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***Shigella* antigen-specific B memory cells are associated with decreased disease severity in subjects challenged with wild-type *Shigella flexneri* 2a**

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Abstract

The role of *Shigella*-specific B memory (B_M) in protection has not been evaluated in human challenge studies. We utilized cryopreserved pre- and post-challenge peripheral blood mononuclear cells and sera from wild-type *Shigella flexneri* 2a (wt-2457T) challenges. Challenged volunteers were either naïve or subjects who had previously ingested wt-2457T or been immunized with hybrid *Escherichia coli-Shigella* live oral candidate vaccine (EcSf2a-2). B_M and antibody titers were measured against lipopolysaccharide (LPS) and recombinant invasion plasmid antigen B (IpaB); results were correlated with disease severity following challenge. Pre-challenge IgA IpaB-B_M and post-challenge IgA LPS-B_M in the previously exposed subjects negatively correlated with disease severity upon challenge. Similar results were observed with pre-challenge IgG anti-LPS and anti-IpaB titers in vaccinated volunteers. Inverse correlations between magnitude of pre-challenge IgG antibodies to LPS and IpaB, as well as IgA IpaB-B_M and post-challenge IgA LPS-B_M with disease severity suggest a role for antigen-specific B_M in protection.

Keywords

Shigella; protection; human challenge; B memory; LPS; IpaB

1. Introduction

Shigella is an important cause of morbidity and mortality from diarrheal diseases among children living in developing countries [1, 2]. The control of shigellosis is impeded by the emergence of antibiotic resistance [3] and lack of a commercially available vaccine. Obstacles in *Shigella* vaccine development include the lack of adequate animal challenge

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models that faithfully reproduce human shigellosis and the complexity of performing human challenge studies or prospective clinical studies in the field [4]. Challenge studies offer a means of studying protective immunity in humans. Three challenge studies were performed at the University of Maryland School of Medicine Center for Vaccine Development (CVD) in the early 1990's to evaluate the efficacy of a live oral hybrid *Escherichia coli-Shigella flexneri* 2a vaccine candidate (EcSf2a-2) developed at the Walter Reed Army Institute of Research [5] and to refine the wild-type challenge model. Efficacy was assessed by measuring the ability of either the vaccine or wild-type infection to prevent illness following experimental challenge with wild-type *S. flexneri* 2a strain 2457T (wt-2457T) [6, 7]. In two studies, volunteers were administered multiple spaced doses of EcSf2a-2 and challenged one month later (along with a group of unvaccinated controls) with wt-2457T. The vaccine induced a modest immune response and conferred 27–36% efficacy against challenge [7]. A subset of these volunteers who developed gastrointestinal symptoms of shigellosis (diarrhea or dysentery) after challenge with virulent *S. flexneri* 2a in bicarbonate buffer agreed to participate in a second challenge study with wt-2457T along with a group of subjects who had not been previously immunized or challenged; the protective efficacy of prior exposure to wt-2457T reached 70% [6]. Based on these studies, IgA anti-LPS antibody secreting cell (ASC) responses have been proposed as a possible correlate of protection [7]. Heretofore, very limited additional putative immune markers, notably IgG serum antibody titers against lipopolysaccharide (LPS) have been found in most, but not all, studies to be statistically correlated with protection against *Shigella* infection [4, 8–11]. Other immune mechanisms that have been proposed to correlate with protection against shigellosis include serum antibody responses against invasion plasmid antigens (Ipa) [4, 6, 11–13] and cell mediated immunity (CMI) [4, 14–16]. However, there is no definitive evidence that these responses by themselves can be considered mechanistic mediators of protection [4]. Therefore, it is important to search for additional correlates of protection that alone or in combination can be used to predict the efficacy of candidate *Shigella* vaccines.

The appearance of ASC ~7 days after immunization suggests immune priming that may also be accompanied by the generation of B memory (B_M) cells. B_M cells are responsible for mounting a rapid anamnestic antibody response (recall response) upon re-exposure to microbial antigens and thus are considered an indicator of long-term protection induced by vaccine- or natural infection [17, 18]. Methodological advances and the availability of purified antigens (including recombinant IpaB) now enable the measurement of B_M cells in cryopreserved peripheral mononuclear cell (PBMC) specimens elicited by orally administered attenuated enteric vaccines or other vaccine candidates. Using this approach we have recently demonstrated the presence of B_M cells in subjects immunized with attenuated strains of *Shigella*, *S. Typhi*, *S. Paratyphi A*, *S. Paratyphi B* and Norovirus [19–23]. Cryopreserved specimens from *Shigella* challenge studies performed in the 1990s offered a unique opportunity to identify potential immune correlates that could not be identified at that time because the technology was not available. Thus, we utilized the limited specimens remaining from those studies to measure B_M cells as well as serum antibodies in specimens collected before and after challenge, and correlated these responses with disease outcome. Our goal was to investigate correlations among pre- and post-challenge LPS- and IpaB-specific B_M and serum antibodies, as well as antibody secreting cells (ASC) with disease outcome to better define the role of specific immune responses in protection.

2. Materials and Methods

2.1. Study design

We analyzed available PBMC specimens cryopreserved in liquid nitrogen and serum cryopreserved at -70°C from the three clinical trials involving subjects challenged with

wt-2457T described in the introduction. All available PBMC specimens from 20 volunteers challenged with wt-2457T were used for B_M cell assays; 13 had prior exposure to *S. flexneri* 2a (pre-exposed group) through vaccination or experimental challenge and the remaining 7 were newly recruited healthy controls (naïve group) who had no history of prior exposure to *S. flexneri* 2a. Following thawing, an average 76% of the originally cryopreserved PBMC/vial were recovered with an average cell viability of 82% as determined by trypan blue exclusion.

PBMC were available from a pre-exposed group, comprised of three volunteers who had been previously challenged with wt-2457T (no immunizations, referred to as “veterans” in previous publications; [6, 7]), and ten subjects who had been previously immunized with 4 doses of EcSf2a-2 (7×10^8 CFU). Serum samples were available from fifteen vaccinated volunteers previously immunized with 4 doses of EcSf2a-2 (7×10^8 CFU). Samples from naïve controls (PBMC: n=7 and serum: n=14) were also available from the same study [7]. These control subjects were healthy volunteers with no history of previous exposure to WT-2457T or EcSf2a-2. All volunteers (including the naïve subjects) were challenged with wt-2457T and samples (PBMC and sera) used in the current study were collected before (day 0) and after (day 28 days) following challenge. Informed, written consent was obtained from participants prior to enrollment in the clinical trials according to the guidelines of the Institutional Review Board of the University of Maryland.

2.2. Disease Index

The design of the inpatient challenge studies is described elsewhere [6, 7]. A categorical outcome-based disease index was created prior to analysis based on body temperature, daily number of bloody stools, daily number of loose stools, and total daily volume of the loose stools. The disease index assigned to each subject is the average grade of all components shown in Table 1. A dichotomous clinical outcome categorization (ill or well) was the one originally described for these clinical challenge studies (objective illness was defined on the basis of fever, diarrhea or dysentery) [6, 7]. The disease index was created for this study to explore the possibility that a categorical variable will define more precisely the severity of the clinical outcome than a dichotomous one, offering a better opportunity to correlate clinical and immunological measurements. When we compared the two methods, of those subjects who were protected or “well” (n=7) according to the previous criteria, all but one (who was graded as grade 1) were graded as 0, in our new disease index (n=6). Those who were previously categorized as “ill” (n=13), were further subdivided into grades (grade 1–3) based on disease severity.

2.3. Antigens

The LPS from *S. flexneri* 2a LPS was purified by the hot aqueous phenol extraction method of Westphal [24] and the *Shigella* IpaB and *Yersinia pestis* LcrV (the negative control protein) antigens were purified as recombinant proteins expressed in *E. coli* as previously described in detail [20].

2.4. B_M assay

B_M responses were measured using an optimized dual color ELISpot assay for the simultaneous measurement of IgG and IgA spot forming cells (SFC). Briefly, PBMC were thawed, expanded and seeded in duplicate wells of plates coated with *Shigella* antigens or total IgG or IgA as previously described [20, 25]. Plates were then incubated with antihuman IgG-alkaline phosphatase and IgA-horseradish peroxidase (Jackson ImmunoResearch, PA). IgA SFC were visualized with nitro-blue tetrazolium chloride (NBT) substrate (Vector laboratories, CA) as blue spots and, after washing, plates were incubated with 3-Amino-9-ethylcarbazole (AEC) substrate (Calbiochem, MA) to visualize IgG SFC as

red spots. SFC were enumerated using an automated ELISPOT reader (Immunospot 3B, Cellular Technologies Ltd, OH) with aid of the Immunospot software version 5.0 (Cellular Technologies Ltd). Total and antigen-specific B_M SFC were calculated as SFC/ 10^6 cells. Isotype-specific frequencies of B_M against specific antigens are expressed as the percentages of specific B_M per total IgA or IgG (specific B_M SFC per 10^6 /total Ig SFC per $10^6 \times 100$).

2.5. Antibody measurements

ELISA was used to measure serum antibody against *Shigella* antigens as previously described [20]. End-point titers were reported as the inverse of the serum dilution that produced an OD of >0.2 above the blank.

2.6. Statistical analysis

Microsoft® Office Excel 2007 and GraphPad Prism 5.0 were used for statistical analysis. Hypotheses were evaluated using non-parametric two-sided tests. Correlations between immune response and disease indices were assessed using Spearman rho. The Wilcoxon signed rank test was used to assess continuous pre- to post-challenge antigen-specific immune responses.

3. Results

3.1. Correlations between LPS and IpaB-specific B_M with disease severity

B_M assays were performed using cryopreserved PBMC from volunteers before (pre) and after (post) challenge with wt-2457T as described in study design. B_M responses to *S. flexneri* 2a LPS were correlated with disease index. As shown in Fig. 1A and 1B as well as Table 2, post-challenge IgA LPS- B_M cell responses were found to correlate negatively ($\rho = -0.64$, $p = 0.02$) with disease index in the pre-exposed, but not in the naïve group. Of note, post-challenge IgA LPS- B_M also showed a significant negative correlation ($r = -0.48$, $p = 0.03$) with disease severity when all subjects were analyzed together ($n = 20$) (data not shown). In contrast, neither pre-challenge IgA and IgG nor post-challenge IgG B_M specific to LPS showed significant correlations with disease index (Table 2).

As mentioned in Methods (section 2.2.), in the original studies performed in the 1990's, the disease outcome following challenge was categorized dichotomously as "well" or "ill" [6]. As observed with disease indices, a negative correlation ($\rho = -0.52$, $p = 0.02$) was also observed between post-challenge IgA LPS- B_M and the dichotomous outcome categorization (data not shown). Moreover, the magnitude (mean $\pm$ SE) of post-challenge IgA LPS- B_M was significantly higher among the "well" ($n = 7$) compared to those who became "ill" ($n = 13$) following challenge (0.46 ± 0.28 vs 0.03 ± 0.02 , $p = 0.03$) (data not shown).

IpaB is an immunogenic *Shigella* antigen which is largely conserved among the various *Shigella* serotypes [11, 20, 21, 26, 27]. Therefore, we measured IpaB- B_M in pre- and post-challenge PBMC to investigate their correlation with disease outcome. Only pre-challenge IgA IpaB- B_M cells were observed to be negatively correlated ($\rho = -0.61$, $p = 0.03$) with disease index (Table 2, Figs 1C, D) among pre-exposed volunteers. In contrast to LPS- B_M , no statistically significant correlations were observed between post-challenge IgA IpaB- B_M and disease index (Table 2). However, both pre- (Figure 1 E, F) and post-challenge IgG IpaB- B_M (Table 2), showed a trend to be negatively correlated with disease index ($\rho = -0.47$ and $\rho = -0.46$, $p = 0.11$, respectively). A trend towards a negative correlation of pre- and post-challenge IgG IpaB- B_M with disease severity was also observed when all subjects were analyzed together ($n = 20$; $p = 0.12$ and $p = 0.09$ respectively, data not shown).

The IpaB-B_M measurements were also compared with dichotomous (i.e., well/ill) disease outcomes. Results showed similar trends to the correlations observed with disease index. However, as observed with LPS-B_M measurements, correlations with disease index generally exhibited higher significance than when the categorical well/ill definition was used (data not shown). The magnitude of pre-challenge IgA IpaB-B_M showed a trend to be higher in those who were “well” (n=7) comparing to those who were categorized as “ill” following challenge (n=13, 0.23 ± 0.04 vs 0.04 ± 0.04 , $p=0.09$). Similar results were also observed with pre-challenge IgG IpaB B_M (0.41 ± 0.20 vs 0.06 ± 0.04 , $p=0.15$) but not with post-challenge IgA and IgG IpaB-B_M. Throughout these studies, B_M responses were not observed to the LcrV antigen used as an experimental negative control (data not shown).

3.2. Correlations between LPS and Ipa-specific ASC with disease severity

In contrast to B_M assays which can be performed using cryopreserved specimens, ASC assays are typically performed on fresh cells. Therefore, LPS and Ipa- specific IgA and IgG ASC data from these 20 volunteers were retrieved from archived records of the original studies. Of note, at that time, Ipa-specific ASC assays were performed with a water-extract preparation [7] while in the present study, B_M assays and serological determinations experiments were performed with a recombinant IpaB protein used as antigen. In the pre-exposed group (n=13, see methods), among the pre-challenge ASC assays performed 7 days post vaccination or experimental challenge (i.e., IgA and IgG ASC specific for both LPS and Ipa), only IgA LPS-ASC showed a trend to be negatively correlated with disease index (n=13, $\rho=-0.45$, $p=0.12$) (data not shown). However, post-challenge ASC measured 7 days following challenge in the same group showed significant positive correlations between IgA LPS ($\rho=0.8$, $p<0.01$), IgG LPS ($\rho=0.57$, $p<0.04$) and IgA Ipa ($\rho=0.67$, $p=0.01$) ASC with disease severity (data not shown).

3.3. Correlations between LPS- and IpaB-specific antibody responses with disease severity

Serum antibody assays were performed with cryopreserved pre-and post-challenge specimens from 29 subjects. Fifteen out the 29 volunteers received 4 doses of EcSf2a-2 vaccine (vaccinated) prior to challenge and 14 were naïve controls as described in Methods. All subjects were challenged with the wt-2457T strain.

The vaccinated group showed a statistically significant negative correlation ($\rho=-0.52$, $p=0.05$) between pre-challenge IgG LPS-antibody with disease index (Table 3, Figure 2A). No significant negative correlations were observed between pre- and post-challenge IgG or IgA LPS-antibody titers with disease index in the naïve group (Table 3, Figure 2B).

Antibody titers against recombinant IpaB in these subjects were also measured and correlated with disease index. In contrast to the results observed with LPS, both pre-and post-challenge IgA IpaB-antibody titers in the vaccinated group, but not in the naïve group, showed a trend to be negatively correlated ($p=0.13$ and $p=0.07$, respectively) with disease index (Table 3, Figure 2C, D). On the other hand, pre-challenge IgG IpaB-antibody titers in vaccinated, but not naïve volunteers, showed a significant negative correlation ($\rho=-0.6$, $p=0.02$) with disease index (Table 3, Figure 2 E, F). Post-challenge IgG IpaB-antibody titers showed similar results with a trend toward a negative correlation with disease index in vaccinees ($\rho=-0.5$, $p=0.06$) (Table 3). Similar results showing negative correlations of pre- and post-challenge IgG IpaB with disease severity was also observed when all subjects were analyzed together (n=29; $p=0.03$ and $p=0.06$ respectively; data not shown).

4. Discussion

The identification of immune correlates of protection can greatly accelerate vaccine development [28, 29]. Immunological responses elicited by vaccines or natural infection that statistically “correlate with protection” may be causally responsible (mechanistic correlate) or may be non-mechanistic, which nevertheless predicts vaccine efficacy through its correlation with other immune response(s) that mechanistically protect [29]. The induction of B_M responses following vaccination or natural infection with enteric pathogens that we [19–23] and others [30, 31] have reported led to the speculation that B_M likely play an important role in protection. Recently, in a case-control study, the presence of *V. cholerae* O1 LPS-specific IgG-B_M cells in PBMC was associated with a decreased risk of infection among household contacts suggesting a role of IgG LPS-B_M in protection [32]. However, the possibility that B_M responses to enteric bacteria can be used as correlates of protection has not been directly addressed in a volunteer challenge model. In the present study we took advantage of a unique collection of cryopreserved specimens collected during clinical studies conducted in the early 1990’s at the CVD and measured B_M and antibody responses against two key *Shigella* antigens (*S. flexneri* 2a LPS and the conserved protein antigen IpaB) to investigate the presence of statistical correlations between these immune responses and protection against disease following challenge with wt-2457T.

These correlations were performed using a new disease categorization that graded the disease severity to explore whether this approach will add power to detect differences. The observation that a higher frequency of IgA LPS-B_M measured 28 days after challenge negatively correlated with disease severity in subjects who were pre-exposed to *Shigella* but not among naïve volunteers, suggests a possible role of IgA LPS-B_M responses in protection. This correlation was observed whether the disease index or well/ill dichotomous categorizations were used in the analysis, although significance levels were somewhat higher when disease index was used in the calculations. Of note, this correlation was absent with IgA LPS-B_M levels measured prior to challenge. It is possible that a considerable proportion of the LPS-B_M induced by exposure to *Shigella* antigens 28 days prior to challenge had already homed to their target sites (e.g., lymph nodes, bone marrow, gut) [33–35]. Upon the subsequent challenge with wt-2457T, a robust recall response involving a new wave of proliferation and differentiation ensued, resulting in the higher levels of LPS-B_M cells present in circulation 28 days later. This suggests that different time points might need to be evaluated to detect these responses. Moreover, the kinetics of B_M in circulation might be different for LPS (a T-cell independent antigen) than for IpaB (a protein antigen).”

The observation that the categorical outcome variable used in these studies as disease index instead of dichotomous determination of clinical outcome resulted in increased power to define immunological correlates of protection, suggests that this approach can be of value in upcoming vaccine and challenge studies.

The clinical studies conducted during the 1990’s suggested IgA-LPS ASC (plasmablasts/plasma cells) may be considered one of the key indicators of protection against wild type *Shigella* infection [7]. Similar to those observations, our post-hoc analysis showed a trend towards a negative correlation between pre-challenge LPS specific IgA-ASC and disease severity. Likewise, post-challenge IgA LPS B_M measurements showed a statistically significant negative correlation with disease severity, while post-challenge IgA LPS-ASC (along with IgG LPS or IgA Ipa-ASC) demonstrated a statistically significant positive correlation. These data suggest that the presence of higher ASC levels following challenge is indicative of a strong primary response to wild-type challenge rather than of a protective secondary response that were observed with IgA LPS-B_M.

Taken together, negative correlations of post-challenge IgA LPS-B_M, and pre-challenge IgA LPS-ASC (but not IgG), with disease severity support the previously advanced notion that mucosal immune responses against LPS may have a dominant role in protection against *Shigella* [7, 20, 21]. The current findings provide, to our knowledge, the first direct evidence that the frequency of IgA LPS-B_M correlates with protection from shigellosis following challenge with wild-type organisms.

The available evidence strongly suggests that serogroup and serotype specific-protection is elicited by natural *Shigella* infection or challenge [4, 6–8, 10, 36–38]; however whether the protection is mediated predominantly by mucosal (sIgA), systemic (IgG) anti-O antigen antibodies, and/or additional effector immune responses remains controversial [4]. The results in the present study showing that IgG but not IgA anti-LPS serum antibody titers negatively correlate with disease severity reinforces the notion that mucosal IgA and serum IgG anti-LPS antibodies play a role in protection. Of note, although our results suggest the lack of a significant role of serum IgA-anti LPS in protection, we observed a positive correlation between pre-vaccination IgA anti-LPS and disease index in the naïve group. It is unclear at present what could be the mechanism(s) that explain this counterintuitive association in naïve subjects. Future studies will be required to confirm these findings and address this issue in detail.

IpaB, one of the conserved components of the *Shigella* type III secreting system [27, 39], has been shown to be highly immunogenic among infected individuals, as well as in candidate vaccine trials [11, 12, 20, 21, 26]. Moreover, we had previously shown the induction of IgG and IgA B_M against IpaB following immunization with *Shigella* candidate vaccines [20, 21]. In the present study, using a highly purified recombinant IpaB preparation, we further expanded these observations by demonstrating for the first time an association of pre-existing anti-IpaB B_M and serum antibody levels with protection in a human challenge model. These results suggest that IpaB could also have a role in protection, providing additional support for the inclusion of this conserved protein as a component of broad based vaccines against *Shigella*.

As per protocol, all volunteers received antibiotics if severely ill or on day 5 post vaccination and challenge. Although at present it is not definitively known what effect, if any, this antibiotic treatment has on the immune response, it is probable that this effect is minimal since in symptomatic individuals as the bacteria has replicated sufficiently to induce disease and in asymptomatic individuals most shedding has disappeared by day 5, likely due to the host's immune response killing of *Shigella*.

A limitation of this study was the relatively low sample size. Future vaccination/challenge studies with larger numbers of volunteers will be required to confirm and expand the results described in these studies. Moreover, due to the limited number of cells available, measurement of immunity against a wide range of *Shigella* antigens (e.g., IpaC, IpaD VirG) was not possible. However, our results support the contention that protection against *Shigella* is likely mediated by multiple antigen-specific immune responses, including IgA B_M cells. This might constitute an advantage of live oral vaccines over subunit vaccines since they are likely to elicit stronger, more persistent and more diverse immune responses that results in enhanced protective immunity.

5. Conclusions

In this manuscript we provide, for the first time, initial evidence of a statistical association between the induction of LPS and IpaB antigen specific B memory cells and protection in a human *S. flexneri* 2a challenge model, suggesting that these responses may be important in

protecting the host from *shigella* infection. We also report significant negative correlations of pre-challenge IgG anti-LPS and anti-IpaB titers in vaccinated volunteers and disease severity upon challenge. These observations suggest that the presence of B_M cells should be considered an important component of the effector immunity to be evaluated in future clinical studies with relevant *Shigella* vaccines.

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Abbreviations

ASC	antibody secreting cell
B_M	memory B cells
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunosorbent spot assay
Ig	immunoglobulin
Ipa	Invasion plasmid antigen
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells
SFC	spot forming cells

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Highlights

- Studied immunity to LPS and IpaB antigens in a human *Shigella* challenge model
- Specific B memory cells inversely correlated with disease severity upon challenge
- LPS and IpaB antibodies also showed negative correlations with disease severity
- Shigella-specific B memory and antibodies might play a protective role in humans

Figure 1. Correlation of specific B_M and disease indices in subjects following challenge with wild-type *S. flexneri* 2a

Antigen specific B_M cells were measured before (Day 0) and after (Day 28) challenge with wild-type *Shigella flexneri* 2a. Percentages of antigen specific B_M among those subjects who were pre-exposed to either vaccine or wild-type *S. flexneri* 2a prior to challenge (details in Materials and Methods) are shown in panels A, C, E and among naïve volunteers are shown in panels B, D, F. The broken lines represent 95% confidence intervals.

Figure 2. Correlation of specific serum antibody titers and disease indices in subjects following challenge with wild-type *S. flexneri* 2a

Antigen specific serum antibody titers were measured before (Day 0) and after (Day 28) challenge with wild-type *Shigella flexneri* 2a. The antibody titers among those who were immunized with EcSf2a-2 (vaccinated) prior to challenge are shown in panels A, C, E and among naïve volunteers are shown in panels B, D, F. The broken lines represent 95% confidence intervals

Table 1

Disease index definitions

Disease index	Illness	Body Temp (°C)	Stool		
			Bloody (times/day)	Loose (times/day)	Vol. (mL) (total/day)
0	None	37.0–37.9	0	0	NA
1	Mild	38.0–38.9	1–2	1–2	<500
2	Moderate	39.0–40.0	3–10	3–10	500–2000
3	Severe	>40	>10	>10	>2000

Table 2.Correlation of disease indices with B_M

	pre-exposed (n=13) p(r)	naïve (n=7) P(r)	pre-exposed (n=13) p(r)	naïve (n=7) p(r)
IgA % B _M /Total	LPS		IpaB	
pre-challenge	0.92 (−0.02)	0.41 (−0.36)	0.03** (−0.61)	0.23 (0.52)
post-challenge	0.02** (−0.64)	0.90 (0.1)	0.80 (−0.05)	0.15 (−0.61)
IgG % B _M /Total	LPS		IpaB	
pre-challenge	0.60 (−0.16)	0.24 (−0.51)	0.11# (−0.47)	0.24 (0.53)
post-challenge	0.75 (−0.13)	0.24 (0.53)	0.11# (−0.46)	0.90 (−0.01)

p=p value; r=Spearman's rho; LPS=lipopolysaccharide; IpaB=invasion plasmid antigen-B pre-exposed: exposed to wild-type *Shigella* (n=3/13) or EcSf2a-2 vaccine (n=10/13) prior to challenge naïve: healthy volunteers without prior exposure to *Shigella*

pre-challenge: prior to challenge with wild-type *Shigella* (day 0)

post-challenge: day 28 following challenge with wild-type *Shigella*

** significant (p<0.05, two tail) with negative correlation

trend (p>0.05, 0.13, two tail) with negative correlation

Table 3

Correlation of disease indices with serum antibody titers

	IgA antibody titer		IgG antibody titer	
	vaccinated (n=15)	naïve (n=14)	vaccinated (n=15)	naïve (n=14)
	p (r)	p (r)	p (r)	p (r)
LPS		LPS		
pre-challenge	0.42 (−0.23)	0.02^{##} (0.61)	0.05^{**} (−0.52)	0.32 (0.29)
post-challenge	0.40 (0.24)	0.22 (0.35)	0.79 (−0.08)	0.19 (0.38)
IpaB		IpaB		
pre-challenge	0.13 [#] (−0.41)	0.72 (−0.11)	0.02^{**} (−0.60)	0.38 (−0.25)
post-challenge	0.07 [#] (−0.48)	0.80 (−0.08)	0.06 [#] (−0.50)	0.55 (−0.17)

p=p value; r=Spearm an's rho; LPS=lipopolysaccharide; Ipa B =invasion plasmid antigen-B vaccinated: immunized with 4 doses of EcSf2a-2

prior to challenge naïve: healthy volunteers without prior exposure to *Shigella*

pre-challenge: prior to challenge with wild-type *Shigella* (day 0)

post-challenge: day 28 following challenge with wild-type *Shigella*

^{**} significant (p<0.05, two tail) with negative correlation

[#] trend (p>0.05, 0.13, two tail) with negative correlation

^{##} significant (p<0.05, two tail) with positive correlation