Immunization with Ty21a live oral typhoid vaccine elicits crossreactive multifunctional CD8 + T-cell responses against *Salmonella enterica* serovar Typhi, *S.* Paratyphi A, and *S.* Paratyphi B in humans

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Previously we have extensively characterized *Salmonella* enterica serovar Typhi (*S.* Typhi)-specific cell-mediated immune (CMI) responses in volunteers orally immunized with the licensed Ty21a typhoid vaccine. In this study we measured *Salmonella*-specific multifunctional (MF) CD8 + T-cell responses to further investigate whether Ty21a elicits crossreactive CMI against *S.* Paratyphi A and *S.* Paratyphi B that also cause enteric fever. Ty21a-elicited crossreactive CMI responses against all three *Salmonella* serotypes were predominantly observed in CD8 + T effector/memory (T$_{EM}$) and, to a lesser extent, in CD8 + CD45RA + T$_{EM}$ (T$_{EMRA}$) subsets. These CD8 + T-cell responses were largely mediated by MF cells coproducing interferon-γ and macrophage inflammatory protein-1β and expressing CD107a with or without tumor necrosis factor-α. Significant proportions of *Salmonella*-specific MF cells expressed the gut-homing molecule integrin α$_4$β$_7$. In most subjects, similar MF responses were observed to *S.* Typhi and *S.* Paratyphi B, but not to *S.* Paratyphi A. These results suggest that Ty21a elicits MF CMI responses against *Salmonella* that could be critical in clearing the infection. Moreover, because *S.* Paratyphi A is a major public concern and Ty21a was shown in field studies not to afford cross-protection to *S.* Paratyphi A, these results will be important in developing a *S.* Typhi/*S.* Paratyphi A bivalent vaccine against enteric fevers.

INTRODUCTION

Typhoid fever caused by *Salmonella* enterica serover Typhi (*S.* Typhi) is responsible for an estimated 21.7 million cases and 200,000 deaths per year worldwide.[1,2] Other significant causative agents of enteric fevers are *S.* Paratyphi A and *S.* Paratyphi B, and rarely *S.* Paratyphi C.[3] Recent reports indicate that the incidence of paratyphoid A fever is on the rise in areas of endemicity (e.g., South and Southeast Asia and China) and among travelers returning from those areas.[1,4–7] The emergence of multiple antibiotic-resistant *Salmonella* strains has further increased the health risks posed by these infections.[8]

To prevent typhoid fever, three licensed vaccines are available, i.e., live attenuated oral vaccine Ty21a (Ty21a), parenteral polysaccharide Vi vaccine (Vi vaccine), and Vi-conjugated vaccine. In contrast, no vaccines are available against paratyphoid fevers. Although a high degree of homology at the DNA level exists among *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi B, a critical virulence factor, the *S.* Typhi Vi polysaccharide, is not expressed either by *S.* Paratyphi A or *S.* Paratyphi B.[9] Therefore, the parenteral Vi vaccines are not expected to provide cross-protection against paratyphoid A and B fevers. The possibility that the live attenuated Ty21a confers cross-protection against *S.* Paratyphi A and/or *S.* Paratyphi B has been studied in several field studies.[10–12] These studies indicate that Ty21a does not protect against *S.* Paratyphi A, although it does confer a moderate degree of protection against *S.* Paratyphi B disease.[13]

Because of the recent increased incidence of enteric fever caused by *S.* Paratyphi A, the need for an effective vaccine against paratyphoid A fever has been emphasized.[14] However,

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the need for an effective vaccine against S. Paratyphi A, as well as more effective vaccines to typhoid fever, requires a better understanding of the immunological basis of the crossreactive and cross-protective responses induced by Ty21a. This is complicated by the fact that S. Typhi is a human host-restricted pathogen and animal models do not faithfully recapitulate human disease. Nevertheless, the S. Typhimurium "typhoid" mouse model has led to important insights into the role that various innate and adaptive effector mechanisms might play in protection from Salmonella infection. For example, resistance to virulent challenge with S. Typhimurium by immunized mice requires production of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) by both CD4 + and CD8 + T cells.[15–18]

In humans, humoral, and most importantly cell-mediated immune (CMI) responses that are induced following vaccination of healthy volunteers with Ty21a and other live oral candidate vaccine strains (i.e., CVD 908, CVD 908-htrA, CVD 909, and MZH09) have been studied extensively by us and others.[19–30] Typically, we observed that following immunization with live oral S. Typhi vaccines, both CD4 + and CD8 + T-cell responses, including cytotoxic T cells (CTLs), were observed depending on the nature of the stimulant used in in vitro or ex vivo experiments.[23,24,27,29–35] Although typically CD4 + T-cell responses were more pronounced to soluble antigens (e.g., flagella), CD8 + T cells were the predominant responders against S. Typhi-infected targets.[24,27,30,32]

Depending on the expression of defined markers, T memory (T_M) cell subsets have been broadly divided into T central memory (T_{CM}: CD45RA − CD62L +), T effector/memory (T_{EM}; CD45RA − CD62L −), and RA + T_{EM} (T_{EMRA}; CD45RA + 62L −).[36,37] Of note, T_{EMRA}, considered to be "terminally differentiated T_{EM} cells,"[36,37] were found to expresses high levels of perforin and granzyme and have been implicated in protection against viral (e.g., HIV, Cytomegalovirus, and Epstein–Barr virus (EBV)) and bacterial (Mycobacterium tuberculosis) infections.[38–42] Regarding immunity to S. Typhi, we have shown that oral immunization with attenuated typhoid vaccines elicits S. Typhi-specific CD8 + T-cell responses, mostly involving the T_{EM} and T_{EMRA} subsets, although lower magnitude responses were also observed in the CD8 + T_{CM} subset.[22,25,26,30] Of interest, we showed that a significant portion of S. Typhi-specific T cells coexpressed the gut-homing molecule integrin $\alpha_4\beta_7$, suggesting their potential to migrate to the primary site of infection.[25,30,43] Taken together, these observations strongly suggest that live oral typhoid vaccines elicit CD8 + CTLs and other CMI responses likely to be the primary mediator(s) of protective immunity, both in clearing acute infection and providing long-term protection against S. Typhi.[32,44] Recent studies further showed that antigen-specific multifunctional (MF) T cells (cells producing two or more cytokines and/or expressing CD107a, a marker of cytotoxic activity),[45] induced in response to various vaccines,[46] including Ty21a,[22,26] might play a key role in long-term protective immunity.

However, in spite of the considerable progress in uncovering S. Typhi-specific responses, very limited information is available on the crossreactive responses induced against S. Paratyphi A or S. Paratyphi B by live oral typhoid vaccines. Recently, we and others have described crossreactive humoral responses induced by Ty21a against S. Typhi and S. Paratyphi A and B.[47–49] Humoral responses induced following immunization with Ty21a were directed predominantly against S. Typhi; however, crossreactive responses were also recorded against S. Paratyphi A and B. We further observed the induction of crossreactive functional vaccine-induced antibodies that were, nevertheless, not sufficient to clear Salmonella infections once they become intracellular.[21,48] Taken together, these observations support the notion that in addition to humoral immunity, CMI responses might be critical for the efficient control of S. Typhi,[22–27,29,30,32,44] as well as for S. Paratyphi A or S. Paratyphi B infections.[47,48]

To address the gaps in knowledge regarding the mechanisms of cross-protective immunity among enteric fevers, we compared the ability of Ty21a to induce crossreactive CMI responses among S. Typhi and S. Paratyphi A and B. We observed, for the first time, that the predominant crossreactive Salmonella-specific responses were observed in the CD8 + T_{EM} subset, whereas lower magnitude responses were also observed in CD8 + T_{EMRA} cells. Moreover, we identified the dominant subsets of MF cells that mediate crossreactive Salmonella-specific responses and show that Salmonella-specific CD8 + T_M populations are composed of cells that express, or not, the gut-homing molecule integrin $\alpha_4\beta_7$. Finally, of importance, we observed that Ty21a-elicited CMI responses against S. Typhi were found to be similar to those observed against S. Paratyphi B-infected but not S. Paratyphi A-infected targets. These observations provide a plausible immunological explanation for the observations of cross-protection between typhoid and paratyphoid B fever in Ty21a-vaccinated subjects in field trials.

RESULTS
The peripheral blood mononuclear cell (PBMC) samples used in this study were collected from volunteers before (day 0) and after (days 42/84) immunization with Ty21a as described in Methods. Routine complete blood counts performed in these blood specimens were used to estimate the absolute numbers of lymphocytes and CD3 + CD8 + cells. We observed that the percentages and absolute lymphocyte counts were similar (i.e., not statistically different, $P > 0.3$) when before (day 0) and after (days 42/84) vaccination were compared (**Supplementary Figure S1** online). Furthermore, we measured the percentages of CD3 + CD8 + T cells in PBMCs by flow cytometry and converted these percentages into approximate absolute counts of CD8 + T cells using available absolute lymphocyte counts from complete blood count analyses. Again, no statistically significant differences ($P > 0.15$) were observed in the calculated absolute counts for CD8 + T cells among specimens collected at days 0, 42, or 84 (**Supplementary Figure S1**).

To measure Salmonella-specific responses, PBMCs were stimulated ex vivo with S. Typhi- and S. Paratyphi A- and

B-infected autologous EBV-B cells (**Supplementary Figure S2**) as described in Methods. Activated CD8+ T cells (i.e., CD8+CD69+ cells) produced IFN-γ (CD69+INF-γ+) and/or expressed CD107a (**Supplementary Figure S3**). Activated cells resided predominantly in the CD62L- T_M subpopulations, i.e., T_{EM} and T_{EMRA} (**Supplementary Figure S3**). A similar phenomenon was also observed in TNF-α-producing cells (data not shown). Based on these observations, subsequent analyses were focused in the CD8+ T_{EM} and T_{EMRA} T-cell subsets.

Evaluation of *Salmonella*-specific multifunctional CD8+ T cells

In response to *S*. Typhi-specific stimulation, activated effector CD8+ T cells from Ty21a vaccinees are capable of producing single cytokines or expressing CD107a only (single positives) or concommitantly producing two or more cytokines and/or expressing CD107a (MF)[22,26] (**Supplementary Figure S4**). We observed that Ty21a immunization elicited increases in CD8+ T cells that produce IFN-γ and/or express CD107a following stimulation with *S*. Typhi- as well as with *S*. Paratyphi A- or B-infected targets (**Figure 1**). A significantly higher proportion of these *Salmonella*-specific IFN-γ-producing cells were MF when compared with single-positive IFN-γ+ cells in both T_{EM} (**Figure 1a**) and T_{EMRA} (**Figure 1b**) subsets. Similarly, significantly higher percentages of *Salmonella*-specific MF cells expressing CD107a were also observed in CD8+ T_{EM} subsets (**Figure 1c**). However, in CD8+ T_{EMRA} subsets,

significant increases in *Salmonella*-specific MF CD107a responses were only observed after stimulation with *S*. Typhi-infected targets ($P<0.01$), whereas a trend was observed with *S*. Paratyphi B ($P=0.08$). No dominance of MF CD107a responses in the T_{EMRA} subset was observed for *S*. Paratyphi A ($P=0.23$; **Figure 1d**). The above described postvaccination increases in *Salmonella*-specific MF CD8+ T_{EM} and CD8+ T_{EMRA} T_M subsets for each individual volunteer are shown in **Supplementary Figure S5**.

Characterization of *Salmonella*-specific multifunctional CD8+ T_{EM} cells

As described above, results indicated that vaccination with Ty21a elicits crossreactive, predominantly MF CD8+ T_{EM} and T_{EMRA} CMI responses against *S*. Typhi-, *S*. Paratyphi A-, and *S*. Paratyphi B-infected targets. To further characterize these MF responses, we first categorized these MF cells into double- (2+), triple- (3+), or quadruple- (4+) positive subsets based on whether they produce IFN-γ, TNF-α, and/or interleukin 2 (IL-2), and/or express CD107a. Results showed that among CD8+ T_{EM} cells the percentages of *Salmonella*-specific MF cells followed the hierarchy 2+ = 4+ > 3+ (**Supplementary Figure S6 A,B,C**). In contrast, among CD8+ T_{EMRA} cells, the hierarchy of *Salmonella*-specific MF cells was 2+ > 3+ > 4+ (**Supplementary Figure S6 D,E,F**). We next evaluated whether unique MF profiles (e.g., production of particular cytokines and/or expression of CD107a combinations) were elicited by Ty21a against *S*. Typhi-, *S*. Paratyphi A-, and *S*. Paratyphi
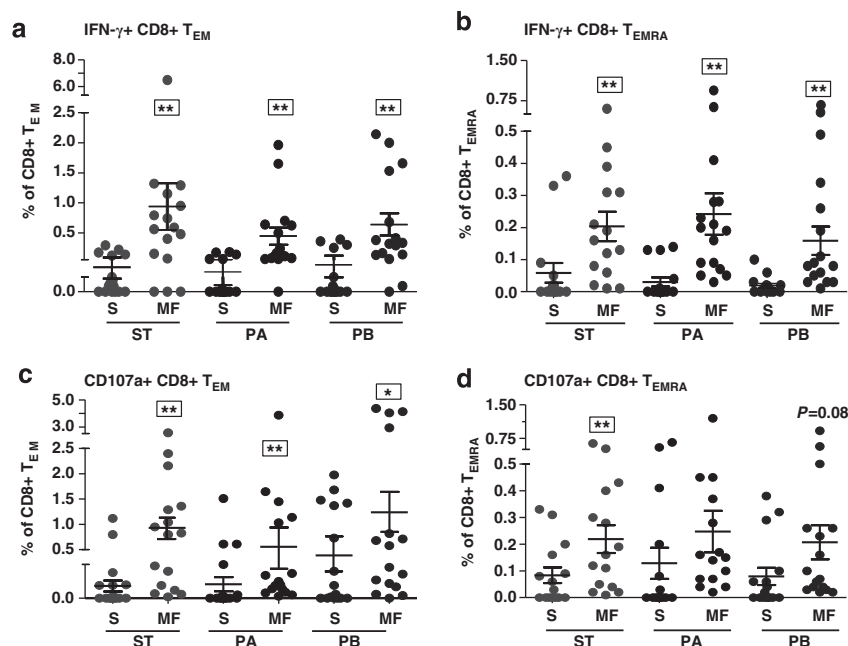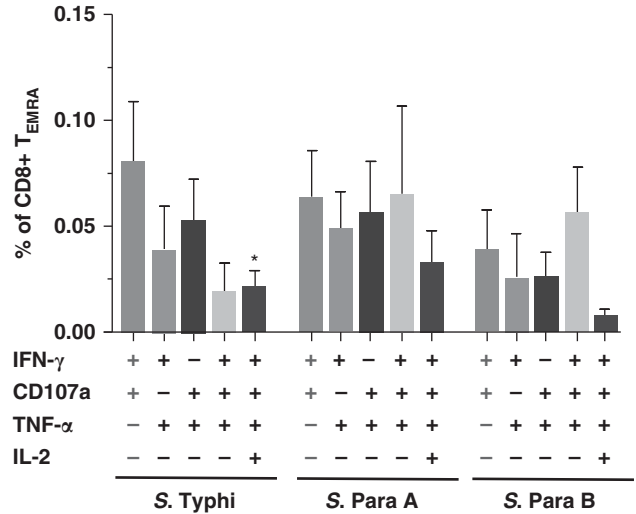

Figure 1 Induction of multifunctional cells in Ty21a vaccinees. Peripheral blood mononuclear cells (PBMCs) collected from Ty21a vaccinees ($n=16$) were stimulated with *S*. Typhi-infected targets and the data analyzed using FCOM (described in the text). Shown are the peak postvaccination increases in single-positive (S) and interferon-γ (IFN-γ)+ (**a,b**) and CD107+ (**c,d**) total multifunctional (MF, the sum of all multifunctional subsets) cells in CD8+ T_{EM} (**a,c**) and CD8+ T_{EMRA} (**b,d**) subpopulations specific for *S*. Typhi (ST)-, *S*. Paratyphi A (PA)-, or *S*. Paratyphi B (PB)-infected targets. Postvaccination peaks: peak of the responses at days 42 or 84 minus prevaccination (day 0) levels. Horizontal bars represent mean ± s.e.m. T_{EM}, T effector/memory; T_{EMRA}, T effector/memory CD45RA+. **$P<0.01$. *$P<0.05$ compared with corresponding single-positive cells by Wilcoxon signed-rank test, two tailed.

B-infected targets. Of all possible combinations (16 for the 4 parameters evaluated), we focused our studies on the 5 dominant MF subsets in CD8 + T_{EM} and T_{EMRA}, all showing net increases of > 0.05% positive cells (**Figures 2 and 3**). Of note, when combined, these 5 selected "high-frequency" MF subsets typically represented > 80% of all MF cells within both the T_{EM} and T_{EMRA} T_M subsets. In CD8 + T_{EM} subsets, postvaccination increases showed a dominance of S. Typhi-specific IFN-γ + CD107a + TNF-α − IL-2 − cells over the next four most frequent S. Typhi-specific MF subsets, i.e., IFN-γ + CD107a − TNF-α + IL-2 − ($P < 0.01$), IFN-γ − CD107a + TNF-α + IL-2 − ($P = 0.07$), IFN-γ + CD107a + TNF-α + IL-2 − ($P = 0.09$), and IFN-γ + CD107a + TNF-α + IL-2 + ($P = 0.02$; **Figure 2a**). Moreover, following Ty21a immunization, the induction of S. Typhi-specific CD8 + T_{EM} IFN-γ + CD107a + TNF-α − IL-2 − cells (0.46 ± 0.18), was significantly higher than those specific to S. Paratyphi A (0.06 ± 0.03, $P = 0.01$) or S. Paratyphi B (0.13 ± 0.06, $P = 0.04$; **Figure 2a**). Of importance, the percentages of subjects ($n = 16$) who were considered responders for IFN-γ + CD107a + TNF-α − IL-2 − CD8$^+$ T_{EM} specific to S. Typhi (56.3%) were similar to those responding to S. Paratyphi B (43.8%, $P = 0.5$) and both were significantly higher than the 12.5% of volunteers responding to S. Paratyphi A ($P < 0.01$ and $P < 0.05$ as compared with S. Typhi and S. Paratyphi B, respectively; **Figure 2b**).

Characterization of *Salmonella*-specific multifunctional CD8 + T_{EMRA} cells

A similar analysis to the one described above for T_{EM} cells was used to characterize the Ty21a-induced crossreactive MF responses in CD8 + T_{EMRA} subsets (**Figure 3**). The specific responses observed in CD8 + T_{EMRA} subsets were generally of lower magnitude; however, their MF profiles were similar to

those observed in T_{EM} subsets. The postvaccination increase of S. Typhi-specific CD107a + IFN-γ + TNF-α − IL-2 − cells were moderately higher than the other subsets among CD8 + T_{EMRA} MF cells, although unlike CD8 + T_{EM} cells, these differences did not reach statistical significance (**Figure 3**).

Figure 3 Postvaccination increases in multifunctional (MF) CD8 + T_{EMRA} subsets in peripheral blood mononuclear cells (PBMCs) from Ty21a vaccinees ($n = 15$). Shown are postvaccination peak increases (peak at days 42 or 84 after vaccination minus the corresponding prevaccination levels) in the five dominant MF subpopulations following stimulation with targets infected with S. Typhi, S. Paratyphi A (S. Para A), or S. Paratyphi B (S. Para B). Bars indicate mean + s.e.m. *$P < 0.05$, compared with IFN-γ + CD107a + TNF-α − IL-2 − MF cells for S. Typhi-infected targets. Wilcoxon signed-rank test, two tailed. IFN-γ, interferon-γ; IL-2, interleukin 2; TNF-α, tumor necrosis factor-α.
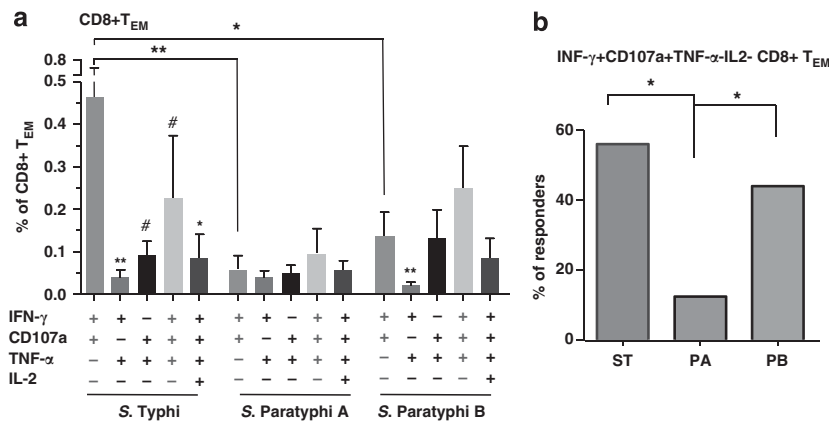
Figure 2 Postvaccination increases in multifunctional (MF) CD8 + T_{EM} cells in peripheral blood mononuclear cells (PBMCs) from Ty21a vaccinees ($n = 16$). Induction of multiple cytokine (interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and/or interleukin 2 (IL-2))-producing and/or expressing CD107a CD8 + $_{TEM}$ cells following stimulation with targets infected with S. Typhi (ST), S. Paratyphi A (PA), or S. Paratyphi B (PB). Data were analyzed using FCOM as described in the text. The postvaccination peak increases (peak level at days 42 or 84 after vaccination minus the corresponding prevaccination levels) in dominant subpopulations are shown as mean ± s.e.m. (**a**). The percentage of responders was calculated as: (number of volunteers with peak postvaccination increases ≥ 0.1% in IFN-γ + CD107a + TNF-α − IL-2 − subsets)/(total volunteers ($n = 16$)) × 100 (**b**). T_{EM}, T effector/memory. Horizontal bars represent mean ± s.e.m. **$P < 0.01$; *$P < 0.05$; #$P < 0.1$ compared with IFN-γ + CD107a + TNF-α − IL-2 − MF cells for each corresponding *Salmonella*-infected target. Other significance values relate to the indicated data sets. Wilcoxon signed-rank test, two tailed (**a**). *$P < 0.05$, by χ^2 test two tailed (**b**).
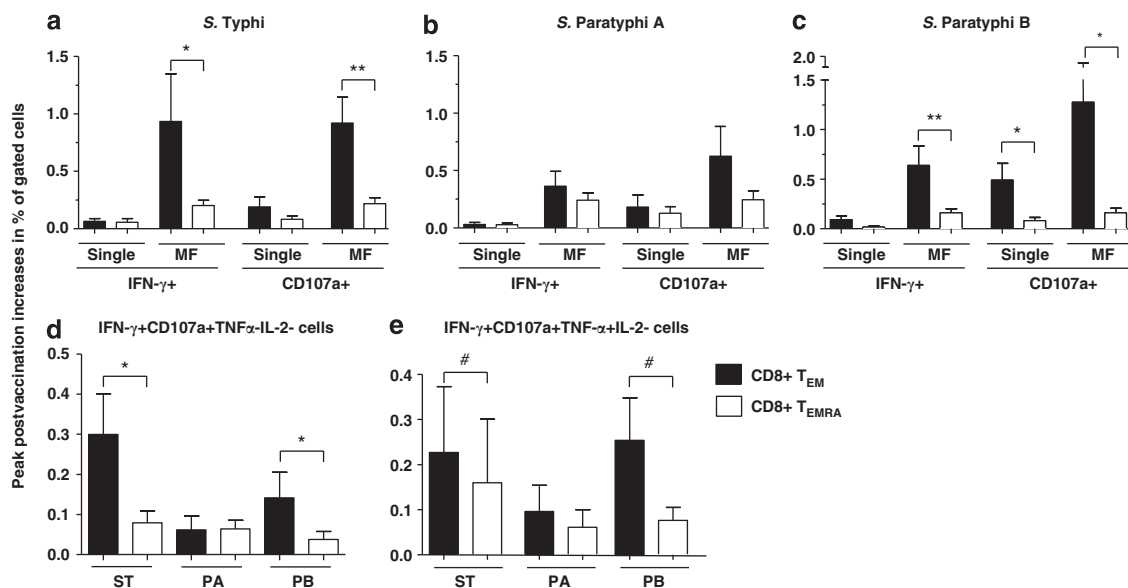
Comparison of MF cells between CD8 + T$_{EM}$ and CD8 + T$_{EMRA}$ subsets

We have described above that Ty21a immunization elicited increases in *Salmonella*-specific responses in CD8 + T cells in both T$_{EM}$ and T$_{EMRA}$ subsets (**Figures 1–3**). We next compared those responses induced in these two T$_M$ subsets of CD8 + T cells (**Figure 4** and **Supplementary Figure S5**). These comparative analyses showed that IFN-γ + and CD107a + MF responses specific to *S.* Typhi (**Figure 4a**) and *S.* Paratyphi B (**Figure 4c**), but not those to *S.* Paratyphi A (**Figure 4b**), were significantly higher in T$_{EM}$ than the corresponding increases in CD8 + T$_{EMRA}$ subsets. Interestingly, increased percentages of single CD107a-expressing cells in T$_{EM}$ over T$_{EMRA}$ were observed in *S.* Paratyphi B- but not *S.* Typhi- and *S.* Paratyphi A-infected targets (**Figure 4a–c**). As described above, *Salmonella*-specific MF cells can be categorized according to their "functional" characteristics into 2 +, 3 +, and 4 + subsets. A comparative analysis showed that the 4 + MF cells specific to all three *Salmonella* strains were elicited at a significantly ($P < 0.01$) higher percentage in CD8 + T$_{EM}$ as compared with T$_{EMRA}$ subsets (**Supplementary Figure S6** online). In contrast, 2 + MF CD8 + T$_{EM}$ cells specific to *S.* Typhi ($P = 0.04$) and *S.* Paratyphi B ($P = 0.08$), but not *S.* Paratyphi A ($P = 0.2$), were induced in lower percentages than the corresponding 2 + MF CD8 + T$_{EMRA}$ cells (**Supplementary Figure S6**). Moreover, postvaccination increases observed in *S.* Typhi- ($P = 0.04$) and *S.* Paratyphi B- ($P = 0.02$)-specific IFN-γ + CD107a + TNF-α – IL-2 – MF cells were higher in T$_{EM}$ compared with T$_{EMRA}$ CD8 + subsets (**Figure 4d**). Although similar effects were also observed with *S.* Typhi- and

S. Paratyphi B-specific IFN-γ + CD107a + TNF-α + IL-2 – CD8 + T$_{EM}$ MF cells, these increases did not reach statistical significance ($P < 0.1$; **Figure 4e**). Of importance, although induction of *S.* Typhi- or *S.* Paratyphi B-specific MF cells were higher in T$_{EM}$ compared with the corresponding responses in CD8 + T$_{EMRA}$ subsets, no such differences were observed with *S.* Paratyphi A (**Figure 4b,d,e** and **Supplementary Figure S6**). Taken together, these comparative analyses between CD8 + T$_{EM}$ and T$_{EMRA}$ subsets revealed that the response patterns elicited to *S.* Typhi were remarkably similar to those of *S.* Paratyphi B, but different than those of *S.* Paratyphi A.

Crossreactive *Salmonella*-specific MIP-1β and IL-17 responses

Macrophage inflammatory protein-1β (MIP-1β) and IL-17 are two critical chemokines/cytokines that have been recently implicated in protection against infections.[50,51] Therefore, we evaluated the induction of MIP-1β and IL-17 production in response to *Salmonella*-infected targets by PBMCs obtained from Ty21a vaccinees ($n = 8$). To this end, we used an optimized 14-color flow cytometry panel (described in Methods) that included additional monoclonal antibodies (mAbs) against MIP-1β and IL-17. Similar to the results described above regarding induction of IFN-γ + or CD107a + MF (**Figure 1**), Ty21a immunization elicited *Salmonella*-specific, predominantly MF, MIP-1β + cells in CD8 + T$_{EM}$ (**Figure 5a**) and T$_{EMRA}$ (**Figure 5b**) subsets. Results from individual volunteers are shown in **Supplementary Figure S7**.

Figure 4 Comparison of the induction of crossreactive multifunctional (MF) cells between CD8T$_{EM}$ and CD8T$_{EMRA}$ subpopulations. Postvaccination peak increases (peak level at days 42 or 84 after vaccination minus the corresponding prevaccination levels) in interferon-γ (IFN-γ) + and CD107a + single and MF subsets in CD8$_{TEM}$ and CD8T$_{EMRA}$ subsets in response to *S.* Typhi (ST), *S.* Paratyphi A (PA), and *S.* Paratyphi B (PB) were compared (**a,b,c**). Similar comparisons with IFN-γ + CD107a + TNF-α – IL-2 – and IFN-γ + CD107a + TNF-α + IL-2 – MF subsets are shown in (**d**) and (**e**), respectively. Bars indicate mean + s.e.m. **P < 0.01, *P < 0.05, #*P* < 0.10 compared with corresponding CD8 T$_{EM}$ subsets. Wilcoxon signed-rank test, two tailed. IL-2, interleukin 2; T$_{EM}$, T effector/memory; T$_{EMRA}$, T effector/memory CD45RA +; TNF-α, tumor necrosis factor-α.

To further our understanding of the MF capabilities of MIP-1β + cells, we evaluated their ability to concomitantly produce other cytokines/chemokines and/or express CD107a. Ty21a elicited MIP-1β + specific MF cells consisting of six dominant MF subsets that were identified as those exhibiting 2 + (MIP-1β + IFN-γ + CD107a − TNF-α − IL-2 − IL-17 − and MIP-1β + IFN-γ − CD107a + TNF-α − IL-2 − IL-17 −), 3 + (MIP-1β + IFN-γ + CD107a + TNF-α − IL-2 − -IL-17 −), 4 + (MIP-1β + IFN-γ + CD107a + TNF-α + IL-2 − IL-17 − and MIP-1β + IFN-γ + CD107a + TNF-α − IL-2 + IL-17 −), or 5 + (MIP-1β + IFN + γ + CD107a + TNF-α + IL-2 + -IL-17 −) functional responses (data not shown). Interestingly, the common characteristics among these different CD8 + T$_{EM}$ MIP-1β + MF subsets was that, besides MIP-1β, all of them

concomitantly produced/expressed one or both of the two key markers of CTLs, i.e., IFN-γ and/or CD107a. Virtually identical profiles of MIP-1β + MF cells were induced following stimulation with *S.* Typhi- or *S.* Paratyphi B-infected targets in both CD8 + T$_{EM}$ and CD8 + T$_{EMRA}$ subsets. However, as described above (**Figure 2**), there was a trend (not statistically significant) toward lower magnitude responses to *S.* Paratyphi A-infected targets (data not shown). Of note, overall, the magnitude of all *Salmonella*-specific MF subsets in CD8 + T$_{EM}$ cells responses were higher than those observed in T$_{EMRA}$ cells (data not shown).

We also included a mAb against IL-17 in our flow cytometry panel to evaluate its role in Ty21a-elicited crossreactive immunity. Postvaccination increases in total IL-17-producing cells above 0.1% in CD8 + T$_{EM}$ or T$_{EMRA}$ subsets following stimulation with *Salmonella*-infected targets were observed in 3 out of 8 volunteers (37.5%) (data not shown). However, because of the low magnitude of IL-17 responses, these data were not deemed adequate for detailed analyses for MF properties of IL-17-producing cells.

Characterization of the gut-homing potential of *Salmonella*-specific MF CD8 + T$_{EM}$ cells

Mucosal immunity elicited in the gut microenvironment following immunization with Ty21a is thought to be an important component of the protective immune response against enteric fevers.[30,43,44,52] Specific effector T cells with the potential to migrate to the gut mucosa can be measured by evaluating the expression of integrin α$_4$β$_7$. Thus, we examined integrin α$_4$β$_7$ expression by *Salmonella*-specific IFN-γ +, CD107a +, and MIP-1β + single and MF cells in PBMCs from 12 volunteers immunized with Ty21a. We focused our studies on the gut-homing patterns of cells in the CD8$^+$ T$_{EM}$ subset as this was found to be the dominant subset of *Salmonella*-specific CD8 + T cells responding to Ty21a immunization. We observed that Ty21a immunization elicited increases in both single and MF *Salmonella*-specific CD8 +
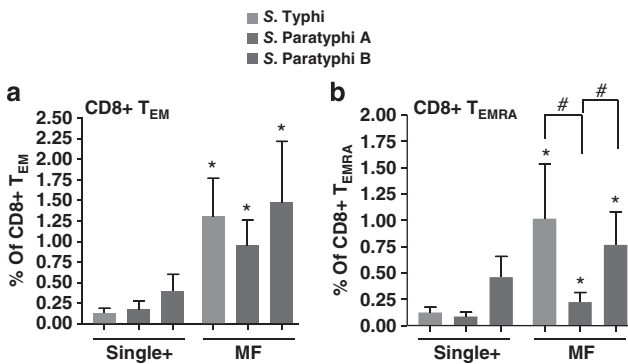
Figure 5 Postvaccination increases in macrophage inflammatory protein-1β (MIP-1β)-producing CD8 + cells in response to *S.* Typhi-, *S.* Paratyphi A-, and *S.* Paratyphi B-infected targets. Postvaccination peak increases in MIP-1β production by CD8 + T$_{EM}$ (a) and CD8 + T$_{EMRA}$ (b) in single positive (single +) for MIP-1β (MIP-1β + IFN-γ − TNF-α − IL-2 − IL-17 −) and all other MIP-1β + that are multifunctional (MF) were analyzed by FCOM in peripheral blood mononuclear cells (PBMCs) obtained from Ty21a vaccinees (*n* = 8). Bars indicate mean + s.e.m. *P < 0.05, MF compared with the corresponding single-positive cells. #P < 0.12. Wilcoxon signed-rank test, two tailed. IFN-γ, interferon-γ; IL, interleukin; T$_{EM}$, T effector/memory; T$_{EMRA}$, T effector/memory CD45RA + ; TNF-α, tumor necrosis factor-α.
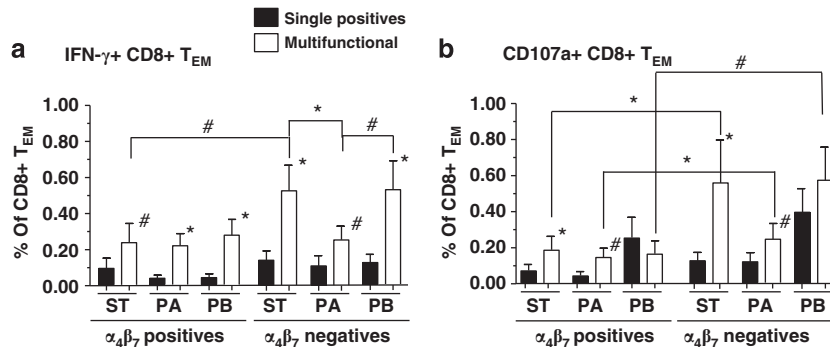
Figure 6 Concomitant expression of the gut-homing molecule integrin α$_4$β$_7$ by *Salmonella*-specific single and multifunctional (MF) CD8 + T effector/memory (T$_{EM}$) cells in Ty21a vaccinees. Peripheral blood mononuclear cells (PBMCs) collected from Ty21a vaccinees were stimulated with *S.* Typhi- (ST), *S.* Paratyphi A- (PA), and *S.* Paratyphi B (PB)-infected targets and the data were analyzed using FCOM (described in the text). Shown are the peak postvaccination increases in (**a**) antigen-specific interferon-γ (IFN-γ) + (*n* = 12), (**b**) CD107a + (*n* = 12), single-positive (closed bars), and the sum of all multifunctional (open bars) cells in CD8 + T$_{EM}$ subpopulations expressing integrin α$_4$β$_7$ (α$_4$β$_7$ positives) or not (α$_4$β$_7$ negatives). Postvaccination peaks: peak responses at days 42 or 84 minus prevaccination (day 0) levels. Bars indicate mean + s.e.m. *P < 0.05, #P ≤ 0.1 compared with corresponding single-positive cells by Wilcoxon signed-rank test, two tailed. Other significance values relate to the indicated data sets.

T$_{EM}$ cells expressing, or not, integrin α$_4$β$_7$ (**Figure 6**). Integrin α$_4$β$_7$+ coexpressing IFN-γ+ (**Figure 6a**) and CD107a+ (**Figure 6b**) CD8+ T$_{EM}$ MF cells elicited by Ty21a immunization were equally responsive to *S.* Typhi-, *S.* Paratyphi A-, or B-infected targets. In contrast, integrin α$_4$β$_7$-negative IFN-γ+ MF cells specific to *S.* Typhi ($P < 0.05$) or *S.* Paratyphi B ($P = 0.12$) showed higher postvaccination increases compared with those specific to *S.* Paratyphi A (**Figure 6a**). Similarly, although the magnitude of integrin α$_4$β$_7$-negative CD107a MF cells specific to *S.* Typhi ($P = 0.26$) or *S.* Paratyphi B ($P = 0.14$) showed higher postvaccination increases than those specific to *S.* Paratyphi A, these differences did not reach statistical significance (**Figure 6b**). Of note, trends were also observed for integrin α$_4$β$_7$-negative IFN-γ+ MF cells specific to *S.* Typhi ($P = 0.12$) and *S.* Paratyphi B ($P = 0.2$), but not to *S.* Paratyphi A ($P = 0.8$), to exhibit higher postvaccination increases compared with the corresponding integrin α$_4$β$_7$+ subsets (**Figure 6a**). On the other hand, in response to all three *Salmonella*-infected targets, integrin α$_4$β$_7$-negative cells coexpressing CD107a, showed a higher postvaccination increase compared with corresponding cells expressing integrin α$_4$β$_7$ (**Figure 6b**). Specific MIP-1β responses were also observed in integrin α$_4$β$_7$-negative and -positive cells (data not shown).

We then investigated integrin α$_4$β$_7$ expression by the dominant *Salmonella*-specific CD8+ T$_{EM}$ MF subsets described in **Figure 2** (e.g., IFN-γ+CD107a+TNF-α−IL-2− and INF-γ+CD107a+TNF-α+IL-2−). We observed that although a significant proportion of these also expressed integrin α$_4$β$_7$, most cells were α$_4$β$_7$ negative (data not shown). This dominance of integrin α$_4$β$_7$-negative MF cells was not observed in MIP-1β+ subsets.

DISCUSSION

Ty21a and other attenuated *S.* Typhi oral vaccine strains elicit a wide array of CMI responses in immunized volunteers[23–26,32,33,44,53] including the induction of *S.* Typhi-specific multifunctional CD8+ T cells.[22,26] In this study we investigated whether Ty21a immunization elicits crossreactive CMI responses against two closely related *Salmonella enterica* serovars, i.e., *S.* Paratyphi A and *S.* Paratyphi B. In addition, by comparing the CD8+ T-cell responses to these three *Salmonella* serovars following Ty21a immunization, we explored whether defined effector CMI responses might help explain field observations showing that Ty21a provides significant cross-protection against *S.* Paratyphi B, but not against *S.* Paratyphi A.[13]

We used PBMCs samples collected from healthy volunteers before (day 0) and after (day 42 and/or day 84) immunization with the live oral typhoid vaccine Ty21a. Measurements of the absolute numbers of lymphocytes and CD3+CD8+ cells based on complete blood counts and the proportions of these cells obtained by flow cytometry revealed that immunization with Ty21a did not significantly affect the levels of these cells in circulation. Thus, it is unlikely that the observed postvaccination increases in the percentages of *Salmonella*-specific CD8+

T-cell subsets have been influenced by fluctuations in absolute cell counts following vaccination.

We have recently reported that healthy subjects who have neither a previous history of exposure to *S.* Typhi, including vaccination, nor have traveled to endemic areas, have variable background immune responses to this organism.[54] These background responses are thought to be the result of the presence of crossreactive T cells acquired during previous infections with other Gram-negative enteric pathogens or by natural exposure to other Gram-negative organisms that form part of the normal gut microbiota. Similar prevaccination responses were observed in the present studies. Thus, as in previous studies, we determined Ty21a-elicited specific "recall" responses by subtracting the background responses before immunization in individual subjects from each postvaccination time point (days 42/84).[24,27,30,53]

Postvaccination increases in specific CD8+ T-cell responses were observed against all three *Salmonella*-infected targets (i.e., *S.* Typhi, *S.* Paratyphi A, or *S.* Paratyphi B), predominantly in the T$_{EM}$ and T$_{EMRA}$ subsets. In contrast, very low *Salmonella*-specific responses were observed in T$_{CM}$ and, as expected, almost no responses in T naive cells. These and previous studies[22,25,26,30] provided the rationale for focusing most our current studies on multifunctional T$_{EM}$ and T$_{EMRA}$ CD8+ T subsets.

CD8+ T cells mediate effector functions by producing various cytokines (e.g., IFN-γ, TNF-α, IL-2, IL-17), chemokines (e.g., MIP-1β), or by releasing perforin and/or granzymes (indirectly measured by the expression of CD107a).[45,55] At the single-cell level, T cells are capable of producing single cytokines/chemokines or simultaneously producing two or more cytokines/chemokines and/or expressing CD107a. The latter have been termed MF cells. It has been shown that these MF T cells produce higher levels of individual cytokines, exhibit enhanced function, and are more likely to correlate with protection from disease when compared with single cytokine-producing cells.[56,57] In fact, induction of MF T cells at a higher magnitudes than single cytokine-secreting cells have also been shown in other disease models, i.e., HIV, *Cytomegalovirus*, vaccinia, and EBV infections,[58] including the evaluation of candidate vaccines against *M. tuberculosis*[46,59] and Ebola virus in humans.[60,61]

MF cells that produce IFN-γ together with other critical cytokines (e.g., TNF-α, IL-2) and/or express CD107a can enhance the killing of intracellular bacteria more efficiently than single cytokine-producing T cells.[58,62] Moreover, specific MF responses by T$_{EM}$, as well as by T$_{EMRA}$ CD8+ T cells, are thought to be associated with protection against various viral and bacterial infections.[63,64] Therefore, the quality of T-cell responses, as measured by their MF capabilities, have the potential to provide a more revealing assessment of vaccine-induced immune responses than single-parameter functional measurements (e.g., only IFN-γ production).[58]

In this study we found that the dominant subsets of specific MF cells were 2+ *S.* Typhi-specific cells that largely comprised IFN-γ+CD107a+TNF-α−IL-2−;

IFN-γ + CD107a − TNF-α + IL-2 − ; and IFN-γ-CD107a + TNF-α + IL-2 − subsets. However, a significant proportion of 3 + MF cells were also induced. Of note, most of the CD8 + MF cells produced IFN-γ, coproducing/expressing CD107a + or TNF-α, whereas a smaller subset also coproduced IL-2 (4 +). These results markedly extend those reported in other infectious diseases showing that antigen-specific MF T_{EM} or T_{EMRA} CD8 + T cells that produce IFN-γ also contained subsets coproducing TNF-α, but very few coproducing IL-2.[58,65] Recently, it has been proposed that during antigen-specific memory cell proliferation and differentiation, TNF-α- and IL-2-producing clones may fade earlier than those secreting IFN-γ. Thus, terminal effector CD8 + memory subsets comprise mostly IFN-γ-secreting cells with less functional heterogeneity.[58] In this context, our observations of a dominance of S. Typhi-specific 2 + (IFN-γ + CD107a + TNF-α-IL-2 −) and 3 + (IFN-γ + CD107a + TNF-α + IL-2 −) CD8 + T_{EM} cells may indicate that Ty21a immunization elicits a heterogeneous population of activated $CD8^+$ T_{EM} that secrete IFN-γ and TNF-α with cytolytic activity (CD107a +), which subsequently become terminal effector cells, maintaining their ability to produce IFN-γ and express CD107a in the absence of TNF-α production.[58] Interestingly, we observed that postvaccination increases in S. Paratyphi A-specific IFN-γ + CD107a + TNF-α − IL-2 − MF cells were less pronounced than those observed to S. Typhi or S. Paratyphi B. However, the significance of this observation is unclear as the exact role of the IFN-γ + CD107a + TNF-α − IL-2 − CD8 + T cells in protection remains undefined.

The observations that S. Typhi- and S. Paratyphi B-specific IFN-γ + and CD107a + MF cells, as well as double- and triple-positive MF subsets, were induced at a higher magnitude in T_{EM} subsets than in T_{EMRA} cells are similar to our previous observations in Ty21a vaccinees.[22,25,26,30,32,44] In contrast, MF cells specific to S. Paratyphi A were induced at lower magnitudes in most subjects and without such predominance of responses in T_{EM} subsets. In the absence of known correlates of protection or knowledge on the functional role of MF cells in protection from S. Typhi infection, the significance of these differences observed between S. Paratyphi A and S. Paratyphi B at present is unclear. However, it is reasonable to speculate that the similarities between S. Typhi- and S. Paratyphi B-specific CMI responses, as well as the differences with S. Paratyphi A, may help explain field trials with Ty21a reporting cross-protection against S. Paratyphi B but not from S. Paratyphi A.[13] Of note, although similar immune responses were observed in the majority of participants, these responses were, to a certain extent, heterogeneous, with a few volunteers exhibiting different dominant patterns. These results highlight the importance of considering cumulative responses, as well as those from individual volunteers, when interpreting data derived from human studies. Further studies are needed to fully understand the role of these T_M subsets in protection from enteric fevers.

Production of β-chemokines (i.e., RANTES, MIP-1α, MIP-1β) by CD8 + T cells has been shown, among others, to play an important role in CTL activity.[66] For example, HIV-antigen specific CD8 + MIP-1β + cells coproducing IFN-γ were related with nonprogressors, suggesting that they might play a role against the infection.[50,67] A recent report also showed that in response to S. Typhi antigens, PBMCs obtained from S. Typhi-infected convalescent patients produced MIP-1β.[68] We have previously shown that MIP-1β is coproduced with other cytokines, i.e., IFN-γ, TNF-α, and IL-2, following vaccination with Ty21a.[22] In this study, we further characterized Ty21a-elicited CD8 + MF MIP-1β T cells specific to S. Typhi, and provide the first evidence that these cells are crossreactive to S. Paratyphi A and S. Paratyphi B. We observed that the majority of these Salmonella-specific MIP-1β + MF cells coproduced/expressed IFN-γ and CD107a, suggesting that Ty21a-elicited MF cells coproducing MIP-1β are likely an important component of a protective CTL response against enteric fevers.

Effector immune responses in the gut microenvironment are expected to be important in protecting the host against S. Typhi and other enteric infections, including those caused by S. Paratyphi A and S. Paratyphi B. In previous studies we have demonstrated that, as expected, a substantial component of the S. Typhi-specific IFN-γ + CD4 + and CD8 + T cells elicited by Ty21a and CVD 909 had the potential to home to the gut as measured by expression of the integrin $\alpha_4\beta_7$ gut-homing molecule.[25,30] In this study we extended these observations by demonstrating that S. Typhi-specific CD8 + MF T cells producing/expressing IFN-γ + , CD107a + , and/or MIP-1β + elicited by Ty21a immunization consisted of cells expressing, or not, integrin $\alpha_4\beta_7$ and are crossreactive with S. Paratyphi A and S. Paratyphi B. Of note, Salmonella-specific integrin $\alpha_4\beta_7$ + CD8 + MF T_{EM} cells were present in circulation at a lower magnitude than integrin $\alpha_4\beta_7$-negative cells. This observation is likely the consequence of the migration of Salmonella-specific integrin α4β7 + cells to the gut mucosa, resulting in a decrease in circulation.

The present studies have a few limitations. These include the relatively limited number of volunteers studied and the availability of only two time points after vaccination (days 42 and 84). The latter might have limited our ability to detect postvaccination increases in Salmonella-specific IL17 + cells in the majority of individuals.

In summary, the present investigations provide insights into the immunological basis underlying the observed cross-protection against S. Paratyphi B, but not S. Paratyphi A, observed in Ty21a field studies.[13] Overall, these observations support the notion that a bivalent S. Typhi/S. Paratyphi A vaccine might be required to protect against enteric fevers.

METHODS

Subjects, immunizations, and isolation of PBMCs. Sixteen healthy adult volunteers (median age 42 years, range 23–52 years) from the Baltimore, MD/Washington, DC area and the University of Maryland Baltimore community who had no history of typhoid fever were recruited for the study with the approval of University of Maryland Baltimore institutional review board. They received four recommended spaced doses of Ty21a vaccine (Vivotif enteric-coated

capsules; Crucell, Bern, Switzerland).[47] Blood samples were drawn prevaccination (day 0) and 42 (day 42) or 84 (day 84) days after vaccination. PBMCs were isolated immediately after blood draws by density gradient centrifugation and were cryopreserved in liquid nitrogen.[33,53]

Target/stimulator cell preparation. EBV-transformed B-LCL (EBV-B cells) were generated from PBMCs obtained from Ty21a vaccinees as previously described.[27,53] Salmonella strains, i.e., wild-type S. Typhi strain (ISP-1820, Vi +, a clinical isolate from Chile), S. Paratyphi A (CV 223, ATCC 9150), and S. Paratyphi B (CV 23, a clinical isolate from Chile) were obtained from the Center for Vaccine Development (CVD), University of Maryland reference stocks. EBV-B cells were incubated with Salmonella strains at the multiplicity of infection of 10:1 (bacteria/cell) as previously described and rested overnight.[27,53] Infected cells were gamma-irradiated (6,000 rad) before being used as "targets" for ex vivo PBMCs stimulation. To confirm the adequacy of the infection with S. Typhi, S. Paratyphi A, or S. Paratyphi B, infected EBV-B cells were stained with anti-Salmonella common structural Ag (CSA-1)-FITC (Kierkegaard & Perry, Gaithersburg, MD) and analyzed by flow cytometry using a customized LSR-II instrument (BD, Franklin Lakes, NJ). The percentage of cells infected with S. Typhi was recorded for each experiment. Infected targets were only used if the infection was detected (CSA-1 positive) in > 40% of viable cells (**Supplementary Figure S2**).

Ex vivo PBMCs stimulation. Frozen PBMCs were thawed, rested overnight, and stimulated with autologous S. Typhi-, S. Paratyphi A-, or B- infected targets at a ratio of 10:1 (PBMCs/target). After 2 h, the protein transport blockers Monensin (1 µg ml^{-1}, Sigma, St Louis, MO) and Brefeldin A (2 µg ml^{-1}; Sigma) were added to the PBMCs and cultures were continued overnight at 37 °C in 5% CO_2. Media alone and uninfected autologous EBV-B cells were used as negative controls. Staphylococcal enterotoxin B (10 µg ml^{-1}; Sigma) was used as a positive control.

Surface and intracellular staining. Surface and intracellular staining was performed as described previously.[22] Briefly, following ex vivo stimulation, PBMCs were first stained for live/dead discrimination using LIVE/DEAD fixable violet dead cell stain kit (Invitrogen, Carlsbad, CA) and then surface stained with a panel of fluorochrome-conjugated mAbs that included CD14-Pacific Blue (TuK4, Invitrogen), CD19-Pacific Blue (SJ25-C1, Invitrogen), CD3-Qdot 655 (UCHT1, BD), CD4- PerCP-Cy5.5 (SK3, BD), CD8-Qdot 705 (HIT8A, Invitrogen), CD45RA-biotin (HI100, BD), CD62L-APC-EF780 (Dreg 56, Invitrogen), integrin $\alpha_4\beta_7$-Alexa 488 (clone ACT-1; conjugated in-house), and CD107a-A647 (eBioH4A3, eBiosciences, San Diego, CA). Of note, to maximize the detection of anti-CD107a, this mAb was added during the overnight ex vivo stimulation. The cells were then fixed and permeabilized with Fix & Perm cell buffers (Invitrogen) according to the manufacturer's recommendations. This procedure was followed by intracellular staining with mAbs against IFN-γ-PE-Cy7 (B27, BD), TNF-α-Alexa 700 (MAb11, BD), IL-2-PE (5344.111, BD), and CD69-ECD (TP1.55.3, Beckman Coulter, CA). For some experiments, a modified panel of mAbs (14 colors) was used to concomitantly detect two additional cytokines, i.e., MIP-1β and IL-17. This modified panel of mAbs included surface staining with Live/DEAD fixable yellow dead-cell staining kit (Invitrogen), CD14-Brilliant violet (BV) 570 (TuK4, Invitrogen), CD19- BV570 (HIB19, Biolegend, San Diego, CA), CD3-BV650 (OKT3, Biolegend), CD4-PE-Cy5 (RPA-T4, BD), CD8-PerCP-Cy5.5 (SK1, BD), CD45RA-biotin (HI100, BD), CD62L-APC-EF780 (Dreg 56, eBioscience), CD107a-FITC (H4A3, BD), and integrin $\alpha4\beta7$-A647 (ACT-1; conjugated in-house). Secondary staining was performed with strepta-vidin Qdot 800 (Invitrogen), followed by intracellular staining with IFN-γ-PE-Cy7 (B27, BD), TNF-α-Alexa 700 (MAb11, BD), IL-2-BV605 (MQ1-17H12, Biolegend), IL-17A-BV421 (BL168, Biolegend), MIP-1β-PE (24006, R&D, Minneapolis, MN), and CD69-ECD or -PE

(TP1.55.3, eBioscience). After staining, cells were fixed in 1% par-aformaldehyde and stored at 4 °C until analyzed. Flow cytometry was performed using a customized LSRII flow cytometer (BD) and data were analyzed using WinList version 7 (Verity Software House, Topsham, ME). Of note, in preliminary experiments we optimized the multichromatic panels used in these studies by performing titration of mAbs alone or in combination, as well as fluorescence minus one staining, to minimize spectral overlap and compensation (data not shown).

Gating protocol. T-cell responses in different live CD8 + (CD3 +, CD8 + CD4 −) T_M subsets were evaluated by their expression of CD45RA and CD62L into T_{CM} (CD62L + CD45RA −), T_{EM} (CD62L − CD45RA −), and T_{EMRA} (CD62L − CD45RA +). Naive T cells were defined as CD62L + CD45RA + (**Supplementary Figure S2**). The FCOM analysis tool (WinList version 7) was used to classify events based on combinations of selected gates in multidimensional space (i.e., whether cells express single or multiple intracellular cytokines and/or CD107a alone or in all possible combinations) for the detection of single or MF cells. Flow cytometric analyses were performed in 300,000–500,000 events collected for each sample, of which 161,700 (128,023–208,752) (median and interquartile range in parenthesis) were within the live lymphocyte gate (**Supplementary Figure S3A1**).

Statistical analyses. The statistical tests used to analyze each set of experiments are indicated in the figure legends. P-values of < 0.05 were considered significant.

DISCLOSURE
The authors declared no conflict of interest.

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