Generation of specific effector and memory T cells with gut- and secondary lymphoid tissue- homing potential by oral attenuated CVD 909 typhoid vaccine in humans

R Wahid[1], R Salerno-Gonçalves[1], CO Tacket[2], MM Levine[1,2] and MB Sztein[1,2]

Induction of effective memory T cells is likely to be critical to the level and duration of protection elicited by novel live oral typhoid vaccines. Using cells from volunteers who ingested *Salmonella Typhi* vaccine strain CVD 909, we characterized the induction of interferon (IFN)-γ-secreting central (T_{CM}, CD45RO$^+$CD62L$^+$) and effector (T_{EM}, CD45RO$^+$CD62L$^-$) memory T populations, and their gut-homing potential based on integrin α4/β7 expression. Both CD4$^+$ T_{EM} and T_{CM} populations secreted IFN-γ. However, although CD4$^+$ T_{EM} expressed, or not, integrin α_4/β_7, CD4$^+$ T_{CM} cells were predominantly integrin $\alpha_4/\beta_7{}^+$. In contrast, IFN-γ-secreting CD8$^+$ cells were predominantly classical T_{EM} and CD45RA$^+$ T_{EM} (T_{EMRA}, CD45RO$^-$CD62L$^-$) subsets. However, although CD8$^+$ T_{EM} expressed, or not, integrin α_4/β_7, CD8$^+$ T_{EMRA} were predominantly integrin $\alpha_4/\beta_7{}^+$. This is the first demonstration that oral immunization of humans with *S.* Typhi elicits diverse IFN-γ-secreting CD4$^+$ and CD8$^+$ T_{CM} and T_{EM} subsets able to migrate to the gut and other lymphoid tissues.

INTRODUCTION

Typhoid fever is caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), a human-restricted pathogen that enters the host through the gut-associated lymphoid tissue. It is estimated that ~21,650,000 cases and 216,500 deaths occur annually worldwide.[1] Current licensed typhoid vaccines include the purified Vi polysaccharide parenteral vaccine and the live oral attenuated Vi-negative *galE* mutant *S.* Typhi strain Ty21a vaccine (henceforth Ty21a).[2] Ty21a, which does not elicit anti-Vi antibodies, is likely to mediate protection by eliciting antibodies to other *S.* Typhi antigens[2,3] and an array of cell-mediated immune responses (CMIs) at the systemic and mucosal levels.[4–11]

Although Ty21a is known to confer a moderate level of long-lived protection (5–7 years),[12] this requires the administration of three doses. Accordingly, others and we have engineered new attenuated typhoid vaccine strains that aim to be as well-tolerated as Ty21a yet immunogenic and protective following ingestion of just a single dose.[2,8,13–23] Strain CVD 908-*htrA*[19] was well-tolerated and immunogenic in clinical trials, eliciting potent antibody responses to *S.* Typhi antigens (other than Vi) and CMI.[8,18,19,21,22] In this regard, we have previously shown in volunteers immunized orally with attenuated strains of *S.* Typhi strains, including Ty21a, CVD 908, CVD 908-*htrA*, the induction of an array of potent CMI responses that might play an important role in host defense against *S.* Typhi.[2,4–8,14,18–23] To develop an attenuated vaccine strain that might also elicit Vi antibodies to enhance protection against typhoid fever, we modified CVD 908-*htrA* to constitutively express Vi, resulting in strain CVD 909.[24] In two clinical trials, CVD 909 was well-tolerated and strongly immunogenic, eliciting robust anti-lipopolysaccharide antibodies. Although serum anti-Vi antibody was not stimulated, IgA antibody-secreting cells to Vi were detected in the majority of vaccinees, indicating mucosal priming to this antigen.[17] We also demonstrated that CVD 909 elicited a wide array of potent CMI, including cytotoxic T cells, interferon (IFN)-γ, tumor necrosis factor-α, and interleukin (IL)-10 (but not IL-2, IL-4, or IL-5) production and proliferation to *S. Typhi* antigens.[25]

However, despite this progress, virtually no information is currently available regarding the induction of CD4$^+$ and CD8$^+$ memory T (T_M)-cell sub-populations by live oral bacterial vaccines and their ability to migrate to appropriate effector and

[1]Department of Pediatrics, Center for Vaccine Development, University of Maryland, Baltimore, Maryland, USA. [2]Department of Medicine, University of Maryland, Baltimore, Maryland, USA. Correspondence: MB Sztein (msztein@medicine.umaryland.edu)

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induction sites. Understanding the contribution of these cells to host defense is critical to improve the induction of long-term protection by typhoid and other vaccines administered through mucosal surfaces. It is generally accepted that T_M cells express the CD45RO, but not the CD45RA isoform.[26–33] Moreover, based on the coexpression of CD62L (L-selectin) surface molecules, T_M cells can be subdivided into two main subsets: central memory (T_{CM}, $CD45RO^+CD62L^+$) and effector memory (T_{EM}, $CD45RO^+CD62L^-$). Some T_{EM}, sometimes referred to as "terminal memory" cells, express CD45RA (T_{EMRA}, $CD45RO^-CD45RA^+CD62L^-$).[26–30] This novel sub-population (T_{EMRA}) of $CD4^+$ and $CD8^+$ cells recently has been described in humans during viral infections.[26–33] The use of these markers also allows the identification of naive T cells ($CD45RO^-CD62L^+$).[26–33] In this study, we evaluated the induction of *S.* Typhi-specific T_{CM} and T_{EM} cell populations and their gut-homing potential, using peripheral blood mononuclear cells (PBMCs) isolated from subjects who were orally immunized with one or two doses of CVD 909 in two phase-1 clinical trials.[17,24,25]

We demonstrate, for the first time, that oral immunization of subjects with an attenuated *S.* Typhi vaccine elicited diverse specific IFN-γ-secreting $CD4^+$ and $CD8^+$ T_{CM} and T_{EM} T cells. We also provide the first available evidence in humans that oral immunization with an attenuated *S.* Typhi vaccine candidate strain elicits a subset of specific T_{EM} that coexpresses CD45RA (T_{EMRA}). Moreover, we observed that these specific T_M populations are composed of cells that express, or not, the gut-homing molecule integrin α4/β7. These data provide novel and unexpected insights into the homing characteristics and memory-effector T-cell sub-populations elicited by Gram-negative bacteria infection in humans and have wide implications for the development of live attenuated bacterial vaccines.

RESULTS

Characterization of IFN-γ-secreting memory T-cell populations in response to soluble and particulate *S.* Typhi antigens

A fundamental tenet of the induction of effective and long-lasting immune responses following immunization is the induction of adequate and persistent memory T-cell responses. Thus, we characterized the IFN-γ-secreting cell populations with regard to their memory-effector characteristics following an overnight *in vitro* stimulation with *S.* Typhi antigens. After stimulation, PBMCs were simultaneously stained with monoclonal antibodies against CD3, CD4, CD8, CD45RO, CD62L, integrin α_4/β_7, and IFN-γ molecules conjugated to different fluorochromes as detailed in Methods. As expected based on our previous observations,[22] $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) T cells were the predominant cell population that secreted IFN-γ in response to soluble *S.* Typhi antigens (e.g., *S.* Typhi flagella). In fact, in the current study, only four of the 10 volunteers studied showed IFN-γ increases in $CD8^+$ ($CD3^+$ $CD4^-$ $CD8^+$) cells, and these increases were of considerably lower magnitude than those of $CD4^+$ cells (data not shown). Thus, we limited the analysis of the characteristics of IFN-γ-secreting cells in response to *S.* Typhi flagella (**Figures 1** and **2**) and TypVac (data not shown) to $CD4^+$ T cells.

We observed positive significant net increases (mean 0.11±0.03%; range 0.05–0.26) in IFN-γ-secreting cells by total $CD4^+$ populations exposed to *S.* Typhi flagella compared to respective "no antigen" controls (costimulants only) in seven of 10 vaccinees ($P=0.007$). Representative dot plots showing the gating strategy used for different sub-populations of T cells are shown in **Figure 1a** and **c**. As shown in **Figure 1b**, the percentage of antigen-specific IFN-γ-secreting $CD4^+$ T cells coexpressing the T_M marker CD45RO in response to *S.* Typhi flagella was almost twice as high as that observed in $CD4^+$ cells that did not secrete IFN-γ (63.3±3.4 vs. 37.3±2.1, respectively, $P<0.001$). When we further subdivided these IFN-γ-secreting cells into the various memory T sub-populations described above and compared them with the percentages of the corresponding T populations that did not secrete IFN-γ, we observed marked increases of $CD4^+$ T central memory (T_{CM}, $CD45RO^+CD62L^+$) ($P=0.003$) and T effector memory cells (T_{EM}, $CD45RO^+CD62L^-$), ($P<0.001$) subsets within IFN-γ-secreting cells (**Figure 1d**). As expected, these changes were accompanied by significantly reduced percentages of the naive ($CD45RO^-CD62L^+$) T subset ($P<0.001$) within IFN-γ-secreting cells. These results showed that *S.* Typhi flagella-specific IFN-γ-secreting $CD4^+$ T cells are mostly T_M cells with marked increases in both T_{CM} and T_{EM} sub-populations. Similar results were observed with TypVac (data not shown). No significant IFN-γ responses were observed in control cultures (absence of stimulation with *S.* Typhi antigens; data not shown).

Characterization of the homing potential of IFN-γ-secreting $CD4^+$ memory T cells in response to soluble antigens

We next evaluated the hypothesis that immunization with CVD 909 elicited $CD4^+$ *S.* Typhi-specific T-cell responses able to home to the gut microenvironment. Selective homing of effector/memory cells to the gut is driven, to a large extent, by the expression of integrin α_4/β_7.[32–35] Thus, we studied the expression of the gut-homing molecule integrin α_4/β_7 using an eight-color multichromatic flow cytometric approach to simultaneously assess the expression of integrin α_4/β_7 among T memory-effector sub-populations. Interestingly, results showed that IFN-γ-secreting $CD4^+$ cells consisted of populations that express, or not, integrin α_4/β_7 (**Figure 2a**). However, stimulation with *S.* Typhi flagella resulted in significant increases in IFN-γ-secreting cells that coexpress integrin α_4/β_7 compared with the IFN-γ-negative populations (34.6±2.6% (range: 23.0–42.6) vs. 20.4±2.3% (range: 12.2–28.2), respectively) ($P=0.001$). To further characterize the expression of integrin α_4/β_7 in IFN-γ-producing cells, we compared the proportions of T_{EM}, T_{CM}, $CD45RA^+$ T_{EM} (T_{EMRA}, $CD45RO^-CD62L^-$), and naive T subsets that express, or not, integrin α_4/β_7 between IFN-γ^+ and IFN-γ^- subsets. The gating strategy is shown in **Figure 2a** and **b**. As shown in **Figure 2c**, among integrin α_4/β_7-negative cells, IFN-γ-secreting cells were significantly increased ($P=0.001$) only in the T_{EM} sub-population. In contrast, among cells expressing integrin α_4/β_7,

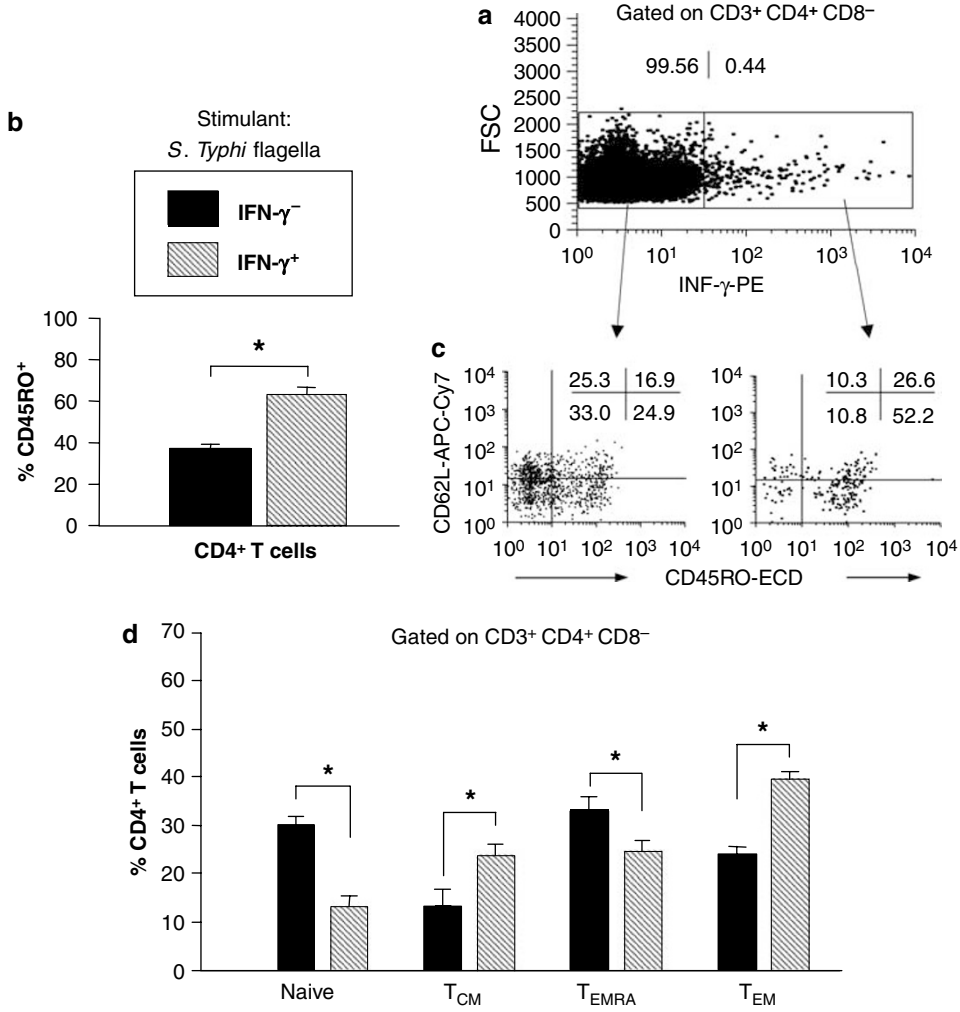

Figure 1 Characterization of the IFN-γ-secreting CD4+ T cells in response to *ex vivo* stimulation with purified *S.* Typhi flagella. Peripheral blood mononuclear cells (PBMCs) from CVD 909-vaccinated volunteers (n=10, from the Ty32004 and Ty35000 studies) 60/91 days after immunization were analyzed by seven-color flow cytometry. (**a** and **c**) Representative dot plots from one of the volunteers (subject no. 32004-4). (**b**) The mean±s.e. of the percentage of T memory cells (CD3+ CD4+ CD8− CD45RO+) from the 10 subjects following stimulation with *S.* Typhi flagella. (**d**) The mean±s.e. distribution of naive (CD45RO− CD62L+), T central memory cell (T_{CM}, CD45RO+ CD62L+), CD45RA-positive T effector memory cell (T_{EMRA}, CD45RO− CD62L−), and T effector memory cell (T_{EM}, CD45RO+ CD62L−) subsets of CD4+ T cells from all subjects following stimulation. One-tail paired *t*-tests were used to analyze the data by comparing IFN-γ-secreting (IFN-γ+) and the corresponding IFN-γ non-secreting (IFN-γ−) sub-populations in **b** and **d**. *P<0.05. IFN-γ, interferon-γ.

significant increases in IFN-γ were observed in both T_{CM} (P=0.011) and T_{EM} (P=0.004) subsets (**Figure 2d**).

Characterization of IFN-γ-secreting memory CD8+ and CD4+ T-cell populations cocultured with *S.* Typhi-infected cells

We have previously reported that the predominant effector population responding to soluble (*S.* Typhi flagella) and particulate (TypVac) *S.* Typhi antigens is composed of CD3+ CD4+ CD8− T cells. In contrast, the effector subsets responding to *S.* Typhi-infected stimulators included both CD3+ CD8+ CD4− (predominant), as well as CD3+ CD4+ CD8− populations.[4–6,8,22] In this study, we confirmed and markedly extended these findings. We used PBMC from six subjects whom we had previously shown to specifically secrete IFN-γ when exposed to *S.* Typhi-infected targets[25] in an eight-color multichromatic flow cytometric approach to characterize in depth these IFN-γ-producing T-cell sub-populations and their homing potential following exposure to *S.* Typhi-infected blasts. To this end, stimulator cells (*S.* Typhi-infected or non-infected autologous blasts as controls) were stained with CD45-biotin followed by streptavidin conjugated with pacific blue before coculture with effectors to enable their exclusion by electronic gating during flow cytometric analysis (i.e., only CD45-negative effector populations were analyzed). This approach eliminated an important source of nonspecific background (see Methods for details). Cells incubated in the presence of *S.* Typhi-infected or non-infected autologous blasts were simultaneously stained with monoclonal antibodies against CD3, CD4, CD8, CD45RO, CD62L, integrin α_4/β_7, and IFN-γ molecules conjugated to different fluorochromes. We then determined by flow cytometry the proportions of T_{EM}, T_{CM}, T_{EMRA}, and T naive subsets in IFN-γ+ and IFN-γ− cells, as well as coexpression of

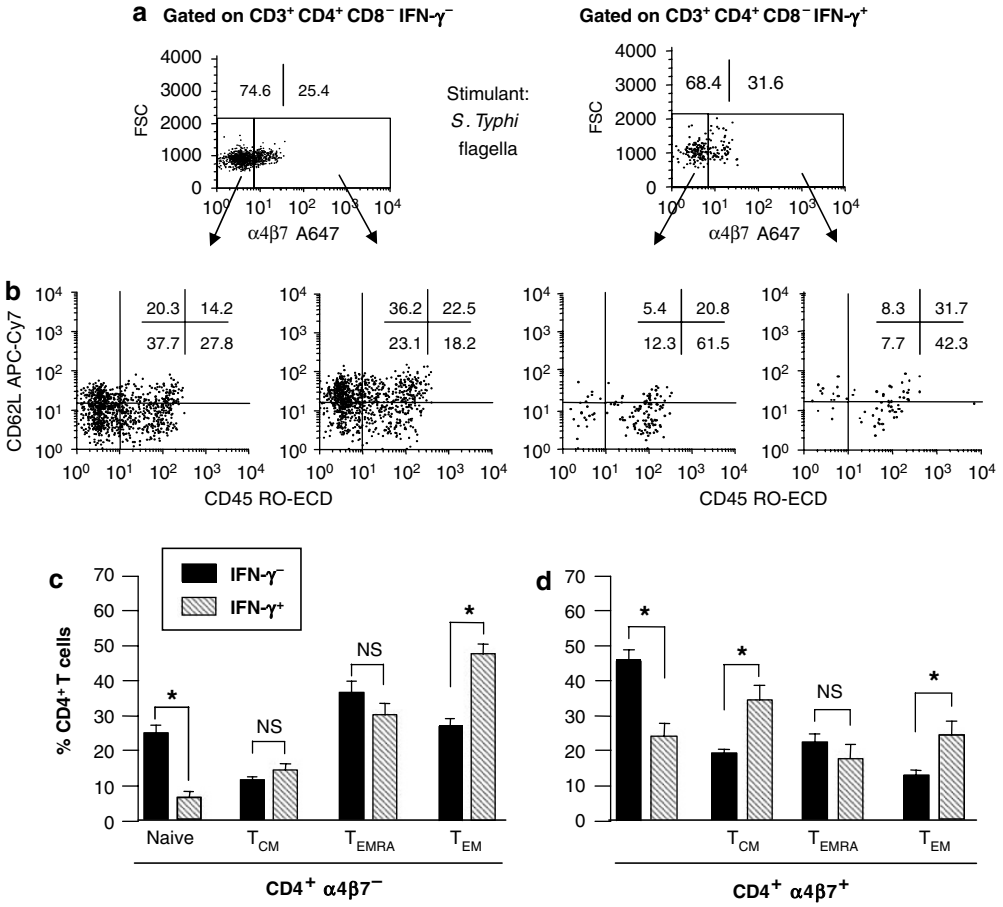

Figure 2 Expression of the gut-homing receptor integrin α_4/β_7 by IFN-γ-secreting $CD4^+$ cells in response to *ex vivo* stimulation with purified *S.* Typhi flagella. PBMCs are from the same 10 volunteers described in **Figure 1**. (**a** and **b**) Representative dot plots from one of the volunteers (subject no. 32004-4). (**c** and **d**) The mean±s.e. distribution of naive, T_{CM}, $T_{EMRA,}$ and T_{EM} $CD4^+$ subsets in integrin α_4/β_7^- and integrin α_4/β_7^+ subsets, respectively, from all subjects following stimulation with *S.* Typhi flagella. One-tail paired *t*-tests were used to analyze the data by comparing IFN-γ-secreting (IFN-γ^+) and the corresponding IFN-γ non-secreting (IFN-γ^-) sub-populations in **c** and **d**. *$P<0.05$. IFN-γ, interferon-γ; PBMCs, peripheral blood mononuclear cells.

integrin α_4/β_7. The gating strategy for these studies is shown in **Figure 3a, b, d** and **e**.

The patterns of cell populations that secrete IFN-γ in response to *ex vivo* stimulation with *S.* Typhi-infected blasts showed a different profile than that exhibited following stimulation with soluble antigens (see above). $CD8^+$ cells were the predominant population that secreted IFN-γ following incubation with *S.* Typhi-infected blasts. The percentages of $CD8^+$ IFN-γ^+ cells were two- to threefold higher (mean net increases 0.46±0.15%; range: 0.26–0.85%) when cocultured with *S.* Typhi-infected autologous blasts than in the presence of non-infected blasts (negative controls) ($P=0.036$). Moreover, a strong trend toward increases in IFN-γ^+ cells, albeit of lower magnitude, was also observed in $CD4^+$ cells from these subjects (mean net increases 0.09±0.04% (range: 0.05–0.21%, $P=0.06$)).

The percentages of antigen-specific IFN-γ-secreting $CD8^+$ T cells coexpressing CD45RO (T_M) following coculture with *S.* Typhi-infected blasts were higher than those observed in $CD8^+$ subsets that did not secrete IFN-γ (42.8±34.9 vs. 22.5±3.1, respectively, $P=0.01$) (**Figure 3c**). Increased proportions of $CD4^+$ $CD45RO^+$ cells were also observed among IFN-γ^+ cells (71.4±4.8 vs. 44.6±6.0, respectively, $P<0.005$) (**Figure 3c**).

When we further subdivided $CD8^+$ IFN-γ^+ cells into the various T sub-populations as described above and compared them with the percentages of the corresponding T populations that did not secrete IFN-γ, we observed marked increases of $CD8^+$ T_{EM} ($P=0.02$) and a strong trend toward increases in T_{EMRA} ($P=0.12$) subsets within IFN-γ-secreting cells. These changes were accompanied by significantly reduced percentages of the naive T subset ($P=0.013$) within IFN-γ-secreting cells. No changes were observed in the T_{CM} subset (**Figure 3f**).

Similar analysis of $CD4^+$ cells revealed marked increases of $CD4^+$ T_{EM} ($P=0.004$) and significantly reduced percentages of the naive $CD4^+$ T subset ($P=0.007$) within IFN-γ-secreting cells. No changes were observed in the $CD4^+$ T_{CM} and T_{EMRA} subsets (**Figure 3g**). No significant IFN-γ responses were observed in control cultures (cocultures with non-infected targets; data not shown).

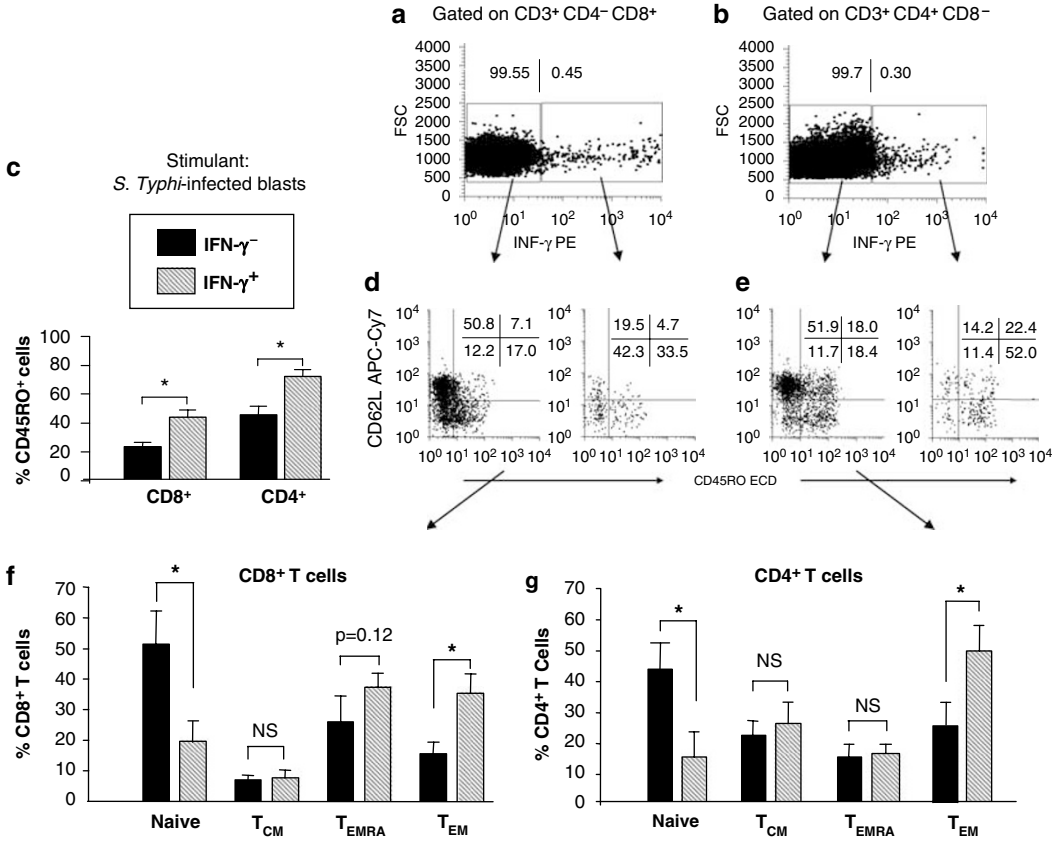

Figure 3 Characterization of IFN-γ-secreting CD8$^+$ and CD4$^+$ T cells following an overnight incubation with *S.* Typhi-infected autologous blasts. PBMCs from six CVD 909-vaccinated volunteers were analyzed by eight-color flow cytometry as described in Methods. (**a**, **b**, **d,** and **e**) Representative dot plots from one of the volunteers. (**c**) The mean±s.e. of the percentage of CD4$^+$ and CD8$^+$ T memory cells (CD3$^+$ CD4$^+$ CD8$^-$ CD45RO$^+$ and CD3$^+$ CD4$^-$ CD8$^+$ CD45RO$^+$, respectively) from all subjects following stimulation. (**f** and **g**) The mean±s.e. distribution of naive, T_{CM}, T_{EMRA}, and T_{EM} subsets of CD8$^+$ and CD4$^+$ T cells, respectively, from all subjects following stimulation with *S.* Typhi-infected autologous blasts. One-tail paired *t*-tests were used to analyze the data by comparing IFN-γ-secreting (IFN-γ$^+$) and the corresponding IFN-γ non-secreting (IFN-γ$^-$) sub-population in **c**, **f**, and **g**. **P*<0.05. IFN-γ, interferon-γ; PBMCs, peripheral blood mononuclear cells.

Characterization of the homing potential of IFN-γ-secreting memory CD8$^+$ and CD4$^+$ T cells cocultured with *S.* Typhi-infected targets

We then studied integrin α_4/β_7 expression on CD8$^+$ and CD4$^+$ T cells that specifically secrete IFN-γ following exposure to *S.* Typhi-infected blasts. Interestingly, IFN-γ-secreting CD8$^+$ cells consisted of populations that express, or not, integrin α_4/β_7 (**Figure 4a** and **b**). Incubation with *S.* Typhi-infected blasts resulted in significant decreases in IFN-γ-secreting cells that coexpress integrin α_4/β_7 compared with the IFN-γ-negative populations (35.0±4.6 (range: 27.8–49.6%) vs. 51.2±6.6 (range: 36.8–64.4), respectively) ($P=0.012$). These results are in contrast with those observed in *S.* Typhi flagella-simulated cultures.

To characterize the expression of integrin α_4/β_7 in CD8$^+$ IFN-γ$^+$ cells, we compared the proportions of T_{EM}, T_{CM}, T_{EMRA}, and naive T subsets that express, or not, integrin α_4/β_7 between IFN-γ$^+$ and IFN-γ$^-$ subsets. As shown in **Figure 4a**, among integrin α_4/β_7-negative cells, IFN-γ-secreting cells were significantly increased ($P=0.023$) only in the T_{EM} sub-population. In contrast, among cells expressing integrin α_4/β_7, significant increases in IFN-γ$^+$ cells were observed in both T_{EMRA} ($P=0.01$) and T_{EM} ($P=0.02$) subsets (**Figure 4b**). The proportion of naive cell populations was significantly lower in both integrin $\alpha_4/\beta_7{}^-$ ($P=0.02$) and integrin $\alpha_4/\beta_7{}^+$ ($P=0.004$) IFN-γ-secreting CD8$^+$ cells. No differences were recorded in T_{CM} subsets regardless of whether they express or not integrin α_4/β_7.

We also characterized the expression of integrin α_4/β_7 in CD4$^+$ IFN-γ$^+$ cells, by comparing the proportions of T_{EM}, T_{CM}, T_{EMRA}, and naive T subsets that express, or not, integrin α_4/β_7 between IFN-γ$^+$ and IFN-γ$^-$ subsets. Somewhat increased percentages of cells expressing integrin α_4/β_7 were observed within the CD4$^+$ IFN-γ-secreting cell populations (34.4±4.6 vs. 27.4±3.8). Specific CD4$^+$ T cells that produce IFN-γ following stimulation with *S.* Typhi-infected blasts followed a pattern similar to that recorded in response to *S.* Typhi flagella. As shown in **Figure 4c**, among integrin α_4/β_7-negative cells, IFN-γ-secreting cells were significantly increased ($P=0.005$) only in the T_{EM} sub-population. In contrast, among cells expressing integrin α_4/β_7, significant increases in IFN-γ$^+$ cells were observed in both T_{CM} ($P=0.005$) and T_{EM} ($P=0.016$) subsets (**Figure 4d**). The proportion of naive cell populations was significantly lower in both integrin $\alpha_4/\beta_7{}^-$ ($P=0.011$) and integrin

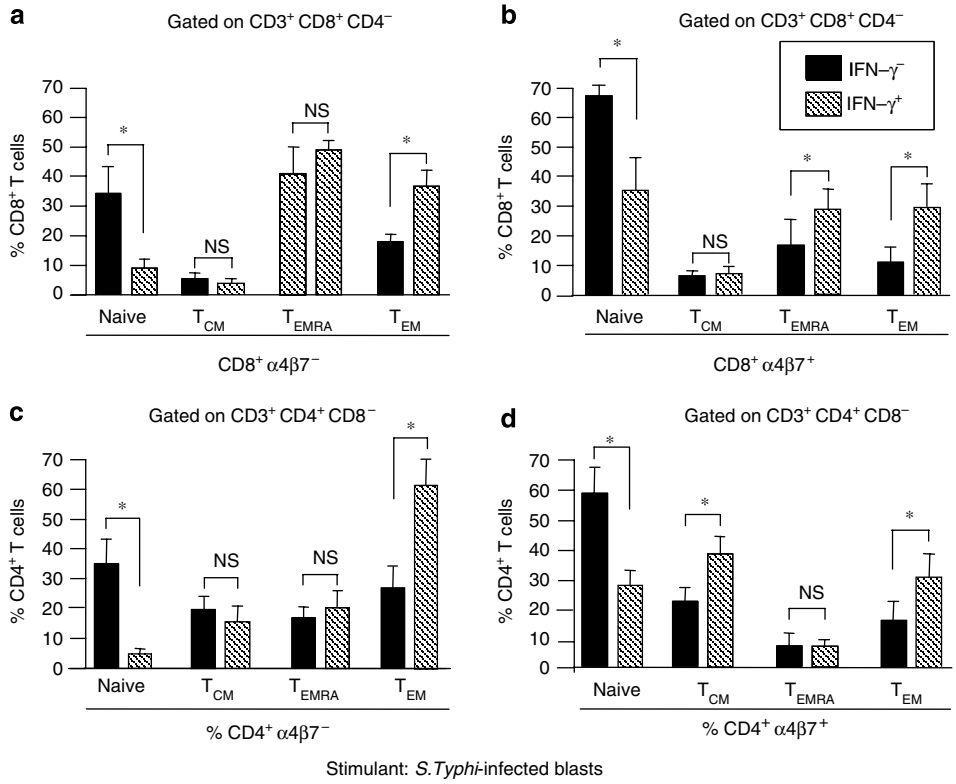

Figure 4 Expression of the gut-homing receptor integrin α4/β7 by $CD8^+$ and $CD4^+$ IFN-γ-secreting cells following an overnight incubation with *S.* Typhi-infected autologous blasts. PBMCs are from the same six volunteers described in **Figure 3**. Shown are the means±s.e. distributions of naive, T_{CM}, $T_{EMRA,}$ and T_{EM} $CD8^+$ (**a** and **b**) and $CD4^+$ (**c** and **d**) populations in integrin α_4/β_7^- (**a** and **c**) and integrin α_4/β_7^+ subsets (**b** and **d**) from all subjects following stimulation with *S.* Typhi-infected autologous blasts. One-tail paired *t*-tests were used to analyze the data by comparing IFN-γ-secreting (IFN-γ^+) and the corresponding IFN-γ non-secreting (IFN-γ^-) sub-populations. *$P<0.05$. IFN-γ, interferon-γ; PBMCs, peripheral blood mononuclear cells.

α_4/β_7^+ ($P=0.001$) IFN-γ-secreting $CD4^+$ cells. No differences were recorded in T_{EMRA} subsets regardless of whether they express or not integrin α_4/β_7.

DISCUSSION

Some of the critical challenges being addressed in the development of new typhoid vaccines are how to achieve strong immunogenicity without increasing reactogenicity and how to elicit long-term efficacy.[36] Enduring protection relies upon the induction of strong, long-lived, broadly based immunological B- and T-cell memory to antigens that correlate with protection, i.e., the ability to recall previous exposures to antigens and to mount enhanced and accelerated effector responses.[36] The development of improved typhoid vaccines is a public health priority. In recent years, a handful of promising new attenuated oral typhoid vaccine candidates have been developed by our group and others that exhibit low reactogenicity and broad immunogenicity, encompassing a wide array of CMI responses including increased proliferative responses and cytokine production (particularly IFN-γ, tumor necrosis factor-α, and IL-10), as well as classical and non-classical class I-restricted cytotoxic T cell activity to purified *S.* Typhi antigens and *S.* Typhi-infected cells.[4–6,8,9,11,14,15,17–19,21–23,25,37] These responses are likely to play an important role in the host defense against *S.* Typhi by several mechanisms, including enhancement of the bactericidal activity of cells of the innate immune response (e.g., macrophages) and antigen presentation, killing of *S.* Typhi-infected cells, and providing help for antibody responses to *S.* Typhi antigens.[4–6,8,22,23,25,38,39]

However, long-term protection depends, to a large extent, on the ability of a vaccine to elicit T_M able to migrate to effector and inductive sites.[36] Here, $CD4^+$ and $CD8^+$ T_M cell populations were evaluated for their expression of CD45RO and the lymph node-homing molecule CD62L (the expression of which has been shown to be highly correlated with that of CCR7).[30,35,40] The T-cell phenotype based on expression of these markers allows one to define central memory T cells (T_{CM}, $CD45RO^+$ $CD62L^+$), effector memory T cells (T_{EM}, $CD45RO^+$ $CD62L^-$), and naive T cells (largely $CD45RO^-$ $CD62L^+$),[26–33] as well as a recently described population of T memory-effectors that express CD45RA (T_{EMRA}, $CD45RO^-$ $CD62L^-$), which appears to play a significant role during viral infections in humans.[26–28,30] It is widely accepted that T_{EM} populations are ready for immediate effector action, whereas T_{CM} are important in the generation of a new wave of effector T cells.[30–33]

In the present studies, we used anti-CD28 antibody and anti-CD49d monoclonal antibodies (mAbs) as costimulators to characterize the various T_M subsets in response to soluble

antigens. Costimulation with these mAbs enhances the detection of low frequencies of antigen-specific memory-effector $CD4^+$ and $CD8^+$ cell populations with minimal or no increase in the levels of nonspecific stimulation.[41,42]

As described in Methods, during the flow cytometric analysis, we followed an electronic-gating strategy designed to eliminate bias in our observations. $CD3^+$ $CD4^+$ $CD8^-$ and $CD3^+$ $CD4^-$ $CD8^+$ cells were analyzed for IFN-γ expression and the expression of integrin α_4/β_7 in each sub-population determined before evaluating the proportions of T_{EM}, T_{CM}, $T_{EMRA,}$ and naive T cells. Using this approach, we first analyzed IFN-γ-secreting cells in response to soluble antigens. In agreement with our previous observations, responses to soluble *S.* Typhi antigens were predominantly mediated by $CD4^+$,[8,22] with only a minor component attributable to $CD8^+$ cells. IFN-γ-secreting cells were mostly $CD4^+$ T_M cells, predominantly T_{EM}. However, it is noteworthy that a sizable proportion of IFN-γ-secreting cells was T_{CM} cells and that even some naive T and T_{EMRA} subsets were found to be IFN-γ^+. These findings could be explained by the marked heterogeneity observed even within these well-defined populations. For example, it has been shown that T_M are heterogeneous for CD62L expression;[30,32,33] thus, some of the cells that are defined in this study as T_{CM} ($CD45RO^+$ $CD62L^+$) could be T_{EM} expressing moderate levels of CD62L. Similarly, some of the cells in the naive population ($CD45RO^-$ $CD62L^+$) may be T_{EMRA} that express variable levels of CD62L. Further studies in which expression of CCR7, CD27, and other molecules[30,32,33] are evaluated in conjunction with the molecules described above using more colors in even more complex multichromatic flow cytometry studies will be required to further define the anti-*S.* Typhi-specific T_M cells elicited by immunization.

It is worth noting that throughout these studies, no significant differences were observed in the proportions of T_{EM}, T_{CM}, T_{EMRA}, and naive T sub-populations among IFN-γ-negative $CD4^+$ or $CD8^+$ T cells from experimental samples (stimulated with *S.* Typhi antigens or *S.* Typhi-infected blasts) and in those observed in negative control cultures (no antigen or non-infected blasts) (data not shown). These results are consistent with the contention that T cells that did not secrete IFN-γ in response to stimulation with *S.* Typhi flagella or *S.* Typhi-infected blasts represent non-responding T-cell populations as measured by this effector function.

We next explored the important hypothesis that immunization with live attenuated oral Salmonella vaccines is likely to induce *S.* Typhi-specific T_M cells with the capability to home to the gut, the site of entry of *S.* Typhi. This was investigated by determining the expression of integrin α_4/β_7 in the various T-cell subsets. Unexpectedly, although there was an increase in the percentages of $CD4^+$ IFN-γ^+ T cells that express integrin α_4/β_7, a significant proportion of IFN-γ^+ cells did not express integrin α_4/β_7. Moreover, we observed differences in the proportions of the $CD4^+$ T_M subsets present in integrin $\alpha_4/\beta_7{}^+$ and integrin $\alpha_4/\beta_7{}^-$ cells. Although IFN-γ^+ T_{CM} and T_{EM} subsets were found to be increased in integrin $\alpha_4/\beta_7{}^+$ cells, only IFN-γ^+ T_{EM} were enriched in integrin $\alpha_4/\beta_7{}^-$ cells. These results indicate that immunization with CVD 909 elicits a complex pattern of $CD4^+$ T_M cells with the capability to home to the lamina propria and Peyer's patches in the gut, as well as to peripheral lymph nodes and perhaps to other sites as well.

We have previously shown that $CD8^+$ cytotoxic T-cell responses, which are restricted by classical and non-classical HLA-E major histocompatibility complex molecules, might play a key role by eliminating cells harboring *S.* Typhi.[4–6,22,23] Here, we studied in detail the various T_M subsets of IFN-γ-secreting cells in response to *S.* Typhi-infected autologous blasts, a response that, although mediated mostly by $CD8^+$ cells, also includes a sizable $CD4^+$ component.[8] It is likely that both $CD4^+$ and $CD8^+$ cells respond to this stimulation because infected targets are able to present *S.* Typhi antigens in the context of both class I and II molecules. The present studies confirmed and extended previous findings by identifying T_M cells ($CD45RO^+$) as the predominant responding populations (**Figure 3**), although some IFN-γ^+ cells lack expression of CD45RO. When we looked at T_M subsets composing the IFN-γ^+ T-cell population, we found differences between the $CD8^+$ and $CD4^+$ cells. Although increases were observed in T_{EM} and T_{EMRA} subsets of $CD8^+$ IFN-γ^+ cells, only increases in T_{EM} subset were recorded in $CD4^+$ populations (**Figure 3**). To our knowledge, this is the first demonstration that T_{EMRA} is a significant component of the T_{EM} memory response to bacteria in humans. This could be very important given that T_{EMRA} are a distinct subset of $CD8^+$ T cells that has been shown to carry the largest amount of perforin and that has been proposed to be among the most active effector T cells.[26–28,30]

Concerning the expression of homing molecules in $CD8^+$ IFN-γ^+ T_M populations, we have observed marked differences between responses to stimulation with *S.* Typhi-infected blasts and those measured to soluble *S.* Typhi antigens. It is important that the proportion of $CD8^+$ T_M cells expressing integrin α_4/β_7 was lower in IFN-γ^+ than in IFN-γ^- subsets. This could be the result of a depletion in peripheral blood of integrin $\alpha_4/\beta_7{}^+$ $CD8^+$ T_M cells, which peaked at earlier times, and were retained in the gut. As a result, when measured 60–90 days after immunization, most recirculating T_M cells might not express integrin α_4/β_7. However, the $CD8^+$ T_M subsets that do persist in circulation showed different compositions among integrin $\alpha_4/\beta_7{}^+$ and integrin $\alpha_4/\beta_7{}^-$ cells. Although increased proportions of T_{EM} were observed in integrin $\alpha_4/\beta_7{}^-$ cells, both T_{EMRA} and T_{EM} were increased among integrin $\alpha_4/\beta_7{}^+$ populations. In contrast, the $CD4^+$ T_M subset distribution was similar to that observed with soluble antigens, i.e., increased T_{EM} in integrin $\alpha_4/\beta_7{}^-$ and increased T_{CM} and T_{EM} responses in integrin $\alpha_4/\beta_7{}^+$ populations. Other homing molecules, e.g., the CCR9 receptor, expressed by subsets of integrin $\beta_7{}^+$ T cells, and its ligand, the chemokine CCL25, have also been shown to play a selective role in effector T-cell homing to the small intestinal mucosa.[35] The expression of this and other important homing molecules will be investigated in future studies to further explore the gut-homing potential of specific effector and memory T cells elicited by live oral *S.* Typhi vaccines in humans.

Taken together, our results in CVD 909 vaccinees showed that the responses elicited are heterogenous with regard to the

S. Typhi-specific T_M subsets stimulated, as well as their capability to migrate both to the gut (lamina propria as well as Peyer's patches) and to the peripheral lymph nodes. However, we observed distinct patterns in $CD4^+$ and $CD8^+$ T cells. Although increases in the proportions of *S.* Typhi-specific $CD4^+$ integrin α_4/β_7^+ T_M cells were largely in T_{EM} and T_{CM} subsets, the $CD8^+$ integrin α_4/β_7^+ T_M subsets preferentially expanded were predominantly T_{EM} and T_{EMRA}. In contrast, increases in integrin α_4/β_7^- $CD4^+$ and $CD8^+$ T_M cells were exclusively on T_{EM} cells. These observations are in contrast to the only other study in the literature, of which we are aware, that has recently reported that immunization with Ty21a induced $CD4^+$ and $CD8^+$ T_M cells that almost exclusively express integrin β_7.[11] However, these disparities could be attributed to several factors, including the use of different vaccines, that responses were observed at days 7 and 14 but not at later time points, that only CD45RA was used to identify T_M cells (an inaccurate marker of T_M in the absence of coexpression of other molecules such as CD62L or CCR7), that an mAb to integrin β_7 instead of integrin α_4/β_7 was used, or to differences in antigenic preparations. Among these variables, the one that is likely to be of particular significance is that only cells that are homing to the gut could be identified at early time points, whereas a more complex mix of T_M subsets homing to various tissues can be found in circulation at later times. We are directly addressing this possibility by carefully studying the kinetics of induction following immunization.

In sum, we have provided novel information concerning specific T_M-cell subsets elicited by immunization with an attenuated *S.* Typhi oral vaccine, including the first demonstration that bacterial infection elicits the appearance of strong $CD8^+$ T_{EMRA} responses in humans. Moreover, we showed that immunization elicited T_M subsets with the potential to migrate to the gut as well as to peripheral lymph nodes that persist for at least 90 days in circulation. These observations have broad implications for the development of attenuated bacterial vaccines, as well as for their use as live vectors to deliver prokaryotic and eukaryotic expression vectors in humans.

METHODS

Subjects, vaccinations, and isolation of PBMC. Peripheral blood mononuclear cells from subjects recruited from the Baltimore metropolitan area participating in two separate CVD 909 phase-1 vaccine trials were used in these studies.[17,25] One study (hereafter Ty32004) consisted of six subjects (median age 28.3 years, range: 20–39 years) who received a single dose of CVD 909 (2.5×10^9 colony-forming units), and blood samples were collected on days 0, 28, and 91 postimmunization. The second study (hereafter Ty35000) included eight healthy subjects (median age 24 years, range 19–39 years) who were immunized with two oral doses of CVD 909 (6.2×10^9 colony-forming units per dose), one dose on day 0, and one dose on day 14. PBMCs from 12 of these volunteers, who have previously shown to specifically secrete IFN-γ in response to *S.* Typhi flagella, *S.* Typhi particulate, or *S.* Typhi-infected targets following immunization[25] were used in these studies. Blood samples from these subjects were obtained on days 0, 28, and 60 postvaccination.[25] PBMCs were isolated immediately after the blood draws by density-gradient centrifugation and cryopreserved in liquid N_2 following standard techniques until used in the assays.[14,22] Volunteers gave signed informed consent. The human experimentation guidelines of the US Department of Health and Human Services and those of the University of Maryland, Baltimore, were followed in the conduct of the present clinical research.

Antigens. *S.* Typhi flagella H-antigen: Flagellar antigen was purified from the rough *S.* Typhi strain Ty2R following standard procedures and used at a concentration of $10\,\mu g\,ml^{-1}$.[7,14]

Heat-phenolyzed whole-cell *S.* Typhi (TypVac): The particulate *S.* Typhi antigen consisted of Ty2 heat-killed, phenol-preserved, phenol-killed, whole-cell bacteria (typhoid vaccine; Wyeth Laboratories, Marietta, PA, lot no. 4978038, ~2.5×10^8 organisms per ml) and was used in the assays as previously described.[14]

Preparation of autologous blasts for use as stimulators in intracellular IFN-γ production assays by flow cytometry: Autologous phytohemagglutinin-stimulated PBMCs (henceforth called "blasts") were used in this study as stimulator cells. Blasts were prepared by stimulation with $1\,\mu g\,ml^{-1}$ phytohemagglutinin-L for 24 h, washed, and cultured with $20\,U\,ml^{-1}$ human recombinant interleukin-2 (Boehringer Gmbh, Mannheim, Germany) for 5–6 days as previously described.[6,25]

Infection of stimulator cells with *S.* Typhi for use in intracellular IFN-γ production assays by flow cytometry: Blasts were incubated in RPMI-1640 (without antibiotics) for 3 h at 37 °C in the absence or presence of wild-type *S.* Typhi strain ISP1820 (wild-type *S.* Typhi) (obtained from J. Nataro, Center for Vaccine Development) at different multiplicities of infection as previously described.[22] After exposure to *S.* Typhi, cells were washed and incubated overnight at 37 °C in RPMI-1640 supplemented with $100\,U\,ml^{-1}$ penicillin, $100\,\mu g\,ml^{-1}$ streptomycin, $50\,\mu g\,ml^{-1}$ gentamicin, 2 mM L-glutamine, 2.5 mM sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 10% heat-inactivated fetal bovine serum (complete RPMI) before being used to stimulate effector PBMC.

Ex vivo stimulation for IFN-γ secretion studies by multichromatic flow cytometry intracellular cytokine analysis: PBMCs resuspended in complete RPMI were stimulated with *S.* Typhi flagella ($10\,\mu g\,ml^{-1}$) or TypVac (2×10^5 particles per well) in the presence of mAbs to costimulatory molecules CD28 (clone 37.51) and CD49d (clone 9F10) ($1.5\,\mu g\,ml^{-1}$ each; BD PharMingen, San Jose, CA), or with *S.* Typhi-infected or uninfected autologous blasts (1:7 to effector PBMC). Unstimulated PBMCs or stimulation with phytohemagglutinin-L ($1\,\mu g\,ml^{-1}$), or PMA ($1\,ng\,ml^{-1}$) plus ionomycin ($500\,ng\,ml^{-1}$) were used as negative and positive controls, respectively. After an overnight incubation, Brefeldin A (Sigma-Aldrich, St Louis, MO) was added at a final concentration of $10\,\mu g\,ml^{-1}$ and cultures were continued for an additional 5–6 h. Cells were then harvested, washed, and stained for seven- or eight-color multichromatic flow cytometric analysis as described below.

Staining procedures and seven- or eight-color multichromatic flow cytometry analysis strategy: Surface staining with monoclonal antibodies to identify T-cell sub-populations and intracellular staining to determine IFN-γ content were performed according to standard methods.[4–6,22] For the experiments done with antigens (*S.* Typhi flagella and TypVac), cells were surface-stained with CD3-PE-Cy7 (clone UCHT1; Beckman-Coulter, Fullerton, CA), CD4-FITC (clone RPA-T4; PharMingen, San Jose, CA), CD8-PerCP-Cy5.5 (Clone SK1; BD PharMingen), CD45RO-ECD (PE-Texas red, Clone UCHL-1; Immunotech, Fullerton, CA), CD62L-APC-Cy7 (Clone DREG-56; Caltag, Carlsbad, CA), and integrin α4β7-Alexa 647 conjugate (the ACT-1mAb was kindly provided by W. Newman, Leukosite, Cambridge, MA), and conjugated to Alexa 647 using an Alexa 647-labeling kit (Molecular probes, Eugene, OR). In some experiments, infected and uninfected autologous blasts were used as stimulators. In the latter studies, target cells were stained with CD45-biotin (a leukocyte common surface antigen, Clone H130; BD PharMingen) and streptavidin-conjugated with pacific blue (Molecular Probes) before exposure to the effectors to enable their exclusion from analysis as previously described.[6] Following surface staining, cells were fixed and permeabilized for intracellular staining with IFN-γ-PE (Clone B27; PharMingen). Samples were run in a MoFlo flow cytometer/cell sorter system (Coulter-Cytomation, Fort Collins, CO) and analyzed using

the WinList flow cytometry software analysis package (Verity Software House, Topsham, ME). Cells present on the lymph gate (defined in forward vs. side scatter cytograms) were gated on $CD3^+$ cells and then on either $CD4^+CD8^-$ or $CD4^-CD8^+$ subsets. Cells in each population were subsequently analyzed for IFN-γ expression and the expression of integrin α_4/β_7 in each sub-population was determined. Finally, the proportions of T_{EM}, T_{CM}, T_{EMRA}, and naive T cells were determined on IFN-γ^+ and IFN-γ^- subsets that express, or not, integrin α_4/β_7.

Statistical analysis. All tests were performed using SigmaStat software (version 3.1; Systat Software, Point Richmond, CA). *P*-values <0.05 were considered significant.

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DISCLOSURE

Drs Sztein and Levine are coinventors of a patent for attenuated *Salmonella enterica* serovar Typhi strains that constitutively express Vi capsular polysaccharide antigen. However, no company has licensed this technology. Drs Wahid, Salerno-Gonçalves, and Tacket declare that they do not have a commercial or other association that might pose a conflict of interest.

REFERENCES

1. Crump, J.A., Luby, S.P. & Mintz, E.D. The global burden of typhoid fever. *Bull. World Health Organ.* **82,** 346–353 (2004).
2. Levine, M.M. *et al.* Attenuated strains of *Salmonella enterica* serovar Typhi as live oral vaccines against typhoid fever. In *New Generation Vaccines* (Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M. & Good, M., eds) 479–486 (Marcel Dekker, New York, NY, 2004).
3. Levine, M.M., Tacket, C.O. & Sztein, M.B. Host–Salmonella interaction: human trials. *Microbes Infect.* **3,** 1271–1279 (2001).
4. Salerno-Goncalves, R., Wahid, R. & Sztein, M.B. Immunization of volunteers with *Salmonella enterica* serovar Typhi strain Ty21a elicits the oligoclonal expansion of CD8+ T cells with predominant Vβ repertoires. *Infect. Immun.* **73,** 3521–3530 (2005).
5. Salerno-Goncalves, R., Fernandez-Vina, M., Lewinsohn, D.M. & Sztein, M.B. Identification of a human HLA-E-restricted CD8+ T cell subset in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. *J. Immunol.* **173,** 5852–5862 (2004).
6. Salerno-Goncalves, R., Pasetti, M.F. & Sztein, M.B. Characterization of CD8(+) effector T cell responses in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. *J. Immunol.* **169,** 2196–2203 (2002).
7. Wyant, T.L., Tanner, M.K. & Sztein, M.B. Potent immunoregulatory effects of *Salmonella typhi* flagella on antigenic stimulation of human peripheral blood mononuclear cells. *Infect. Immun.* **67,** 1338–1346 (1999).
8. Sztein, M.B. Cell-mediated immunity and antibody responses elicited by attenuated *Salmonella enterica* Serovar Typhi strains used as live oral vaccines in humans. *Clin. Infect. Dis.* **45** (Suppl 1), S15–S19 (2007).
9. Kilhamn, J., Lundin, S.B., Brevinge, H., Svennerholm, A.M. & Jertborn, M. T- and B-cell immune responses of patients who had undergone colectomies to oral administration of *Salmonella enterica* serovar Typhi Ty21a vaccine. *Clin. Diagn. Lab. Immunol.* **10,** 426–430 (2003).
10. Viret, J.F. *et al.* Mucosal and systemic immune responses in humans after primary and booster immunizations with orally administered invasive and noninvasive live attenuated bacteria. *Infect. Immun.* **67,** 3680–3685 (1999).
11. Lundin, B.S., Johansson, C. & Svennerholm, A.M. Oral immunization with a *Salmonella enterica* serovar typhi vaccine induces specific circulating mucosa-homing CD4(+) and CD8(+) T cells in humans. *Infect. Immun.* **70,** 5622–5627 (2002).
12. Levine, M.M. *et al.* Duration of efficacy of Ty21a, attenuated *Salmonella typhi* live oral vaccine. *Vaccine* **17** (Suppl 2), S22–S27 (1999).
13. Hindle, Z. *et al.* Characterization of *Salmonella enterica* derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers. *Infect. Immun.* **70,** 3457–3467 (2002).
14. Sztein, M.B. *et al.* Cytokine production patterns and lymphoproliferative responses in volunteers orally immunized with attenuated vaccine strains of *Salmonella typhi*. *J. Infect. Dis.* **170,** 1508–1517 (1994).
15. Kirkpatrick, B.D. *et al.* The novel oral typhoid vaccine M01ZH09 is well tolerated and highly immunogenic in 2 vaccine presentations. *J. Infect. Dis.* **192,** 360–366 (2005).
16. Hohmann, E.L., Oletta, C.A. & Miller, S.I. Evaluation of a phoP/phoQ-deleted, aroA-deleted live oral *Salmonella typhi* vaccine strain in human volunteers. *Vaccine* **14,** 19–24 (1996).
17. Tacket, C.O., Pasetti, M.F., Sztein, M.B., Livio, S. & Levine, M.M. Immune responses to an oral typhoid vaccine strain that is modified to constitutively express Vi capsular polysaccharide. *J. Infect. Dis.* **190,** 565–570 (2004).
18. Tacket, C.O. *et al.* Phase 2 clinical trial of attenuated *Salmonella enterica* serovar typhi oral live vector vaccine CVD 908-htrA in US volunteers. *Infect. Immun.* **68,** 1196–1201 (2000).
19. Tacket, C.O. *et al.* Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. *Infect. Immun.* **65,** 452–456 (1997).
20. Tacket, C.O. *et al.* Clinical acceptability and immunogenicity of CVD 908 *Salmonella typhi* vaccine strain. *Vaccine* **10,** 443–446 (1992).
21. Tacket, C.O. *et al.* Safety and immune responses to attenuated *Salmonella enterica* serovar Typhi oral live vector vaccines expressing tetanus toxin fragment C. *Clin. Immunol.* **97,** 146–153 (2000).
22. Salerno-Goncalves, R. *et al.* Concomitant induction of CD4(+) and CD8(+) T cell responses in volunteers immunized with *Salmonella enterica* serovar Typhi strain CVD 908-htrA. *J. Immunol.* **170,** 2734–2741 (2003).
23. Sztein, M.B., Tanner, M., Polotsky, Y., Orenstein, J.M. & Levine, M.M. Cytotoxic T lymphocytes after oral immunization with attenuated vaccine strains of *Salmonella typhi* in humans. *J. Immunol.* **155,** 3987–3993 (1995).
24. Wang, J.Y., Noriega, F.R., Galen, J.E., Barry, E. & Levine, M.M. Constitutive expression of the Vi polysaccharide capsular antigen in attenuated *Salmonella enterica* serovar Typhi oral vaccine strain CVD 909. *Infect. Immun.* **68,** 4647–4652 (2000).
25. Wahid, R., Salerno-Goncalves, R., Tacket, C.O., Levine, M.M. & Sztein, M.B. Cell-mediated immune responses in humans after immunization with one or two doses of oral live attenuated typhoid vaccine CVD 909. *Vaccine* **25,** 1416–1425 (2007).
26. Geginat, J., Lanzavecchia, A. & Sallusto, F. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* **101,** 4260–4266 (2003).
27. Champagne, P. *et al.* Skewed maturation of memory HIV-specific CD8T lymphocytes. *Nature* **410,** 106–111 (2001).
28. Oswald-Richter, K. *et al.* Identification of a CCR5-expressing T cell subset that is resistant to R5-tropic HIV infection. *PLoS. Pathog.* **3,** e58 (2007).
29. Kedzierska, K., La Gruta, N.L., Turner, S.J. & Doherty, P.C. Establishment and recall of CD8+ T-cell memory in a model of localized transient infection. *Immunol. Rev.* **211,** 133–145 (2006).
30. Sallusto, F., Geginat, J. & Lanzavecchia, A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* **22,** 745–763 (2004).
31. Harari, A. *et al.* Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol. Rev.* **211,** 236–254 (2006).
32. Rodrigo, M.J. & von Andrian, U.H. Specificity and plasticity of memory lymphocyte migration. *Curr. Top. Microbiol. Immunol.* **30,** 83–116 (2006).
33. Lefrancois, L. Development, trafficking, and function of memory T-cell subsets. *Immunol. Rev.* **211,** 93–103 (2006).
34. Hamann, A., Andrew, D.P., Jablonski-Westrich, D., Holzmann, B. & Butcher, E.C. Role of α4-integrins in lymphocyte homing to mucosal tissues *in vivo*. *J. Immunol.* **152,** 3282–3293 (1994).
35. Johansson-Lindbom, B. & Agace, W.W. Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol. Rev.* **215,** 226–242 (2007).

36. Levine, M.M. & Sztein, M.B. Vaccine development strategies for improving immunization: the role of modern immunology. *Nat. Immunol.* **5,** 460–464 (2004).
37. Hohmann, E.L., Oletta, C.A., Killeen, K.P. & Miller, S.I. phoP/phoQ-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J. Infect. Dis.* **173,** 1408–1414 (1996).
38. Mastroeni, P., Villarreal Ramos, B. & Hormaeche, C.E. Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent *salmonellae* in mice vaccinated with live attenuated aro- *Salmonella* vaccines. *Microb. Pathog.* **13,** 477–491 (1992).
39. Tite, J.P., Dougan, G. & Chatfield, S.N. The involvement of tumor necrosis factor in immunity to *Salmonella* infection. *J. Immunol.* **147,** 3161–3164 (1991).
40. Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401,** 708–712 (1999).
41. Ott, P.A. *et al.* CD28 costimulation enhances the sensitivity of the ELISPOT assay for detection of antigen-specific memory effector CD4 and CD8 cell populations in human diseases. *J. Immunol. Methods* **285,** 223–235 (2004).
42. Waldrop, S.L., Pitcher, C.J., Peterson, D.M., Maino, V.C. & Picker, L.J. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J. Clin. Invest.* **99,** 1739–1750 (1997).