

Oral typhoid vaccination with Ty21a generates Ty21a-responsive and heterologous influenza-responsive CD4⁺ and CD8⁺ T-cells at the human intestinal mucosa

Shaun H. Pennington^{1,2}, Ameeka L. Thompson^{1,2}, Adam K.A. Wright², Daniela M. Ferreira², Kondwani C. Jambo², Angela D. Wright², Brian Faragher², Jill W. Gilmour³, Stephen B. Gordon², Melita A. Gordon^{1,‡}

¹Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, UK

²Department of Clinical Sciences, Liverpool School of Tropical Medicine, UK

³International AIDS Vaccine Initiative (IAVI), Human Immunology Laboratory, Imperial College, London, UK

[‡]Corresponding Author: Melita A Gordon, Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, UK, 0151 705 3725, magordon@liverpool.ac.uk

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ABSTRACT

BACKGROUND: Oral vaccination with live-attenuated *S. Typhi* (Ty21a) is modestly efficacious, however the mechanisms of protection are currently unknown. While humoral and cellular immune responses are well-described in peripheral blood, the cellular response at the intestinal mucosa has never been directly assessed.

METHODS: We vaccinated healthy adults with Ty21a and assessed humoral and cellular immunity in vaccinated volunteers and controls after 18 days. Immunoglobulin levels were assessed in peripheral blood by enzyme-linked immunosorbent assay. Cellular responses were assessed in peripheral blood and at the duodenal and colonic mucosa by flow cytometry.

RESULTS: We demonstrate the generation of Ty21a-responsive and heterologous influenza-responsive CD4⁺ and CD8⁺ T-cells at the duodenal mucosa. All duodenal responses were consistently correlated and no responses were observed at the colonic mucosa. Peripheral anti-lipopolysaccharide IgG and IgA responses were significantly correlated with duodenal responses. The assessment of integrin β_7 expression intensity among peripheral and duodenal T-cell subsets revealed varied capacities for mucosal homing and residence.

CONCLUSIONS: The breadth of duodenal cellular responses was not reflected peripherally. The direct evaluation of mucosal immune defence may yield functional correlates of protection, and could provide insight into mechanisms which may be manipulated in order to enhance vaccine immunogenicity.

INTRODUCTION

Salmonella enterica serovar Typhi (S. Typhi) is a human host-restricted, intracellular pathogen, and the causative agent of typhoid fever. Following ingestion, the bacteria cause systemic illness following invasion via the mucosal surface of the small intestine.[1] In the 1970s, through chemical mutagenesis of pathogenic S. Typhi strain Ty2, a live-attenuated oral typhoid vaccine was developed – Ty21a.[2] Vaccination with three doses of Ty21a is moderately protective and, although estimates of efficacy vary,[3-5] a recently published review calculated a cumulative efficacy of 48% three years following vaccination.[6]

Ty21a is able to induce humoral and cellular immune responses, both of which have been implicated in protection against disease. While opsonophagocytic antibody function,[7] cellular cytotoxicity, proliferation, and cytokine production functionality have been assessed in peripheral blood following vaccination with Ty21a,[8-13] cellular immunity at the human intestinal mucosa has never been directly assessed. Numerous studies have demonstrated that cellular immune responses generated through vaccination with Ty21a are primed for mucosal homing,[13-15] highlighting the importance of mucosal immunity in defence against disease. Furthermore, it has been demonstrated that the assessment of vaccine immunogenicity by peripheral sampling alone provides an incomplete reflection of vaccine immunogenicity.[16]

It has previously been observed in murine models that previously primed T-cells of heterologous specificities are recruited to the lung during influenza infection.[17] Although this phenomenon has not been observed in humans, we hypothesised that vaccination with Ty21a could enhance T-cell responses to heterologous antigens at the mucosal surface by a similar mechanism.

Through the direct assessment of immunity at the intestinal mucosa, it may be possible to identify mechanisms involved in the induction of protective immunity, which may be manipulated in order to improve oral vaccine immunogenicity. Here, we have assessed cellular immunity in vaccinated volunteers and controls at the duodenal and colonic mucosa and in peripheral blood after 18 days. We have compared and correlated peripheral and mucosal cellular responses with accepted peripheral humoral measures of vaccine efficacy, providing a unique insight into the relationship between human mucosal and peripheral immune defence.

RESULTS

Serum immunoglobulin specificity

Ty21a-mediated protection is dependent upon the expression of lipopolysaccharide (LPS)[2] and, in field trials, humoral responses to LPS were shown to correlate with vaccine efficacy.[18] We compared levels of serum anti-LPS IgG and IgA prior to and following vaccination. We also measured levels of serum IgG and IgA specific to influenza – a common naturally-encountered pathogen – in order to assess the impact of vaccination on humoral immunity to a heterologous pathogen. Influenza was selected since the majority, if not all, volunteers would have been exposed to this pathogen in the community.

While levels of anti-LPS serum IgG and IgA among unvaccinated subjects were not different between day 18 and day 0, levels among the vaccinated were higher at day 18 than at day 0 (Figure 1A; $P=0.03$ and $P=0.01$). Levels of anti-influenza serum IgG or IgA among the unvaccinated and the vaccinated were not different between day 18 and day 0 (Figure 1B).

Peripheral blood and gut mucosal cellular responses

We compared the frequency of Ty21a-responsive T-cells in vaccinated volunteers and controls, at the duodenal and colonic mucosa and in peripheral blood. We also measured the frequency of influenza-responsive T-cells.

A combinatorial gating strategy was used to identify the proportion of CD4⁺ and CD8⁺ T-cells positive for any combination of IFN γ $\pm$ TNF α $\pm$ IL2 (Figure 2). Cytokine production in non-stimulated samples (negative control) was minimal, not different between the vaccinated and the unvaccinated, and was subsequently subtracted from other conditions. Cytokine production in Staphylococcal enterotoxin B (SEB)-stimulated samples (positive control) was high, and was not different in the vaccinated compared to the unvaccinated.

At day 0, in peripheral blood, the frequency of Ty21a-responsive and heterologous influenza-responsive CD4⁺ and CD8⁺ T-cells in the vaccinated group was not different from the unvaccinated control group (Figure S1). These data suggest that groups were well-matched for prior exposure to Ty21a and influenza antigens, and that any differences observed thereafter may be attributed to an effect of vaccination with Ty21a. Paired comparisons between day 0 and day 18 were not made in peripheral blood, as overnight fasting, required prior to endoscopy, is known to influence cytokine production in response to restimulation with bacterial and viral antigens.[19]

At day 18, at the duodenal mucosa, the frequency of Ty21a-responsive CD4⁺ T-cells was three-fold higher, and the frequency of Ty21a-responsive CD8⁺ T-cells five-fold higher in vaccinated compared to unvaccinated volunteers (Figure 3A; $P=0.007$ and $P<0.0001$). The frequency of heterologous influenza-responsive CD4⁺ T-cells was five-fold higher, and the frequency of heterologous influenza-responsive CD8⁺ T-cells six-fold higher in vaccinated

compared to unvaccinated volunteers (Figure 3A; $P=0.005$ and $P=0.01$). At the colonic mucosa, there was no significant difference between the frequencies of Ty21a-responsive or heterologous influenza-responsive T-cells in vaccinated compared to unvaccinated volunteers (Figure 3B).

In peripheral blood, the frequency of Ty21a-responsive CD4⁺ T-cells was four-fold higher in vaccinated compared to unvaccinated volunteers (Figure 3C; $P=0.019$). Vaccination did not influence the frequency of peripheral Ty21a-responsive CD8⁺ T-cells, nor the frequency of heterologous influenza-responsive CD4⁺ or CD8⁺ T-cells at day 18.

Characteristics and functionality of cellular responses

Polyfunctional T-cells – those which express multiple cytokines simultaneously – have been shown to correlate with vaccine mediated protection against other intracellular infections.[20, 21] We compared the cytokine expression profile of vaccinated volunteers with that of unvaccinated volunteers to assess the functionality of responses to Ty21a and influenza antigens.

At the duodenal mucosa, the CD4⁺ T-cell response to Ty21a was functionally heterogeneous, with the frequency of cells expressing one, two or three cytokines being higher in vaccinated compared to unvaccinated volunteers (Figure 4A; $P=0.006$, $P=0.018$ and $P=0.031$). The duodenal CD8⁺ T-cell response to Ty21a antigens was, in contrast, largely attributable to cells expressing just one cytokine (Figure 4A; $P=0.0004$). Duodenal CD4⁺ and CD8⁺ T-cell responses to influenza antigens were also functionally heterogeneous, with the frequency of cells expressing one, two or three cytokines being higher in vaccinated compared to unvaccinated volunteers (Figure 4B; CD4⁺ $P=0.008$, $P=0.005$ and $P=0.005$, respectively; CD8⁺ $P=0.0008$, $P=0.010$ and $P=0.011$). In peripheral blood, the CD4⁺ T-cell response to

Ty21a antigens was functionally heterogeneous, with the frequency of cells expressing one, two or three cytokines being higher in vaccinated compared to unvaccinated volunteers (Figure 4C; $P=0.026$, $P=0.028$ and $P=0.008$). Analysis of expression profiles by individual cytokine demonstrated that responses to Ty21a and influenza antigens were attributable to cells producing combinations of IFN γ , TNF α and IL2, but that no one cytokine predominated (Figure S2).

Correlations between cellular populations at the duodenal mucosa

We explored the relationship between the generation of Ty21a-responsive and heterologous influenza-responsive T-cells at the duodenal mucosa.

The frequencies of Ty21a-responsive CD4⁺ and CD8⁺ T-cells were correlated (Table 1; $r^2=0.537$, $P=0.015$), as were the frequencies of heterologous influenza-responsive CD4⁺ and CD8⁺ T-cells (Table 1; $r^2=0.930$, $P<0.0001$). The frequencies of Ty21a-responsive CD4⁺ and CD8⁺ T-cells also correlated robustly with the frequencies of corresponding influenza-responsive T-cells (Table 1; CD4⁺ $r^2=0.74$, $P=0.0003$ and CD8⁺ $r^2=0.677$, $P=0.001$).

Correlations between peripheral and mucosal immunity

We explored the relationship between the generation of peripheral and duodenal immune responses.

The frequency of peripheral Ty21a-responsive CD4⁺ T-cells was modestly correlated with the frequency of duodenal Ty21a-responsive CD4⁺ T-cells (Table 1; $r^2=0.530$, $P=0.024$), but not with the frequency of duodenal Ty21a-responsive CD8⁺ T-cells nor with the frequency of heterologous influenza-responsive CD4⁺ and CD8⁺ T-cells.

Levels of serum anti-LPS IgG and IgA correlated with the frequency of duodenal Ty21a-responsive CD4⁺ T-cells (Table 1; IgG $r^2=0.542$, $P=0.014$ and IgA $r^2=0.710$, $P=0.0004$).

Levels of serum IgA specific to LPS also correlated with the frequency of duodenal heterologous influenza-responsive CD4⁺ and CD8⁺ T-cells (Table 1; CD4⁺ $r^2=0.512$, $P=0.025$ and CD8⁺ $r^2=0.585$, $P=0.008$). Levels of anti-influenza IgG and IgA did not, however, correlate with either the frequency of Ty21a-responsive or heterologous influenza-responsive T-cells at the duodenal mucosa in either subset.

Cellular potential for mucosal homing and residence

Integrin β_7 plays a prominent role in mucosal cellular immune defence; $\alpha_4\beta_7$ facilitates peripheral trafficking to the intestinal mucosa and $\alpha_e\beta_7$ helps maintain resident intraepithelial lymphocyte (IEL) populations.[22, 23] Since duodenal CD4⁺ and CD8⁺ T-cell responses to Ty21a and influenza antigens were closely correlated, we assessed the cellular capacity for mucosal homing and residence, this being a potential mechanism by which Ty21a vaccination may have influenced trafficking to the duodenal mucosa. We assessed integrin β_7 expression intensity, a component of both $\alpha_4\beta_7$ in peripheral blood and $\alpha_e\beta_7$ at the duodenal mucosa. A combinatorial gating strategy was used to determine the level of integrin β_7 expression among CD4⁺ and CD8⁺ T-cell populations (Figure 2).

At the duodenal mucosa, integrin β_7 expression was more intense among the total CD4⁺ T-cell population than among the total CD8⁺ T-cell population (Figure 5A; $P<0.0001$).

Integrin β_7 expression intensity among Ty21a-responsive and influenza-responsive CD4⁺ T-cell subpopulations was not different to that among the total mucosal CD4⁺ T-cell population.

In contrast, integrin β_7 expression intensity among Ty21a-responsive and influenza-responsive CD8⁺ T-cell subpopulations was greater than that among the total mucosal CD8⁺ T-cell population (Figure 5A; $P=0.0002$ and $P<0.0001$).

In contrast with observations made at the duodenal mucosa, integrin β_7 expression was more intense among the total $CD8^+$ T-cell population than among the total $CD4^+$ T-cell population in peripheral blood (Figure 5B; $P=0.0005$). While integrin β_7 expression intensity among Ty21a-responsive $CD4^+$ T-cell subpopulation was greater than that among the total $CD4^+$ T-cell population (Figure 5B; $P=0.002$), expression intensity among influenza-responsive $CD4^+$ T-cell subpopulation was not different to that among the total $CD4^+$ T-cell population. Integrin β_7 expression intensity among Ty21a-responsive and heterologous influenza-responsive $CD8^+$ T-cell subpopulations was greater than that among the total $CD8^+$ T-cell population (Figure 5B; $P=0.004$ and $P=0.041$).

DISCUSSION

We have described, for the first time, the cellular response to oral vaccination at the human intestinal mucosa. We demonstrate that Ty21a vaccination generates both polyfunctional Ty21a-responsive and heterologous influenza-responsive T-cells at the duodenal mucosa. The frequency of Ty21a-responsive and influenza-responsive T-cells at the duodenal mucosa were robustly and consistently correlated. In contrast, cellular and humoral measurements made in peripheral blood were poorly correlated with one another, and did not provide insight into the induction of heterologous responses at the duodenal mucosa.

While robust Ty21a and heterologous-influenza $CD4^+$ and $CD8^+$ T-cell responses were observed at the duodenal mucosa, no cellular response was observed at the colonic mucosa to either antigen in either T-cell subset. This suggests that duodenal responses were compartmentalised to the embryological mid-gut, which includes the terminal ileum – the likely site of active invasion by Ty21a.[1] Interestingly, the frequency of Ty21a-responsive

CD4⁺ and CD8⁺ T-cells correlated robustly with one another and with the frequency of heterologous influenza-responsive CD4⁺ and CD8⁺ T-cells at the duodenal mucosa. This suggests that the mechanisms responsible for the generation of these mucosal responses were localised and capable of influencing T-cells of different phenotypes and specificities.

Polyfunctionality among Ty21a-responsive T-cells at the duodenal mucosa and in peripheral blood may point towards an important mechanism through which Ty21a confers protection against disease.[20, 21] Further, the generation of anti-LPS immunoglobulin, an accepted indication of vaccine efficacy, was closely associated with cellular responses at the duodenal mucosa, suggesting that mucosal cellular activity may be associated with the generation of protective immunity.

In murine models, influenza infection results in the recruitment of previously-primed heterologous T-cells to the lung.[17] We propose that a similar mechanism was responsible for the generation of heterologous responses at the duodenal mucosa. Although only two individuals had previously been vaccinated against influenza all were likely to have been exposed to this mucosal pathogen in the community – indeed, at baseline, anti-influenza IgG and IgA, as well as influenza responsive CD4⁺ T-cells were detected in all volunteers, not just those who had been previously vaccinated against influenza.

Trafficking to the mucosa is largely dependent upon the expression of $\alpha 4\beta 7$ and its ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1), as well as C-C chemokine receptor (CCR)9 and its ligand, chemotaxis chemokine ligand (CCL)25.[24] We hypothesise that vaccination with Ty21a upregulated the expression of mucosal homing ligands resulting in the non-specific migration of previously-primed heterologous influenza-responsive T-cells, carrying mucosal homing receptors, to the duodenal mucosa. This effect may or may not be restricted to influenza responsive T-cell subsets, and further study is warranted.

We observed that integrin β_7 expression intensity was generally higher among both Ty21a-responsive and influenza-responsive cells, compared to total cell populations, in peripheral blood and at the intestinal mucosa. Multiphasic peripheral CD8⁺ T-cell responses have previously been described and attributed to the trafficking of immune cells from peripheral blood to the mucosa.[8, 9] In keeping with this, integrin β_7 expression among peripheral CD8⁺ T-cells, particularly antigen-specific subpopulations, was more intense than that among peripheral CD4⁺ T-cells. Integrin β_7 expression among CD4⁺ T-cell subsets was highly polarised, being relatively low among peripheral subsets and high among duodenal subsets. Differential integrin β_7 expression among antigen-specific CD4⁺ T-cell populations may ensure that some peripheral T-cells persist in blood and avoid sequestration. We and others have demonstrated the presence of intracellular intravascular and bone-marrow bacteria in early, late and recurrent invasive *Salmonella* disease.[25, 26] Thus, while T-cells trafficking to the mucosa likely play a key role in the early response to invasion, CD4⁺ T-cell populations which persist in peripheral blood may help to prevent intravascular dissemination and persistence at secondary systemic sites of infection.

Prior to vaccination, all volunteers had detectable baseline levels of serum immunoglobulin specific to *S. Typhi* LPS. This is likely the result of antibody binding to the core, or as a result of environmental exposure to non-typhoidal strains bearing the same LPS O-antigens as *S. Typhi* (O-9 and O-12); *S. Enteritidis* expresses both O-9 and O-12 antigens and *S. Typhimurium* expresses the O-12 antigen.[27] Not all vaccinated volunteers generated peripheral humoral anti-LPS responses; likely a reflection of the limited efficacy of this vaccine. It was interesting that mucosal cellular responses correlated strongly with serum anti-LPS immunoglobulin responses, as humoral responses to LPS are associated with vaccine efficacy.[18] This suggests that in individuals in whom Ty21a vaccination is not

efficacious, the failure encompasses both cellular and humoral mechanisms of defence. It has previously been demonstrated that the generation of immunoglobulin following oral vaccination is dependent upon $\alpha_4\beta_7$. [28] Thus, the close association between cellular and humoral immune responses suggests that the strength of each volunteer's cellular and humoral response to vaccination was influenced by a mucosal mechanism, possibly the expression intensity of $\alpha_4\beta_7$ and/or MAdCAM-1 at the time of vaccination.

It is possible that through toll-like receptor (TLR) engagement, vaccination may have non-specifically activated mucosal antigen-presenting cells (APCs) and T-cells, resulting in enhanced cytokine production following experimental restimulation. [29, 30] However, as background cytokine production in the negative control was minimal, and as cytokine production in the positive control was not different in the vaccinated compared to the unvaccinated, we believe this is unlikely to be the primary factor responsible for the observed heterologous cellular response. Due to the invasive nature of endoscopic biopsy, we were limited to sampling from intestinal sites at a single time point. Data presented here, and elsewhere, [8] indicate that the assessment of mucosal immunity at alternate time points could provide further insight into the generation of protective immune responses. We assessed the expression of just three cytokines and, as a result, the T-cell populations identified here are unlikely to represent the responsive populations in their entirety.

In future studies, we plan to study the effect of oral vaccination with Ty21a on the cellular response to a range of heterologous antigens. We will comprehensively assess cellular phenotype and functionality through the inclusion of additional surface markers and a broader repertoire of cytokines/chemokines. We also plan to further investigate the timing and longevity of peripheral and mucosal immune responses.

Taken together, our data demonstrate that oral vaccination with Ty21a generates Ty21a-responsive T-cells as well as heterologous influenza-responsive T-cells at the duodenal mucosa. We propose that heterologous influenza-responsive T-cells, previously primed through natural exposure, were recruited to the duodenal mucosa through compartmentalised upregulation of homing ligands. The direct evaluation of mucosal cellular immune defence provides the opportunity to identify functional correlates of protection, and may offer new insights into mechanisms which may be manipulated to improve oral vaccine immunogenicity, either through the development of oral adjuvants, or the development of multivalent *Salmonella*-based vectors.

MATERIALS AND METHODS

Ethical approval, recruitment and study protocol

All volunteers provided written informed consent. This study was approved by the UK National Research Ethics Service (10/H1005/20). Twenty-three healthy adult volunteers were enrolled into the study. Eleven volunteers (6 males and 5 females (2 previously vaccinated against influenza); median age 24 years) were randomly selected for oral vaccination with live-attenuated *S. Typhi* (Ty21a, Vivotif®). A single oral capsule was taken on days 0, 2 and 4, approximately one hour before a meal, with a cold or lukewarm drink. Twelve volunteers (3 males and 9 females (3 previously vaccinated against influenza); median age 23 years) were randomly assigned to an unvaccinated control group.

Mucosal mononuclear cell (MMC) isolation

Mucosal samples were acquired at day 18. Nasal O₂ was administered and saturation monitored throughout. Sedation was offered to all volunteers; those who requested sedation

were given up to 5mg midazolam intravenously. Using large capacity forceps (Boston Scientific), 12-15 single-bite mucosal biopsies were acquired during flexible video-endoscopy, from the duodenal mucosa at D2-D3 (n=20) and from the sigmoid colon at 20-25cm insertion (n=17). MMCs were isolated from biopsies using a modified version of a previously described method.[31] Full details are presented in Supplementary Materials and Methods.

Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood samples were collected in sodium heparin Vacutainers™ (BD Biosciences) at day 0 (n=23) and, after overnight fasting, at day 18 (n=21). PBMCs were isolated using Lymphoprep™ (Axis-Shield), according to the manufacturers instructions. Full details are presented in Supplementary Materials and Methods.

Antigenic stimulation and incubation

PBMCs (1×10^6 cells/mL) and MMCs (no less than 5×10^6 cells/mL) were seeded in complete medium in 96 well flat-bottom plates. Cells were stimulated with either 5×10^6 colony forming units (cfu)/mL heat-killed (suspended in Dulbecco's PBS, quantified using Miles and Misra technique, and incubated at 95°C for 30 minutes) *Salmonella* Typhi Ty21a (Vivotif®), or 0.5ng/mL influenza haemagglutinin and neuraminidase antigens (Influvac® containing, in equal quantities, a A/Brisbane/59/2007 H₁N₁-like strain, a A/Brisbane/10/2007 H₃N₂-like strain, and a B/Brisbane/60/2008-like strain). One positive control well was stimulated with 500ng/mL staphylococcal enterotoxin B (SEB) (Sigma-Aldrich). One negative control well was left untreated to adjust for non-antigen-specific background cytokine production. Cells were then incubated at 37°C in 5% CO₂. After two hours, 5µL/mL

brefeldin A (BD GolgiPlug™; BD Biosciences) was added to each well, and the plate incubated for a further 16 hours at 37°C in 5% CO₂.

Flow cytometric analyses

Following incubation, PBMCs and MMCs were washed, stained for viability, surface phenotype and, following fixation and permeabilisation, stained for intracellular cytokine production. Details of the antibodies which were used are presented in Supplementary Materials and Methods. Cells were washed, resuspended and stored in the absence of light at 4°C until data were acquired using a LSR II flow cytometer (BD Biosciences). Compensation beads (BD Biosciences) were used to create compensation matrices and sequential cell isolation used to identify populations of interest (Figure 2). Full details are presented in Supplementary Materials and Methods.

Enzyme-linked immunosorbent assay (ELISA)

Each well of Maxisorp® flat-bottom 96 well plate (Nunc) was coated with 100µL carbonate-bicarbonate buffer containing either 5µg/mL *S. typhi* lipopolysaccharide (Sigma-Aldrich) or 10ng/mL influenza haemagglutinin and neuraminidase antigens (Influvac® containing, in equal quantities, a A/Brisbane/59/2007 H₁N₁-like strain, a A/Brisbane/10/2007 H₃N₂-like strain, and a B/Brisbane/60/2008-like strain) and incubated at 4°C overnight. Plates were washed three times with phosphate buffered saline-tween (PBS-tween). Plates were blocked with 1.0% bovine serum albumin (BSA) and incubated for two hours at room temperature. A standard was created using serum obtained from a convalescent patient diagnosed with typhoid. Volunteer samples were diluted four times across an optimised range for optimum comparison against the standard. Plates were washed, samples were added in duplicate and incubated at 4°C overnight. For detection of IgG, plates were washed and incubated with

1:4,000 anti-human-IgG-alkaline-phosphatase (Sigma-Aldrich) for two hours. For detection of IgA, plates were washed and incubated with 1:4,000 anti-human-IgA (AbD Serotec) for two hours; plates were washed again and then incubated with 1:2,000 streptavidin:alkaline phosphatase (AbD Serotec) for one hour. For detection of both IgG and IgA, plates were washed and incubated with 100 μ L *p*-nitrophenyl phosphate (*p*NPP; Sigma-Aldrich). Optical density was measured at 405nm using a FLUOstar Omega ELISA plate reader (BMG Labtech).

Statistical analyses

Comparisons were made using paired and unpaired *t* tests, as indicated. Associations were measured using Pearson's correlation coefficient. Statistical analyses were performed using Prism v5.03 (GraphPad). *P* values are two-tailed and considered significant at *P*<0.05.

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FIGURE 1. Serum-specificity among IgG and IgA populations. The levels of IgG and IgA specific to *S. Typhi* LPS (A) and influenza (B) in serum expressed in arbitrary units (AU). For control (C; closed squares and circles) and vaccinated (V; open squares and circles) volunteers, paired comparisons were made between day 0 and day 18 values (paired *t* tests were performed using logarithmically transformed data).

FIGURE 2. Representative flow cytometric gating strategy. Dot plots are shown for cells isolated from the duodenal mucosa (A) and peripheral blood (B). Dead cells, B cells and monocytes were removed by staining for viability (Vivid®), CD19 and CD14 and gating on the negative population. T-cells were identified according to the expression of CD3. T-cells were classified according to the expression of CD4 and CD8 and the expression of TNF α , IL2 and IFN γ cytokine assessed in non-stimulated (NS) and in Ty21a, influenza and SEB stimulated samples.

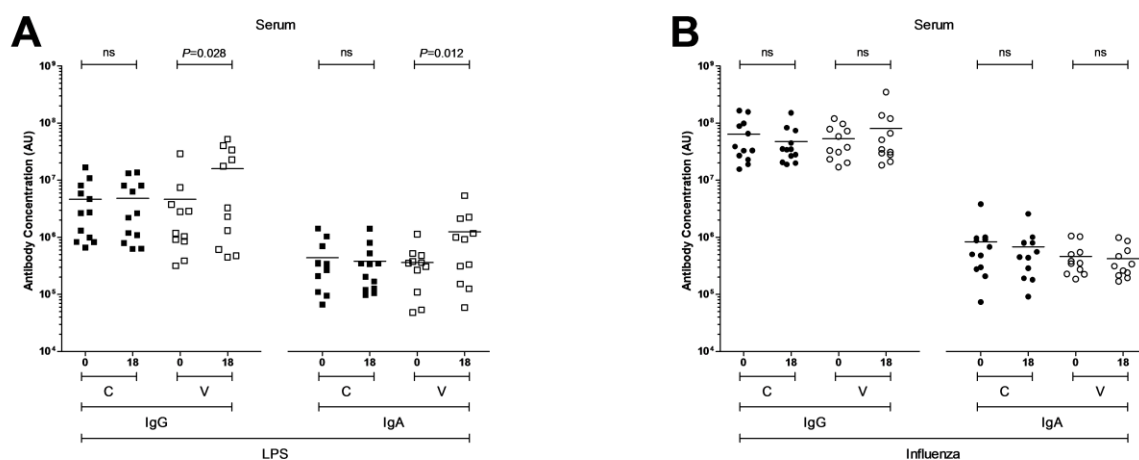
FIGURE 3. Antigen-specific cytokine-producing populations at day 18. The frequency of CD4⁺ and CD8⁺ Ty21a-responsive and heterologous influenza-responsive populations expressing any combination of IFN γ $\pm$ TNF α $\pm$ IL2 above background. SEB-stimulated control data is also included. For control (C; closed squares, circles and triangles) and vaccinated (V; open squares, circles and triangles) volunteers, measurements were made at the duodenal mucosa (A), the colonic mucosa (B), and in peripheral blood (C). Horizontal bars represent mean values (comparisons were made using unpaired *t* tests).

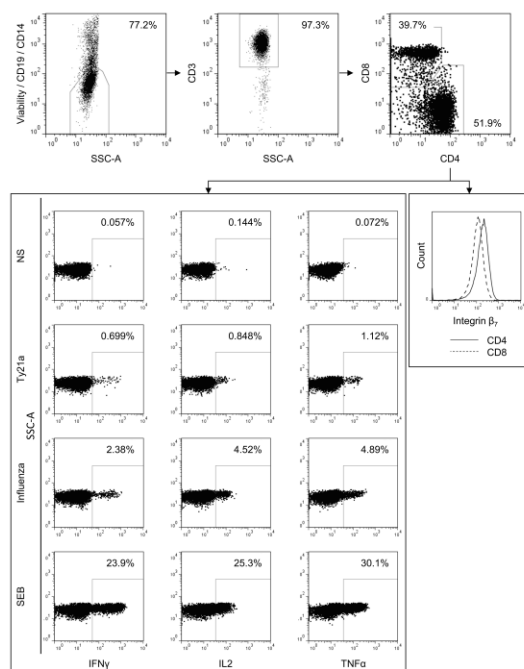
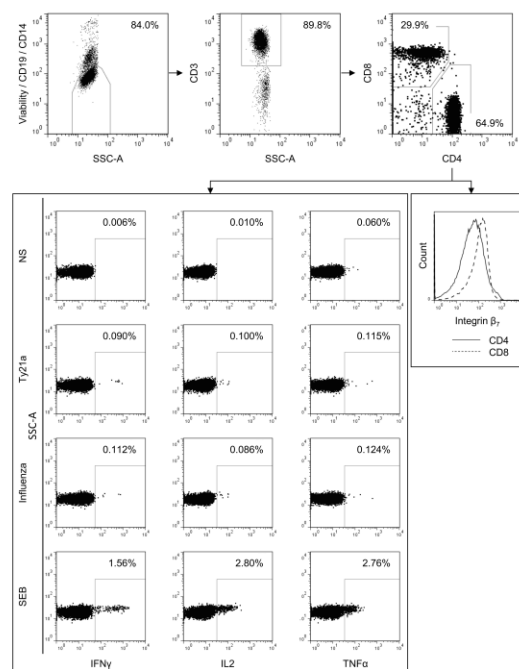
FIGURE 4. Combinations of antigen-specific cytokine production at day 18. The frequency of CD4⁺ and CD8⁺ Ty21a-responsive and heterologous influenza-responsive populations expressing one (+), two (++) or three (+++) cytokines (IFN γ $\pm$ TNF α $\pm$ IL2) above background. For control (C; closed squares, circles and triangles) and vaccinated (V; open squares, circles and triangles) volunteers, measurements were made at the duodenal

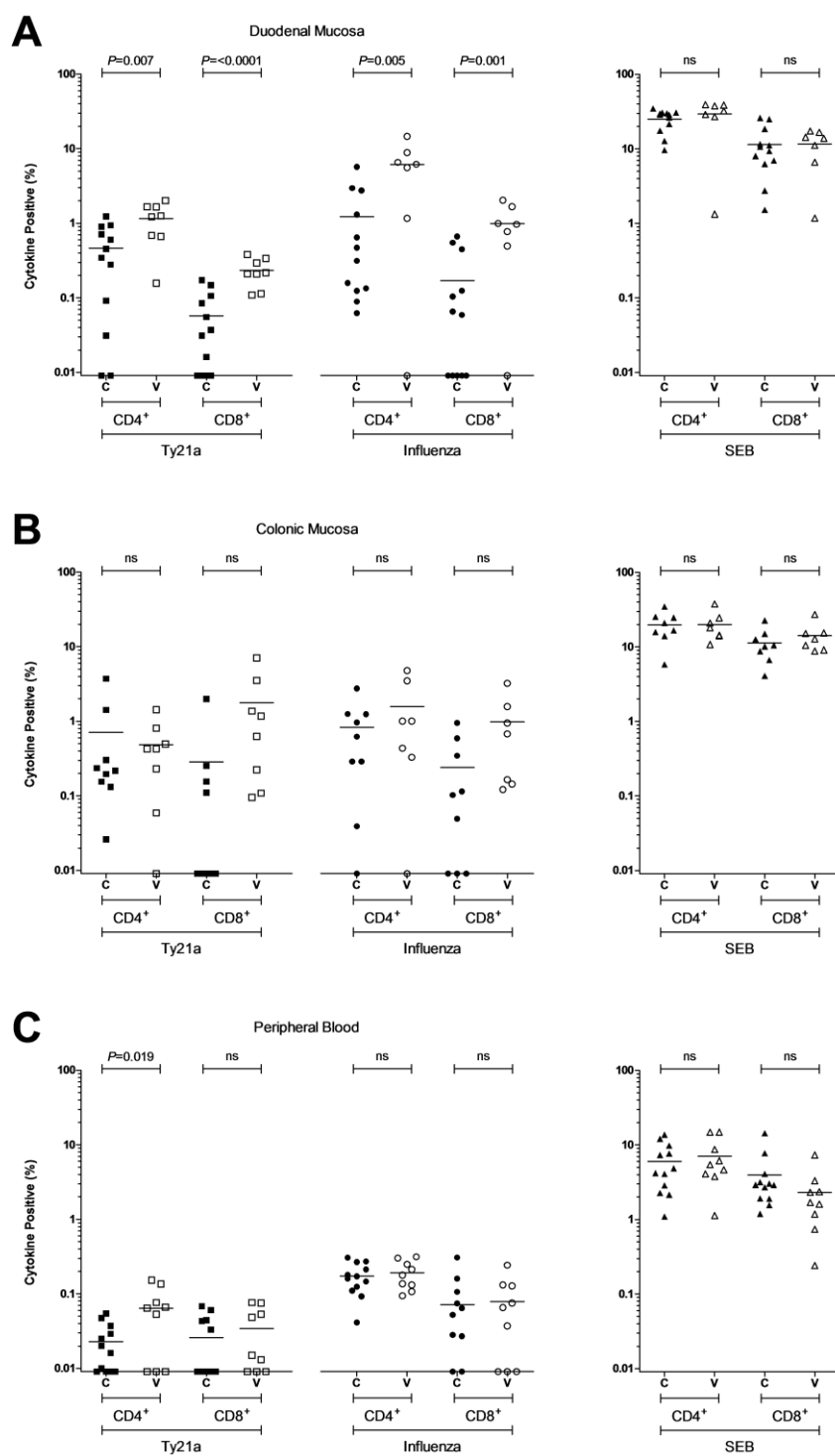
mucosa (**A and B**), and in peripheral blood (**C and D**). Horizontal bars represent mean values (comparisons were made using unpaired *t* tests).

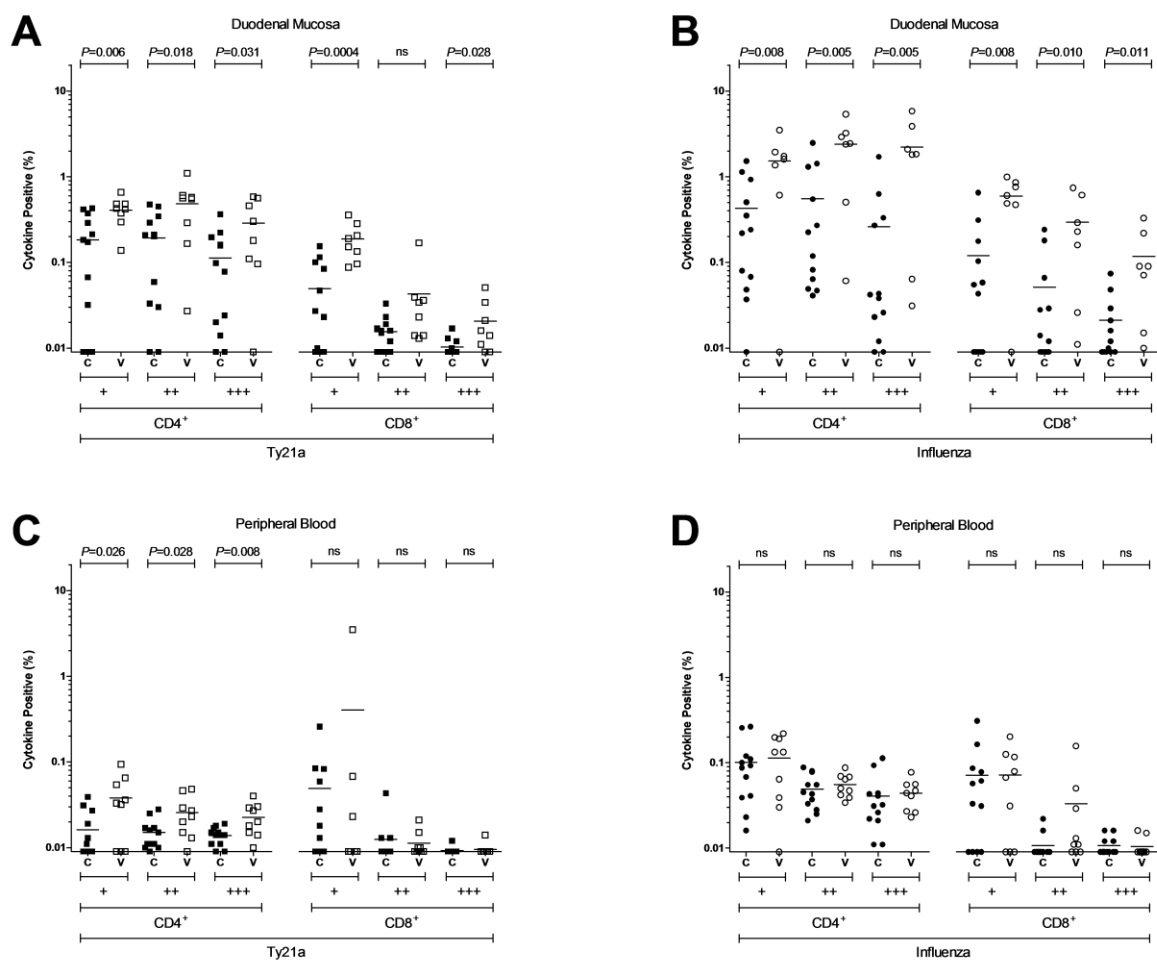
FIGURE 5. Integrin β_7 expression intensity among T-cell populations at day 18.

Geometric mean fluorescence intensity (MFI) expression of integrin β_7 among non-stimulated total CD4⁺ and total CD8⁺ T-cell populations (control volunteers closed diamonds and vaccinated volunteers open diamonds), and in Ty21a-stimulated (control volunteers closed squares and vaccinated volunteers open squares) and heterologous-influenza-stimulated (control volunteers closed circles and vaccinated volunteers open circles) cytokine-producing subpopulations. Measurements were made at the duodenal mucosa (**A**), and in peripheral blood (**B**). Horizontal bars represent mean values (comparisons were made using unpaired *t* tests).



A**B**





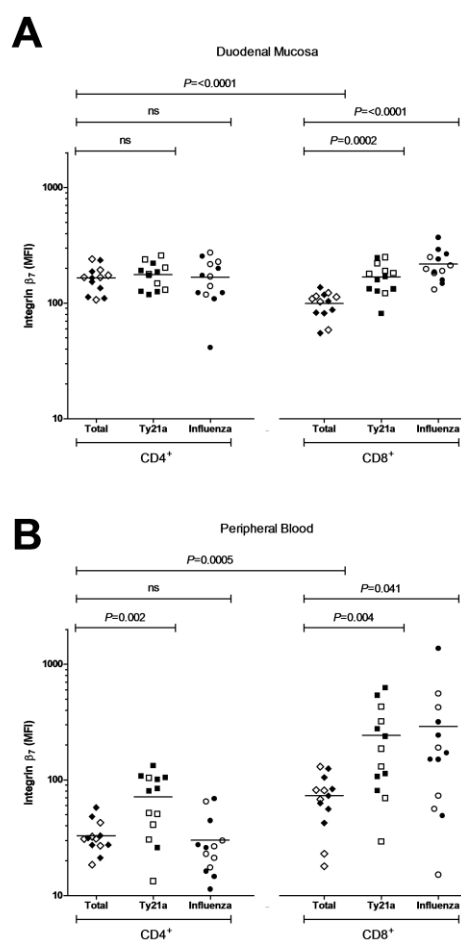


Table 1. Pearson correlation analysis of cellular and humoral immune responses.

		DUODENAL MUCOSA				PERIPHERAL BLOOD				SERUM				
		Ty21a		Influenza		Ty21a		Influenza		S. Typhi LPS		Influenza		
		CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺	IgG	IgA	IgG	IgA	
DUODENAL MUCOSA	Ty21a	CD4 ⁺	1	0.537 *	0.741 **	0.724 **	0.530 *	0.319 ns	-0.102 ns	-0.223 ns	0.542 *	0.710 *	0.160 ns	0.008 ns
		CD8 ⁺		1	0.587 **	0.677 **	0.395 ns	0.126 ns	-0.117 ns	-0.232 ns	0.315 ns	0.396 ns	0.177 ns	-0.348 ns
	Influenza	CD4 ⁺			1	0.929 **	0.327 ns	0.299 ns	-0.025 ns	-0.122 ns	0.392 ns	0.512 *	-0.245 ns	-0.228 ns
		CD8 ⁺				1	0.394 ns	0.515 ns	0.099 ns	0.080 ns	0.392 ns	0.585 **	-0.149 ns	-0.300 ns

P*<0.05 (light grey); *P*<0.001 (dark grey)