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Vibrio cholerae O1 Infection Induces Proinflammatory CD4⁺ T-Cell Responses in Blood and Intestinal Mucosa of Infected Humans

Alison Kuchta, Taibur Rahman, Erica L. Sennott, Tauquier R. Bhuyian, Taher Uddin, Rasheduzzaman Rashu, Fahima Chowdhury, Ashraf I. Kahn, Mohammad Arifuzzaman, Ana A. Weil, Michael Podolsky, Regina C. LaRocque, Edward T. Ryan, Stephen B. Calderwood, Regina C. LaRocque, and Jason B. Harris

Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts; Department of Pediatrics, Harvard Medical School, Boston, Massachusetts; International Centre for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh; Department of Medicine, Harvard Medical School, Boston, Massachusetts; Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts; and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts

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Vibrio cholerae O1 is a noninvasive enteric pathogen and serves as a model for studies of mucosal immunity. Although symptomatic V. cholerae infection induces durable protection against subsequent disease, vaccination with oral killed whole-cell V. cholerae stimulates less long-lasting protection against cholera. In this study, we demonstrated that cholera induces an early proinflammatory cellular immune response that results in priming of Th1- and Th17-type cytokine responses to ex vivo antigenic stimulation and an increase in the ratio of Th1 to Th2 CD4⁺ T-cell responses. Comparable priming of Th1 and Th17 responses, with an increased ratio of Th1 to Th2 CD4⁺ T-cell responses, was not observed in subjects who received two doses of the oral cholera vaccine Dukoral (a whole-cell cholera toxin B subunit containing [WC-CTB] vaccine). These findings suggest that natural V. cholerae infection induces an early, proinflammatory cellular immune response, despite the apparent lack of clinical signs of inflammation. The failure of the WC-CTB vaccine to activate equivalent, CD4⁺ T-cell responses is a potential explanation for the shorter duration of protection following immunization with this vaccine. Additional studies are needed to determine whether these early T-cell-mediated events predict the subsequent duration of immunologic memory.

Vibrio cholerae causes 3 million to 5 million cases of diarrhea and over 100,000 deaths annually (2). Organisms colonize the epithelial surface of the small intestine and elaborate cholera toxin (CT), an essential virulence factor for all pandemic strains of V. cholerae (30). Because V. cholerae is a prototypical noninvasive pathogen, it serves as a model for the study of mucosal immunity (17).

Natural infection with V. cholerae provides greater than 90% protection against subsequent disease for at least 3 years in U.S. volunteer studies (the maximum period studied) and an average of 3 to 8 years on the basis of epidemiological studies in areas of endemicity (5, 19, 21). The mechanism(s) of protective immunity against cholera is not well understood but has been hypothesized to depend on anamnestic responses of memory B cells (16). Such long-lasting memory B cells against V. cholerae protein antigens could play a role in mediating anamnestic responses and protection against subsequent infection.

In contrast, a currently licensed cholera vaccine, Dukoral (a whole-cell CT B subunit [WC-CTB] vaccine), consists of killed V. cholerae O1 supplemented with recombinant cholera toxin B subunit (rCTB) and provides over 60% protection. However, protection wanes by 3 years after vaccination, and the vaccine provides limited direct protection in children (6, 7). A second vaccine, licensed as Shanchol, is a bivalent preparation that includes a mixture of killed V. cholerae O1 as well as the O139 serogroup but does not include rCTB. The protective efficacy of this vaccine is greater than 65% over a 2- to 3-year observation period in India in all age groups, but longer-term protection has not yet been characterized (33). Recently, we observed that memory-B-cell responses against V. cholerae antigens were substantially less following vaccination with Dukoral than following natural infection, despite initially similar antibody-secreting cell and plasma antibody responses (1). Because of its toxicity, both licensed cholera vaccines lack the ADP-ribosylating cholera toxin A subunit (29).

Because of the critical role of CD4⁺ T cells in follicular B-cell differentiation, we hypothesized that the induction of CD4⁺ T-cell responses might be critical to the subsequent development of memory-B-cell responses and for long-term
CD4+ T-cell responses to *V. cholerae* infection and their role in subsequent memory development, we analyzed canonical lineage-specific cytokine responses of T cells to *ex vivo* stimulation with a *V. cholerae* membrane preparation (MP) after natural *cholera* and compared these to the responses seen following vaccination with the licensed WC-CTB vaccine.

**MATERIALS AND METHODS**

**Study design and subject enrollment.** Patients presenting to the hospital at the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), with severe acute watery diarrhea with stool cultures positive for *V. cholerae* O1 were eligible for this study. Patients with significant comorbid conditions were excluded. Blood specimens were collected on the day after presentation (day 2) to allow for clinical stabilization and confirmation of a positive stool culture result. Adults from an unplanned urban area with demographic patterns similar to those observed in cholera patients and with no history of diarrhea during the previous 1 month were included as healthy controls. Additionally, healthy adult volunteers from a similar urban area were enrolled and vaccinated with two doses of the WC-CTB oral cholera vaccine on day 0 and day 14.

Because of the volume of blood required for the immunological assays, separate cohorts of patients, healthy controls, and vaccinees were enrolled for different components of the study. For the comparison of cytokine secretion in response to *V. cholerae* MP stimulation of whole blood, 7 patients and 5 vaccinees between the ages of 17 and 38 years were enrolled. The median age was 29 years in both groups. For the study of cytokine expression using intracellular cytokine staining/flow cytometry, 12 patients and 11 vaccinees between the ages of 16 and 50 years were enrolled. The median ages were 29 years in patients (mean, 29.9 ± 8.3 years) and vaccinees (28.6 ± 4.8 years) and 27 years in the control group (27.0 ± 5 years). A total of 14 cholera patients were enrolled in the endoscopy portion of the study for collection of duodenal pinch biopsy specimens. Specimens obtained from 5 healthy individuals with asymptomatic *Helicobacter pylori* infection enrolled in a separate and ongoing study were also utilized as controls. This cohort ranged in age from 22 to 45 years (median age, 30 years). All these studies were approved by the Research and Ethical Review Committee of the ICDDR,B and the Institutional Review Board of Massachusetts General Hospital, and all participants provided written informed consent. The human experimentation guidelines of the U.S. Department of Health and Human Services were followed during the conduct of this research.

**Microbiologic examination of stools.** Stool from patients with a characteristic *V. cholerae* colonization was plated directly on taurocholate-tellurite-gelatin agar (Oxoid, England) and incubated for 24 h at 37°C. Colonies were counterstained with hematoxylin, dehydrated, and mounted. QWin software (Leica Microsystems, Switzerland) with the Leica (DMLB) microscope was used for measuring the intensity of the IL-17-positive area. The intensity of IL-17 staining was analyzed using an acquired computerized image analysis (ACIA) score. This metric has been described previously and is defined as the total positively stained area measured times the mean intensity of the positive area divided by the total area measured, including both negatively or positively stained tissues.

Another biopsy specimen was used for extraction of lamina propria lymphocytes. Pinch biopsy specimens 0.1 cm2 in size were obtained as described previously from the first part of the duodenum from selected study participants (28). A portion of the biopsy specimens was immediately fixed in 10% formalin in 60% TBS. All the biopsy specimens were embedded in paraffin (TechPlain, Morris Plains, NJ), embedded in paraffin, and sectioned at 5 μm. Antigen retrieval was performed by heating the sections in a microwave oven in sodium citrate buffer (pH 6.0). The sections were incubated with antibody against IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA). Horse-radish peroxidase-conjugated mouse anti-rabbit antibody was used as the secondary reagent, and the sections were developed with diaminobenzidine as the substrate. The sections were counterstained with hematoxylin, dehydrated, and mounted. QuantiSoft software (Leica Microsystems, Switzerland) with the Leica (DMLB) microscope was used for measuring the intensity of the IL-17-positive area. The intensity of IL-17 staining was analyzed using an acquired computerized image analysis (ACIA) score.

**Stimulation of whole-blood cultures and measurement of cytokine production.** The methods for stimulation of whole-blood cultures and measurement of cytokine production used in these studies have been described previously (36). Briefly, venous blood was collected in lithium-heparin-coated tubes from patients on the second day of hospitalization (day 2) and again on days 7 and 30 after the onset of disease. Single blood samples were collected from the healthy controls. Venous blood from patients was collected on the day of vaccination (day 0) and 7 days following each dose of vaccination (days 7 and 30). Whole blood was diluted 1:8 in Dulbecco modified Eagle medium supplemented with 1% gentamicin, 1% mercaptoethanol, and 10% heat-inactivated fetal calf serum (FCS). Then, 100 μl of stimulating antigen, *V. cholerae* MP (10 μg/ml) prepared from the *V. cholerae* O1 El Tor strain N16961 grown under toxin-inducing conditions, was added. The protein composition of *V. cholerae* MP was defined by mass spectroscopy and was previously described in detail (36). Phytosmagsulinoglutinin (PHA; 1.0 μg/ml) was used as a positive control, and nonstimulated cultures were used as negative controls. Culture supernatants obtained after 5 days of incubation at 37°C in 5% CO2 were used to measure concentrations of cytokines (gamma interferon [IFN-γ]), interleukin-13 [IL-13], IL-17, IL-10) using a Miliplex human cytokine/chemokine kit following the manufacturer’s recommended protocols (Millipore Corp., Billerica, MA). Although they are not uniquely expressed by a specific cell lineage, the cytokines IFN-γ, IL-13, IL-17, and IL-10 were chosen because they are among the canonical functional cytokines of major CD4+ T-cell subtypes (Th1, Th2, Th17, and regulatory T cells, respectively) and are expressed at high levels in these subsets.

**Intracellular cytokine staining.** Peripheral blood mononuclear cells (PBMCs) and plasma were separated by differential centrifugation on Ficoll-Isoaque gradients (Pharmacia, Piscataway, NJ). PBMCs were resuspended at a concentration of 2 × 106 cells/ml in RPMI medium (Gibco, Carlsbad, CA) supplemented with 10% FCS (Gibco) and cultured in U-bottom tissue culture plates (Nunc, Denmark) in the presence of *V. cholerae* MP (10 μg/ml) or phorbol myristate acetate (PMA)-ionomycin at concentrations of 5 ng/ml and 1.0 μg/ml, respectively. Samples containing unstimulated cells were included as a negative control. Purified anti-CD28 (clone 2.6.2, BD Pharmingen) and anti-CD49d (clone 9F10; BD Pharmingen) at a concentration of 1.0 μg/ml were used for costimulation.

PBMCs and antigens were incubated for 2 h at 37°C in 5% CO2. After 2 h, brefeldin A (BFA; Sigma), (10 μg/ml) was added and cell cultures were incubated for an additional 4 h (15). Following the 6-h stimulation, cells were washed with phosphate-buffered saline (PBS) and 2% FCS. Washed pellets were stained for 30 min at 4°C with anti-human monoclonal antibodies: anti-CD3–peridinin-chlorophyll-protein complex (PK3; Becton Dickinson Immunocytometry Systems [BD], San Jose, CA). Following surface staining, cells were washed and then incubated with fluorescence-activated cell sorter (FACS) lysing solution (BD Bioscience, San Jose) for 10 min, washed, and then permeabilized with FACS permeabilizing solution (BD Bioscience, San Jose, CA) for 10 min at room temperature. Permeabilized cells were washed and then stained for 30 min at 4°C with fluoro-chrome-conjugated intracellular antibodies (i.e., anti-IFN-γ, anti-IL-13, anti-IL-10, and anti-IL-17) (BD Bioscience). Following staining, cells were washed and fixed in formamide before flow cytometry was performed using a FACS Calibur apparatus (BD Bioscience) (15). The lymphocyte population was identified in a forward- versus side-scatter plot, and then the CD3+ CD4+ subpopulation was gated, followed by identification of the CD4+ cytokine-positive subpopulation. Net stimulation was calculated by subtracting the frequency of parental (FOP) of the nonstimulated cells from the FOP of the antigen-stimulated cells.

**Duodenal biopsies, immunohistochemistry, and lamina propria lymphocyte cultures.** Pinch biopsy specimens 0.1 cm2 in size were obtained as described previously from the first part of the duodenum from selected study participants (28). A portion of the biopsy specimens was immediately fixed in 10% formalin in 60% TBS. All the biopsy specimens were embedded in paraffin (TechPlain, Morris Plains, NJ), embedded in paraffin, and sectioned at 5 μm. Antigen retrieval was performed by heating the sections in a microwave oven in sodium citrate buffer (pH 6.0). The sections were incubated with antibody against IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated mouse anti-rabbit antibody was used as the secondary reagent, and the sections were developed with diaminobenzidine as the substrate. The sections were counterstained with hematoxylin, dehydrated, and mounted. QuantiSoft software (Leica Microsystems, Switzerland) with the Leica (DMLB) microscope was used for measuring the intensity of the IL-17-positive area. The intensity of IL-17 staining was analyzed using an acquired computerized image analysis (ACIA) score. This metric has been described previously and is defined as the total positively stained area measured times the mean intensity of the positive area divided by the total area measured, including both negatively or positively stained tissues (31).
Biosciences). Plates were incubated at 37°C in 5% CO₂ for 5 days. Culture supernatants were used to obtain the concentrations of cytokines (IL-6, IL-17, and IL-1H9252) using a Milliplex human cytokine/chemokine kit following the manufacturer’s recommended protocols (Millipore Corp., Billerica, MA). The concentration of IL-8 was measured using a human CXCL8/IL-8 DuoSet kit following the manufacturer’s recommended protocol (R&D Systems, Inc., Minneapolis, MN). Transforming growth factor β1 (TGF-β1) concentrations were measured using a Milliplex human TGF-β1 kit following the manufacturer’s recommended protocols (Millipore Corp).

Statistical analyses. To measure the statistical significance of the observed cytokine responses, the Mann-Whitney U test was used to evaluate comparisons of immune responses between groups of patients, vaccinees, and healthy controls. The Wilcoxon test was used to compare responses within groups at different time points. To evaluate trends in the immune responses after one and two doses of vaccine, a nonparametric trend test was used (9). A two-tailed P value of ≤0.05 was considered the cutoff for a significant difference.

RESULTS

Cytokine responses to ex vivo V. cholerae antigens in cell culture supernatants. To compare the cytokine responses to V.
cholerae antigen stimulation of circulating T cells in patients with cholera and in recipients of the WC-CTB vaccine, we measured the concentrations of IFN-γ, IL-10, IL-13, and IL-17 in supernatants collected from cultures of whole blood, stimulated by the addition of V. cholerae MP, using methods described previously (34, 36). Cytokine levels were compared in patients and vaccinees in response to antigenic stimulation; unstimulated whole blood and PHA-stimulated whole blood were used as negative and positive controls, respectively.

As shown in Fig. 1, cholera patients demonstrated a significant increase in production of IFN-γ and IL-17 in response to ex vivo stimulation with V. cholerae MP. There was also a trend toward an increase in IL-13 in V. cholerae MP-primed cells (P = 0.06). There was no significant increase seen in IL-10. Similarly, there were no significant differences in cytokine levels in the unstimulated controls (see Fig. S2 in the supplemental material) or the PHA-stimulated controls at different time points, indicating that the priming of IFN-γ and IL-17 secretion in peripheral blood demonstrated on day 7 was V. cholerae antigen specific (see Fig. S1 in the supplemental material). The increased IFN-γ response to V. cholerae antigen stimulation remained detectable in the peripheral circulation through day 30 after infection. In contrast, subjects who received the WC-CTB vaccine did not develop a significant increase in IFN-γ, IL-13, or IL-17 production in response to ex vivo stimulation of whole blood with V. cholerae MP. There was a modest trend suggestive of increasing IL-10 responses after two doses of the cholera vaccine, but this did not reach statistical significance (P = 0.10).

Cytokine responses of specific V. cholerae antigen-stimulated CD4+ T-cell populations. Because the measurement of cytokine concentrations in cell culture supernatants does not distinguish the precise cellular origin of individual cytokines, we also performed flow cytometry-based intracellular cytokine staining (ICS) on PBMCs stimulated with V. cholerae MP to compare the proportions of individual CD4+ T cells expressing cytokines characteristic of Th1 (IFN-γ) and Th2 (IL-13) lineages.

Previous studies have focused on the ratio of Th1/Th2 responses as an important determinant of the mucosal immune response (12, 13, 26). As shown in Fig. 2C, infection with V. cholerae resulted in a shift toward a Th1 to Th2 response to V. cholerae MP stimulation, with a >2 fold increase in the ratio of IFN-γ-expressing/IL-13-expressing CD4+ T cells on day 7 following cholera compared to the ratios on day 2 (P = 0.008) as well compared to the ratios for healthy controls (P < 0.001). In contrast, no change in the ratio of CD4+ T cells expressing IFN-γ/IL-13 in response to stimulation with V. cholerae MP was seen at any time point after vaccination compared to the baseline measurement (P = 0.248 at day 7 and P = 0.203 on day 21). Furthermore, no change in the ratio of IFN-γ-expressing/IL-13-expressing cells was seen in patients or vaccinees in response to PHA stimulation, indicating that the increased Th1 and Th2 response observed in patients was specific to V. cholerae antigenic stimulation.

Confirmation of a mucosal IL-17 response in cholera patients. Cholera is a prototypical noninvasive noninflammatory diarrheal illness, and therefore, we had not anticipated a significant IL-17 response to V. cholerae antigen stimulation in peripheral blood of cholera patients. To confirm the induction of Th17 responses in mucosal tissues in cholera patients, we performed immunohistochemistry to examine IL-17 expression in duodenal biopsy specimens of patients recovering from cholera. As shown in Fig. 3, increased expression of IL-17 in situ was demonstrated in the lamina propria during acute cholera (day 2) compared to the expression on subsequent days and in control subjects, suggesting that infection with V. cholerae induces mucosal Th17 responses in lamina propria cells during the acute phase of illness that can subsequently be detected in the circulation by measuring responses of CD4+ T cells to V. cholerae antigenic stimulation.

We further recovered LPLs from duodenal biopsy specimens obtained immediately during acute illness (day 2), later in convalescence (day 30), and in healthy controls. LPL cell culture supernatants were used to measure cytokine expression, as cytokines secreted by the cultured LPLs on day 2 likely reflect the functional phenotype of these cells stimulated in vivo during V. cholerae O1 infection. As shown in Fig. 4, there was a trend toward an elevation of IL-17 in acute cholera (P = 0.07 compared to day 30, P = 0.09 compared to healthy controls), as well as increases in other proinflammatory cytokines, including IL-6 (P = 0.007 compared to day 30, P = 0.04 compared to healthy controls), which, along with TGF-β, is essential for the initial development of Th17 responses. Despite the lack of a clinically significant systemic inflammatory
response in cholera, IL-8 was also increased during acute illness ($P = 0.032$ compared to day 30). In contrast, TGF-β was expressed by LPLs at similar levels in acute- and convalescent-phase cholera samples.

**DISCUSSION**

Infection with *V. cholerae* provides long-term protection against subsequent disease (5, 19, 21). Although the mechanism(s) of protective immunity is not well understood, protection may be mediated by anamnestic immune responses to *V. cholerae* antigens (16, 25, 35). In support of this, memory-B-cell responses to *V. cholerae* protein antigens remain detectable for at least 1 year after infection, longer than circulating antibodies or circulating memory-B-cell responses to *V. cholerae* O1 lipopolysaccharide (16). Because helper T cells play a critical role in the development of long-lived memory B cells directed against protein antigens (8), we have hypothesized that the initial CD4$^+$ T-cell response to *V. cholerae* infection may contribute to long-term protection against cholera.

**FIG. 3.** Natural infection with *V. cholerae* O1 induces a mucosal IL-17 response. Duodenal biopsy specimens were stained for IL-17 expression, and the distribution and intensity of staining were compared in healthy controls (HC; $n = 5$) and cholera patients ($n = 14$) on days 2, 30, and 180. (A) Representative anti-IL-17-stained samples from a healthy control (HC) and an infected patient on day 2 (D2) are shown. (B) During acute infection (day 2), cholera patients demonstrated a significant increase in IL-17 staining in the lamina propria (LP) compared to healthy controls and paired specimens obtained after convalescence (day 180), as measured by ACIA. No significant difference in IL-17 staining was observed in the surface epithelium (SE).

**FIG. 4.** Infection with *V. cholerae* O1 induces mucosal IL-17, IL-6, IL-1β, and IL-8 responses. Cytokines (IL-17, IL-6, IL-1β, TGF-β1, and IL-8) excreted by unstimulated cultured lamina propria lymphocytes from duodenal biopsy specimens of cholera patients ($n = 5$, color) were measured during the acute (day 2) and convalescent (day 30) phases of infection and compared to those secreted by biopsy specimens obtained from healthy controls ($n = 5$, black). Data are shown color coded by patient, with significant $P$ values noted.
Previously, we observed that patients recovering from cholera develop gut-homing central and effector memory CD4+ T-cell immune responses to *V. cholerae* protein antigens that peak in the circulation 7 days after cholera (3, 36). In this study, we characterized the functions of the cellular immune responses to *V. cholerae* O1 in cholera patients and vaccinees by measuring the lineage-specific CD4+ T-cell responses to *ex vivo* antigenic stimulation.

The paradigmatic cellular immune response to mucosal infection involves Th2-regulated IgA production; however, there are examples where Th1 or Th17 responses may be critical determinants of protective immunity against potentially invasive mucosal pathogens (12, 13, 23, 26). Because cholera is prototypical of a noninflammatory, toxin-mediated diarrhea (17), it was plausible that the disease would induce a CD4+ T-cell response skewed predominantly toward a noninflammatory Th2 response to *V. cholerae* antigens.

However, the findings of this study do not support this hypothesis. Instead, infection with *V. cholerae* primed Th1 and Th17 responses, with a shift toward Th1 to Th2 CD4+ T-cell responses. Despite the absence of overt pyogenic infection and gross histological changes in the gut during cholera, these responses are preceded by a mucosal innate immune response to infection that includes increases in IL-1β, IL-6, and IL-8 secretion by lamina propria lymphocytes. These results are consistent with increasing evidence that early responses to *V. cholerae* O1 infection include the upregulation of expression of proteins with presumed bacterial inhibitory properties, including lactoferrin and long palate, lung, and nasal epithelium clone 1 (LPLUNC1) (14, 28), and the migration of neutrophils to the lamina propria and epithelium during acute cholera (14, 28). It was previously shown that IL-1β expression is increased during acute cholera, a finding reproduced in this study. However, the finding that IL-6, a critical bridge between innate and adaptive immunity through the promotion of B-cell IgA class switch differentiation and T-cell differentiation (Th17 lineage), was increased in acute cholera is an extension of these findings with potential implications for the development of long-term immunologic memory.

Despite the similar antigenic composition of the WC-CTB vaccine and live *V. cholerae* O1 organisms encountered in natural infection, we observed significant differences in the CD4+ T-cell response in cholera patients and vaccinees. Compared to natural infection with *V. cholerae* O1, vaccination resulted in limited priming of cellular immune responses to *V. cholerae* antigens. In addition, while subjects recovering from cholera developed a shift toward increased Th1/Th2 CD4+ T-cell responses, a comparable shift in the Th1/Th2 ratio of T-cell cytokine production was not observed in subjects who received two doses of the vaccine.

The finding that natural cholera induces proinflammatory CD4+ T-cell responses greater than those seen in WC-CTB vaccine recipients is consistent with *in vitro* and murine models of the mechanisms of the adjuvant effects of CT holotoxin. In mice, the effectiveness of CT as an adjuvant is dependent on the presence of CD4+ T cells (18). The CT holotoxin induces a broad spectrum of CD4+ T-cell responses, including Th1, Th2, Th17, and regulatory T-cell responses, to coadministered antigens (20, 22). Recent evidence, also from a murine model, suggests that the induction of Th17 responses is essential for the mucosal adjuvant activity of CT holotoxin; specifically, the induction of an IL-17 response by CT is required to generate protective mucosal IgA antibody responses to coadministered antigens (11). Our clinical observations here similarly demonstrate that humans with natural cholera (and, hence, exposure to CT holotoxin) also develop *V. cholerae* antigen-specific mucosal IL-17 responses, despite the absence of overt pyogenic infection.

In contrast to the adjuvant effect of the CT holotoxin, the mucosal application of only CTB, the GM1 ganglioside-binding portion of the toxin, results in the differentiation of both adaptive and natural regulatory T cells that produce IL-10 and suppress Th1, Th2, and Th17 responses and promote tolerance to coadministered antigens (10, 22, 32). Thus, the presence of CTB in the WC-CTB vaccine may provide a possible mechanism by which vaccine-induced CD4+ T-cell responses to *V. cholerae* MP may be diminished or skewed toward the development of a Th2 T-cell phenotype. This may also explain why in initial clinical trials, the protective efficacy of the whole-cell-only vaccine (without CTB) against cholera was more persistent and ultimately afforded protection that exceeded the protection afforded by the WC-CTB vaccine by the third year of follow-up (62% for the whole-cell-only vaccine compared to 40% for the WC-CTB vaccine) (6, 7). Because the more recently licensed Shanchol vaccine does not include CTB, it may elicit a different profile of cellular immune responses to *V. cholerae* protein antigens than the WC-CTB vaccine and requires additional study.

In this study, we observed robust CD4+ T-cell immune responses in cholera patients that fell outside the predicted model for a noninvasive mucosal infection. However, in future studies, it will be critical to gauge the significance of these early CD4+ T-cell responses to *V. cholerae*. Because memory-B-cell responses to protein antigens persist longer than serum antibody responses, it may be useful to evaluate the association between early T-cell responses and the subsequent development and maintenance of long-term memory B cells. In addition, future studies are needed to examine the role of the CXC/R5-expressing follicular helper T cells, which likely play a particularly important role in regulating B-cell proliferation and class switching (24, 27). These studies may help further define the role of early T-cell responses in determining longlasting immunity to cholera.

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