Activated T follicular helper-like cells are released into blood after oral vaccination and correlate with vaccine specific mucosal B-cell memory

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T follicular helper (Tfh)-like cells with potent B-cell helping ability are mobilized into human circulation after parenteral vaccination and are generally held to reflect ongoing germinal center reactions. However, whether mucosal vaccination induces systemic Tfh responses and how such responses may relate to IgA production are unknown. We investigated the frequencies, phenotype and function of circulating Tfh-like CD4+ CXCR5+ T cells (cTfh) in adults receiving an oral inactivated enterotoxigenic Escherichia coli vaccine. Subjects were classified as vaccine responders or weak/non-responders based on their intestine-derived antibody-secreting cell (ASC) IgA responses to major vaccine antigens. Oral immunization induced significantly increased proportions of cTfh cells expressing the cTfh activation marker inducible costimulator (ICOS) in ASC responders, but not in weak/non-responders. Vaccination also enhanced the expression of IL-21, Th17 markers and integrin β7 by activated cTfh cells, supporting functionality and gut homing potential. cTfh cells promoted total and vaccine specific IgA production from cocultured B cells. Magnitudes of cTfh responses assessed within a week after primary vaccinations correlated with memory intestine-derived vaccine specific IgA responses 1–2 years later. We conclude that activated ICOS+ Tfh-like cells are mobilized into blood after oral vaccination and may be used as biomarkers of vaccine specific mucosal memory in humans.

Protection against non-invasive enteric infections such as cholera and enterotoxigenic Escherichia coli (ETEC) diarrhoea is mainly mediated through antigen-specific secretory IgA (SIgA) antibodies produced locally in the mucosa. Such antibodies can be induced both by natural infection and by oral vaccination. However, little is currently known about the longevity of mucosal IgA responses in humans, including how such responses are initiated and maintained on a cellular level. This is largely due to the limited access to mucosal samples and scarcity of validated methods for measuring immunological memory in humans. Therefore, identification of easily assessable biomarkers associated with induction of protective and long-lasting IgA responses may improve our understanding of mucosal immunity in humans and facilitate the development and evaluation of new mucosal vaccines.

CD4+ T follicular helper (Tfh) cells promote long-lived humoral immunity by providing help to B cells in germinal centers (GCs) in secondary lymphoid organs. Although more fully characterized in mice, Tfh cells with potent B cell helping ability have also been observed in human tonsils. GC Tfh cells express the chemokine receptor CXCR5, which guides their migration into B cell follicles in response to the CXCL13 ligand, as well as inducible costimulator (ICOS), a molecule essential for the secretion of IL-21, which potently promotes class-switching and B cell differentiation into plasma cells and memory B cells. So far, the evaluation of Tfh responses in humans has been hampered by the difficulty to obtain secondary lymphoid tissue. However, a sub-set of circulating CD4+ CXCR5+ T cells which shares both phenotypic and functional properties with GC Tfh cells has been identified in both humans and mice. Although the vast majority of these circulating Tfh-like (cTfh) cells are in a resting state, studies show that ongoing GC reactions in peripheral lymph nodes result in the emergence of activated CD4+ CXCR5+ cTfh cells, characterized by high expression of ICOS and programmed cell...
death protein 1 (PD-1) and low expression of CCR7, in peripheral blood\(^{10,11}\). Furthermore, the cTfh responses appear to reflect the development of protective high affinity antibody responses after parenteral immunization in both mice and humans\(^{12-15}\). However, little is currently known about Tfh responses in GC or blood during mucosal immune responses in humans and whether cTfh responses can be used to monitor the processes leading to generation of long-lasting and protective mucosal IgA responses.

We have recently demonstrated the capacity of a novel oral inactivated whole cell vaccine against ETEC diarrhea (ETVAX) to induce both primary and memory mucosal IgA responses in two large Phase I trials in Swedish adults, using vaccine-specific IgA antibody secreting cell (ASC) responses in peripheral blood as well as SlgA antibody levels in fecal extracts as correlates of intestinal SlgA responses\(^{16,17}\). In these trials, we showed that the multivalent ETEC vaccine, consisting of four inactivated recombinant \(E.\ coli\) strains over-expressing the major ETEC colonization factors (CFs) CFA/I, CS3, CS5 and CS6 mixed with a heat labile toxin binding-subunit (LTB) related toxoid, LCTBA\(^{18}\), was safe and induced mucosal IgA responses to all CFs and to LTB in a majority of subjects\(^{16}\). Importantly, we also demonstrated that the vaccine induced long-lasting IgA responses, since a late booster dose administered 1–2 years after primary vaccinations induced significantly more rapid blood ASC responses than a single dose vaccination given to naive subjects\(^{17}\). Administration of the vaccine with the mucosal adjuvant double mutant heat labile toxin (dmLT) enhanced primary IgA ASC responses to antigens present in low amounts in the vaccine, but did not influence memory B cell responses\(^{16,17}\). In this study we utilized blood samples collected in the two previously reported vaccine trials for exploratory analyses to determine if oral vaccination gives rise to cTfh responses. We also investigated the potential relation between cTfh responses and memory IgA responses elicited in the intestinal mucosa.

**Results**

**Oral vaccination induced cTfh responses.** To determine if oral vaccination induces release of activated Tfh-like cells into peripheral blood, we selected frozen peripheral blood mononuclear cell (PBMC) samples from a subset of ETEC vaccinees, including subjects who had responded strongly as well as weakly/not at all to the five major vaccine antigens (CFA/I, CS3, CS5, CS6 and LTB) in the antibodies in lymphocyte supernatants (ALS) assay used for ASC assessment after primary and booster vaccinations (See Supplementary Fig. S1 and Materials and Methods for detailed study design and responder criteria)\(^{16,17}\). Blood ASCs induced by oral vaccination express the gut homing marker integrin \(\alpha 4\beta 7\) and correlate with ASC responses in intestinal mucosa as well as with IgA responses in fecal and lavage samples and are thus good surrogate measures of mucosal immune responses\(^{19-24}\). In the ALS assay, ex vivo secreto antibodies from plasma blasts/plasma cells (i.e. ASCs) are analyzed in culture supernatants, and provide comparable results to the more traditional ELISPOT assay for assessment of intestine-derived ASC responses to oral vaccination\(^{16,25-28}\). Subjects were defined as weak/non-responders to the vaccine based on the combined ALS response index, defined as the sum of the magnitudes (maximal fold rises above prevaccination baseline IgA levels) of IgA ALS responses to the five major vaccine antigens (Supplementary Fig. S1).

Since ICOS has previously been shown to consistently reflect cTfh activation after parenteral vaccination and has been used to monitor cTfh responses in several different studies\(^{14,15,29,30}\) we focused our analysis on ICOS-expressing cTfh cells, defined as CD4\(^+\)CXCR5\(^+\) T cells, in selected samples before and after oral ETEC vaccination by flow cytometric (FCM) analysis (the gating strategy is shown in Supplementary Fig. S2). We found significantly increased proportions of ICOS\(^+\) cells among CD4\(^+\)CXCR5\(^+\) cTfh cells in post- compared to pre-vaccination samples in subjects receiving two primary oral doses of ETEC vaccine, two weeks apart (Fig. 1a). Comparable increases in ICOS expression on cTfh cells were also detected after administration of a single, late booster vaccine dose 1–2 years later (Fig. 1b). Data shown in Fig. 1b is from a separate set of subjects than the primary vaccination subjects in Fig. 1a). In contrast, the frequencies of ICOS\(^+\) cells among CD4\(^+\)CXCR5\(^+\) T cells did not change after vaccination (Fig. 1a and b). Increased ICOS expression on cTfh cells was often detected both after the first (day 7) and second dose (day 5), with a majority (70%) of strong ALS responders to the vaccine having higher responses at the later time point. The ICOS expression decreased in most subjects on day 7 compared to day 5 after the second dose. After administration of the late booster dose, increased ICOS expression compared to pre booster levels was commonly observed both on day 4/5 and day 7, with a majority (60%) of strong ALS responders having higher responses on day 4/5 compared to day 7. Maximal increases in ICOS expression on cTfh cells detected at any time point after primary (one or two doses) or booster vaccinations are indicated in the graphs (Fig. 1a–c). The maximal cTfh responses observed after administration of the late booster dose were of similar magnitudes as the maximal responses after the primary vaccine doses (Fig. 1a and b). We also found a small, but significant increase in CXCR5 expression among CD4\(^+\) T cells (13.8 vs 15.3%, \(P = 0.02, n = 14\)) in post-compared to prevaccination samples in subjects receiving two primary oral doses of ETEC vaccine. However, the frequencies of CXCR5\(^+\) cells did not change in response to booster vaccination 1–2 years later (9.0 vs 9.2%, \(P = 0.2, n = 10\)).

Since ICOS expression increased to comparable extent both with regard to magnitude of responses and frequencies of responders after primary and booster vaccination, data from the two sets of subjects were pooled and analyzed in relation to vaccine-specific intestine-derived IgA ALS responses (Fig. 1c). cTfh responses, henceforth defined as increased ICOS expression after vaccination compared to prevaccination baseline levels, were primarily detected in subjects classified as strong ALS responders, whereas responses in weak/non-responders were not significant. cTfh responses were observed in subjects receiving primary vaccine doses with as well as without dmLT adjuvant (Group B versus C or D; Fig. 1a). A large majority of the ICOS\(^+\) cTfh cells expressed the Tfh-associated marker PD-1, consistent with a cTfh phenotype\(^{31}\) and the PD-1 expression among ICOS\(^+\) cTfh cells was significantly lower (Fig. 1d and e). The vast majority (>90%) of the ICOS\(^+\) cTfh cells were CD27\(^+\)CD45RO\(^+\) central memory cells (Supplementary Fig. S2).
Previous studies show that cTfh cells produce IL-21 after activation and that this cytokine is critical for their ability to aid antibody production. To analyze the functional properties of cTfh cells in ETEC vaccinees, IL-21 production was analyzed after stimulation of PBMCs with phorbol myristate acetate (PMA) and ionomycin. Consistent with their activated Tfh-like phenotype, ICOS$^+$ CD4$^+$ CXCR5$^+$ T cells produced IL-21 while...
ICOS+ cells produced little IL-21 (Fig. 1f and g). Notably, ETEC vaccination induced increased expression of IL-21 among ICOS+ CD4 T cells, but not among ICOS+ CD8 T cells (Fig. 1f and g), in all tested individuals.

Collectively, these results demonstrate that oral ETEC vaccination induces the appearance of activated ICOS+ CD4 CXCR5+ cells with Th-like characteristics in peripheral blood after both primary and booster mucosal immunizations in individuals with a strong IgA ASC response to the vaccine.

**cTfh responses correlated with vaccine specific IgA responses after oral vaccination.** Previous studies show that after parenteral vaccination, activated cTfh cells promote the differentiation of plasmablasts, which emerge in peripheral blood at the same time as activated cTfh cells14,30,33,34. Our ALS data indirectly demonstrate that oral ETEC vaccination also induces the release of plasmablasts into peripheral blood, since vaccine specific ASC responses in blood induced by oral vaccination are mainly derived from gut homing plasmablasts19,23,24. Consistently, we found significantly increased frequencies of circulating CD27+ CD38hi plasmablasts in subjects both after primary and booster vaccinations (Supplementary Figs S3 and S4). As would be expected, increased frequencies of plasmablasts were primarily detected in strong ALS responders (Fig. 2a and b, maximal response magnitudes are shown). The kinetics of plasma blast responses, as detected by FCM, was consistent with previous reports of the ALS response kinetics after primary and booster vaccinations16,37. Most subjects (75%) had higher responses after the second compared to the first vaccine dose, with maximal responses detected on day 5 after the second dose in a majority of subjects. After the late booster dose, 90% of subjects had higher responses on day 4/5 compared to day 7. The maximal plasmablast responses to the primary vaccine doses and the late booster dose were of similar magnitudes (Figure 2a and Supplementary Fig. S4). In contrast to the observed plasmablast responses, frequencies of CD19+ B cells or CD19+ CD27+ memory B cells did not change in response to vaccination (Supplementary Fig. S4). Comparable magnitudes of plasmablast responses were observed after vaccination with or without dLT (Supplementary Fig. S4). A large proportion of plasmablasts expressed IgA (Fig. 2c).

Although the frequencies of total plasmablasts increased after vaccination, the relative proportions of IgA+ cells among the plasmablasts did not change. The magnitudes of IgA+ plasmablast responses (fold rises in plasmablast frequencies) correlated significantly with the combined magnitudes of vaccine specific IgA ALS responses to the five major vaccine antigens (Fig. 2d). The expression of IgG or IgM on plasmablasts was not analyzed due to limited cell numbers and technical difficulties to analyze such cells.

Given the importance of Tfh cells for plasma cell development, we next evaluated the relation between the cTfh and plasmablast and ALS responses induced by ETEC vaccination in a larger number of vaccinees from the primary and booster vaccination studies. The magnitudes of the cTfh responses correlated significantly with both the IgA+ plasmablast responses (Fig. 3a, R = 0.77), the total plasma blast responses (R = 0.70, not shown) and the combined index of ALS responses against the five major vaccine antigens (Fig. 3b, R = 0.60).

Taken together, these results demonstrate that IgA+ plasmablasts and activated cTfh cells are released into peripheral blood in parallel after oral vaccination, and that the magnitudes of these two types of responses correlate. These data indicate that a functional relationship may exist between cTfh activation and plasmablast induction after oral vaccination.

**cTfh cells from ETEC vaccinees promoted vaccine specific IgA antibody production.** To evaluate the functional capacity of cTfh cells from ETEC vaccinees to influence B cell antibody production, interactions between cTfh cells and B cells were mimicked *in vitro* by culturing sorted CD4+ CXCR5+ cTfh or CD4+ CXCR5- T cells with CD19+ CD27+ memory B cells isolated from the same cell samples from three strong responders to the vaccine (Supplementary Fig. S5). After 6 days of coculture in the presence of the superantigen staphylococcus enterotoxin B (SEB), ≥93% of the T cells were alive. cTfh cells strongly promoted B cell survival and/or expansion, since cultures with CXCR5+ cells contained significantly higher proportions of live CD19+ B cells than cultures with CXCR5- cells (≥83% vs ≤18%). In cultures with cTfh cells memory B cells differentiated into IgA expressing plasmablasts, whereas almost no live IgA+ plasmablasts were detected in cultures with CXCR5- cells (Fig. 4a). Increased proportions of live IgA+ plasmablasts were present in cultures with cells collected post-coculture and compared to prevaccination in all three individuals tested (Fig. 4a). Furthermore, relatively high levels of IgA antibodies were detected in supernatants from cTfh cocultures, and the production increased in response to vaccination in all subjects, whereas much less IgA was detected in cocultures with CXCR5- cells isolated at any time point (Fig. 4b). Due to the low number of cells and small volumes of culture supernatants available from one of the three subjects tested, samples from only two volunteers could be analyzed for vaccine specific IgA antibodies or total IgG antibodies. Importantly, our results from these subjects suggest that cTfh cells could also promote the production of vaccine specific antibodies, since increased levels of LTB-specific IgA antibodies were detected in cocultures with post vaccination memory B cells (Fig. 4c). Some LTB-specific antibodies were also detected in cultures with CXCR5- cells using the highly sensitive GM1-LTB ELISA method, but at about 4-fold lower levels than in cTfh cultures from the same volunteers. IgG antibodies were also primarily detected in cTfh cocultures and levels increased after vaccination (Fig. 4d). Due to the low numbers of weak responders to the vaccine, the functional capacity of cTfh cells from this study group could not be evaluated.

Collectively, these results support the conclusion that cTfh cells from ETEC vaccinees can actively promote plasmablast differentiation and IgA production.

**Activated cTfh cells and plasmablasts induced by oral vaccination expressed integrin α4.** Integrin α4β7 is an important homing receptor recruiting B and T cells to intestinal lymphoid tissue and lamina propria25. To evaluate the gut homing properties of cTfh cells activated by ETEC vaccination, the expression of integrin α4β7 was analyzed on ICOS+ cTfh cells from ETEC vaccinees. Among the activated cTfh cells, 15–60% expressed α4β7 before vaccination and the expression increased significantly in response to both primary and booster vaccinations (Supplementary Fig. S6). The up-regulation of integrin α4β7 was only observed in activated ICOS+ cTfh cells,
Figure 2. Oral ETEC vaccination induced increased frequencies of circulating plasmablasts. (a and b) Frequencies of plasmablasts (CD27+CD38hi) among CD19+ B cells in PBMCs collected before (preVacc) and after (postVacc) vaccination were analysed by FCM. (a) Data for strong and weak/non-responders (data pooled from the primary and booster vaccination study; the same subjects as in Fig. 1a–c). (b) Representative plasmablast staining from one strong ALS responder. (c) Frequencies of IgA+ cells among plasmablasts in post vaccination samples from strong and weak/non-ALS responders from the primary and booster studies (symbols as in (a), filled symbols; strong responders and open symbols; weak/non-responders). (d) Correlation between the combined response index of vaccine specific IgA ALS responses against the five major vaccine antigens and the magnitudes (fold rises) of IgA+ plasmablast responses (n = 24; same subjects as in a). (a,c and d) Each symbol represents one individual. (a and c) Bars indicate median values.
whereas the frequencies of $\beta_7^+$ cells among ICOS$^-$ cTfh cells or CD4$^+$CXCR5$^-$ T cells did not change (Fig. 5a and Supplementary Fig. S6). Interestingly, increased $\beta_7$ expression was detected on activated cTfh cells from both strong and weak/non-ALS-responders (Fig. 5a). The expression of $\beta_7$ also increased on circulating plasmablasts in both strong and weak/non-ALS-responders (Fig. 5c and d). In contrast, vaccination did not influence $\beta_7$ expression in CD19$^+$B cells or CD19$^+$CD27$^+$ memory B cells (Supplementary Fig. S6).

In conclusion, these results support increased intestinal homing potential of both cTfh cells and plasmablasts in response to oral ETEC vaccination in a majority of vaccinees.

Oral vaccination enhanced the expression of Th17 associated markers on activated cTfh cells. Previous studies show that cTfh cells, like non-cTfh CD4$^+$ T cells, can be subdivided into Th1, Th2 and Th17 subsets, characterized by production of the signature cytokines IFN-\(\gamma\), IL-4/IL-5/IL-13 and IL-17A/IL-22, respectively\(^{10,12,32}\). These subsets can be distinguished phenotypically by expression of the chemokine receptors CXCR3 and CCR6. Notably, Th17 (CXCR3$^-$CCR6$^+$) and Th2 (CXCR3$^+$CCR6$^-$) tTfh cells can efficiently induce both naive and memory B cells to become plasmablasts in vitro, while Th1 (CXCR3$^+$CCR6$^+$) tTfh cells may primarily support memory B cell differentiation\(^{10,12,14,31}\). In addition, the Th17 tTfh subset strongly promotes switch to IgA production in naive human B cells\(^{12}\). To evaluate whether oral ETEC vaccination influences cTfh cell polarization, we analyzed CXCR3 and CCR6 expression on T cells from strong responders to the ETEC vaccine (Fig. 6a–d). The proportions of activated cTfh cells expressing Th17 markers significantly increased whereas the expression of Th1 markers decreased in response to both primary vaccination and booster vaccination (Fig. 6b–d). The largest increase in expression of Th17 associated markers was observed among activated ICOS$^+$ cTfh cells, but a smaller increase was also noted among ICOS$^-$ cTfh cells (Supplementary Fig. S7). A very small, but significant increase was also observed among CD4$^+$CXCR5$^-$ cells. Corresponding decreases in Th1 associated markers were detected in ICOS$^+$ and ICOS$^-$ cTfh cells as well as the whole CD4$^+$ T cell population (Supplementary Fig. S7). Expression of integrin $\beta_7$ was significantly higher in Th17 compared to Th1 or Th2 subsets (Fig. 6e). Due to the low numbers of weak responders to the vaccine, and the low numbers of ICOS$^+$ cTfh cells in this study group, the polarization of ICOS$^+$ cTfh cells could not be evaluated among weak vaccine responders.

The down-regulation of Th1 characteristics in cTfh cells in response to vaccination was supported by decreased frequencies of IFN-\(\gamma\) producing cells detected among activated cTfh cells after PMA stimulation in post vaccination samples among all individuals tested (Fig. 6f). In contrast, the production of IFN-\(\gamma\) among ICOS$^+$ cTfh cells did not change in response to vaccination. PMA stimulation only induced IL-17A expression in a very small proportion of CD4$^+$ T cells which prevented analysis of IL-17A expression in the cTfh subset.

Taken together, these results indicate that ETEC vaccination primarily promotes the development of Th17 associated properties of activated cTfh cells and that these cells have mucosal homing potential.

cTfh responses to primary vaccination correlated with vaccine specific memory IgA responses. To specifically evaluate the relation between cTfh responses and B cell memory, we investigated the correlation

**Figure 3.** cTfh responses induced by oral ETEC vaccination correlated with blood plasmablast and vaccine specific ALS responses. Correlation between the magnitudes (fold rises) of activated cTfh cell (ICOS$^+$CD4$^+$CXCR5$^+$) and (a) IgA$^+$ plasmablast (IgA$^+$CD27$^+$CD38$^+$CD19$^+$) responses and (b) the combined response index of vaccine specific IgA ALS responses against the five major vaccine antigens. Each symbol represents one individual; n = 31 in (a) (18 subjects from the primary and 13 from the booster vaccination study) and n = 34 in (b) (20 subjects from the primary and 14 from the booster vaccination study) and data is displayed in a log-log scale plot.
between cTfh responses detected after primary vaccination and the vaccine specific memory IgA responses induced by a late booster dose administered 13–23 months later in all subjects from whom samples were available for analysis both after primary and booster vaccinations. We have recently shown that the strong vaccine specific ALS IgA responses detected on day 4/5 after administration of a single booster ETEC vaccine dose reflect a mucosal memory IgA response, since unvaccinated subjects respond very weakly or not at all to a single dose of vaccine at this early time point. We found that the magnitudes of cTfh responses after primary vaccination correlated significantly with the combined vaccine specific IgA ALS response index after the booster immunization (Fig. 7), i.e. with vaccine specific IgA antibodies produced by plasmablasts derived from mucosal memory B cells reactivated by the booster vaccination and transiently migrating through peripheral blood. These results suggest that activated ICOS⁺ cTfh cells mobilized into peripheral blood early after primary mucosal vaccination may be useful and easily assessable biomarkers for memory B cell induction.

Discussion

In this study, we have shown that oral vaccination of healthy volunteers resulted in increased frequencies of activated ICOS⁺ CD4⁺ CXCR5⁺ Tfh-like cells in the peripheral circulation. To our knowledge, this is the first demonstration of a cTfh response in blood after mucosal vaccination, suggesting that monitoring of cTfh cells may provide information about GC responses in the intestinal mucosa. The increased expression of integrin β7 detected on the activated cTfh cells in response to vaccination supports that these cells have gut homing properties.

Figure 4. cTfh cells from ETEC vaccinees promoted plasmablast formation and IgA antibody production. CXCR5⁺ CD4⁺ CD3⁺ cTfh cells, CXCR5⁻ CD4⁺ CD3⁺ T cells and memory B cells were sorted from PBMCs collected from three strong ALS responders before (preVacc) and after (postVacc) ETEC vaccination. cTfh or CD4⁺ CXCR5⁻ cells were coincubated with memory B cells collected at the same time point and stimulated with SEB for 6 days. The frequencies of IgA⁺ CD27⁺ CD38hi plasmablasts among CD19⁺ B cells (a) and the levels of total IgA (b), LTB-specific IgA (c) and total IgG (d) antibodies in culture supernatants were analyzed by FCM (a) or ELISA (b, c and d), respectively. Each symbol represents one subject (Subj.) and bars indicate median values (n = 2–3).
Importantly, we also showed that the magnitudes of cTfh responses to primary vaccination correlated significantly with both primary and memory intestine-derived vaccine specific IgA responses, providing further support that monitoring of cTfh cells may increase our understanding about how human mucosal B cell responses are generated and maintained after enteric antigen exposure.

Tfh cells can be a limiting factor for the GC reaction and antibody production. B cells compete for Tfh help in the GC, and B cells that are able to efficiently bind and present antigen to Tfh cells are given survival signals, leading to affinity maturation and the formation of long lived plasma blasts and memory B cells. The exact ontogeny of cTfh cells is still unclear, but several lines of evidence suggest that these cells may be committed to the Tfh lineage, but may leave the peripheral lymphoid organ before they enter the GC. Thus, both humans and mice deficient in signaling lymphocytic activation molecule associated protein (SAP), a key molecule for interaction between Tfh cells and B cells in the GC required for Tfh differentiation, have normal proportions of cTfh cells, but low antibody production, supporting that cTfh cells are Tfh precursors. This is also suggested by the parallel kinetics of cTfh responses in blood and in lymph node GCs in mice after parenteral vaccination. Thus, cTfh cells may be early lymph node emigrants, spreading to additional lymphoid tissue via the blood.

Our observation of increased integrin β7 expression specifically on activated cTfh cells after oral vaccination suggests that these cells are migrating back to the intestinal mucosa, where the α4β7 receptor binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed by endothelial cells in Peyer’s patches (PPs), mesenteric lymph nodes as well as in lamina propria. In mice, a single oral dose of a nitrophenyl-cholera toxin (NP-CT) conjugate was shown to activate B cells in GCs only in a few PPs, but the activated B cell clones then

Figure 5. Oral ETEC vaccination induced increased expression of integrin β7 on activated cTfh cells and circulating plasmablasts. Expression of integrin β7 was analysed among activated cTfh cells (ICOS+CXCR5+) in PBMCs collected before (preVacc) and after (postVacc) vaccination. Data pooled from the primary and booster vaccination study. Each symbol represents one individual (the same subjects as in Figs 1a–c and 2a). Bars indicate median values. Representative stainings from one strong ALS responder.
Figure 6. Oral ETEC vaccination induced increased expression of Th17 associated markers on activated cTfh cells. Activated cTfh cells (ICOS+CD4+CXCR5+) were analysed for the expression of CXCR3 and CCR6 by FCM, as illustrated by the representative staining of cells from one strong responder (a). Frequencies of activated cTfh cells expressing Th1 (CXCR3−CCR6+) (b) and Th1 (CXCR3−CCR6+) (c) associated markers among strong ALS responders before (preVacc) and after (postVacc) vaccination (n = 12, subset of the 15 strong responders displayed in Figs 1a–c, 2a and 5a and c). (d) Proportions of cTfh cells expressing Th1, Th2 and Th17 markers. (e) Expression of integrin β7 among activated cTfh cells expressing Th1, Th2 and Th17 markers from strong ALS responders (n = 9). (f and g) IFN-γ expression among activated cTfh cells from strong responders after stimulation of PBMCs with PMA and ionomycin (n = 5). (b,c,e,f) Each symbol represents one individual and bars indicate median values.
In our cTfh analyses, a relatively high proportion of weak or non-responders two doses of the ETEC vaccine administered two weeks apart induced ASC responses to the primary vaccine antigen to monitor the development of potentially protective IgA antibody responses in humans. We have recently shown that specific IgA responses induced by oral vaccination suggest that cTfh cells may be used to as biomarkers to monitor the development of potentially protective IgA antibody responses in humans. We have recently shown that specific IgA ALS responses against the five major vaccine antigens elicited by booster vaccination. Each symbol represents one individual (n = 11) and data is displayed in a log-log scale plot.

From a practical perspective, our observations that the magnitudes of cTfh responses correlate with vaccine specific IgA responses induced by oral vaccination suggest that cTfh cells may be used to as biomarkers to monitor the development of potentially protective IgA antibody responses in humans. We have recently shown that two doses of the ETEC vaccine administered two weeks apart induced ASC responses to the primary vaccine antigen to monitor the development of potentially protective IgA antibody responses in humans. We have recently shown that specific IgA responses induced by oral vaccination suggest that cTfh cells may be used to as biomarkers to monitor the development of potentially protective IgA antibody responses in humans. We have recently shown that specific IgA ALS responses against the five major vaccine antigens elicited by booster vaccination. Each symbol represents one individual (n = 11) and data is displayed in a log-log scale plot.

At present, cTfh responses have been monitored after a few different types of parenteral immunizations in humans, including influenza, human papillomavirus, pneumococcal polysaccharide or HIV vaccinations. Similar to our findings, increased frequencies of ICOS expressing cTfh cells were observed in the circulation after such parenteral vaccinations. Expanded populations of ICOS+CD4+CXCR5+ T cells have also been observed both in blood and draining lymph nodes in mice after parenteral vaccination with NP-ovalbumin. cTfh responses to vaccine have also been analyzed using alternative combinations of cell surface markers. A majority of activated ICOS+CD4+CXCR5+ cells in blood are CCR7low PD-1high, whereas resting cTfh cells, representing the majority of cTfh cells, are ICOS+CCR7lowPD-1low. Using the CCR7lowPD-1high phenotype to distinguish cTfh with efficient effector functions, He et al. demonstrated cTfh responses to influenza vaccination with similar kinetics as reported using ICOS to define activated cTfh cells. A corresponding expanded subset of CCR7lowPD-1high cells capable of maturing into mature and functional GC Tfh cells upon antigen reexposure was also observed in peripheral blood of mice after parenteral vaccination. In our hands, analysis of ICOS expression gave more consistent results than analysis of the CCR7lowPD-1high cell population, but similar trends were obtained in subjects where cell numbers allowed parallel analysis of all markers. Recently, circulating Tfh-like cells in humans and non-human primates have also been analyzed using expression of other
and malaria may be due to preferential activation of Th1 Tfh cells \cite{14,42}. The Th17 type subset of cTfh cells is also
activated by STAT3 and cytokines IL-17A and IL-22 \cite{12,32}. This cell subset is also a potent producer of IL-21, which is essential for the ability
to promote affinity maturation, plasmablast formation and development of memory B cells \cite{12,32,46}. However, there were no apparent differences in the magnitude of cTfh responses or expression of Th1, Th2 or Th17 associated markers in subjects immunized with or
without dmLT adjuvant in our study, although this needs to be confirmed in a larger number of volunteers in
later trials. Our recent results in adults suggest that dmLT only enhanced magnitudes of ASC responses to the
antigens present in the lowest amounts in the ETEC vaccine, particularly CS616, which is consistent with the
dose sparing effect of dmLT observed in mice \cite{46}, and that dmLT did not affect memory development \cite{47}.Since a high dose of the vaccine was administered in this study, it is possible that the adjuvant was not able to further
promote ThB and B cell responses to most antigens. In continued trials testing the immunogenicity and efficacy of the ETEC vaccine with and without dmLT adjuvant, it will be important to specifically monitor the Th17 cTfh subset, since \textit{in vitro} as well as \textit{in vivo} data support that dmLT may promote Th17 responses \cite{47–49}, and, as previously mentioned, the Th17-like cTfh subset has been shown to be the most potent inducer of IgA responses \textit{in vitro} \cite{42}, and Th17 polarization of cTfh cells has been associated with induction of protective antibody responses in different settings \cite{31,41}.

In conclusion, we have shown that oral vaccination with a killed whole cell ETEC vaccine induced the appearance of ICOS\textsuperscript{+} activated Th1-like T cells with a Th17 and gut homing phenotype in peripheral blood. cTfh cells from vaccinees had potent B cell helping capacity and could promote both total and vaccine specific IgA production. Our demonstration of functional and gut homing cTfh response to ETEC vaccination indicates that the vaccine induces potent GC reactions in the intestinal mucosa. Our observation that the magnitudes of primary cTfh cell responses correlate with both primary and memory vaccine specific mucosal IgA responses provide further support of a functional role of these cells and also suggest that cTfh responses may be used as early and easily assessable biomarkers to predict long-lived memory responses induced by mucosal vaccination in humans.
Methods

Immunizations and sample collection. Detailed descriptions of the two clinical vaccine trials have been published elsewhere, together with results from the primary endpoint analyses of these trials. Briefly, in the primary vaccination study previously unvaccinated Swedish adults received two oral doses of the ETVAX ETEC vaccine with or without 10 or 25 µg dmLT and in the booster vaccination study, random subsets of subjects previously immunized with ETVAX with or without 10 µg dmLT received a single oral booster dose of vaccine alone 13–23 months later (See supplementary Fig. S1 for additional information about the study design and sample collection schedules). PBMCs were isolated from venous blood by density-gradient centrifugation using Ficoll-Paque and cryopreserved as described by Kreher et al. All studies were approved by the Ethical Review Board in the Gothenburg Region and the Swedish Medical Product Agency. Written informed consents were obtained from all volunteers before participation. All experiments and procedures were performed according to relevant guidelines and regulations. The clinical trials are registered in the ISRCTN data base (ISRCTN91136076 and ISRCTN27096290). Preliminary data from the present study was presented at the Vaccines for enteric diseases (VED) conferences in Edinburgh in 2015 and in Albufeira 2017 and the Correlates of enteric vaccine-induced protection meeting (Fondation Merieux) in Annecy 2016.

Vaccine. The multivalent ETEC vaccine ETVAX consists of four inactivated recombinant E. coli strains which overexpress the Cfs CFA/I, CS3, CS5 and CS6, respectively, mixed with LCTB4, a recombinantly produced LTB/cholera toxin B subunit hybrid protein. The vaccine was administered orally alone or together with 10 or 25 µg dmLT adjuvant in 150 ml bicarbonate buffer. dmLT (R192G/L211A) is an LT-derived protein which contains two genetic substitutions in the A subunit which eliminates the enterotoxic activity without removing the adjuvant activity.

ALS analyses. Previously published results from ALS analyses were used for comparisons between ASC and cTfh responses in this paper. Briefly, 2 × 10⁶ fresh PBMCs per well were cultured in 96-well plates in the ALS assay and supernatants were collected after 72 hours. Antibody levels in ALS specimens were analyzed by ELISA using plates coated with CFA/I, CS3, CS5 or CS6 or with GM1 plus LTB.

Study subjects. PBMCs isolated from 14 subjects, aged 18–32 years (median 24 years, 5 females) in the primary vaccination study and 10 subjects in the booster vaccination study (separate from the subjects evaluated after primary vaccination) aged 22–45 years (median 26 years, 8 females) were included in the basic analyses presented in Figs 1, 2, 5 and 6 and the corresponding supplementary figures in the present study (total n = 24). Subjects were selected based on the availability of frozen cells and their combined ALS response index calculated from ALS measurements reported previously. Subjects with a combined ALS response index, defined as the sum of the magnitudes (maximal fold rises above the prevaccination baseline) of IgA ALS responses to the five major vaccine antigens LTB, CFA/I, CS3, CS5 and CS6, of ≥150 were defined as strong responders and subjects with an index ≤ 100 as weak/non-responders (Supplementary Fig. S1). Additional subjects from the primary vaccination study (n = 7, median age 24, 20–31 years, 3 females) and the booster vaccination study (n = 7, median age 25, 23–30 years, 3 females) were included in functional (Figs 1f, 4 and 6f) and correlation (Figs 3 and 7) analyses.

Flow cytometric analysis. FCM analysis was performed using an LSRII flow cytometer (BD Pharmingen, San Diego, CA) and FlowJo analysis software (Version 10, Treestar Inc, Ashland, OR).

For phenotypic FCM analyses of cTfh cells, cryopreserved PBMCs (2 × 10⁶ cells/sample) were stained with anti-CD3-Pacific Blue (clone UCHT1), anti-CD4-APC-Cy7 (RPA-1T), anti-CD27-PerCP-Cy5.5 (DX9), anti-CCR6-PECy7 (11A9), anti-CXCR3-Alexa Fluor 700 (1G6) anti-Integrin β7-APC (FIB504), anti-CD27-Alexa Fluor 700, anti-CD45RO-PECy7 (UCHL1), anti-CCR7-PECy7 (150503), anti-CD62L-Fluor780 (SK11) (all from BD Biosciences, San Jose, CA) and anti-CD20-APC (clone 2H7). Cells were stimulated with 50 ng/mL of PMA and 1 µg/mL of ionomycin in the presence of brefeldin A (10 µg/mL) for 5 hours. After live/dead staining, PBMCs were stained with anti-CD3-Pacific Blue Fluor 700 (UCHT1), anti-CD4-APCCy7 (RPA-T4), anti-CXCR5-Alexa Fluor 647 (RF682) and anti-ICOS-PerCPCy5.5, the cells were fixed, permeabilized (Fixation/Permeabilization Solution Kit, BD Biosciences, San Jose, CA), and stained intracellularly with anti-IL-21-PE (eBio3A3-N2), anti-IFN-γ-V450 (B27) and anti-IL-17-PECy7 (eBio64DEC17) (all from eBiosciences, San Diego, CA).
Cell sorting and coculture experiments. To sort cTfh and memory B cells for coculture experiments, cryopreserved PBMCs were stained with anti-CD3-Pacific Blue, anti-CD4-Alexa 700 (RPA-T4), anti-CXCR5-Alexa Fluor 647, anti-CD27-PE (M-T271) (all from BD Biosciences, San Jose, CA) and anti-CD19-APC-Cy7. Aqua fluorescent live/dead was included for exclusion of dead cells. cTfh (CD3+CD4+CXCR5+) cells and memory B cells (CD3+CD19+CD27+) were sorted (Supplementary Fig. S5) using a BD FACSAria IIIu cell sorter (BD Pharmingen). The purity of the sorted cell populations was always ≥94%.

Sorted CD4+CXCR5+ or CD4+CXCR5− cells (2 × 10⁶ cells/well) were cocultured with CD19+CD27+ memory B cells (2 × 10⁶ cells/well) in RPMI 1640 medium supplemented with 50 µg/mL penicillin/streptomycin/gentamycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 µM 3-mercaptoethanol and 10% heat inactivated fetal calf serum (all from Gibco-Invitrogen Life Technologies, Carlsbad, CA) in the presence of 1 µg/mL SEB in 96-well U-bottomed plates, as described. Culture supernatants were collected after 6 days and the presence of CD3+CD19+CD27+CD38hiIgA+ plasmablasts were analyzed among the recovered cells after staining with with anti-CD3-Pacific Blue, anti-CD4-FITC (RPA-T4), anti-CXCR5-Alexa Fluor 647, anti-CD27-Alexa Fluor 700, anti-CD38-PerCP-Cy5.5 (all from BD Biosciences, San Jose, CA), anti-CD19-APC-Cy7 and anti-IgA-PE. Total IgA and IgG concentrations in culture supernatants were measured by human Ready-SET-GO! ELISA (E Bioscience, San Diego, CA). LTB-specific IgA titers were measured using ELISA plates coated with GM1 gangliooside plus LTB, as described.

Statistical analyses. Data was evaluated with GraphPad Prism (Version 6.0, GraphPad Software, Inc.). Wilcoxon signed rank test was used to evaluate differences between pre- and postvaccination samples and for evaluation of η expression in different cTfh subsets (with Bonferroni correction, when applicable). Spearman test was used for correlation analyses. P values of <0.05 were regarded as significant.

Data availability. All data generated or analysed during this study are included in this article and its supplementary information files.

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Author Contributions
A.C. and A.L. initiated and designed the studies, analyzed the data and wrote the manuscript. M.M. and M.Q.J. provided scientific and technical advice and A.C. and M.M. performed the experiments. All authors participated in the interpretation of results, contributed to the critical review of the manuscript and have approved the final version.

Additional Information
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