD27^+$ $CD20^-$ integrin α_4/β_7^+ IgA^+ cells (a phenotype typically associated with plasmablasts)[29]. While B_M only express surface Ig, expression of Ig in plasmablasts is largely cytoplasmic with lower levels of surface Ig expression [41]. Because this manuscript is focused on B_M and sufficient cells were not available for independent staining panels (i.e., extracellular and intracellular IgA and/or IgG staining), we stained extracellularly for Ig. Thus, it is likely that the smaller differences observed in integrin α_4/β_7^+ IgA^+ plasmablasts in responders as compared to non-responders are the result of a lower efficiency in the detection of IgG^+ and IgA^+ plasmablasts. Future studies using intracellular Ig staining will allow this issue to be addressed directly. In contrast to the increases in IgA^+ cells, this phenomenon was not observed in $CD19^+$ α_4/β_7^+ IgG B_M cells (irrespective of expression of CD27 and/or CD20). In sum, we found that IgA^+, but not IgG^+, $CD19^+$ B cells expressing the gut homing receptor integrin α_4/β_7 increased 28 days after oral vaccination, and that gating on subsets based on the expression of CD27 and CD20 enhanced the ability to identify this population. This observation is consistent with Crotty's original description of the B_M cell ELISPOT where sorting experiments were used to identify that B_M have a $CD27^+$ $CD20^+$ phenotype [25]. The present results suggest an increase in circulating *Shigella* specific IgA B_M and plasmablasts with gut homing potential in individuals in whom immunization elicited anti-*Shigella* humoral responses.

A limitation of this study is the relatively small number of volunteers who could be evaluated, which is largely due to the fact that it employed "convenience" specimens based on availability. In spite of this limitation, we observed strong, statistically significant, specific anti-LPS IgA B_M responses and associations with anti-LPS IgA antibody and ASC levels. However, it is very likely that the small sample size provided insufficient power to adequately estimate the presence and association of anti-IpaB IgA B_M cells and seroresponses. Future studies will address this issue by evaluating larger numbers of subjects and vaccine candidates.

Virulent *Shigella* target the M cells that overlie gut-associated lymphoid tissue and rapidly attain an intracellular niche within epithelial cells. SIgA anti-*Shigella* antibodies can prevent mucosal

invasion. Moreover, wild type organisms coated with such antibody elicit less profound inflammatory responses than *Shigella* not coated with antibody [42]. It is not known how long sIgA intestinal and serum IgG anti-*Shigella* antibodies stimulated by clinical infection or oral immunization with *Shigella* vaccines remain elevated at levels adequate to prevent invasion and clinical illness. If antibody levels decline to non-protective levels, it falls upon B_M cells to mount a sufficiently rapid anamnestic response to limit the clinical consequence of exposure to virulent *Shigella*. Experimental challenge studies with immunologically-naïve healthy adult volunteers and epidemiologic investigations of point source outbreaks suggest that for most *Shigella* serotypes the usual incubation is ~1–3 days from the point of ingestion of virulent organisms until the onset of clinical illness. Thus, an effective anamnestic response mediated by B_M cells must occur within a mere 1–2 days. Few reports have directly addressed the duration of immunity that follows an initial clinical *Shigella* infection in the absence of repetitive boosting (as would occur in an endemic situation). Similarly, to our knowledge the only controlled studies that describe the duration of protection conferred by immunization with live oral *Shigella* vaccines were those of Mel et al. who immunized children living in an endemic area where *Shigella* was highly seasonal [43,44]. Oral immunization with four doses over two weeks of streptomycin-dependent live oral *Shigella* vaccines led to significant serotype-homologous protection that endured for one year [44]. However, the administration of just a single oral booster dose was able to prolong the protection for an additional year [44].

In summary, oral vaccination with a single oral dose of a live-attenuated *Shigella* vaccine elicited antigen-specific IgA B_M responses among IgA seroresponders and sIgA mucosal responders with a high degree of correlation between antigen-specific B_M cells and both serum and mucosal titers as well as antigen-specific ASC in peripheral circulation. Mucosal sIgA responses are more difficult to measure than serum antibody, as is the measurement of small number of IgA B_M cells. Nevertheless, we observed positive correlations between antigen-specific anti-LPS B_M cells and antigen-specific anti-LPS sIgA/total IgA in stool. As *Shigella* invades via the gastrointestinal mucosa, fecal sIgA responses likely play an important role as the first line of defense in protection. Antigen-specific IgA B_M cells should be further studied in natural infection and challenge studies to determine their role in the anamnestic responses and protection against this important and devastating pathogen. For expedited identification of future candidate vaccines, it will be necessary to identify reliable surrogates of protection against symptomatic infection. A strong correlation between anti-LPS IgA B_M cells and peak anti-LPS IgA antibody and ASC responses further reinforces our contention that B_M cells may be an important indicator for long-term humoral immunity and a possible surrogate of protection against shigellosis.

5. Conclusions

Oral vaccination with live-attenuated *S. flexneri* 2a elicited detectable IgA B_M cells to LPS and, to a lesser extent, to IpaB. Positive correlations were observed between anti-LPS IgA B_M and both anti-LPS secretory IgA /total IgA in stool and anti-LPS IgA in serum as well as circulating ASC. In addition, flow cytometric analyses revealed significant differences in IgA^+ B_M subsets expressing integrin α_4/β_7 pre to post vaccination when comparing seroresponders to non-responders. Our results support the contention that B_M cells may be an important indicator for long-term humoral immunity and a possible surrogate of protection against shigellosis.

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