RESEARCH ARTICLE
Host-Microbe Biology

Immune Response Characterization after Controlled Infection with Lyophilized *Shigella sonnei* 53G

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ABSTRACT *Shigella* is a major cause of moderate to severe diarrhea largely affecting children (<5 years old) living in low- and middle-income countries. Several vaccine candidates are in development, and controlled human infection models (CHIMs) can be useful tools to provide an early assessment of vaccine efficacy and potentially support licensure. A lyophilized strain of *S. sonnei* 53G was manufactured and evaluated to establish a dose that safely and reproducibly induced a ≥60% attack rate. Samples were collected pre- and postchallenge to assess intestinal inflammatory responses, antigen-specific serum and mucosal antibody responses, functional antibody responses, and memory B cell responses. Infection with *S. sonnei* 53G induced a robust intestinal inflammatory response as well as antigen-specific antibodies in serum and mucosal secretions and antigen-specific IgA- and IgG-secreting B cells positive for the $\alpha 4\beta 7$ gut-homing marker. There was no association between clinical disease outcomes and systemic or functional antibody responses postchallenge; however, higher lipopolysaccharide (LPS)-specific serum IgA- and IgA-secreting memory B cell responses were associated with a reduced risk of disease postchallenge. This study provides unique insights into the immune responses pre- and postinfection with *S. sonnei* 53G in a CHIM, which could help guide the rational design of future vaccines to induce protective immune responses more analogous to those triggered by infection.

IMPORTANCE Correlate(s) of immunity have yet to be defined for shigellosis. As previous disease protects against subsequent infection in a serotype-specific manner, investigating immune response profiles pre- and postinfection provides an opportunity to identify immune markers potentially associated with the development of protective immunity and/or with a reduced risk of developing shigellosis postchallenge. This study is the first to report such an extensive characterization of the immune response after challenge with *S. sonnei* 53G. Results demonstrate an association of progression to shigellosis with robust intestinal inflammatory and mucosal gut-homing responses. An important finding in this study was the association of elevated *Shigella* LPS-specific serum IgA and memory B cell IgA responses at baseline with reduced risk of disease. The increased baseline IgA responses may contribute to the lack of dose response observed in the study and suggests that IgA responses should be further investigated as potential correlates of immunity.

KEYWORDS *Shigella*, immunogenicity, controlled human infection model, antibody, immunological memory, gut-homing responses, *Shigella sonnei*, gut homing

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Shigella is a significant cause of bacillary dysentery, causing moderate to severe diarrhea (MSD) in travelers, as well as in children in low- and middle-income countries (LMIC) (1–4). *Shigella* has also been shown to be a significant cause of watery diarrhea using more sensitive molecular methods (5). In 2016, *Shigella* was identified as the second leading cause of diarrhea-associated mortality across all age groups, with an increased disease burden among children under the age of 5 (6, 7). Children are also at risk of impaired development after repeated enteric infections (8–12). Infection with *Shigella* species can lead to reduced gut permeability, causing a reduction in intestinal absorption of nutrients and subsequently leading to childhood cognitive and physical stunting (11, 13). Children stunted from the heavy burden of *Shigella* infection are also at higher risk of dying from other infectious diseases (14). *Shigella* remains a high-priority vaccine target for the World Health Organization because of its high global burden and increasing antibiotic resistance (15).

Although several vaccine candidates are in clinical development, there is currently no licensed *Shigella* vaccine. A tool that is useful for early assessment of vaccine efficacy and, at times, utilized to support licensure is the controlled human infection model (CHIM) (16–18). In response to recent calls for model standardization (19–24), a lyophilized strain of *Shigella sonnei* 53G was produced using current good manufacturing practice (cGMP) and evaluated in a dose-escalation manner to determine a dose that safely and reproducibly yielded a ≥60% attack rate for shigellosis (25). Disease outcomes and attack rates with the lyophilized strain of *S. sonnei* 53G are described elsewhere (25).

That study offered a unique opportunity to characterize systemic and mucosal immune responses given the repeated collection of multiple samples throughout the study. Although *S. sonnei* is traditionally associated with infection in industrialized nations, recent reports have indicated a paradigm shift in the incidence of *S. sonnei* infection across developing nations with impacts on adult, child, and infant health (26–30). For this reason, intestinal inflammatory responses were also characterized pre- and postinfection, given its association with stunting and enteric enteropathy in infants and young children in low-resource settings. Since prior disease caused by *Shigella* infection can result in protection from subsequent infection in a serotype-specific manner (31–35), investigation of the immune responses associated with multiple disease outcomes postinfection could provide further insights into potential mechanistic or nonmechanistic immune correlates of protection. Investigating correlates, or surrogates, of protection can also help guide vaccine development and interpretation of results in future *Shigella* CHIMs aimed at assessing vaccine efficacy.

As previously reported, lipopolysaccharide (LPS)-specific serum and fecal IgG and IgA responses postchallenge were not associated with challenge dose or clinical disease outcomes, including dysentery, diarrhea severity, and disease severity score (25). These findings highlight potential gaps in understanding the contribution of other immunological or nonimmunological protective mechanisms and may also confirm the importance of immune responses to nonpolysaccharide antigens (36–38).

Finally, a subset of orally challenged volunteers did not exhibit disease symptoms postchallenge, regardless of dose received (567 to 1,760 CFU [25]). Therefore, a more comprehensive evaluation of the *Shigella*-specific immune responses at baseline and at several time points postchallenge was undertaken. Investigating potential immunological mechanisms associated with observed resistance to infection and subsequent lack of association between immune responses and disease outcomes may also provide insights into potential correlates of protection.

RESULTS

Fecal inflammatory marker responses. Fecal calprotectin and myeloperoxidase concentrations were determined in stool samples from volunteers with ($n = 18$) or without ($n = 26$) shigellosis. A substantial increase in calprotectin and myeloperoxidase (all $P \leq 0.002$; 2-way analysis of variance [ANOVA]) concentrations was observed 3 days postchallenge, regardless of shigellosis outcome, with concentrations returning to

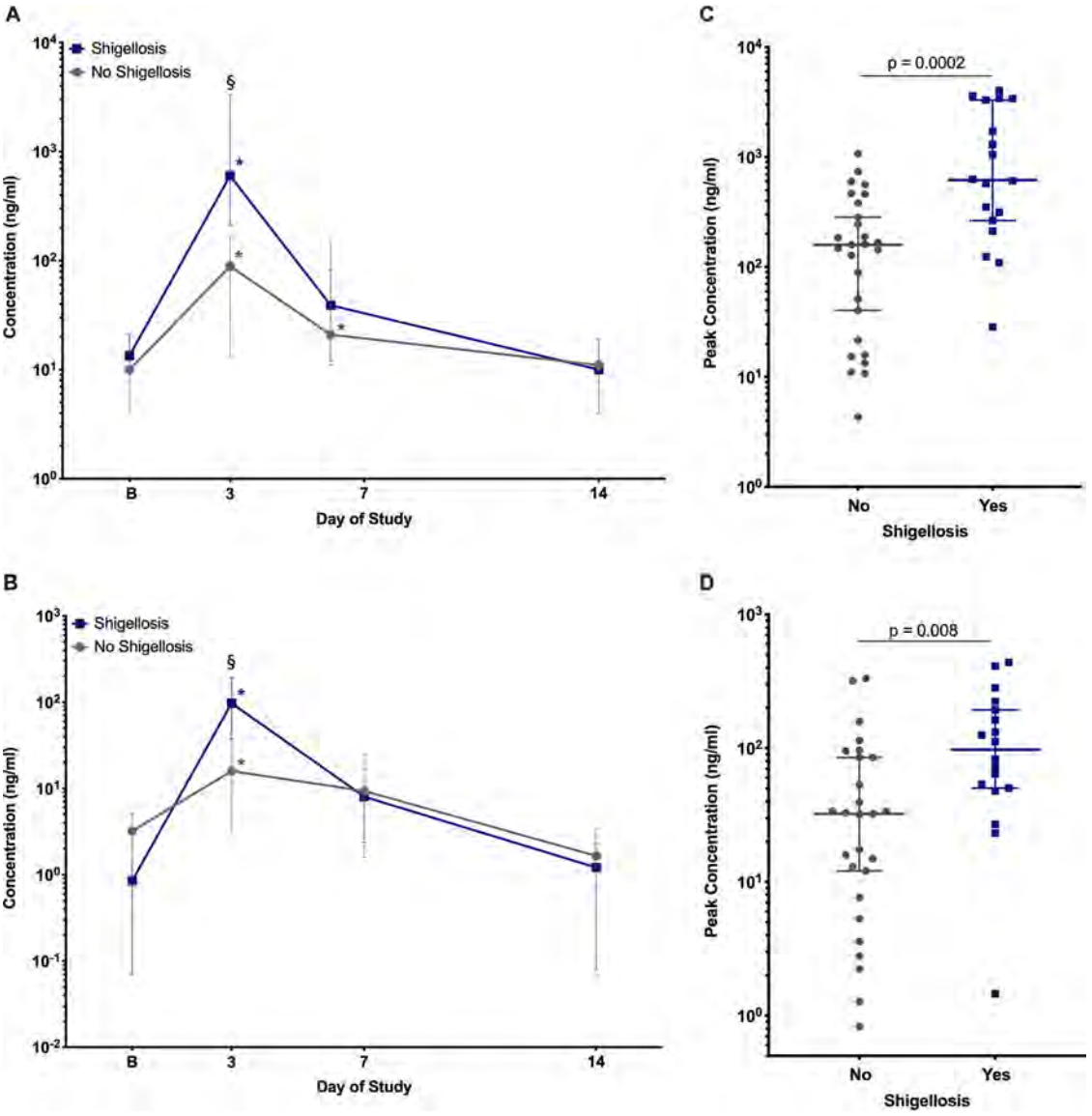

FIG 1 (A and B) Median fecal calprotectin (A) and myeloperoxidase (B) concentrations with 95% confidence intervals at baseline ("B" on the *x* axis) and 3, 7, and 14 days postchallenge, grouped by volunteers with ($n = 18$) or without ($n = 27$) shigellosis. *, significant difference compared to baseline concentrations within shigellosis outcome group; §, significant difference in concentrations between shigellosis groups at the same time point. Significance was determined by 2-way ANOVA of log-transformed concentrations with a Bonferroni *post hoc* test. (C and D) Individual peak fecal calprotectin (C) and myeloperoxidase (D) concentrations with group medians and 95% confidence intervals. *P* values were determined by *t* test of log-transformed concentrations.

baseline levels by day 14 (Fig. 1A and B). Volunteers with shigellosis had significantly higher concentrations of both fecal inflammatory markers whether day 3 concentrations (all $P \leq 0.018$; 2-way ANOVA) (Fig. 1A and B) or peak (observed on day 3 or 7) concentrations ($P = 0.0002$ [calprotectin] and $P = 0.008$ [myeloperoxidase]; *t* test) (Fig. 1C and D) were compared, demonstrating an association of increased intestinal inflammation with progression to shigellosis. In addition, fecal inflammatory marker peak concentrations were associated with multiple other disease outcomes, including diarrhea severity, disease severity score, and dysentery (Fig. S1 and S2).

Serum antibody responses. *Shigella* antigen-specific serum IgG, IgA, IgM, and IgG subclass responses were determined for all volunteers and compared across groups with or without shigellosis. LPS-specific serum IgG, IgA, and IgM levels peaked 14 days postchallenge and remained elevated over baseline levels 14 and 28 days postchallenge (all $P \leq 0.003$; 2-way ANOVA) (Fig. 2). There were no significant differences in

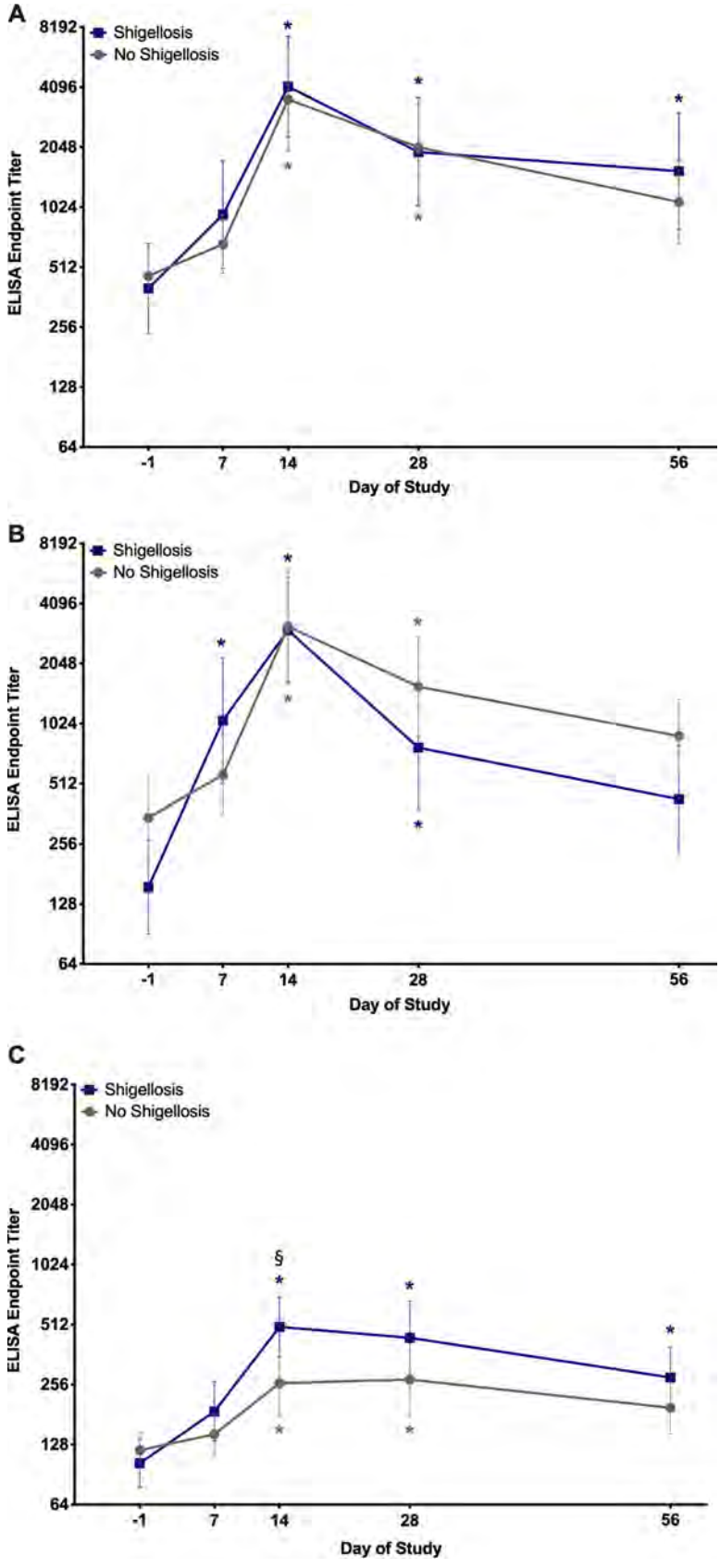

FIG 2 *S. sonnei* LPS-specific serum IgG (A), IgA (B), and IgM (C) geometric mean ELISA endpoint titers and 95% confidence intervals prior to challenge (day −1) and 7, 14, 28, and 56 days postchallenge, grouped

(Continued on next page)

LPS-specific serum IgG or IgA titers postchallenge between volunteers with or without shigellosis (Fig. 2A and B). LPS-specific serum IgM levels on day 14 were higher ($P = 0.033$; 2-way ANOVA) in volunteers with shigellosis (Fig. 2C). Serum IgG and IgA responses directed to additional antigens, including the native Invaplex (IVP) antigen (39) and invasion plasmid antigens IpaB and IpaC, were investigated; however, no differences in peak serum titers postinfection were observed across shigellosis outcome groups (Table S1). IVP-specific titers showed the highest magnitude of response, followed by IpaB and IpaC. Although a moderate increase ($P = 0.0008$; 1-way ANOVA) in LPS-specific serum IgG1 titers was observed by day 14 across all volunteers, there was no difference in peak LPS-specific serum IgG1 responses across volunteers with or without shigellosis (Table S1). There were minimal or undetectable increases in LPS-specific serum IgG2, IgG3, and IgG4 responses postinfection and no differences in peak titer in these IgG subclass responses across shigellosis outcome (Table S1).

$\alpha4\beta7$ ALS responses. To determine antibodies in lymphocyte supernatant (ALS) responses, robust LPS- and IVP-specific $\alpha4\beta7^+$ IgA (all $P \leq 0.0001$; 2-way ANOVA) (Fig. 3A and C) and IgG (all $P \leq 0.0006$; 2-way ANOVA) (Fig. 3B and D) responses were observed 7 days postinfection, regardless of shigellosis outcome. Volunteers with shigellosis had significant increases over baseline $\alpha4\beta7^+$ responses by day 5 postinfection across all antigens and isotypes (all $P \leq 0.048$; 2-way ANOVA) (Fig. 3). Although lower in magnitude, similar trends in the $\alpha4\beta7^-$ populations were observed with peak responses 7 days postinfection. With the exception of the LPS-specific IgA responses, volunteers with shigellosis had higher LPS-specific IgG and IVP-specific IgG and IgA $\alpha4\beta7^-$ titers on day 7 compared to baseline (all $P \leq 0.006$; 2-way ANOVA) (Fig. 3B to D).

Across both shigellosis outcome groups, day 7 LPS- and IVP-specific $\alpha4\beta7^+$ IgA and IgG responses were higher than $\alpha4\beta7^-$ responses (all $P < 0.0001$; 2-way ANOVA) (Fig. 3). LPS-specific $\alpha4\beta7^+$ IgG and IVP-specific $\alpha4\beta7^+$ IgA responses were also increased compared to $\alpha4\beta7^-$ responses 5 days postchallenge ($P = 0.037$ and $P = 0.033$, respectively; 2-way ANOVA) (Fig. 3B and C).

Volunteers progressing to shigellosis had substantially higher $\alpha4\beta7^+$ responses on day 7 than volunteers without shigellosis (all $P < 0.0001$; 2-way ANOVA) (Fig. 3). Interestingly, IVP-specific $\alpha4\beta7^-$ IgA and IgG responses on day 7 were significantly increased ($P = 0.011$ and $P < 0.0001$, respectively; 2-way ANOVA) (Fig. 3C and D) in volunteers with shigellosis, but this difference was not observed in the LPS-specific $\alpha4\beta7^-$ antibody responses (Fig. 3A and B). In addition to the association with shigellosis, LPS- and IVP-specific $\alpha4\beta7^+$ IgA responses were associated with diarrhea severity, disease severity score, and dysentery (Fig. S3 and S4). LPS- and IVP-specific $\alpha4\beta7^+$ IgG responses showed similar trends and were also associated with all aforementioned disease outcomes at similar levels of significance (data not shown).

Fecal antibody responses. Fecal IgA and IgG responses were determined for all volunteers and compared across shigellosis outcome groups. In volunteers with shigellosis, peak titers of LPS-specific fecal IgA and IgG were observed 7 days postinfection, and although they were lower by day 14, they remained elevated over baseline (all $P < 0.0001$; 2-way ANOVA) (Fig. 4A and B). In contrast, LPS-specific fecal IgA and IgG responses in volunteers without shigellosis continued to increase through study day 14 (all $P \leq 0.049$; 2-way ANOVA) (Fig. 4A and B). LPS-specific fecal IgA and IgG titers on day 7 were higher in volunteers with shigellosis than volunteers without (all $P \leq 0.045$; 2-way ANOVA) (Fig. 4A and B), with similar trends and levels of significance observed in the IVP-specific fecal antibody responses (data not shown). Fecal antibody responses were compared with $\alpha4\beta7^+$ ALS antibody responses, revealing a moderate correlation

FIG 2 Legend (Continued)
by volunteers with ($n = 22$) or without ($n = 34$) shigellosis. *, significant difference compared to baseline titers within shigellosis group; §, significant difference in titers between shigellosis groups at the same time point. Significance was determined by 2-way ANOVA of log-transformed titers with a Bonferroni *post hoc* test.

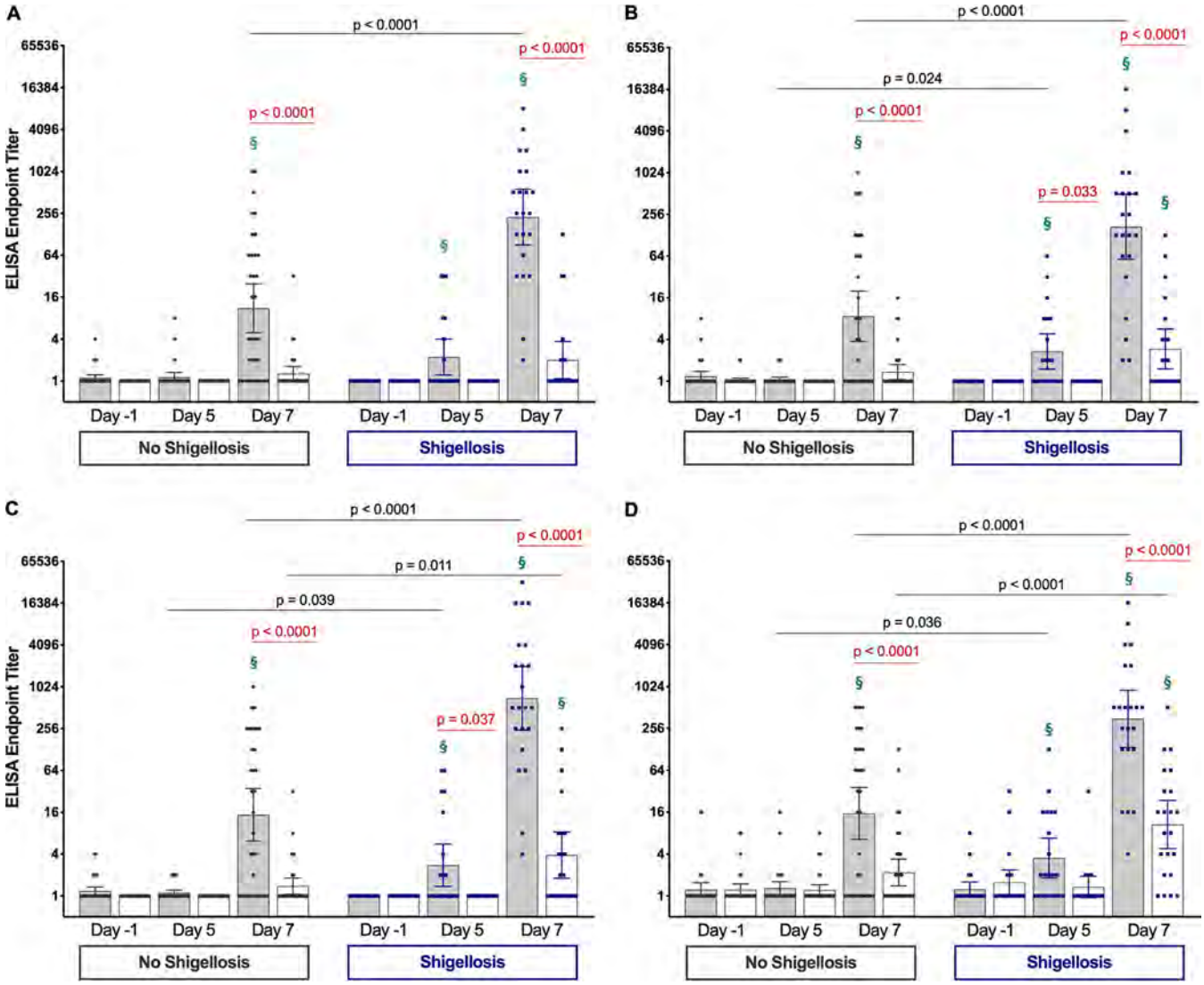

FIG 3 Individual ALS IgA and IgG ELISA endpoint titers with group geometric mean and 95% confidence intervals for $\alpha4\beta7^+$ (gray bars) and $\alpha4\beta7^-$ (white bars) populations prior to challenge (day −1) and 5 and 7 days postchallenge, grouped by volunteers with ($n = 22$) and without ($n = 34$) shigellosis. (A) *S. sonnei* LPS-specific ALS IgA; (B) *S. sonnei* LPS-specific ALS IgG; (C) *S. sonnei* IVP-specific ALS IgA; (D) *S. sonnei* IVP-specific ALS IgG. §, significant compared to matched $\alpha4\beta7^+$ or $\alpha4\beta7^-$ baseline titers within the shigellosis group. *P* values in red compare $\alpha4\beta7^+$ and $\alpha4\beta7^-$ titers on a given study day within the shigellosis group. *P* values in black compare matched $\alpha4\beta7^+$ or $\alpha4\beta7^-$ titers on a given study day across shigellosis groups. Significance across all parameters was determined by 2-way ANOVA of log-transformed ALS titers with a Bonferroni *post hoc* test.

when peak rise (fold) in fecal IgA or IgG was compared with peak rise in $\alpha4\beta7^+$ ALS IgA or IgG ($r = 0.51$ [IgA], $r = 0.53$ [IgG]; $P < 0.0001$; Spearman correlation) (Fig. 4C and D).

Memory B cell responses. ALS titers of LPS- and IVP-specific memory B cell IgG and IgA on day 28 were elevated over baseline (all $P \leq 0.013$; 2-way ANOVA), and although responses decreased by day 56, ALS titers remained elevated over baseline levels (data not shown). With the exception of the LPS-specific memory B cell IgG responses, volunteers with shigellosis had larger rises in LPS-specific IgA and IVP-specific IgG and IgA memory B cell ALS titers than volunteers without shigellosis (all $P \leq 0.027$; Mann-Whitney U test) (Fig. 5).

SBA responses. Peak *S. sonnei*-specific serum bactericidal activity (SBA) was observed 14 days postchallenge; however, levels were increased over baseline by day 7 (all $P < 0.0001$; 2-way ANOVA) (Fig. 6A) and remained elevated through study day 56 (all $P \leq 0.024$; 2-way ANOVA) (Fig. 6A), regardless of shigellosis outcome. There were no differences in peak *S. sonnei*-specific bactericidal activity across volunteers with or

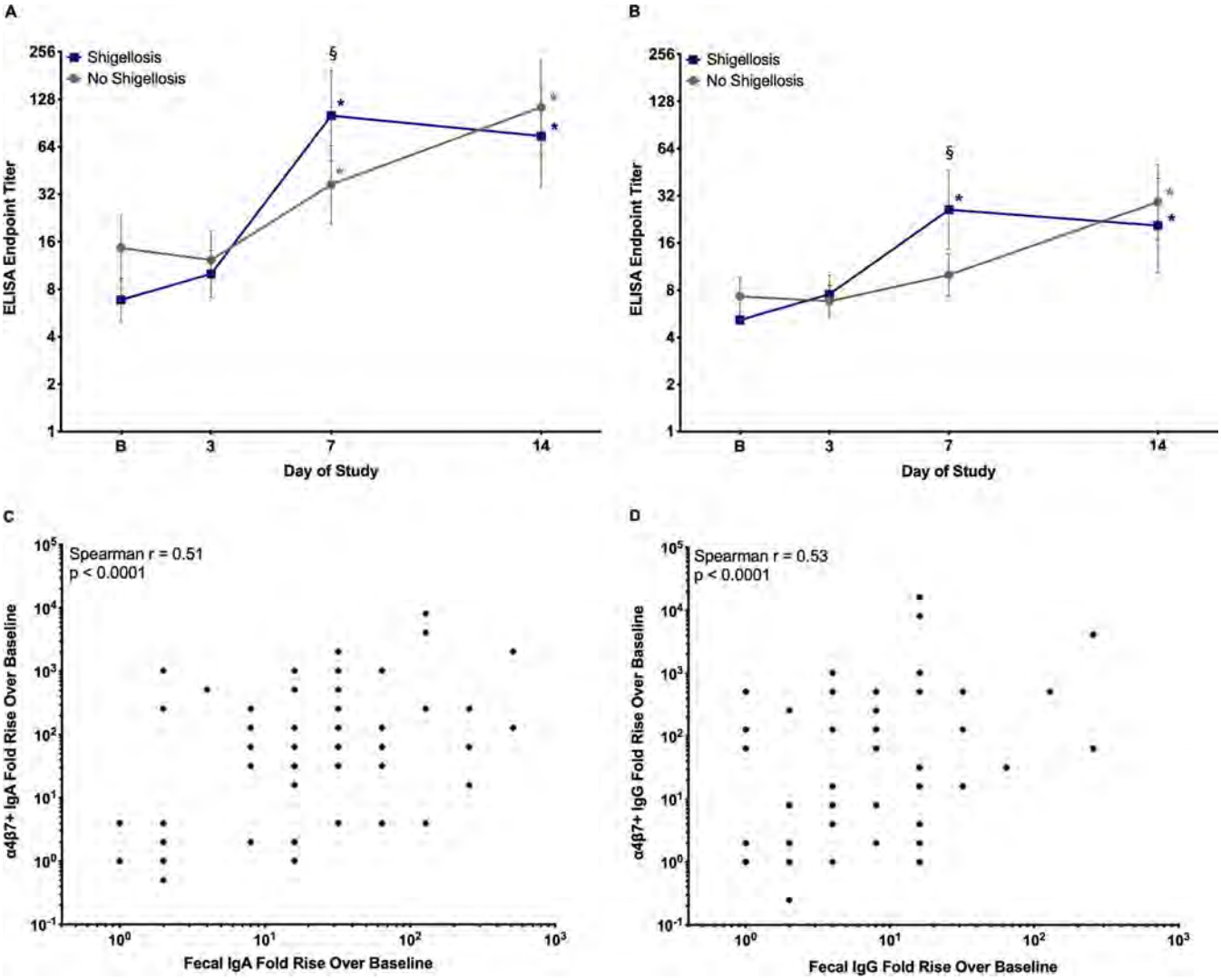

FIG 4 (A and B) *S. sonnei* LPS-specific fecal IgA (A) and IgG geometric mean ELISA endpoint titers (B) with 95% confidence intervals at baseline ("B" on the *x* axis) and 3, 7, and 14 days postchallenge, grouped by volunteers with ($n = 22$) or without ($n = 34$) shigellosis. *, significant difference compared to baseline titers within the shigellosis group; §, significant difference in titers between shigellosis groups at the same time point. Significance was determined by 2-way ANOVA of log-transformed titers with a Bonferroni *post hoc* test. (C and D) Spearman correlation of peak fold rise in *S. sonnei* LPS-specific $\alpha4\beta7^+$ ALS and fecal IgA titers (C) and *S. sonnei* LPS-specific $\alpha4\beta7^+$ ALS and fecal IgG titers (D).

without shigellosis (Fig. 6B), and although the difference was not significant, it is interesting that volunteers without shigellosis had higher *S. sonnei*-specific bactericidal titers 28 and 56 days postinfection (Fig. 6A). Minimal increases in *Shigella flexneri* 2a-specific SBA over baseline were observed, and there were no differences in peak titer (data not shown) or peak rise over baseline (Fig. 6C) across shigellosis outcome groups. A total of 8 volunteers had a 4-fold or greater rise over baseline in *S. flexneri* 2a-specific bactericidal titers, and interestingly, of these 8 volunteers, 7 did not progress to shigellosis (Fig. 6C).

Baseline immune responses. A thorough evaluation of pre-existing immunity was conducted to better understand the lack of association between disease severity and certain immune parameters (Fig. 7) in this study population. As described in the accompanying article (25), 171 volunteers were screened for study inclusion. After exclusions based on other criteria, 130 volunteers were screened for *S. sonnei* LPS-specific serum IgG titers between study days −45 to −2. Of these 130, 4 volunteers (~3%) had titers greater than the 2,500 cutoff used for study inclusion and were excluded from the study (Table S2).

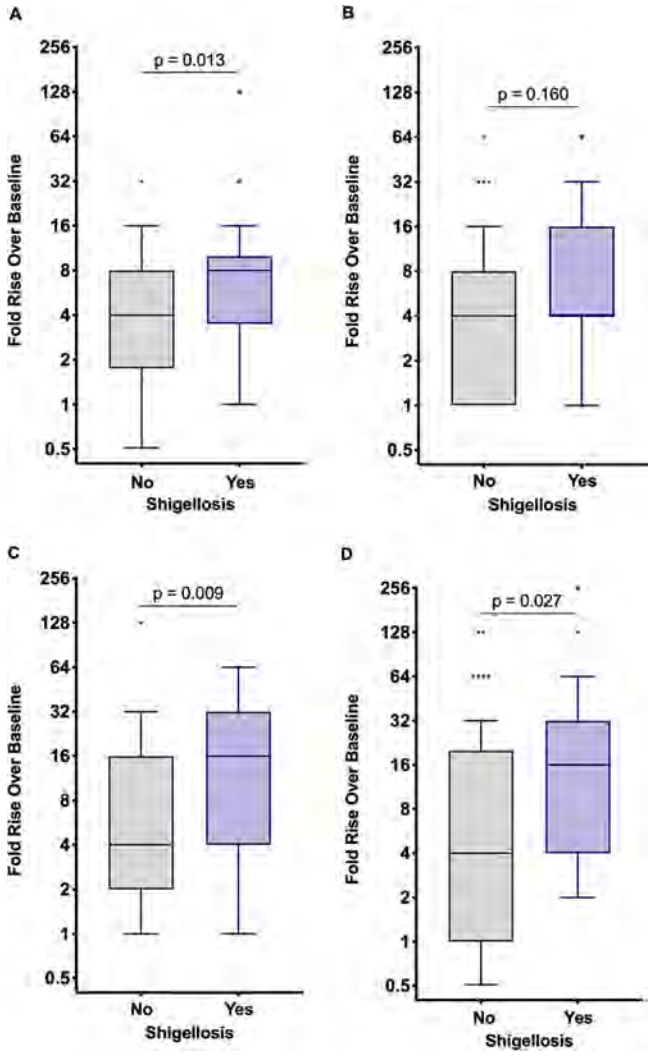

FIG 5 Tukey box-and-whisker plots (whiskers present first and third quartiles) of fold rise over baseline memory B cell ALS ELISA endpoint titers, grouped by volunteers with ($n = 22$) and without ($n = 34$) shigellosis. (A) *S. sonnei* LPS-specific memory B cell ALS IgA; (B) *S. sonnei* LPS-specific memory B cell ALS IgG; (C) *S. sonnei* IVP-specific memory B cell ALS IgA; (D) *S. sonnei* IVP-specific memory B cell ALS IgG. *P* values were determined by the Mann-Whitney U test.

Of the enrolled volunteers, there were no differences in the LPS-specific serum IgG titers at baseline (study day −1) across shigellosis outcome group, and with the exception of one individual, all volunteers had baseline LPS-specific serum IgG titers within 2-fold of the 2,500 cutoff for inclusion (Fig. 7A). In contrast, increased LPS-specific serum IgA baseline titers were associated with volunteers not progressing to shigellosis ($P = 0.027$; *t* test) (Fig. 7A). A similar association was observed with baseline *S. sonnei*-specific SBA ($P = 0.038$; Mann-Whitney U test) (Fig. 7B) as well as LPS-specific fecal IgA and IgG titers at baseline ($P = 0.027$ and $P = 0.035$, respectively; Mann-Whitney U test) (Fig. 7C). There was also a significant association of increased LPS-specific memory B IgA baseline titers with volunteers not progressing to shigellosis ($P = 0.034$; *t* test) (Fig. 7D); however, the same association was not observed in the LPS-specific memory B IgG baseline titers ($P = 0.082$; *t* test) (data not shown). LPS-specific serum IgA baseline titers were also inversely associated with diarrhea severity, dysentery, and disease severity score (Fig. S5). Logistic regression analyses were used to investigate the association of increasing baseline LPS-specific serum IgG or IgA enzyme-linked immunosorbent assay (ELISA) titers with progression to either MSD or shigellosis (Table 1). Neither the unadjusted nor adjusted models showed an association of

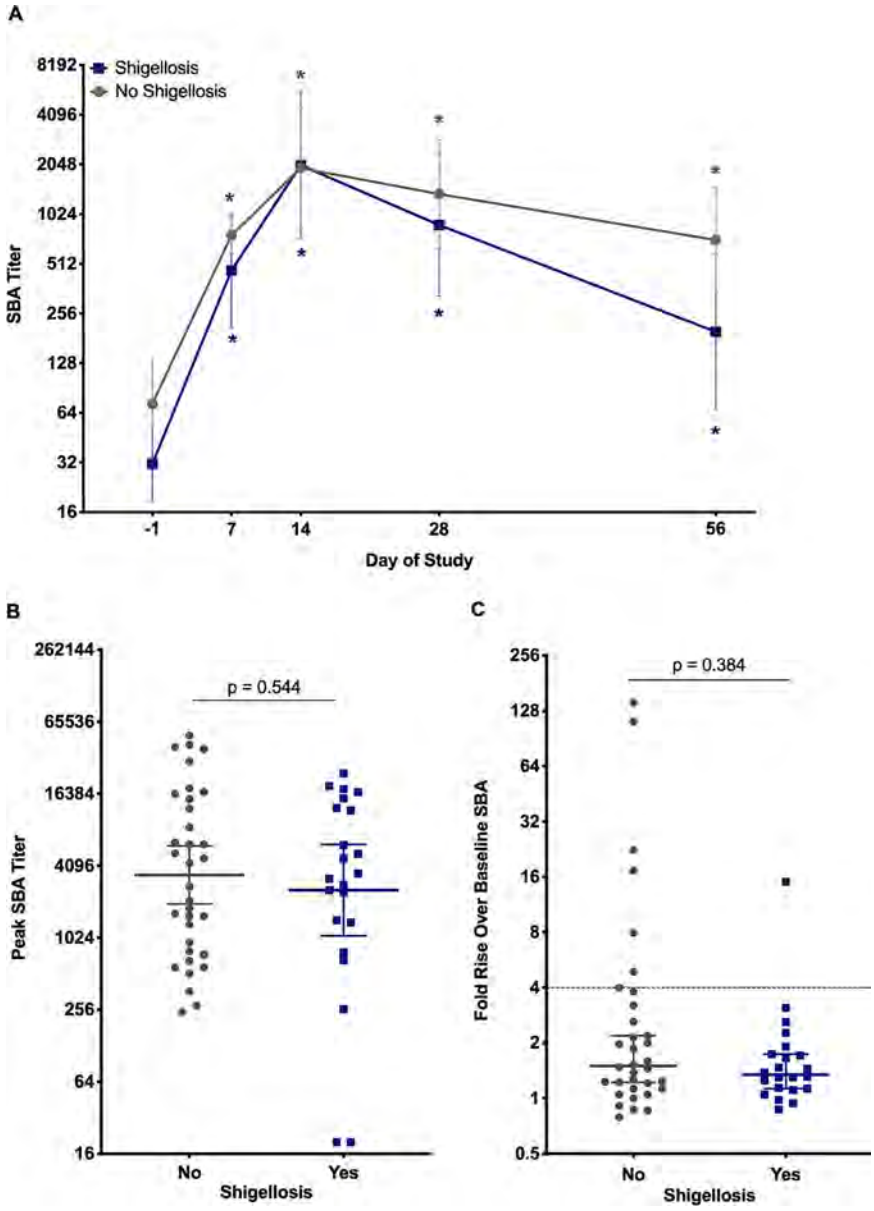

FIG 6 (A) Geometric mean *S. sonnei*-specific serum bactericidal titers (GMT) and 95% confidence intervals prior to challenge (day −1) and 7, 14, 28, and 56 days postchallenge, grouped by volunteers with ($n = 22$) or without ($n = 34$) shigellosis. *, significant difference compared to baseline titers within shigellosis group. Significance was determined by 2-way ANOVA of log-transformed titers with a Bonferroni *post hoc* test. (B) Individual peak *S. sonnei*-specific SBA titers with group GMT and 95% confidence intervals, grouped by volunteers with ($n = 22$) and without ($n = 34$) shigellosis. *P* values were determined by *t* test of log-transformed titers. (C) Individual fold rise in *S. flexneri* 2a-specific SBA titers over baseline with group median and 95% confidence interval grouped by volunteers with ($n = 22$) and without ($n = 34$) shigellosis (the line at 4 indicates the cutoff for definition of a responder). *P* values were determined by Mann-Whitney U test.

increasing baseline LPS-specific serum IgG with either outcome of interest. In contrast, both the unadjusted and adjusted models showed a significant association between progression to shigellosis or MSD and increasing baseline LPS-specific serum IgA titers. In the adjusted model, each fold increase in baseline LPS-specific serum IgA titer resulted in nearly a 40% reduction in odds of progression to shigellosis (odds ratio [OR] = 0.61; 95% confidence interval [CI] = 0.40 to 0.94) (Table 1), with an area under the receiver operating characteristic (ROC) curve of 0.777. Similarly, increasing baseline LPS-specific serum IgA titers resulted in nearly a 50% reduction in odds of progression

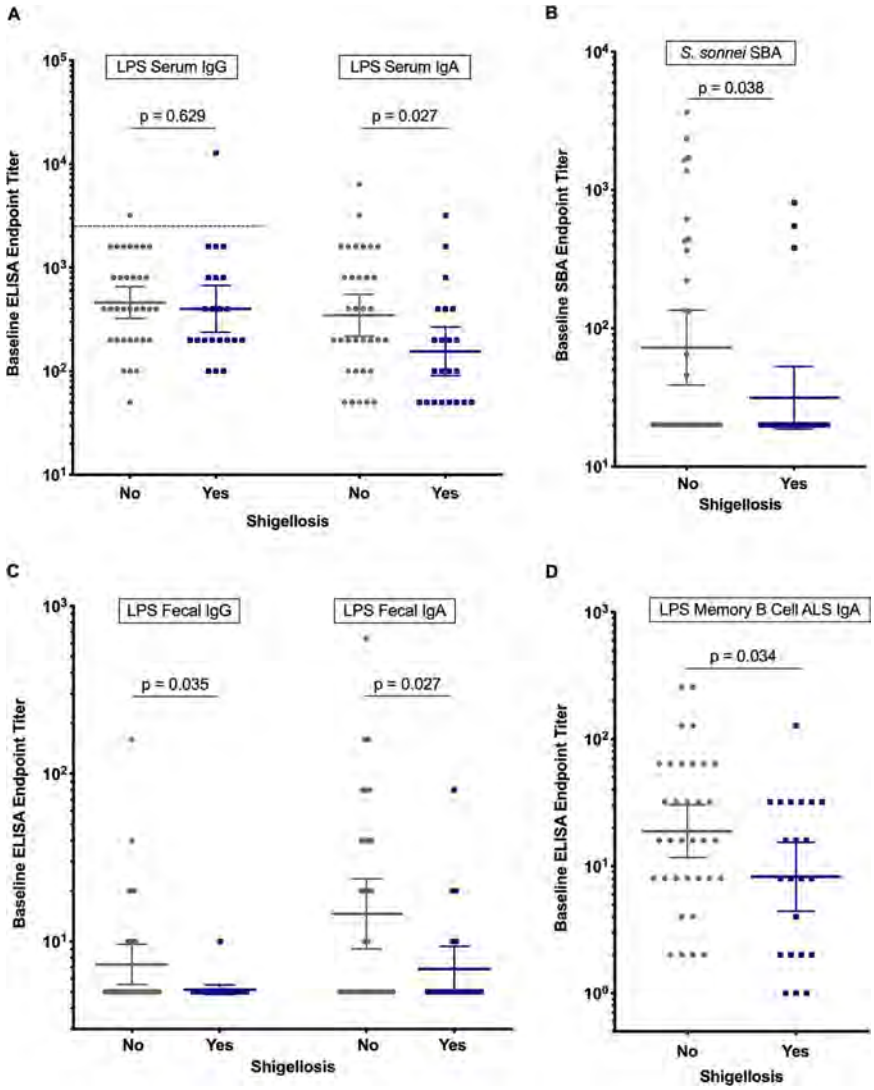

FIG 7 Individual ELISA or SBA endpoint titers, with group geometric means and 95% confidence intervals, prior to challenge (baseline), grouped by volunteers with ($n = 22$) and without ($n = 34$) shigellosis. (A) *S. sonnei* LPS-specific serum IgG (the dashed line indicates the 2,500 cutoff titer used for study inclusion) and *S. sonnei* LPS-specific serum IgA (*P* values were determined by *t* test of log-transformed titers); (B) *S. sonnei* serum bactericidal activity (*P* values were determined by Mann-Whitney U test); (C) *S. sonnei* LPS-specific fecal IgG and *S. sonnei* LPS-specific fecal IgA (*P* values were determined by Mann-Whitney U test); (D) *S. sonnei* LPS-specific memory B cell ALS IgA (*P* values were determined by *t* test of log-transformed titers).

to MSD (OR = 0.51; CI = 0.32 to 0.80) (Table 1) with an area under the ROC curve of 0.831. Additional cut-point analyses suggest LPS-specific serum IgA titers of ≤300 and ≤150 as optimal cut-points for predicting progression to MSD or shigellosis, respectively (data not shown).

DISCUSSION

The primary objective of the *S. sonnei* 53G CHIM was to determine a dose of a cGMP-manufactured lyophilized strain that would safely and reproducibly induce a ≥60% shigellosis rate in challenged volunteers (25). The study also provided a unique opportunity to extensively characterize the *Shigella*-specific systemic, mucosal, and functional antibody immune responses before and after infection with *S. sonnei* 53G in a more robust manner than previous studies. The more expansive immune response characterization further facilitates the understanding of host resistance to infection and

TABLE 1 Logistic regression models investigating the association of progression to shigellosis or moderate-severe diarrhea with baseline *S. sonnei* LPS-specific serum IgG and IgA ELISA titers[a]

Outcome of interest	Unadjusted OR (CI)	*P* value	Adjusted OR (CI)[b]	*P* value
Shigellosis				
Gender				
Male (ref)	1.00		1.00	
Female	0.55 (0.18–1.63)	0.277	0.37 (0.09–1.47)	0.158
Age (yr)				
18–25 (ref)	1.00		1.00	
26–49	0.69 (0.20–2.42)	0.564	0.54 (0.11–2.60)	0.439
Dose (CFU)	**7.65 (1.44–40.6)**	**0.017**	**15.3 (1.91–122.0)**	**0.010**
Baseline LPS-specific serum IgG	0.89 (0.61–1.29)	0.534	1.27 (0.72–2.24)	0.404
Baseline LPS-specific serum IgA	**0.70 (0.51–0.97)**	**0.033**	**0.61 (0.40–0.94)**	**0.026**
Moderate-severe diarrhea				
Gender				
Male (ref)	1.00		1.00	
Female	1.03 (0.35–2.98)	0.961	1.61 (0.40–6.53)	0.504
Age (yr)				
18–25 (ref)	1.00		1.00	
26–40	1.39 (0.36–5.30)	0.633	1.40 (0.23–8.51)	0.712
41–49	2.40 (0.52–11.0)	0.259	7.21 (0.87–59.6)	0.067
Dose (CFU)	**5.08 (1.10–23.5)**	**0.038**	**15.9 (1.95–129.5)**	**0.010**
Baseline LPS-specific serum IgG	0.79 (0.55–1.15)	0.216	1.18 (0.66–2.09)	0.582
Baseline LPS-specific serum IgA	**0.60 (0.43–0.85)**	**0.004**	**0.51 (0.32–0.80)**	**0.004**

[a]OR, odds ratio; CI, confidence interval. Boldface indicates outcomes that are statistically significant.
[b]Model adjusted for gender, age category, log-transformed dose, and baseline LPS-specific serum IgG, and IgA ELISA titer.

could lead to determining immune markers that may be associated with recovery from shigellosis and/or protection against illness upon re-exposure. While the study did not include uninfected or placebo-challenged controls, comparisons of postchallenge immune responses to baseline responses strengthened the analyses of immune response associations with disease outcomes. Infection with *S. sonnei* induced robust intestinal inflammatory and mucosal responses, as observed by sharp increases in fecal inflammatory markers and $\alpha4\beta7^+$ ALS antibody responses in diseased volunteers, and both of these immune parameters were significantly associated with multiple disease outcomes. Interestingly, there was a lack of association with postinfection systemic immune responses (serum antibodies and bactericidal activity) and disease outcomes. The relationship between intestinal inflammatory markers and disease outcomes noted above is intriguing and deserves further investigation into its potential relationship to immune outcomes that are associated with recovery from acute illness or may be predictive of a reduced risk of illness upon re-exposure to *Shigella sonnei*.

Fecal IgA responses are often used as a measure of the mucosal immune response postinfection with *Shigella*. In this study, fecal IgG responses were also evaluated. Antibody responses from B cells positive for the gut homing marker $\alpha4\beta7$ were also determined and presented as a measure of the mucosal immune response. Because $\alpha4\beta7^+$ cells home to the intestinal mucosa and secrete IgA and IgG antibodies, which are actively transported or passively transudated into the lumen of the large intestine, the correlation of these two mucosal measurements was of particular interest. The observed correlation of $\alpha4\beta7^+$ ALS IgG and fecal IgG suggests that the IgG-secreting $\alpha4\beta7^+$ cells may be partially responsible for the antigen-specific IgG detected in stool samples in addition to IgG transudate from systemic circulation.

Antecedent shigellosis reduces the risk of subsequent infection in a serotype-specific manner, indicating a role for LPS-specific immune responses in protection (31–33, 40). LPS-specific serum IgG has been indicated as an immune correlate of protection responsible for this observed serotype-specific protection (41, 42); however, several of these studies have investigated serum IgG levels after parenteral immunization with conjugate vaccines, which would be expected to induce large amounts of

LPS-specific serum IgG (41, 43–46). Furthermore, immunological analyses in many previous studies has been limited with incomplete analysis of other immune parameters outside serum IgG. In contrast, results from this study and other studies evaluating mucosal routes of immunization or infection (42, 47–49) suggest that LPS-specific IgA also contributes to protection. Together, these data provide evidence that there exist alternate mechanisms to induce a protective immune response, depending on the route of antigen exposure.

Interestingly, increased LPS-specific serum IgA, IgA-secreting memory B cells, and SBA titers at baseline were inversely associated with risk of shigellosis. Increased baseline serum IgA titers have also been associated with reduced risk of disease in enterotoxigenic *Escherichia coli* (ETEC) CHIMs (50) as well as reduced susceptibility to cholera infection in endemic settings (49). In an effort to recruit immunologically naive volunteers, exclusion criteria for this study included recent travel to areas where *Shigella* is endemic, known history of culture-confirmed *Shigella* infection, and a titer of *S. sonnei* LPS-specific serum IgG, determined by ELISA, of >2,500; however, LPS-specific serum IgA responses were not assessed during study screening. The serum IgG exclusion criterion used in this study has been applied to several *Shigella* vaccine studies and CHIMs (51–53); however, this immunoassay-based exclusion criterion may be insufficient, even when combined with exclusion based on previous *Shigella* exposure and travel.

As outlined above, memory B cell IgA responses directed to LPS correlated with resistance to shigellosis. In addition, LPS-specific serum IgA responses and SBA also were associated with decreased risk of shigellosis, albeit at a lower magnitude. These results demonstrate that immune parameters other than serum IgG may contribute to resistance to shigellosis. Given these associations with resistance to shigellosis, it may be reasonable to expand the immunological parameters utilized for excluding volunteers from *Shigella* vaccine studies or CHIMs when a naive population is critical. While data presented here suggest that LPS-specific IgA-secreting memory B cell responses at baseline may be the most suitable predictor of resistance to shigellosis, a balance needs to be maintained between volunteer exclusion criteria and the ability to successfully recruit volunteers. Therefore, impractical, costly, or technically difficult screening assays must be weighed against study objectives. Functional immune responses at baseline, as determined by SBA, could also be considered; however, the association of baseline SBA with disease outcomes was less robust than LPS-specific serum IgA responses, and the assay is also more technically challenging and time-consuming than an ELISA. Collectively, these results suggest that LPS-specific serum IgA titers should be considered a potentially useful and practical tool to more reliably exclude volunteers with pre-existing immunity to *Shigella*, similar to ETEC CHIMs (54). Setting additional or more stringent exclusion criteria may be problematic in the development of CHIMs in LMICs; however, an alternative strategy may be to stratify and/or randomize volunteers based on baseline immunity.

Logistic regression analyses indicated approximate 40% and 50% reductions in odds of progression to shigellosis or MSD, respectively; however, these associations were not observed with baseline LPS-specific serum IgG titers. This lack of association with baseline LPS-specific serum IgG and progression to shigellosis or MSD may be due to the fact that volunteers with high baseline serum IgG titers were excluded from the study, potentially providing a biased population after study enrollment. However, of the 130 volunteers that underwent LPS-specific serum IgG screening, only 4 (3.1%) were excluded based on high *S. sonnei* LPS-specific serum IgG titers (Table S2). Additionally, of the 56 enrolled volunteers, 41 (73%) had a LPS-specific serum IgG screening titer of ≤625.

ROC analyses revealed an optimal baseline LPS-specific serum IgA cut-point of ≤300 for predicting progression to MSD. In an effort to investigate the lack of a dose response observed in this CHIM (25), systemic immune responses were reanalyzed by approximate *Shigella* dose (500, 1,000, or 1,500 CFU), excluding volunteers with a baseline LPS-specific serum IgA titer of >400; however, removal of these volunteers did not

result in a dose-dependent LPS-specific serum IgG response or SBA response. This lack of association could be a function of the sample size ($n = 40$) resulting in insufficient power to determine significant differences, or it could imply that there are other host factors, such as microbiome or genetics, that have not yet been measured or explored.

There are multiple immune mechanisms that could be employed to protect against *Shigella*, which can be effective during the intracellular and extracellular phases of the bacterial infection. Protective immune responses could be driven, in part, by antibodies in the lumen preventing transcytosis of the bacteria across the epithelial cell layer (whether secretory IgA or IgG transudate), complement activation in the lamina propria, or antibody-dependent cellular cytotoxicity once *Shigella* has reached the intracellular phase of its life cycle. While assessing mechanistic correlates of protection is important, investigating immunological surrogates that represent the mechanistic correlates of protection is also informative. Additionally, rather than one immune parameter being responsible for protection, there may be multiple immune parameters working in concert, effectively defining a protective immune profile (55; R. W. Kaminski and K. A. Clarkson, unpublished data). Furthermore, depending on the initial route of antigenic exposure, there may be distinct protective immune profiles generated. Additional analyses, including *Shigella*-specific salivary antibody responses, determination of Fc glycosylation patterns, *Shigella* microarray analyses, transcriptomics, and systems serology, are currently planned to parse out more nuanced differences in immune responses across the disease spectrum. Similarly, ongoing analyses and determination of a protective immune profile in the context of parenterally administered *Shigella* vaccines could offer additional insights.

Disease outcomes and immune responses postinfection may also be affected by the duration of antigenic exposure. In the *S. sonnei* 53G CHIM, the majority of volunteers were administered antibiotics 5 days postinoculation (or sooner if clinically indicated [$n = 9$]), which often does not occur during natural infection. Interrupting the infection with antibiotics may reduce the amount of and exposure time to *Shigella* antigens, potentially impacting the magnitude, specificity, duration, maturation, and phenotype of the immune response induced in a CHIM (56), compared to settings where the disease is endemic. Depending on the duration of antigenic stimulation, more robust or perhaps different immune responses could occur, including generation of antibodies with higher affinity or avidity, antibodies with different Fc glycosylation patterns, or increased activation of cell-mediated immune mechanisms.

Additional immune response analyses by time and length of shedding *S. sonnei* in stool samples could also provide important insights into the relationship between *Shigella* infection and immune responses. Unfortunately, the present study was not powered to detect differences in immune responses across culture-confirmed *S. sonnei* shedding, as the majority of volunteers had culture-negative stools within 3 to 5 days after their first culture-positive stool sample. Efforts are under way to quantify *S. sonnei* in stool samples using quantitative PCR (qPCR) detection methods. As qPCR is highly sensitive compared to culture detection methods, these analyses may allow better comparisons between immune responses and *S. sonnei* shedding in stool samples postinfection.

Other nonimmunological factors may also contribute to the resistance to infection and lack of association of some immune parameters with disease outcomes observed in this study. While a CHIM attempts to control for many variables, such as challenge dose and age, there remain several uncontrolled factors in both CHIMs and natural infection settings that could contribute to differences in disease outcomes. Population genetics, prior exposure, and contemporaneous host immune status or coinfections, as well as microbiome and nutritional status may affect immune response profiles and disease outcomes postinfection (57–60). Finally, this study was conducted in healthy, North American adults, who constitute a distinctly different population than the target population of children <2 years of age living in LMICs. Infant immune systems do not fully mature until approximately 24 months of age and have been shown to have lower levels of circulating immunoglobulin and complement effectors, leading to different

immune responses being required to induce protection in this population (61). Furthermore, infant health and immune status can vary greatly in settings where the disease is endemic, so caution is required when postinfection immune responses in healthy North American adults are extrapolated to other populations.

Nonetheless, data collected in this study are invaluable, as comparable characterization of the immune response following a *Shigella* CHIM in the target population is unlikely. In addition to the immune response characterization postinfection, this study provides important information about immune status preinfection. Increased levels of serum IgA may be a contributing factor to resistance after oral *Shigella* challenge, and while additional investigations with increased group numbers are important, this study has been able to provide some guidance on immunological screening assays for use in future *Shigella* CHIMs.

Ideally, protective immunity against shigellosis would be assessed in the context of a challenge-rechallenge CHIM study design; however, the current study was designed and powered to address only the primary clinical outcome. Nonetheless, as prior disease results in protective immunity against subsequent infection with the same *Shigella* serotype, volunteers in this trial provide an important population for the investigation of potential immune correlates or surrogates of protection, as those with moderate to severe disease would likely be protected against reinfection (31, 32, 35, 40). With the dose of *S. sonnei* 53G now established, next steps should focus on the conduct of a challenge-rechallenge study in order to fully elucidate the immunological mechanisms responsible for protection from shigellosis. Additionally, a heterologous challenge-rechallenge study to investigate the potential of cross-protection provided across different *Shigella* species is in the planning stages. The *S. sonnei* 53G CHIM has confirmed the relevance of robust mucosal immune responses postinfection and their potential role as a mechanistic correlate of protection in this model (36), while suggesting that systemic immune responses may play a lesser role, as they were not as reflective of disease outcome and severity in this study. The observations that baseline levels of LPS-specific serum IgA and IgA-secreting memory B cells are associated with reduced odds of disease point to these immune measures as being potentially more sensitive markers for underlying protective immunity, and therefore, they should be further studied. Together, these data may help guide the rational design of future *Shigella* vaccines while representing a framework for future studies and functioning as a benchmark for comparisons across CHIMs.

MATERIALS AND METHODS

Study design and inoculation. The study was an open-label dose-finding CHIM designed to determine a target dose of lyophilized cGMP *S. sonnei* 53G for use in future *Shigella* CHIMs that would induce shigellosis in ≥60% of volunteers, as described elsewhere (25). Five days postchallenge (or sooner if clinically warranted), all volunteers initiated antibiotic treatment and were discharged from the inpatient facility after producing 2 culture-negative stool samples. Volunteers returned 14, 28, and 56 days postchallenge for additional blood and stool sample collections.

Blood processing. Whole blood was collected on study days −1, 5, 7, 14, 28, and 56. Serum samples were stored at −80 ± 10°C until assayed. Peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll gradient with Leucosep tubes, frozen using BioCision Cool Cells, and stored in liquid nitrogen until used in immunoassays.

Stool processing. Stool samples for immunoassays were collected on study days −1, 0, 3, 7, and 14 and immediately frozen at −80 ± 10°C. Archived stool samples were thawed and processed for immunoassays by resuspending 2 g of stool in a protease inhibitor buffer (Roche cOmplete EDTA-free protease inhibitor tablets, 1× phosphate-buffered saline [PBS], 0.05% Tween 20, 0.1% bovine serum albumin [BSA]), vortexed, and centrifuged for 30 min at 4°C at ~1,900 × *g*. Fecal extract supernatants were collected and frozen at −80 ± 10°C. Stool samples for inflammatory marker analyses were processed in myeloperoxidase or calprotectin-specific collection and processing kits according to the manufacturer's instructions (Epitope Diagnostics) and frozen at −80 ± 10°C.

Fecal inflammatory markers. Stool samples processed for inflammatory marker analyses were assayed by enzyme-linked immunosorbent assay (ELISA) to determine calprotectin and myeloperoxidase concentrations per manufacturer's instructions (Epitope Diagnostics). Inflammatory marker concentrations were interpolated from a standard curve, with values below the assay limit of detection (LOD) being assigned a value of half the lowest concentration in the standard curve (1/2 LOD). As this was a *post hoc* analysis, only stool from volunteers consenting the use of their samples in future or additional investigations were used for this analysis. The assessment of fecal inflammatory marker concentrations in stool

samples was a *post hoc* analysis and therefore limited to a smaller subset of volunteers (i.e., 45 of the 56 volunteers).

ALS generation. PBMCs used to generate antibodies in lymphocyte supernatant (ALS) were suspended in complete RPMI medium (10% heat-inactivated fetal calf serum, 1× penicillin-streptomycin, 1× glutamine) at 5×10^6 cells/ml, plated in a sterile 24-well tissue culture plate (1 ml/well) and cultured for 4 days at 37 ± 1°C with 5% CO_2. Supernatants were collected and frozen at −80 ± 10°C until assayed by ELISA.

$\alpha4\beta7$ PBMC separation. Frozen PBMCs from study days −1, 5, and 7 were thawed, washed, and suspended at 1×10^7 cells/ml in complete RPMI medium prior to incubation with an Alexa Fluor 647 (AF647)-conjugated anti-$\alpha4\beta7$ monoclonal antibody (Act-1; NIH AIDS Reagent Program) for 10 min at 4 ± 2°C, protected from light. After washing, the cells were incubated with anti-AF647 MicroBeads (Miltenyi) for 15 min at 4 ± 2°C, protected from light. PBMCs were then washed and passed through a 30-μm cell strainer prior to separation of $\alpha4\beta7$-positive and -negative PBMC populations using a Miltenyi AutoMACS cell separator. Both the $\alpha4\beta7^+$ and $\alpha4\beta7^-$ populations were adjusted to 5×10^6 cells/ml and cultured as described above to collect $\alpha4\beta7^+$ and $\alpha4\beta7^-$ ALS. Immediately prior to ALS culture, 100 μl of both the $\alpha4\beta7^+$ and $\alpha4\beta7^-$ populations was removed to assess population purity by flow cytometry. As $\alpha4\beta7^+$ cells were bound by the AF647-conjugated anti-$\alpha4\beta7$ monoclonal antibody, it was expected that only the positive population would fluoresce when analyzed by flow cytometry. The $\alpha4\beta7^+$ and $\alpha4\beta7^-$ populations for a given volunteer and time point were analyzed using a FACSCanto II flow cytometer and determined to be ≥90% pure postseparation (data not shown).

Memory B cell expansion and quality control. Frozen PBMCs from study days −1, 28, and 56 were thawed, washed, and expanded as previously described (62) with minor modifications. Briefly, cells were suspended at 1×10^6 cells/ml in complete RPMI medium with 2-mercaptoethanol and polyclonal mitogens: pokeweed mitogen extract at a 1:20,000 dilution (Sigma), CpG-ODN-2006 at 6 μg/ml (Invivogen), and *Staphylococcus aureus* Cowan at a 1:10,000 dilution (Sigma). Cells were plated in a sterile 6-well tissue culture plate (3 to 5 ml/well) and cultured for 6 days at 37 ± 1°C with 5% CO_2. After expansion, the cells were washed twice with mitogen-free complete RPMI medium, adjusted to 5×10^6 cells/ml, and cultured as described above to collect memory B cell ALS. A novel method was developed to characterize the memory B cell expansion and establish acceptability criteria in order to objectively determine successful expansion of memory B cell populations (R. W. Kaminski and K. A. Clarkson, unpublished data). Briefly, samples were analyzed by flow cytometry before and after polyclonal mitogen stimulation using T and B cell-specific markers (CD3-BV711, CD19-BV421, CD27-APC, and CD20-PE-Cy7; BD Biosciences) and analyzed by flow cytometry using a FACSCanto II flow cytometer. Acceptance criteria for a successful expansion included assessing cell viability and cell concentrations pre- and postexpansion as well as ensuring an overall increase postexpansion in the percentage of cells positive for the CD19 B cell marker. Additionally, an expansion was considered successful if B cell populations postexpansion showed a ≥20% increase in B cells positive for the CD27 memory marker, compared to pre-expansion B cell populations.

Enzyme-linked immunosorbent assay. Serum, stool extracts, and ALS samples were assayed by ELISA to determine *Shigella* antigen-specific antibody endpoint titers as previously described (39), with the exception of the use of Immulon 1-B ELISA plates and human-specific secondary antibodies (reserve allophycocyanin [AP]-conjugated goat-anti-human IgG, IgA, or IgM [Seracare]; AP-conjugated mouse anti-human IgG1, IgG2, IgG3, or IgG4 [Southern Biotech]). Samples that were negative at the starting dilution were assigned a titer corresponding to half the starting dilution (1/2 LOD). Immune responders were defined *a priori* as having a ≥4-fold increase over their baseline titer.

Serum bactericidal activity. Antibody functionality was assessed by determining *Shigella*-specific serum bactericidal activity (SBA) as previously described (63). Serum samples were assayed starting at a 1:40 dilution, and titers were interpolated from a standard curve using NICE software (64). A titer of 20, corresponding to half of the lowest serum dilution tested (1/2 LOD), was assigned to samples not exhibiting detectable serum bactericidal activity at the starting dilution. Immune responders were defined *a priori* as those with a ≥4-fold increase in SBA titer over baseline. Bactericidal activity directed to *S. sonnei* Moseley was determined, as well as that to the *S. flexneri* 2a strain 2457T to investigate the cross-reactivity of functional antibodies with other *Shigella* serotypes.

Disease outcomes and definitions. Immune responses pre- and postchallenge were compared across shigellosis outcome to determine the association of these immune parameters with progression to and severity of disease postchallenge. The outcome of shigellosis was chosen because it is often the focus of *Shigella* vaccine development. However, immune responses demonstrating differences across additional disease outcomes are also presented in the supplemental material. Diarrhea severity, dysentery (25), and disease severity score (19) were defined as previously described. While the primary clinical shigellosis endpoint was defined *a priori*, all analyses were conducted using an alternative shigellosis definition developed through a convening of *Shigella* CHIM experts to standardize *Shigella* CHIM clinical endpoints (23). All disease outcomes used for analyses are also described in Table S3.

Statistical analyses. All immune response data in the current study were assessed for normality using distribution plots. Normally distributed continuous immune response data were analyzed using appropriate parametric tests (*t* test or ANOVA) with Bonferroni *post hoc* analyses as applicable. Nonnormally distributed data were $\log_{10}$ transformed in order to meet the assumptions for the parametric tests. If the log transformation did not correct the distribution of the data set, then nonparametric statistical tests were used (Mann-Whitney U test) (51, 65). A multivariate logistic regression model was developed to assess the association between various immune parameters at baseline and the odds of progressing either to shigellosis MSD or to no or mild diarrhea. Continuous variables were analyzed for assumption

of linearity by plotting the variable against the log odds of the outcome. As age did not demonstrate a linear relationship with either outcome, it was appropriately categorized (for MSD, categories were 18 to 25, 26 to 40, and 41 to 49 years of age; for shigellosis, they were 18 to 25 and 26 to 49 years of age). Covariates of interest (gender, age category, race, and log-transformed dose) were investigated in univariate models ($\alpha = 0.20$) and included in the final model if they were significantly associated ($P \leq 0.05$) with the outcome of interest or influenced the effect estimate of baseline immune responses by ≥10% (gender, age category, and log-transformed dose). Race did not meet either inclusion criterion and was excluded from the multivariate model. Cut-point analyses were performed by plotting receiver operating characteristics (ROC) and investigating sensitivity and specificity across different areas under the curve. Additional cut-point analyses were performed using Liu and Youden cut-point methods. All statistical tests were interpreted in a two-tailed fashion ($\alpha = 0.05$), with P values of ≤0.05 being considered statistically significant in either Stata (version 14 for Mac) or Prism (version 7 for Mac).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, DOCX file, 0.1 MB.

FIG S2, DOCX file, 0.1 MB.

FIG S3, DOCX file, 0.1 MB.

FIG S4, DOCX file, 0.1 MB.

FIG S5, DOCX file, 0.5 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.02 MB.

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