Young children, older children, and adults develop comparable levels and durations of immunity following cholera. In comparison, young children receiving oral killed cholera vaccines (OCV) develop a lower level and shorter duration of protection than those of older children and adults. The reasons for this are unclear. We investigated OCV-induced memory T cell responses in younger and older children and compared responses to those in children with cholera. We found that patients with cholera developed significant levels of toxin-specific effector memory T cells (TEM) with follicular helper and gut-homing characteristics. Older children (6 to 14 years of age) receiving two doses of OCV containing recombinant cholera toxin B subunit (rCTB) had more modest TEM responses with follicular helper and gut-homing characteristics, but younger vaccinees (24 to 71 months of age) did not develop TEM responses. The TEM response correlated positively with subsequent IgG memory B cell responses specific to rCTB in older vaccinees. Cytokine analyses indicated that cholera patients developed significant Th1, Th17, and Th2 responses, while older children receiving vaccine developed more modest increases in Th1 and Th17 cells. Younger vaccinees had no increase in Th1 cells, a decrease in Th17 cells, and an increase in regulatory T (Treg) cells. Our findings suggest that T cell responses to cholera toxin as a mucosal adjuvant (11). Furthermore, using intracellular cytokine staining of individual T cells following stimulation with V. cholerae membrane preparations (MP), we have shown that T cell responses following naturally acquired infection are more durable than those to T cell-independent antigens (20). We have also shown the presence of mucosal interleukin 17 (IL-17) responses following naturally acquired infection, which is required to induce protective B cell responses to cholera toxin as a mucosal adjuvant (11). Furthermore, using intracellular cytokine staining of individual T cells following stimulation with V. cholerae membrane preparations (MP), we have shown that T cell responses following infection in adults are shifted toward a Th1 type of response, while this...
shift is not evident in adult vaccines (24). On the other hand, in children, we have demonstrated that CD4+ T cell responses to a mutant cholera toxin B subunit (CTB) antigen are detectable after vaccination while responses to MP are not found (2). These findings suggest that T cell responses in vaccinees are different from those associated with naturally acquired infection and that responses differ between adults and children.

Memory T cells are heterogeneous and are divided into central memory T cells (T<sub>CM</sub>), which migrate in lymphoid organs, and effector memory T cells (T<sub>EM</sub>), which migrate to peripheral tissues. T cell responses may be functionally classified into Th1, Th2, Th17, regulatory T cell (Treg), and the more recently recognized memory T cells (TCM), which migrate in lymphoid organs, and d30 indicate the early and late convalescent phases, respectively. (d, day; MBC, memory B cell).

**MATERIALS AND METHODS**

**Study subjects.** The study was conducted in Dhaka, Bangladesh. Following informed consent of parents/guardians, healthy children without diarrhea in the preceding 3 months and from the Mirpur area of Dhaka were assigned into older (6 to 14 years of age) and younger (2 to 14 months of age) groups for vaccination with two doses of Dukoral at a 2-week interval. Pediatric patients (2 to 14 years of age) admitted to the International Centre for Diarrheal Disease Research, Dhaka, Bangladesh (icddr,b), Dhaka hospital with acute diarrhea, whose stool culture was positive for V. cholerae O1 (33, 35), were also enrolled in this study for comparison. For patients, d2 indicates the acute stage of infection; d7 and d30 indicate the early and late convalescent phases, respectively. (d, day; MBC, memory B cell).

**Antigen.** We used the flow cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA) to detect lymphoblast populations in response to antigenic stimulation as previously described (16, 42, 45). For this method, we collected whole blood in a lithium-heparinized tube and diluted the sample 1:8 in Dulbecco modified Eagle medium (Gibco, NY) supplemented with 1% gentamicin, 1% mercaptoethanol, and 10% heat-inactivated fetal calf serum. We then added 100 μl of a stimulating antigen, control antigen, or antigenic mixture to each 400 μl of diluted blood in a 5-ml polystyrene tube. The final concentrations of each stimulatory antigen and the control antigen were 1 and 1 μg/ml, respectively. After 6 days of in vitro culture in an atmosphere containing 5% CO<sub>2</sub> at 37°C, we recovered the culture supernatant by centrifugation, mixed it with a protease inhibitor cocktail (aprotinin, 0.15 μM; leupeptin, 10 μM; sodium azide, 15 μM; and 4-(amino-ethyl) benzene sulfonyl fluoride, 0.2 μM) and then stored this at −70°C for subsequent cytokine analysis. We stained the pellet of whole-blood cells with anti-CD3-phycocerythrin (PE) Texas Red (Invitrogen, CA), anti-CD4-Amycan, anti-CD45RA-V450, anti-integrin β7-PE, anti-CXCR5-AP488, anti-CCR7-PE-Cy7, and anti-CCR9-AF647 monoclonal antibodies (BD Bioscience, San Jose, CA). We also stained a subset of samples with propidium iodide to determine the percentage of dead cells (see Fig. S1 in the supplemental material). In addition, we analyzed and excluded doublets (see Fig. S2a in the supplemental material). Erythrocytes were lysed with ammonium chloride (Sigma) solution containing potassium chloride and EDTA for 5 min, followed by centrifugation, removal of supernatant, and washing of the pellet. We resuspended cells in a BD stabilizing fixative (BD Bioscience, San Jose, CA) and stored them in the dark at 4°C. Cells were acquired within 12 h by fluorescence-activated cell sorting for standardized 2-min intervals using a FACSaria III instrument (BD Bioscience, San Jose, CA) and the FACSDiva software program. We analyzed the data by using the FlowJo software program (TreeStar, Inc., Oregon). We excluded dead cells, and we gated lymphocyte and lymphoblast populations using forward and side scatterers. Lymphocyte and lymphoblast populations were then analyzed separately for the presence of memory T cells and their subtypes. CD3<sup>+</sup> CD4<sup>+</sup> T cells from either lymphocytes or lymphoblasts...
we were selected for memory by the absence of the naive surface marker CD45RA, and effector memory T cells were selected based on the absence of the central memory marker CCR7. TFH cells were gated from the TEM population by a high level of CXCR5 expression. Antibodies to integrin β7 and CCR9 were used to gate gut-homing single-positive cells (integrin β7+ CCR9− or integrin β7− CCR9+) by gating of TEM cells (see Fig. S2b in the supplemental material). The cellular proliferative responses are presented as the ratio of lymphoblast count with antigenic stimulation to the count without stimulation (42). The ratio is referred to as the stimulation index (SI). An SI value equal to “1” indicates that stimulation is equal in samples with or without a V. cholerae antigen, and “>1” indicates V. cholerae antigen-specific stimulation.

**Cytokine analysis.** We measured concentrations of cytokines in FASCIA culture supernatants using the Milliplex human cytokine/chemokine kit (Millipore Corp., MA) and the Bio-Plex 200 system (Bio-Rad, Pennsylvania), per the manufacturers’ instructions. The cytokines analyzed were chosen on the basis of their known or possible role in infection, their expression at various stages of cell differentiation, and their ability to be detected within the limit of the experiment.

**Detection of MBC responses in blood.** We assessed rCTB-specific memory B cell (MBC) responses using an enzyme-linked immunosorbent spot (ELISPOT) technique as previously described (3, 20). We expressed results as the percentage of antigen-specific IgG or IgA memory B cells by ELISPOT out of the total IgG or IgA memory B cell population.

**Statistical analysis.** We used the software program Graphpad Prism 5.0 for statistical analyses and preparation of figures. We used paired t tests or Wilcoxon signed-rank tests to compare immunologic responses of cholera patients and vaccinees on different study days. We used Pearson’s correlation to assess the relationship between T and B cell responses. All reported P values are two tailed. We used a P value of ≤0.05 as the predetermined threshold for statistical significance.

**RESULTS**

**Study population.** We enrolled 40 children for vaccination, and 38 completed all follow-up visits. The median age of all children was 6 years (25th and 75th percentiles were 5 and 10 years, respectively). We enrolled 20 younger vaccinees (24 to 71 months of age; median, 60 months) and 20 older vaccinees (6 to 14 years of age; median, 10 years). In the younger group of vaccinees, 9 of 20 were female, and in the older group, 10 of 20. None of the vaccinees had adverse events after vaccination. To compare immune responses, we also enrolled eight children with acute cholera (5 females; median age, 66 months; 25th and 75th percentiles of 42 and 102 months, respectively); of these, four were 24 to 71 months of age (median, 42 months) and four were 6 to 14 years of age (median, 8.5 years). As part of the initial experiments to determine the abilities of different cholera toxin derivatives to stimulate T cells in a FASCIA analysis, we also enrolled nine adult cholera patients (5 females; median age, 25 years). All cholera patients were infected with the V. cholerae O1 Ogawa serotype.

**Selection of antigens for T cell stimulation.** We compared the antigen-specific memory T cell responses between the acute (day 2) and early convalescent (day 7) stages, utilizing different cholera toxin/toxoid antigens for T cell stimulation. We found that all antigens were able to produce significantly higher memory T cell responses on day 7 than on day 2 (Fig. 2). Among the antigens, mCT and dLT produced responses in all patients tested; however, we selected mCT for further experiments for its origin with V. cholerae and its corresponding higher magnitude of responses. We also included stimulation with MP as an antigen because this has been previously shown.
to stimulate memory T cells in individuals following cholera (24, 45).

**Distribution of memory subtypes in antigen-stimulated whole blood.** After 6 days of stimulation, more than 95% of lymphocytes were alive, and lymphoblast death was less than 20% (see Fig S1 in the supplemental material). As reported earlier (45), lymphoblasts had a higher memory cell-to-naive cell ratio than lymphocytes when stimulated in vitro for 6 days, and the majority of memory CD4+ T cells were TEM (see Fig. S2b). In lymphocytes, the gut-homing integrin β7-positive subset was larger in TEM cells (median, 20%) than in naive (median 3.5%; data not shown) T cells. In comparison, CCR9+ lymphocytes were found mostly in the naive T cell population (median, 11%, compared to ~1% in TEM cells; data not shown). Since T cell lymphoblasts were mostly of the TEM phenotype after stimulation, they expressed a higher percentage of integrin β7 and less of CCR9. Five to ten percent

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**FIG 3** Comparison of different toxin derivatives as T cell stimulatory antigens in younger children receiving vaccine (n = 20), older children receiving vaccine (n = 20), and patients with naturally acquired cholera (n = 8). Mean stimulation indexes of T effector memory cells and their subtypes, with error bars representing standard errors of the means, are given. An asterisk (*) denotes a significant difference (P ≤ 0.05) from the baseline (day 2 for patients and day 0 for vaccinees). An “∧” indicates a significant increase (P ≤ 0.05) in responses on day 7 for patients compared to day 21 responses in older vaccinees. The G33D variant of CT holotoxin (mCT), membrane preparation of V. cholerae O1 (MP), T effector memory cells (TEM), and T follicular helper cells (TFH) are analyzed.
CD4+ lymphocytes were CXCR5+ Tfh cells, and the percentages of these were similar in both TEM and TCAM cells (data not shown). Although the lymphoblast population had more TEM cells overall after stimulation, the percentage of follicular helper effector memory T cells (Tfh TEM) was less in the lymphoblasts than in the lymphocytes (data not shown). We focused on the phenotypes of lymphoblasts following antigen stimulation and further analyzed the effector memory T cells for their follicular helper and gut-homing subtypes.

mCT- and MP-specific memory T cell responses. In children infected with V. cholerae, mCT-specific T cell responses were significantly higher on day 7 than on day 2 of infection, including both the Tfh and gut-homing subsets (Fig. 3). In the older children following vaccination, we also found much more modest but statistically significantly higher Tfh and integrin B7 gut-homing subsets of TEM cells on day 21 than on day 0 (P < 0.02) (Fig. 3, top right panel). Younger vaccinees, however, did not demonstrate any increases in any subsets of TEM cells following vaccination (Fig. 3, top left panel). Cholera in children produced significant TEM responses to stimulation with MP, including the integrin B7-positive gut-homing subset at day 7 (Fig. 3, bottom right panel). However, MP did not induce T cell proliferation in blood from either older or younger children following vaccination (Fig. 3, middle panels).

Correlation between early memory T cell responses and late memory B cell responses in vaccinees. To determine the association between memory B and T cell responses specific to a particular cholera antigen, we compared rCTB-specific memory B cell responses at day 42 to mCT-specific TEM responses at day 21 in the same older vaccinees (median age, 10 years). We were able to compare data from 9 vaccinees for the IgG rCTB-specific responses and from 13 vaccinees for the IgA rCTB-specific memory B cell responses. We found that rCTB-specific IgG secreting memory B cells at day 42, 28 days after the second dose of vaccine, correlated with the values of both mCT-specific TEM responses on day 21, 7 days after the second dose of vaccine (Pearson correlation coefficient r = 0.89; P = 0.002) (Fig. 4), and also its follicular helper subtype (Pearson correlation coefficient r = 0.7; P = 0.03) (data not shown). We did not find any such correlation with early TEM responses and subsequent memory B cell responses of the IgA isotype.

Cytokine responses in culture supernatants of whole blood stimulated with mCT (G33D), determined by multiplex assay. To compare the functionality of the T cell responses after vaccination and infection, we compared cytokines in supernatants of whole blood stimulated with mCT on days 0 and 21 after vaccination and on days 2 and 7 in six infected patients (Fig. 5). We found that older vaccinees had small elevations of gamma interferon (IFN-γ) at day 21 compared to responses in younger vaccinees and of IL-17 compared to levels at day 0 but no significant changes in IL-13 or IL-10. In contrast, younger children receiving vaccine had no change in levels of IFN-γ or IL-13, a fall in the level of IL-17, and an increase in the level of IL-10 at day 21 compared to results at day 0, suggesting a significantly different T cell response in younger versus older vaccinees. On the contrary, patients with naturally acquired disease had elevated levels of IFN-γ, IL-17, and IL-13 at day 7 compared to those at day 2 (or compared to responses in vaccinees, for IL-17) and no significant change in IL-10.

DISCUSSION

Memory CD4+ T cells can differentiate into different subtypes and subsequently migrate to lymphoid or peripheral compartments. Upon reexposure to antigen, CD45RO+ CCR7+ central memory Th cells convert into effector memory cells (36). Tfh cells are an independent Th cell lineage distinct from Th1, Th2, or Th17 cells (31) that have central memory characteristics. Antigen-specific effector Tfh cells appear in circulation as CXCR5+ CCR7− Th cells (14) and, upon interaction with dendritic cells, express CCR7 and home to germinal center B cell zones, where they interact with antigen-primed B cells either by direct contact or by cytokine release (19). In this study, we have characterized memory Tfh cells, most of which develop into TEM cells after 6 days of culture (45). We found significant mCT-specific CXCR5+ CCR7− Tfh cell responses in older children receiving vaccine and in infected children but did not find these cells in younger children receiving vaccine. The absence of this response in younger children receiving vaccine may relate to the lower level and shorter duration of protection that they achieve following oral cholera vaccine administration compared to responses in older recipients (39, 46). Furthermore, the absence of MP-specific memory T cell responses in the vaccinees supports our previous findings (2, 24) and indicates a difference in the mechanism of protection between naturally acquired infection and vaccination, especially in children.

In this study, we also established a correlation between development of early antigen-specific TEM and Tfh cells and subsequent antigen-specific IgG memory B cell responses in vaccinees, suggesting that early T cell events may contribute to subsequent B cell memory. Tfh cells have been shown to regulate B cell maturation and proliferation, class switching, and somatic hypermutation (5, 10, 14, 29), and the presence of Tfh cells has been shown to be associated with increases in antibody production (7, 22). Our findings support a correlation seen earlier of CTB-specific T cell responses and serum antitoxin antibody responses in individuals receiving cholera vaccine (8) and suggest that specific T cell help may be necessary for robust B cell responses.

Oral vaccines have been shown to elicit gut-homing T cells expressing integrin B7 or CCR9 (28, 34). Integrin B7 and CCR9 bind to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) and CCL25 (TECK), respectively, which are expressed by endothelial
cells in lamina propria and Peyer’s patches, submucosal high endothelial venules, and intestinal epithelial cells (1, 6, 17). We detected significant mCT (G33D)-induced integrin β7 expressing gut-homing TEM cells both in older children receiving vaccine and in infected patients. As with the TFH subset, the absence of gut-homing TEM cells in younger children receiving vaccine may in part explain differences in protective efficacy afforded by OCV in younger versus older children. The absence of significant memory T cell responses in younger children receiving vaccine may relate to the lower frequency of these cells during early childhood and the gradual increase in this cell population during the first 5 years of life (43, 44). In studies of a novel tuberculosis vaccine, a lower frequency of cytokine-secreting CD4+ T cells was also observed in younger children than was seen in adolescents (38).

In our cytokine analysis, we chose representative cytokines for Th1, Th2, Th17, and Treg subsets, IFN-γ, IL-13, IL-17, and IL-10, respectively. We found substantial elevations of IFN-γ, IL-17, and IL-13 in patients and more modest elevations of IFN-γ and IL-17 in older vaccinees but no changes in IFN-γ or IL-13, a decrease in IL-17, and an increase in IL-10 in younger children receiving vaccine. The results for IFN-γ in infected patients and older vaccinees suggest activation of Th1 cells, which have been shown to be involved in the immunogenicity of vaccines (15). Younger children receiving vaccine did not develop a similar Th1 response and had decreases in the Th17 cytokine, IL-17. At the same time, the younger children receiving vaccine had elevated levels of IL-10, suggesting a possible increase in Treg cells that might be another contributing factor for lessened immunogenicity of cholera vaccine in younger children. IL-10 has been shown previously to suppress vaccine-mediated immunity (40).
A potential shortcoming of this study is that we did not assess the effect of H. pylori, which is associated with strong Treg responses in mice and humans and suppression of Th17 responses in mice (21, 27), although infection with H. pylori is common in Bangladesh and is unlikely to be differently distributed in the three groups we analyzed. Furthermore, the low volume of blood we obtained from children did not allow us to analyze intracellular cytokines, and the 6-day culture supernatant might not detect very early cytokines. However, our preliminary comparison of FASCIA supernatants at different time points (e.g., 4, 5, 6, and 7 days of culture) indicated 6-day culture to be optimum for detection of most cytokines of our interest (unpublished data). In addition, the pattern of the cytokine responses in patients of this study was comparable to that shown previously for adult patients by intracellular cytokine assay (24), suggesting that these cytokines were secreted mostly from corresponding T cell subtypes and suggesting a similarity of cytokine responses between adult and child cholera patients. We were also unable to enroll larger numbers of very young vaccinees (less than 36 months of age), an age group that would have been particularly informative. Immune responses in this even younger age group might be even more distinct than those we observed in the vaccinees in this study.

In summary, our results show that naturally acquired cholera infection is associated with robust TEm responses, including subsetss of TFH cells and cells expressing gut-homing markers. The cytokine profile following antigen stimulation showed strong IFN-γ, IL-13, and IL-17 production. Older children receiving vaccine had more modest TEm responses in the Tfh subset and in those expressing gut-homing markers. The cytokine profiles of these stimulated cells also showed more modest production of IFN-γ and IL-17. In contrast, the younger children receiving vaccine did not develop any TEm responses following vaccination, and the cytokine profile of stimulated T cells from this group showed no increase in IFN-γ, a decrease in IL-17, and an increase in IL-10, suggesting a possible increase in regulatory T cell populations specialized for B cell help or tissue inflammation. Nat. Immunol. 2, 876–881.

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