

## Memory T-Cell Responses to *Vibrio cholerae* O1 Infection<sup>∇</sup>

Ana A. Weil,<sup>1†</sup> Mohammad Arifuzzaman,<sup>1†</sup> Taufiqur R. Bhuiyan,<sup>1</sup> Regina C. LaRocque,<sup>2,3</sup>  
Aaron M. Harris,<sup>1</sup> Emily A. Kendall,<sup>1</sup> Azim Hossain,<sup>1</sup> Abdullah A. Tarique,<sup>1</sup>  
Alaullah Sheikh,<sup>1</sup> Fahima Chowdhury,<sup>1</sup> Ashraf I. Khan,<sup>1</sup> Farhan Murshed,<sup>2</sup>  
Kenneth C. Parker,<sup>4</sup> Kalyan K. Banerjee,<sup>5</sup> Edward T. Ryan,<sup>2,3,6</sup> Jason B. Harris,<sup>2,7‡</sup>  
Firdausi Qadri,<sup>1‡</sup> and Stephen B. Calderwood<sup>2,3,8\*‡</sup>

International Centre for Diarrheal Disease Research, Dhaka, Bangladesh<sup>1</sup>; Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts<sup>2</sup>; Department of Medicine, Harvard Medical School, Boston, Massachusetts<sup>3</sup>; Virgin Instruments, Sudbury, Massachusetts<sup>4</sup>; Division of Biochemistry, National Institute of Cholera and Enteric Diseases, Kolkata, India<sup>5</sup>; Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts<sup>6</sup>; Department of Pediatrics, Harvard Medical School, Boston, Massachusetts<sup>7</sup>; and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts<sup>8</sup>

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***Vibrio cholerae* O1 can cause diarrheal disease that may be life-threatening without treatment. Natural infection results in long-lasting protective immunity, but the role of T cells in this immune response has not been well characterized. In contrast, robust B-cell responses to *V. cholerae* infection have been observed. In particular, memory B-cell responses to T-cell-dependent antigens persist for at least 1 year, whereas responses to lipopolysaccharide, a T-cell-independent antigen, wane more rapidly after infection. We hypothesize that protective immunity is mediated by anamnestic responses of memory B cells in the gut-associated lymphoid tissue, and T-cell responses may be required to generate and maintain durable memory B-cell responses. In this study, we examined B- and T-cell responses in patients with severe *V. cholerae* infection. Using the flow cytometric assay of the specific cell-mediated immune response in activated whole blood, we measured antigen-specific T-cell responses using *V. cholerae* antigens, including the toxin-coregulated pilus (TcpA), a *V. cholerae* membrane preparation, and the *V. cholerae* cytolysin/hemolysin (VCC) protein. Our results show that memory T-cell responses develop by day 7 after infection, a time prior to and concurrent with the development of B-cell responses. This suggests that T-cell responses to *V. cholerae* antigens may be important for the generation and stability of memory B-cell responses. The T-cell proliferative response to VCC was of a higher magnitude than responses observed to other *V. cholerae* antigens.**

*Vibrio cholerae* is a gram-negative bacterium that can cause a severe, acute secretory diarrhea. Serological differentiation of *V. cholerae* strains is based on the O-side chain of the lipopolysaccharide (LPS) component of the outer membrane. Of the more than 200 serogroups of *V. cholerae* identified, only the O1 and O139 serogroups can cause epidemic cholera (44). These pathogens are noninvasive and colonize the mucosal surface of the small intestine (44).

Natural infection with *V. cholerae* is known to provide protection against subsequent disease, but the mechanism of this protective immunity is not fully characterized. The vibriocidal antibody is a complement-dependent bactericidal antibody that is associated with protection from infection. However, no known threshold level of the vibriocidal antibody confers complete protection from *V. cholerae* infection, and some individuals with low serum vibriocidal antibody titers are still protected. This suggests that the vibriocidal titer may be a

surrogate marker (16, 45). Elevated serum immunoglobulin A (IgA) antibody levels specific for the B subunit of cholera toxin (CTB), the major structural subunit of a type IV pilus (TcpA), and LPS are also associated with protective immunity in areas where cholera is endemic (19). However, after natural infection, the serum levels of these antibodies wane more rapidly than protective immunity (19). Patients with cholera develop memory B-cell responses of both the IgG and the IgA isotype to at least two *V. cholerae* protein antigens, CTB and TcpA. These responses are detectable for at least 1 year after infection and persist even after *V. cholerae* antigen-specific antibody-secreting cells and serum antibody titers have returned to baseline (18). B-cell memory responses also develop for the T-cell independent antigen LPS, but these responses wane more rapidly than memory B-cell responses to protein antigens, suggesting that durable memory B-cell responses to some *V. cholerae* antigens may be T-cell dependent (18).

We have recently demonstrated that cholera patients mount a primed T-cell response in the mucosa after *V. cholerae* O1 infection (6). We hypothesize that protection from cholera may be mediated by memory B cells capable of anamnestic response in the gut mucosa and that these memory B cells may depend on stimulation provided by memory T cells for their development and maintenance. T cells may contribute to the activation of B cells during *V. cholerae* infection by secreting

\* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114. Phone: (617) 726-3811. Fax: (617) 726-7416. E-mail: scalderswood@partners.org.

† A.A.W. and M.A. contributed equally as first authors for this work.  
‡ J.B.H., F.Q., and S.B.C. contributed equally as senior authors for this work.

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TABLE 1. Proteins represented in the *V. cholerae* membrane preparation and abundance of selected virulence proteins

Protein identifier	Protein name	Gene no.	Quantity rank	Spectral count
<b>Most abundant proteins</b>				
OMPU_VIBCH	Outer membrane protein U precursor porin OMPU	VC0633	1	1,897
EFTU2_VIBCH	Elongation factor tu-b	VC0362	2	435
CH601_VIBCH	Chaperonin 1 (protein cpn60 1)	VC2664	3	314
ATPA_VIBCH	ATP synthase subunit $\alpha$	VC2766	4	299
O31154_VIBCH	Putative outer membrane porin OMPA	VC2213	5	295
ATPB_VIBCH	ATP synthase subunit $\beta$	VC2764	6	282
Q9KUB8_VIBCH	Aconitate hydratase 2	VC0604	7	281
PPCK_VIBCH	Phosphoenolpyruvate carboxykinase	VC2738	8	212
DNAK_VIBCH	Chaperone protein DNAK	VC0855	9	206
Q9KT62_VIBCH	Long-chain fatty acid transport protein	VC1043	10	199
Q9KQB1_VIBCH	Succinate dehydrogenase, flavoprotein subunit	VC2089	11	195
Q9KNS5_VIBCH	Fumarate reductase, flavoprotein subunit	VC2656	12	194
MDH_VIBCH	Malate dehydrogenase	VC0432	13	192
Q9KUF5_VIBCH	Protease do	VC0566	14	191
LAMB_VIBCH	Maltoporin precursor (maltose-inducible porin)	VCA1028	15	158
EFG2_VIBCH	Elongation factor G2	VC2342	16	155
Q9KNN3_VIBCH	Aspartate ammonia-lyase	VC2698	17	150
ENO_VIBCH	Enolase	VC2447	18	145
Q9KTZ9_VIBCH	Antioxidant, ahpc/tsa family	VC0731	19	142
DLDH_VIBCH	Dihydrolipoyl dehydrogenase	VC2412	20	139
<b>Selected virulence proteins</b>				
HLYA_VIBCH	Hemolysin precursor	VCA0219	90	21
TCPA1_VIBCH	Toxin-coregulated pilin precursor	VC0828	100	11
TCPC_VIBCH	Toxin-coregulated pilus biosynthesis outer membrane protein C	VC0831	106	5

stimulatory cytokines and direct contact with B cells in lymph nodes. Therefore, T cells may have an important role in protective immunity to *V. cholerae* infection.

We characterized the memory T-cell responses to *V. cholerae* antigens following natural *V. cholerae* infection and compared these with serological responses to the same antigens. Previously, our group has studied various *V. cholerae* antigens, including mannose-sensitive hemagglutinin, TcpA, CTB, and LPS (22, 33, 37). We also included in the present study responses to a novel antigen, *V. cholerae* cytolysin/hemolysin (VCC) (31, 32). The *hly* gene that encodes the VCC protein is widespread across both pathogenic and environmental strains of *V. cholerae*, suggesting that VCC may impart an advantage to the organism (42). Although the precise role of VCC in *V. cholerae* infection is unknown, VCC is the primary virulence factor in *V. cholerae* infection with non-O1, non-O139 strains that do not produce cholera toxin (12, 46). The immune response to VCC is not well understood; however, recent studies suggest that VCC may promote a Th2 response in *V. cholerae* infection (2). In addition, the cytolytic activity of VCC may generate epithelial destruction that allows other *V. cholerae* antigens to penetrate the mucosa and promote the inflammatory response observed in *V. cholerae* infection (35, 39).

#### MATERIALS AND METHODS

**Study subjects and overview.** The International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) Dhaka hospital cares for over 20,000 cholera patients annually, most of whom are residents of Dhaka city. Patients older than 6 months who had a positive stool culture for *V. cholerae* and were without significant comorbid conditions were eligible for inclusion in the present study. All of the participants included presented with severe, acute watery diarrhea and required treatment with intravenous fluids. The study participants were also treated with antibiotics. Individuals with similar socioeconomic backgrounds as patients and who experienced no diarrheal illness in the previous 3 months were

selected as healthy controls. For patients, blood samples were obtained during acute infection (the second day of hospitalization) and on days 7 and 30 during the convalescent period. At each time point, T-cell lymphoblast proliferation was measured in response to ex vivo antigenic stimulation with a membrane preparation from *V. cholerae* O1 (MP), TcpA, VCC, or *V. cholerae* LPS. These antigens are described in detail below. In addition, vibriocidal titers and serologic responses to antigens TcpA, VCC, and the homologous LPS (*V. cholerae*-specific serotypes Inaba or Ogawa) were performed. Study participants were enrolled after providing written informed consent. The Research and Ethical Review Committee of the ICDDR,B and the Institutional Review Board of Massachusetts General Hospital approved the present study. The human experimentation guidelines of the U.S. Department of Health and Human Services were followed during the conduct of this research.

**Bacteriological examination of patient stools.** Cases were confirmed by culturing stool onto taurocholate-tellurite-gelatin and MacConkey agar (28). After overnight incubation of plates, suspected *V. cholerae* colonies were serologically confirmed by slide agglutination with specific monoclonal antibody for Ogawa or Inaba serotypes (34, 41).

***V. cholerae*-specific stimulating antigens and controls.** A *V. cholerae* MP was made from the sequenced O1 El Tor strain N16961 grown in vitro in AKI medium (21). Specifically, a culture of N16961 was inoculated into 1 liter of AKI medium (21). The culture was grown in nonaerating conditions to an optical density at 600 nm of 0.3 and then transferred to shaking conditions for growth to stationary phase. Bacteria were pelleted by centrifuging and then sonicated. The sonicated mixture was centrifuged at  $1,400 \times g$  for 10 min, and the remaining supernatant was then centrifuged at  $14,900 \times g$  for 30 min. The pellet containing the membrane fraction was then suspended in  $MgCl_2$ -Tris buffer (5 mM  $MgCl_2$ , 10 mM Tris [pH 8.0]) for subsequent experiments. Mass spectrometry analysis indicated that the MP contains a mixture of bacterial proteins, and the most abundant of these proteins are listed in Table 1.

The VCC monomer was used for *V. cholerae*-specific antigenic stimulation at a concentration of 2.5 ng/ml. We used VCC purified from a non-O1 clinical strain of *V. cholerae*; this protein is known to be immunologically and biochemically identical to *V. cholerae* O1 VCC (50). Isolation and purification was conducted as previously described (10). Recombinant TcpA, prepared as described previously, was used at a concentration of 2.5  $\mu g/ml$  (3). *V. cholerae* O1 Inaba or Ogawa LPS, matched to the case serotype, was used at a concentration of 2.5  $\mu g/ml$ . Preparation of *V. cholerae* O1 LPS was conducted as previously described (33). Positive controls for the assay included purified protein derivative (Statens

Serum Institut, Copenhagen, Denmark) and phytohemagglutinin (Murex, Remel, Sweden) at concentrations of 5 and 1  $\mu\text{g/ml}$ , respectively. Samples containing unstimulated cells were also included.

**Measurement of *V. cholerae* antigen-specific antibodies in serum and lymphocyte supernatants.** Vibriocidal antibody assays were performed as previously described using guinea pig complement and the homologous matched serotype of *V. cholerae* El Tor Ogawa (X-25049) or El Tor Inaba (T-19479) as the target organism (38). The vibriocidal titer was defined as the reciprocal of the highest serum dilution resulting in >50% reduction of the optical density compared to the optical density of control wells without serum. Seroconversion was defined as a  $\geq 4$ -fold increase in vibriocidal titer after acute dehydrating diarrhea.

LPS-, TcpA-, and VCC-specific IgG and IgA in serum samples were quantified by using standardized enzyme-linked immunosorbent assay (ELISA) procedures as previously described (36, 40). For anti-TcpA detection, ELISA plates were coated with TcpA (1  $\mu\text{g/ml}$ ) in carbonate buffer (50 mM, pH 9.6). For LPS- and VCC-specific responses, ELISA plates were coated with 2.5  $\mu\text{g}$  of antigen/ml in phosphate buffered saline (PBS; 10 mM, pH 7.2). Sera were diluted 1:100 for TcpA and VCC antibody testing and 1:50 for LPS, with 0.1% bovine serum albumin in PBS-Tween (10 mM PBS [pH 7.2] containing 0.05% Tween 20). Then, 100  $\mu\text{l}$  of diluted serum/well was added for each antigen. Horseradish peroxidase-conjugated secondary antibodies to human IgG or IgA were applied in separate wells. Plates were washed and developed with ortho-phenylene diamine substrate (Sigma, St. Louis, MO) and 0.012% hydrogen peroxide in 0.1 M sodium citrate buffer. The optical density was measured kinetically at 450 nm for 5 min. The maximal rate of optical density change was expressed as milli-optical density absorbance units per minute. ELISA units were normalized by calculating the ratio of the test sample to a standard of pooled convalescent-phase serum from recovered cholera patients added to each plate (33).

For the collection of lymphocyte supernatants, heparinized blood was diluted in PBS (1:1). Peripheral blood mononuclear cells (PBMC) and serum were isolated by differential centrifugation with Ficoll-Isopaque (Pharmacia, Piscataway, NJ). PBMC were resuspended at a concentration of  $10^7$  cells/ml in RPMI medium (Gibco, Carlsbad, CA) and supplemented with 1% penicillin, 1% streptomycin, 1% L-glutamine, 1% sodium-pyruvate, and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). PBMC were incubated for 48 h at 37°C in 5% CO<sub>2</sub> in 24-well tissue culture plates without mitogen. Plates were centrifuged at  $1,200 \times g$  for 10 min, and the supernatants were collected. A protease inhibitor cocktail containing aprotinin (0.15  $\mu\text{M}$ ), leupeptin (10  $\mu\text{M}$ ), sodium azide (15  $\mu\text{M}$ ), and 4-(amino-ethyl) benzene sulfonyl fluoride (0.2  $\mu\text{M}$ ) was added at a concentration of 10  $\mu\text{l/ml}$  of supernatant, and sample aliquots were preserved at -70°C. Supernatants were assayed at a 1:2 dilution in 0.1% bovine serum albumin in PBS-Tween in an ELISA as described above, and responses were detected with rabbit anti-human IgA- and IgG-horseradish peroxidase conjugate. Responses were measured kinetically as previously described (33).

**FASCIA.** The FASCIA (flow cytometric assay of specific cell-mediated immune response in activated whole blood) method was used as previously described to determine lymphoblast formation in response to antigenic stimulation (14, 48). Briefly, whole blood was collected in a lithium heparin-coated tube and diluted 1:8 in Dulbecco modified Eagle medium supplemented with 1% gentamicin, 1% mercaptoethanol, and 10% heat-inactivated fetal calf serum. Then, 100  $\mu\text{l}$  of stimulating antigen, control antigen, or additional medium was added to 400  $\mu\text{l}$  of diluted blood. After 6 days of in vitro culture at 37°C in 5% CO<sub>2</sub>, supernatant was preserved for cytokine analysis, and cells were stained with anti-CD3-FITC, anti-CD4-PerCP, anti-CD8-APC, and anti-CD45R0-PE monoclonal antibodies (Becton Dickinson Immunocytometry Systems [BD], Stockholm, Sweden). An erythrocyte lysing solution of 1.0  $\mu\text{l}$  of ammonium chloride (Orthumune lysing reagents; Ortho Diagnostics, Stockholm, Sweden) was added for 6 min, followed by incubation at room temperature, followed in turn by centrifugation, the removal of supernatants, and washing. Cells were suspended in 2% paraformaldehyde and stored in the dark; within 12 h, acquisition was conducted by fluorescence-activated cell sorting (FACSCalibur [BD]) for standardized 2-min intervals using CellQuest Pro software (Becton Dickinson, San Jose, CA). Analysis was performed with FlowJo software (TreeStar, Inc.). The results are presented as the ratio of lymphoblast count with antigenic stimulation to the count without stimulation (stimulation index). A value of "1" indicates that stimulation is equal in samples with or without *V. cholerae* antigenic stimulation.

**Additional investigation of memory T-cell phenotype.** To further define the character of lymphoblasts proliferating in response to *V. cholerae*-specific antigens, we examined the peripheral blood of five cholera patients for additional T-cell phenotypic markers. On day 2 and day 7 after infection, we used the FASCIA method as described above and identified cell surface markers with anti-CD4-PerCP, anti-CD45R0-PE, anti-CD45RA-FITC, and anti-CCR7-APC monoclonal antibodies (BD).

TABLE 2. Demographic and clinical characteristics of cholera patients and healthy controls

Demographics and clinical characteristics	Patients (n = 16)	Healthy controls (n = 10)
No. of female (% total)	8 (50)	5 (50)
Median age in yr (range)	34 (13–50)	33 (18–45)
<i>V. cholerae</i> O1 serotype	Ogawa (n = 11), Inaba (n = 5)	
Mean $\pm$ SD		
Duration of diarrhea (h)	21 $\pm$ 3.7	
Amt of fluids required (liters)	8.6 $\pm$ 1.6	
Duration of hospitalization (h)	31 $\pm$ 3.3	

**Statistical analyses.** The Wilcoxon signed-rank test was used to compare immunological responses of cholera patients on different study days, and the Mann-Whitney U test was used to compare immune responses between patients and healthy controls. All reported *P* values are two tailed. A cutoff of  $P \leq 0.05$  was the predetermined threshold for statistical significance. Analyses and figure preparation were performed on GraphPad Prism 4.0 and Stata version 9.0 (Stata Corp., Inc., College Station, TX). Geometric means with 95% confidence intervals are shown in figures unless otherwise stated.

## RESULTS

**Study population.** Sixteen patients were enrolled in the study, and fifteen completed 30 days of follow-up. All participants had cholera and severe dehydration upon initial clinical evaluation. Ten apparently healthy adults were included as healthy controls. Demographic and clinical features comparing cholera patients and healthy controls are shown in Table 2.

**Vibriocidal and antigen-specific antibody responses.** VCC, LPS, and TcpA-specific antibody responses and vibriocidal titers were measured in sera on days 2, 7, and 30 and are shown in Table 3. On day 2 of infection, the geometric mean vibriocidal titer was 35 (95% confidence interval, 13 to 97), and this increased to 4,300 (3,000 to 6,300) by day 7 after infection ( $P < 0.001$ ). All 16 patients demonstrated seroconversion.

We observed significant humoral responses to *V. cholerae*-specific antigens. VCC-specific IgG antibody titers peaked on day 30 and were significantly higher than titers on day 2 ( $P = 0.027$ ). TcpA- and *V. cholerae* O1 LPS-specific IgG levels were highest on day 7 ( $P < 0.001$  and  $P < 0.001$  compared to day 2). Patients demonstrated peak IgA responses on day 7 for all antigens, and these levels were significantly higher than day 2 measurements ( $P = 0.001$  for TcpA- and LPS-specific antibodies,  $P < 0.001$  for VCC-specific antibodies). LPS-, VCC-, and TcpA-specific antibody responses and vibriocidal titers were significantly greater on day 7 compared to responses observed in healthy controls.

**Memory T-cell responses.** Using the FASCIA method to measure lymphoblast proliferation, we observed significant increases in *V. cholerae*-specific memory T cells on day 7 after infection; these results are shown in Fig. 1. T-cell memory responses after stimulation by VCC and MP peaked on day 7 and decreased by day 30 ( $P = 0.013$  and  $P = 0.001$  for day 7 compared to day 2). Proliferation in response to TcpA increased by day 7 ( $P = 0.013$ ) and remained elevated until day 30. Compared to healthy controls, the proliferation of memory

TABLE 3. Serum vibriocidal and antibody responses in patients and healthy controls

Immune response	Antigen	Geometric mean antibody level (95% CI) for <sup>a</sup> :					
		Healthy controls	Study patients				
			Day 2	Day 7	<i>P</i> (day 2 vs day 7)	Day 30	<i>P</i> (day 2 vs day 30)
Vibriocidal	NA <sup>b</sup>	42 (20–91)	35 (13–97)	4,300 (3,000–6,300)*	0.0004	1,280 (900–1,800)*	0.0007
IgA	TcpA	16 (12–21)	13 (8.3–20)	38 (21–67)*	0.001	22 (15–35)	0.0007
	LPS	29 (15–56)	15 (9.4–22)	100 (62–180)*	0.001	34 (23–50)	0.0037
	VCC	35 (21–61)	52 (41–65)	150 (110–200)*	0.0004	88 (70–100)*	0.0008
IgG	TcpA	52 (39–70)	47 (34–64)	87 (64–120)*	0.0009	84 (58–120)	0.0008
	LPS	69 (29–46)	42 (29–62)	150 (110–220)*	0.0005	110 (86–152)*	0.0007
	VCC	150 (110–200)	190 (160–220)	230 (190–280)*	0.056	260 (220–300)*	0.027

<sup>a</sup> See Materials and Methods. CI, confidence interval. \*, *P* ≤ 0.05 compared to healthy controls.

<sup>b</sup> NA, not applicable.

T cells in response to VCC was significantly elevated on day 7 and day 30 (*P* < 0.001 and *P* < 0.001). Differences in proliferation between HC and patient lymphoblasts were significant on both day 7 (*P* < 0.001) and day 30 (*P* = 0.035). Stimulation with *V. cholerae* O1 LPS, a T-cell independent antigen, did not generate any significant differences in memory T-cell proliferation during the 30-day period after infection.

**Immune responses to VCC.** The antigen VCC generated a more robust T-cell memory lymphoblast response than that observed to other *V. cholerae* antigens assayed (Fig. 1). In addition, high-magnitude humoral responses to VCC were measured (Table 3). We also used the antibody in lymphocyte supernatant assay to assess mucosal immune responses to VCC (9). On day 7 after infection, lymphocytes stimulated in the gut mucosa transiently circulate in the peripheral blood (40). At this time, we observed a significant increase in VCC-specific antibodies secreted from circulating lymphocytes compared to day 2. These results are shown in Fig. 2.

**Memory T-cell function and phenotype.** We used known markers of T-cell function to further determine the nature of the memory T-cell populations after *V. cholerae* infection. The CD45RA marker was used to exclude intermediate memory populations (CD45R0<sup>+</sup> CD45RA<sup>+</sup> cells) from the analysis (17). The memory T-cell CD4<sup>+</sup> CD45R0<sup>+</sup> CD45RA<sup>-</sup> popu-

lation was on average 80% CCR7<sup>-</sup> after 6 days ex vivo stimulation, and a representative flow cytometry plot is shown in Fig. 3. Lymphoblast populations displaying these markers indicate an effector memory population. The remaining CD4<sup>+</sup> CD45R0<sup>+</sup> CD45RA<sup>-</sup> cells were CCR7<sup>+</sup>, markers consistent with a central memory T-cell phenotype.

**DISCUSSION**

After natural *V. cholerae* O1 infection, an adaptive immune response provides protection from subsequent disease, but the mechanism of this protection is incompletely understood. In North American volunteers, infection with *V. cholerae* provided over 90% specific protection from rechallenge with the same biotype, and this protection lasted for at least 3 years (24). An observational study showed that dehydrating *V. cholerae* El Tor infection in areas of endemicity conferred significant protection from subsequent disease for approximately 5 years (15).

Compared to natural infection, protective immunity generated by vaccine candidates has been incomplete and often short-lived. In addition, protection mediated by vaccination has declined more rapidly in persons living in areas of endemicity than in *V. cholerae*-naive persons. An oral, inactivated *V. cholerae* vaccine containing CTB (rCTB-WC) resulted in 60% protective efficacy in North American volunteers (7). When

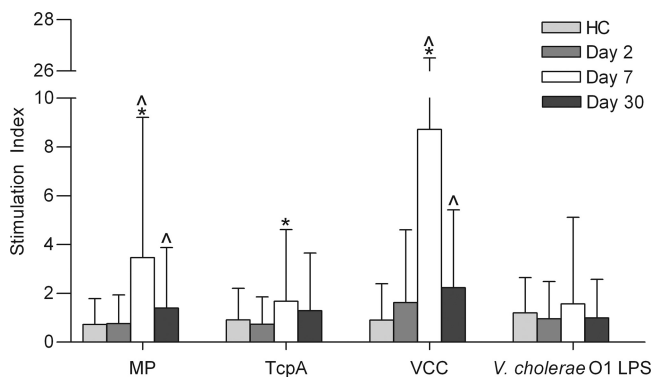


FIG. 1. Memory T-cell responses to *V. cholerae* antigens. Memory T cells are defined as CD4<sup>+</sup>/CD45R0<sup>+</sup> cells. \*, *P* ≤ 0.05 for day 2 compared to day 7 or day 30; Δ, *P* ≤ 0.05 for healthy controls (HC) compared to day 7 or day 30.

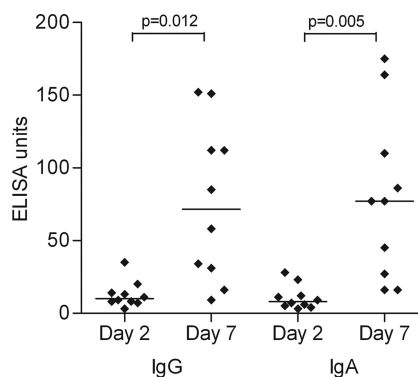


FIG. 2. VCC-specific antibody responses in supernatants of lymphocytes circulating on days 2 and 7 after infection.



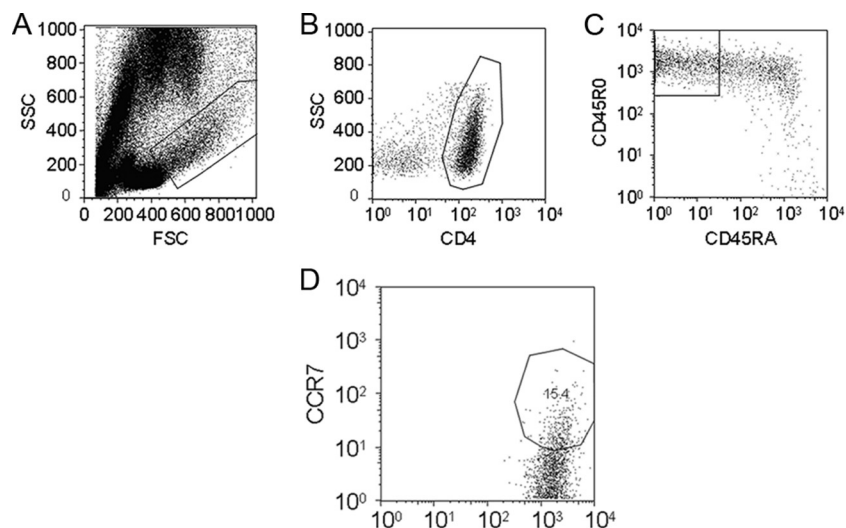


FIG. 3. Proportion of central memory cells in the proliferating memory T-cell population. On average, 20% of  $CD4^+ CD45R0^+ CD45RA^-$  memory T cells had the  $CCR7^+$  marker, a finding consistent with the central memory phenotype. Plots above are representative of the five patients sampled. (A) Lymphoblast and lymphocyte population after 6-day ex vivo stimulation with *V. cholerae* MP. (B) Lymphoblast gated  $CD4^+$  population. (C)  $CD4^+$  gated  $CD45R0^+ CD45RA^-$  population. (D)  $CD4^+ CD45R0^+ CD45RA^-$  gated  $CCR7^+$  population.  $CCR7^+$  gates were placed by comparing a  $CCR7$  unstained control to a  $CCR7$  stained plot.

tested in Bangladesh, this effect waned by 1 year and provided little protection in children (11). Similarly, a live attenuated vaccine strain (CVD-103HgR) gave minimal protection in Indonesian field trials after encouraging results were observed in North American volunteers (43, 49). The reasons for the differences in immune responses between vaccinees and cholera patients and for the discrepancies in the degree of protection between vaccinees in areas where the cholera is endemic versus areas where it is not endemic are not known.

The vibriocidal antibody response is the best characterized of the immune responses to *V. cholerae* infection. Although the magnitude of vibriocidal titer roughly correlates with protection from disease, this relationship is inconsistent. Since *V. cholerae* is noninvasive, it is unclear how a serum protein such as the vibriocidal antibody may provide protection. Instead, vibriocidal titers may serve as a proxy measurement for protective immunity. In a study of household contacts of cholera patients, a logistic regression model demonstrated that levels of vibriocidal antibody and CTB-specific IgA were associated with a protective effect independent of other immunologic factors (19). However, the duration and extent of protection mediated by these factors declines more rapidly than the protection observed after natural infection, suggesting that other longer-lasting immunologic responses are necessary for protection.

In order to identify additional factors that may play a role in protective immunity, we studied responses to VCC, an antigen for which immune responses have not previously been studied in humans. We observed that VCC is strongly immunogenic and generates both B- and T-cell responses of a higher magnitude than those observed to other *V. cholerae* antigens. Further studies are needed to characterize the role of VCC in both infection and protective immunity.

In addition to antigen-specific antibody responses, patients with cholera develop memory B cells specific for *V. cholerae*

antigens CTB, TcpA, and LPS (18, 22). Responses to protein antigens CTB and TcpA persisted for more than 1 year, whereas memory B-cell responses to LPS, a carbohydrate antigen, waned more quickly after infection (18). Memory B-cell responses to LPS do not require T-cell recognition, and this may explain the more rapidly declining memory B-cell response to LPS compared to responses generated by CTB and TcpA. Development of humoral responses to protein antigens are dependent on T-cell responses (25).  $CD4^+$  cell cytokine secretion and costimulation are the primary determinants of the quality and duration of memory B-cell responses to protein antigens (25). The direct binding of T and B cells in secondary lymphoid tissue facilitates CD40 and CD40L interactions critical for B-cell proliferation and isotype switching (13, 26). The stability of memory B-cell responses after exposure to TcpA and CTB, in contrast to T-cell-independent antigens such as LPS, is likely the result of T-cell contributions to memory B-cell activation.

Our results suggest that T-cell responses to *V. cholerae* infection may play a role in protective immunity, and several recent studies support this hypothesis. Cytokine responses, including an increase in IL-13 secretion by proliferating T cells, have been observed in acute cholera, suggesting a Th2 polarized T-cell response (6). Differences in cytokine levels in the serum and fecal extracts of parasite coinfecting cholera patients compared to parasite-uninfected patients suggest that T-cell responses contribute to differences observed between vaccinee responses in areas where cholera is endemic, where parasitic infection is common, versus areas where it is not endemic (20).

Previously observed T-cell responses following *V. cholerae* infection were hypothesized to represent memory T-cell responses because people living in areas of endemicity develop immune responses to *V. cholerae* as children (6, 16, 29, 30). Memory T cells are a heterogeneous population and, unlike B cells, changes in T cells induced by exposure to antigen may be

reversible (4, 27). Therefore, there is a lack of consensus on the classification of the populations that result from memory T-cell proliferation upon exposure to antigen. Generally, subsets are divided into effector memory cells that secrete cytokines and home to peripheral sites, and central memory cells that secrete fewer cytokines and home to lymph nodes (1, 47). We differentiated these populations using the CCR7 marker, a chemokine receptor active in T-cell migration (8). The absence of CCR7 on memory T cells is associated with the ability to secrete cytokines (1, 23). In our study, CD4<sup>+</sup> CD45RO<sup>+</sup> CD45RA<sup>-</sup> CCR7<sup>-</sup> cells, classified as effector memory cells, were the most abundant T-cell type after 6 days of in vitro stimulation. CD4<sup>+</sup> CD45RO<sup>+</sup> CD45RA<sup>-</sup> CCR7<sup>+</sup> cells were classified as central memory cells, and this subtype is known to respond to antigen upon subsequent exposure (5, 47). It is uncertain whether the prolonged in vitro stimulation affects the relative abundance of T-cell memory subtypes we observed.

We hypothesize that protective immunity to *V. cholerae* infection is mediated by an anamnestic memory B-cell response mounted in the intestinal mucosa and that memory T cells are necessary for developing and maintaining this response. Our results show significant memory T-cell responses to a variety of *V. cholerae* antigens by day 7 after infection, at a time prior to and concurrent with the development of B-cell responses. The majority of the memory T cells observed were of the effector memory phenotype, while a substantial minority were central memory T cells. These results suggest that T-cell responses to *V. cholerae* antigens may be important for generation and stability of memory B-cell responses.

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