



Deposited via The University of Sheffield.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/id/eprint/166461/>

Version: Published Version

---

**Article:**

Rapaka, R.R, Wahid, R., Fresnay, S. et al. (2020) Human Salmonella Typhi exposure generates differential multifunctional cross-reactive T-cell memory responses against Salmonella Paratyphi and invasive nontyphoidal Salmonella. *Clinical & Translational Immunology*, 9 (9). e1178. ISSN: 2050-0068

<https://doi.org/10.1002/cti2.1178>

---

**Reuse**

This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here:

<https://creativecommons.org/licenses/>

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.

## ORIGINAL ARTICLE

# Human *Salmonella* Typhi exposure generates differential multifunctional cross-reactive T-cell memory responses against *Salmonella* Paratyphi and invasive nontyphoidal *Salmonella*

Rekha R Rapaka<sup>1,2</sup>, Rezwanul Wahid<sup>1,3</sup>, Stephanie Fresnay<sup>1,3</sup>, Jayaum S Booth<sup>1,3</sup>, Thomas C Darton<sup>4</sup>, Claire Jones<sup>4</sup>, Claire S Waddington<sup>4</sup>, Myron M Levine<sup>1,2,3</sup>, Andrew J Pollard<sup>4</sup> & Marcelo B Szein<sup>1,2,3</sup>

<sup>1</sup>Center for Vaccine Development and Global Health, University of Maryland School of Medicine, Baltimore, MD, USA

<sup>2</sup>Department of Medicine, University of Maryland School of Medicine, Baltimore, MD, USA

<sup>3</sup>Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

<sup>4</sup>Oxford Vaccine Group, Department of Paediatrics, University of Oxford and the NIHR Oxford Biomedical Research Centre, Oxford, UK

## Correspondence

MB Szein, Health Sciences Facility I, Room 480, Center for Vaccine Development and Global Health, University of Maryland School of Medicine, 685 West Baltimore Street, Baltimore, MD 21201, USA.  
E-mail: mszein@som.umaryland.edu

## Present address

Stephanie Fresnay,  
GlaxoSmithKline, Rockville, MD, USA  
Thomas C Darton,  
University of Sheffield Medical School,  
Sheffield, UK  
Claire S Waddington,  
University of Cambridge, Cambridge, UK

Received 18 February 2020;

Revised 16 August 2020;

Accepted 17 August 2020

doi: 10.1002/cti.1178

*Clinical & Translational Immunology*  
2020; 9: e1178

## Abstract

**Objective.** There are no vaccines for most of the major invasive *Salmonella* strains causing severe infection in humans. We evaluated the specificity of adaptive T memory cell responses generated after *Salmonella* Typhi exposure in humans against other major invasive *Salmonella* strains sharing capacity for dissemination. **Methods.** T memory cells from eleven volunteers who underwent controlled oral challenge with *wt S. Typhi* were characterised by flow cytometry for cross-reactive cellular cytokine/chemokine effector responses or evidence of degranulation upon stimulation with autologous B-lymphoblastoid cells infected with either *S. Typhi*, *Salmonella* Paratyphi A (PA), *S. Paratyphi* B (PB) or an invasive nontyphoidal *Salmonella* strain of the *S. Typhimurium* serovar (iNTSTy). **Results.** Blood T-cell effector memory (T<sub>EM</sub>) responses after exposure to *S. Typhi* in humans evolve late, peaking weeks after infection in most volunteers. Induced multifunctional CD4<sup>+</sup> Th1 and CD8<sup>+</sup> T<sub>EM</sub> cells elicited after *S. Typhi* challenge were cross-reactive with PA, PB and iNTSTy. The magnitude of multifunctional CD4<sup>+</sup> T<sub>EM</sub> cell responses to *S. Typhi* correlated with induction of cross-reactive multifunctional CD8<sup>+</sup> T<sub>EM</sub> cells against PA, PB and iNTSTy. Highly multifunctional subsets and T central memory and T effector memory cells that re-express CD45 (T<sub>EMRA</sub>) demonstrated less heterologous T-cell cross-reactivity, and multifunctional Th17 elicited after *S. Typhi* challenge was not cross-reactive against other invasive *Salmonella*. **Conclusion.** Gaps in cross-reactive immune effector functions in human T-cell memory compartments were highly dependent on invasive *Salmonella* strain, underscoring the importance of strain-dependent vaccination in the design of T-cell-based vaccines for invasive *Salmonella*.

**Keywords:** human challenge, iNTS, invasive *Salmonella*, Paratyphi, *S. Typhi*, T-cell memory

## INTRODUCTION

Invasive *Salmonella* infection, caused by typhoidal *Salmonella* (most commonly *S. Typhi* or *S. Paratyphi A*) or invasive nontyphoidal *Salmonella* (iNTS), is spread by faecal–oral route and is responsible for millions of infections yearly and over 600 000 deaths annually, with most mortality caused by iNTS.<sup>1–3</sup> There are no vaccines in clinical use known to prevent disease from *S. Paratyphi A* (PA) or iNTS. Dominant *Salmonella* Typhimurium and *S. Enteritidis* iNTS strains evolving within the last 60 years, identified in sub-Saharan Africa, have significant genomic changes associated with invasive disease absent from typical nontyphoidal *Salmonella* (NTS) strains producing localised gastroenteritis.<sup>4–6</sup> iNTS disease, often presenting with bacteraemia and focal infection such as meningitis or pneumonia, is associated with a case fatality rate of over 20% and is seen in young children with risk factors such as malnutrition and anaemia, or in the setting of co-infections with malaria or HIV.<sup>7,8</sup> Three serovars, *S. Typhi*, PA and *S. Paratyphi B* (PB), are the predominant causes of enteric fever (typhoid and paratyphoid disease) globally, and genomic differences between typhoidal serovars are underscored by studies demonstrating their convergent evolution.<sup>9–11</sup> PA incidence is rapidly increasing in South-East Asia, and an epidemic of extensively drug-resistant *S. Typhi* is ongoing in South Asia since 2016.<sup>12–14</sup> Given high incidence of invasive *Salmonella* infection, low-sensitivity diagnostics and high rates of antimicrobial use for suspected infection, antimicrobial resistance against invasive *Salmonella* is increasing.<sup>12,13,15–18</sup> Improving vaccination against all major invasive *Salmonella* by preventing severe and fatal infections would counteract the threat posed by emerging antibiotic-resistant strains of *Salmonella* and decrease mortality associated with these infections.

While there are data that invasive *Salmonella* host defence in humans involves antibody production,<sup>19</sup> the importance of T-cell-dependent host defence mechanisms against these intracellular bacteria is supported by studies demonstrating increased human susceptibility to

invasive *Salmonella* disease in the presence of specific STAT4 or HLA II polymorphisms or disruptions in IL-12 or IL-23 signalling pathways in humans.<sup>20–22</sup> Murine models of invasive *Salmonella* infection additionally demonstrate a critical role for T cells in host defence.<sup>23</sup> Studies in a human challenge model in which volunteers were orally challenged with wild-type *S. Typhi* demonstrate that high baseline frequencies of multifunctional CD8<sup>+</sup> T<sub>EM</sub> cell responses correlated with protection from the development of typhoid disease.<sup>24</sup> *Salmonella* Typhi vaccines administered in humans generate CD8<sup>+</sup> T cells with the capacity to kill *Salmonella*-infected cells *in vitro*, with killing function correlating with expression of CD8<sup>+</sup> T-cell IFN- $\gamma$  production.<sup>25–27</sup> HIV infection and decreased CD4<sup>+</sup> T-cell counts correlate with susceptibility to invasive nontyphoidal *Salmonella* infection rather than disease with typhoidal strains.<sup>28</sup> These observations demonstrate that the characteristics of human T-cell responses and the genetics of the *Salmonella* strain fundamentally impact risk of invasive *Salmonella* infection in humans.

There are three vaccines currently in clinical use against *S. Typhi*, but two of these are based on the Vi capsular polysaccharide found in *S. Typhi* and absent in other major prevalent invasive *Salmonella* strains. The live-attenuated Ty21a vaccine (generated from an *S. Typhi* strain by nondirected mutagenesis) does not confer cross-protection against PA disease,<sup>29</sup> but was moderately protective against PB.<sup>30</sup> After human Ty21a vaccination, low frequencies of highly multifunctional PA cross-reactive peripheral CD8<sup>+</sup> T cells are elicited compared to frequencies of reactive CD8<sup>+</sup> T cells against *S. Typhi*.<sup>31</sup> It is unknown whether wild-type *S. Typhi* contains antigens able to elicit both cross-reactive CD4<sup>+</sup> and CD8<sup>+</sup> T-cell effector functions against other major invasive *Salmonella* for which vaccines are lacking, such as iNTS strains and PA, or how biologic differences between different invasive *Salmonella* strains impact T-cell priming and the development of T-cell memory and effector functions in humans.

Mice inherently resist mucosal and systemic infection with *S. Typhi* and develop systemic

infection with *Salmonella* strains that typically cause only local gastroenteritis in humans. Given that host species and *Salmonella* strain are important in host susceptibility to disease and disease pathogenesis, we sought to evaluate elements of T-cell cross-reactivity in humans against invasive *Salmonella*. The human challenge model directs acquisition of infection through oral–mucosal exposure and characterisation of systemic memory immune responses in individuals longitudinally, without previous history of invasive *Salmonella* infection. Here, in a controlled human oral challenge model of *S. Typhi* infection, we dissect the cross-reactive immune effector functions of induced memory T cells within different human memory T-cell subsets, against PA, PB and an *S. Typhimurium* isolated in Mali of the ST313 multilocus sequence type (iNTSTy), as a representative iNTS of serovar *Typhimurium* circulating in sub-Saharan Africa.

## RESULTS

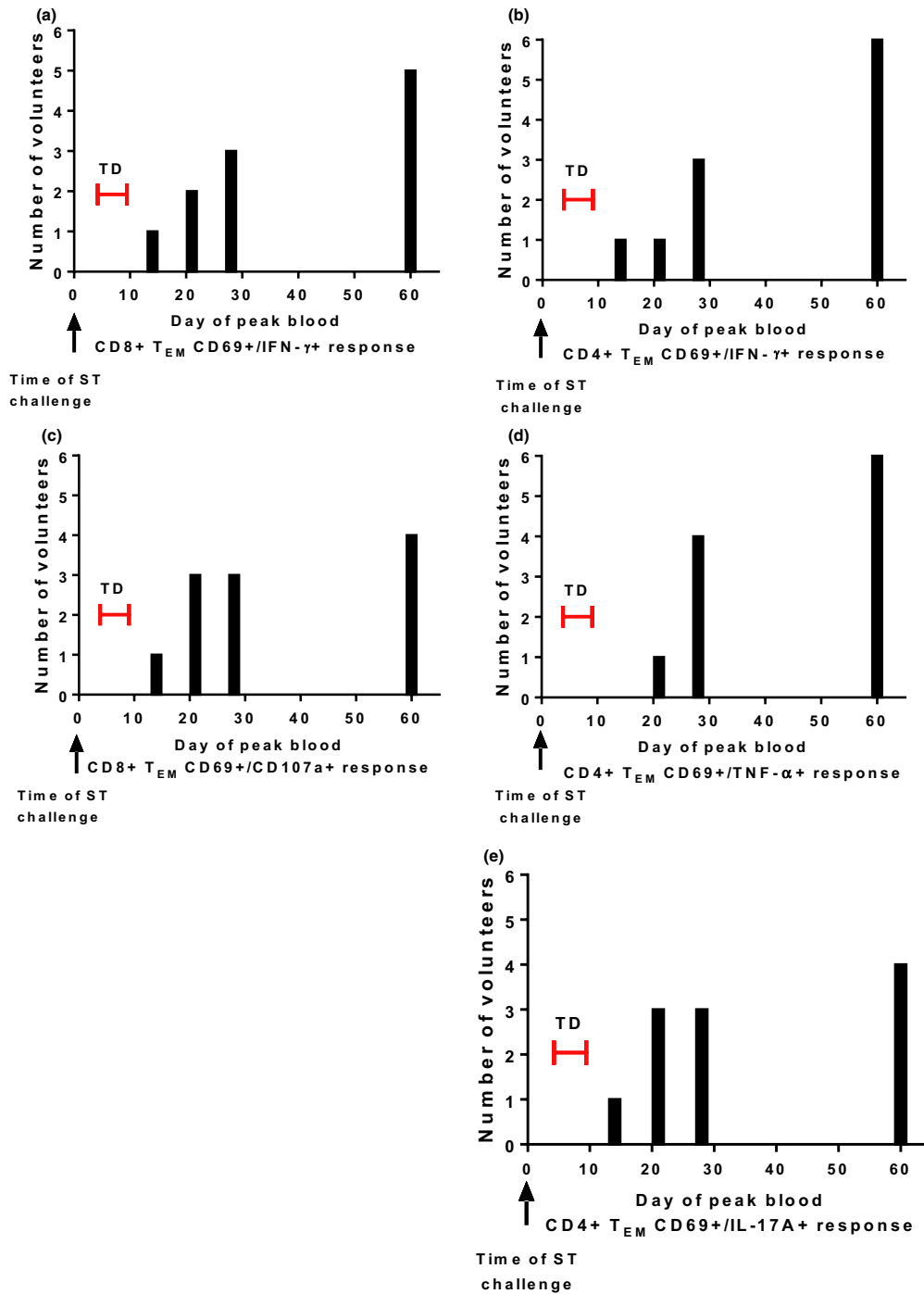
Volunteers ( $n = 11$ ) developed typhoid disease approximately 9 days after oral *wt S. Typhi* challenge. On average, volunteers exhibited classic clinical features of acute systemic *S. Typhi* infection such as decreases in platelets and serum haemoglobin, as summarised in Supplementary table 1. At the time of typhoid diagnosis, volunteers immediately started a 2-week curative antibiotic regimen for *S. Typhi* infection.

Peripheral blood mononuclear cells (PBMCs) collected from volunteers prior to challenge (day 0), and at days 14, 21, 28 and 60 after challenge, were stimulated with autologous B-LCL infected with *S. Typhi*. Frequencies of activated  $CD8^+$  T effector memory cells ( $CD8^+$  T<sub>EM</sub>,  $CD69^+/CD62L^-/CD45RA^-$ ) were analysed for expression of CD107a or IFN- $\gamma$  production. Net responses were subtracted from responses to uninfected B-LCL at all timepoints to determine the peak response per cytokine or effector function. Eight of 11 volunteers had peak IFN- $\gamma$  effector responses 28 days or later after challenge, and 7 of 11 volunteers had peak frequencies of *S. Typhi*-reactive  $CD8^+$  T<sub>EM</sub> cell CD107a expression (a degranulation marker) 28 days or later after challenge (Figure 1a and c). Activated  $CD4^+$  T<sub>EM</sub> cell responses ( $CD4^+$  T<sub>EM</sub>,  $CD69^+/CD62L^-/CD45RA^-$ ) were also evaluated following stimulation with *S. Typhi*-infected autologous B-LCL for individual frequencies of IFN- $\gamma$ , TNF- $\alpha$  or IL-17A expression

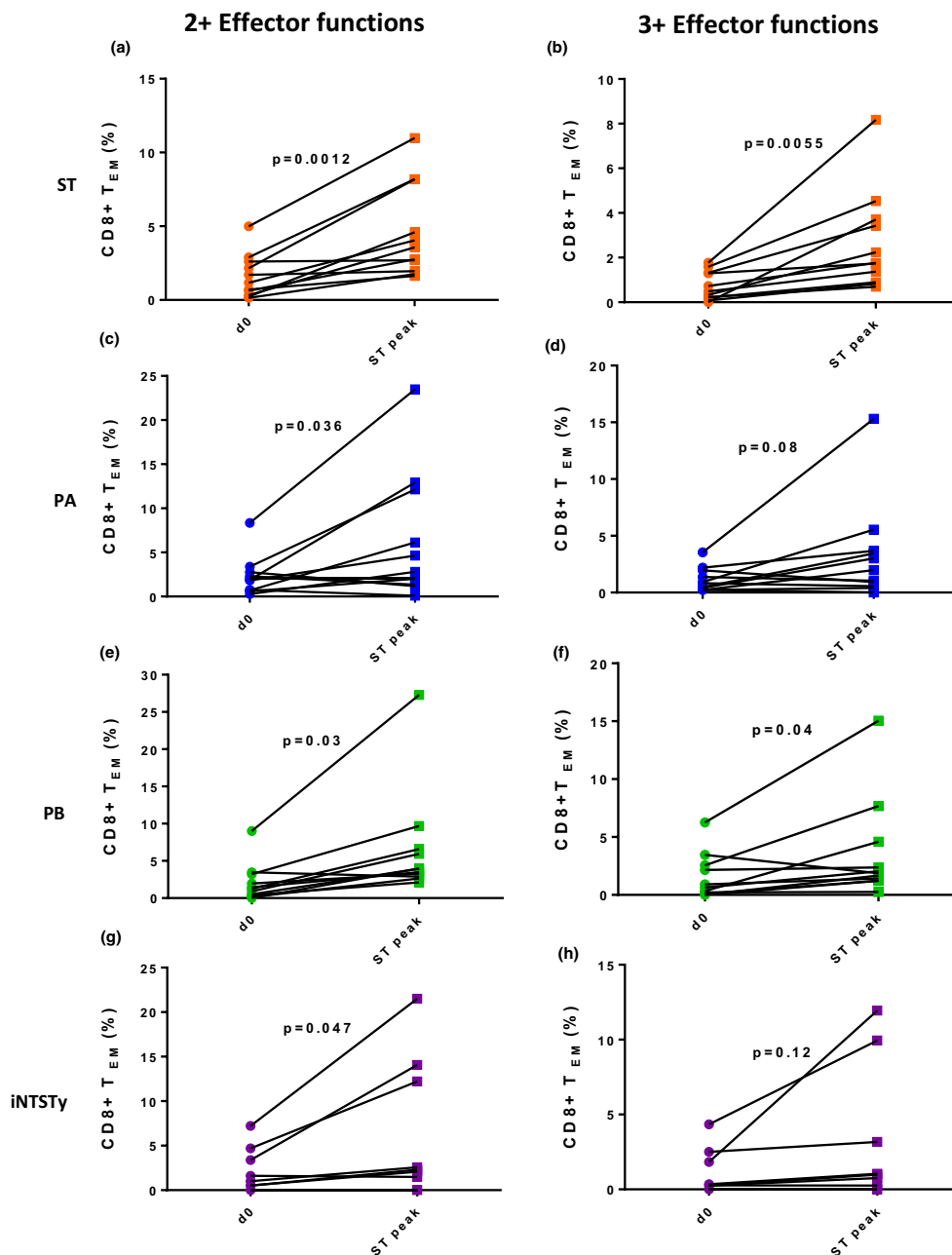
(Figure 1b, d and e). For these cell subsets as well, most volunteers develop a peak response for these individual effector functions 28 days or later after challenge, occurring in 9 of 11 volunteers for activated  $CD4^+$  T<sub>EM</sub> cells expressing IFN- $\gamma$ . Peak blood  $CD4^+$  and  $CD8^+$  T-cell responses after *S. Typhi* challenge occurred long after the diagnosis of typhoid disease and after completion of antibiotic treatment in most volunteers (Figure 1).

We next assessed for the presence of cross-reactive T-cell responses against other invasive *Salmonella* elicited after *S. Typhi* challenge. PBMCs from individual volunteers collected prior to or at various timepoints after challenge were stimulated with autologous B-LCL-infected with either *S. Typhi* (ST), *S. Paratyphi A* (PA), *S. Paratyphi B* (PB) or *S. Typhimurium* iNTS with the ST313 genotype (sub-Saharan African iNTS, iNTSTy). A representative volunteer's  $CD4^+$  T<sub>EM</sub> and  $CD8^+$  T<sub>EM</sub> cell effector responses (expression of TNF- $\alpha$ , IFN- $\gamma$ , IL-2, MIP-1 $\beta$ , IL-17A or surface CD107a expression) are shown over time against four different invasive *Salmonella* strains, as measured following stimulation of B-LCL-infected targets (Supplementary figure 1). For each of the 11 volunteers, the timepoint of the peak of the  $CD8^+$  T<sub>EM</sub> cells IFN- $\gamma$  response was identified against ST (ST peak). At ST peak,  $CD8^+$  T<sub>EM</sub> cells were evaluated for simultaneous expression of two or more effector functions upon stimulation with B-LCL infected with either PA, PB or iNTSTy. Volunteers demonstrated an induced  $CD8^+$  T<sub>EM</sub> cell cross-reactive response, with high frequencies of multifunctional  $CD8^+$  T<sub>EM</sub> cells with two or more concomitant effector functions against typhoidal and invasive nontyphoidal *Salmonella* (Figure 2a, c, e and g). When these same  $CD8^+$  T<sub>EM</sub> cells were evaluated for reactivity against PA, PB or iNTSTy with 3 or more concomitant effector functions, we observed that while there was an induced cross-reactive response against PB, there were limited induced cross-reactive responses against PA or iNTSTy (Figure 2b, d, f and h). These data suggest that while multifunctional cross-reactive  $CD8^+$  T<sub>EM</sub> cells are induced against other invasive *Salmonella* after oral *S. Typhi* challenge in humans, highly multifunctional  $CD8^+$  T<sub>EM</sub> subset responses occur only against *S. Typhi* and *S. Paratyphi B*.

Given the strong correlation of human  $CD8^+$  T-cell production of IFN- $\gamma$  against *Salmonella*-infected cells and human *in vitro* cytolytic activity of *Salmonella*-infected cells,<sup>26</sup> we assessed the hypothesis that  $CD8^+$  T<sub>EM</sub> cells induced after



**Figure 1.** Late development of peak peripheral blood T-cell effector memory responses in humans orally challenged with *wt Salmonella* Typhi. CD8<sup>+</sup> T<sub>EM</sub> or CD4<sup>+</sup> T<sub>EM</sub> cells from individual volunteers were evaluated for CD107a expression, and production of IFN-γ, TNF-α or IL-17A upon stimulation with autologous *S. Typhi* (ST)-infected target cells on days 0, 14, 21, 28 and 60 days after challenge. Background responses from uninfected targets were subtracted. The timepoint of highest effector responses per volunteer (*n* = 11) per effector function was determined for **(a)** CD8<sup>+</sup>/CD69<sup>+</sup>/IFN-γ<sup>+</sup> T<sub>EM</sub> cells, **(b)** CD8<sup>+</sup>/CD69<sup>+</sup>/CD107a<sup>+</sup> T<sub>EM</sub> cells, **(c)** CD4<sup>+</sup>/CD69<sup>+</sup>/IFN-γ<sup>+</sup> T<sub>EM</sub> cells, **(d)** CD4<sup>+</sup>/CD69<sup>+</sup>/TNF-α<sup>+</sup> T<sub>EM</sub> cells or **(e)** CD4<sup>+</sup>/CD69<sup>+</sup>/IL-17A<sup>+</sup> T<sub>EM</sub> cells. Each measurement was performed once per volunteer per effector function. TD: typhoid disease, red bar indicating range of days observed in volunteers for time of TD diagnosis. Volunteers initiated a curative antibiotic course at the time of TD diagnosis.



**Figure 2.** Oral *wt Salmonella* Typhi challenge in humans induces multifunctional CD8<sup>+</sup> T<sub>EM</sub> cells that are cross-reactive with other *Salmonella* serovars. PBMCs from individual volunteers collected prior to (d0) and at the peak of the total CD8<sup>+</sup> T<sub>EM</sub> IFN- $\gamma$  response after *S. Typhi* challenge (ST peak) were stimulated with targets infected with either ST (a, b), PA (c, d), PB (e, f) or iNTSTy (g, h). CD8<sup>+</sup> T<sub>EM</sub> cells were analysed for simultaneous expression of the following effector functions (production of TNF- $\alpha$ , IL-17A, IFN- $\gamma$ , MIP-1 $\beta$ , IL-2 and/or expression of CD107a). Frequencies of reactive multifunctional CD69<sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cells with at least two concomitant effector functions (a, c, e, g); and multifunctional CD69<sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cells exhibiting at least three concomitant effector functions (b, d, f, h).  $n = 11$  volunteers for a-f,  $n = 8$  volunteers for g and h. Paired responses from each volunteer are indicated by lines, and one measurement was performed for each volunteer per timepoint. Data were analysed by a paired Student's *t*-test; *P*-values are displayed.

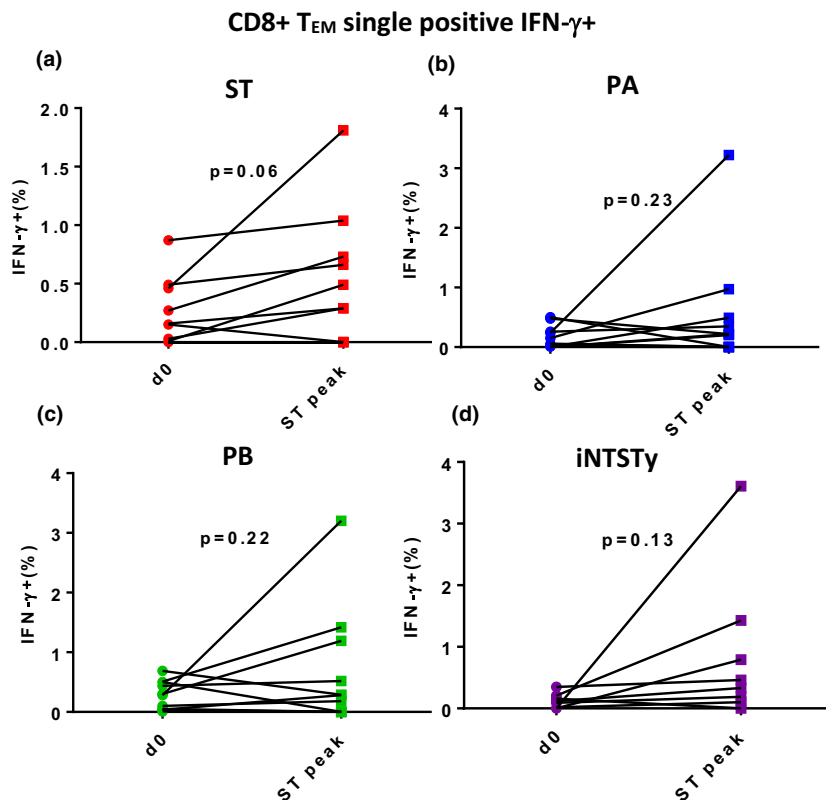
*S. Typhi* challenge predominate in IFN- $\gamma$ <sup>+</sup> multifunctional subsets. To this end, we measured frequencies of CD8<sup>+</sup> T<sub>EM</sub> cells expressing IFN- $\gamma$

alone, that is, not simultaneously expressing any of the other measured effector functions (TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , IL-17A or surface CD107a). At the

peak of the CD8<sup>+</sup> T<sub>EM</sub> cell response against *S. Typhi*, although a trend was apparent, we found that CD8<sup>+</sup> T<sub>EM</sub> cells expressing only IFN- $\gamma$  were not significantly induced after oral challenge (Figure 3a). In addition, there was no significantly induced cross-reactive response of cells with this phenotype against other typhoidal or invasive nontyphoidal *Salmonella* strains (Figure 3b–d).

In contrast, high frequencies of CD8<sup>+</sup> T<sub>EM</sub> cells simultaneously producing IFN- $\gamma$  and TNF- $\alpha$ , and expressing the degranulation marker CD107a, with or without simultaneous expression of MIP-1 $\beta$ , were induced following *S. Typhi* challenge. These induced responses were significantly cross-reactive against PA and PB, but not against the more genetically distant iNTSTy (Figure 4a). Of note, this multifunctional CD8<sup>+</sup> T<sub>EM</sub> population induced by human *S. Typhi* challenge is of higher magnitude

than the induced CD8<sup>+</sup> T<sub>EMRA</sub> population, identified as CD62L<sup>-</sup>/CD45RA<sup>+</sup> (Figure 4b). The CD8<sup>+</sup> T<sub>EMRA</sub> population is notable for re-expression of CD45RA and is a more highly differentiated human T memory cell population, with lower proliferative capacity, than CD8<sup>+</sup> T<sub>EM</sub> cells. We have previously shown that multifunctional *S. Typhi*-specific CD8<sup>+</sup> T<sub>EMRA</sub> cells are induced after Ty21a vaccination<sup>31</sup> and also after human oral *S. Typhi* challenge,<sup>24</sup> albeit at lower frequencies than those observed in the CD8<sup>+</sup> T<sub>EM</sub> population. Here, we observed significant increases in the frequencies of this population against ST, but no significant induction of cross-reactive CD8<sup>+</sup> T<sub>EMRA</sub> cells with the IFN- $\gamma$ <sup>+</sup>/TNF- $\alpha$ <sup>+</sup>/CD107a<sup>+</sup>/MIP-1 $\beta$ <sup>+/-</sup> phenotype against PA, PB or iNTS. (Figure 4b). Hence, this multifunctional CD8<sup>+</sup> T<sub>EMRA</sub> subset elicited after human oral *S. Typhi* challenge is induced at lower frequencies and is



**Figure 3.** Lack of induction of serovar cross-reactive CD8<sup>+</sup> T<sub>EM</sub> cells producing IFN- $\gamma$  in the absence of simultaneous production of either TNF- $\alpha$ , MIP-1 $\beta$ , IL-17A, IL-2 or surface expression of CD107a in the blood of *Salmonella* Typhi-challenged volunteers. PBMCs from *S. Typhi*-challenged participants were stimulated with target cells infected with either (a) ST, (b) PA, (c) PB or (d) iNTSTy. Boolean gating was performed on CD69<sup>+</sup>CD8<sup>+</sup> T<sub>EM</sub> cells to evaluate individual cell expression of IFN- $\gamma$  in the absence of other cytokines above or expression of CD107a in individual cells. Frequencies are shown for CD8<sup>+</sup> T<sub>EM</sub> cells prior to challenge (d0) and at the peak of the CD8<sup>+</sup> T<sub>EM</sub> cell IFN- $\gamma$  response (ST peak).  $n = 11$  volunteers for a–c,  $n = 8$  volunteers for d. Paired responses are indicated by lines, and one measurement was performed for each volunteer per timepoint. Data were analysed by a paired Student's *t*-test; *P*-values are displayed.

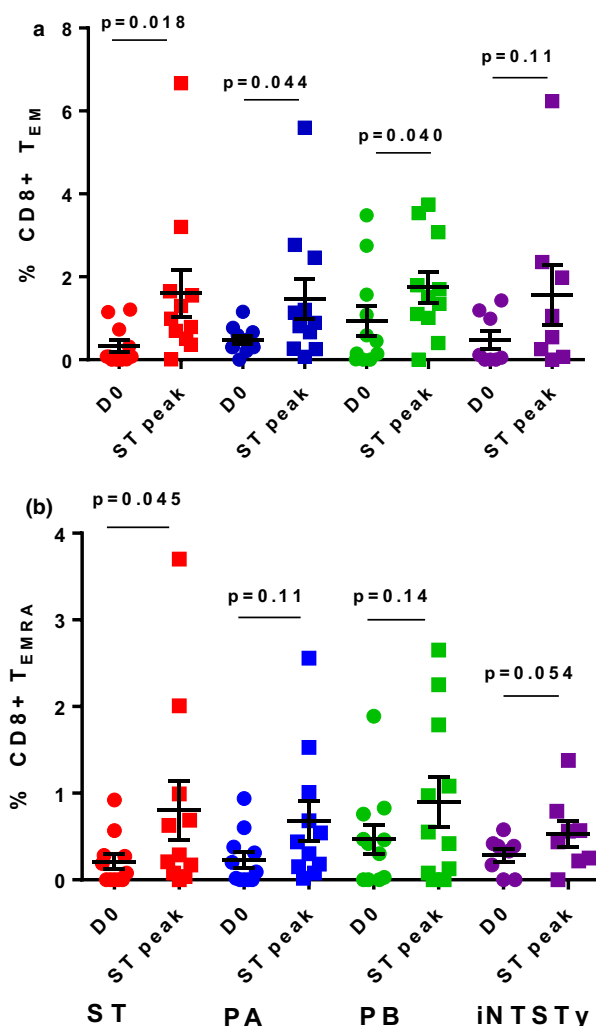
minimally cross-reactive with other invasive *Salmonella*.

The  $T_{CM}$  population ( $CD62L^+$ ,  $CD45RA^-$ ) is classically associated with homing to central lymphoid organs, with high proliferative potential and functional IL-2 expression, supporting growth, expansion and survival of antigen-specific memory T cells. We observed that  $CD8^+$   $T_{CM}$  and  $CD4^+$   $T_{CM}$  cells elicited after *S. Typhi* challenge are induced against *S. Typhi* and cross-reactive only with PA (Figure 5a and b). Notably, there were no significant induced  $T_{CM}$  responses cross-reactive against PB and iNTSTy.

Given the magnitude of the induced  $CD8^+$   $T_{EM}$  subset after human *S. Typhi* challenge, and the identification of cross-reactive multifunctional  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$ , we examined the relationship of pre-existing frequencies of  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  reactive against different invasive *Salmonella* strains to peak  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  responses after human *S. Typhi* challenge. We observed that ST, PA and iNTSTy all had strong correlations in magnitude of frequencies of  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cells at baseline with the magnitude of the peak of the  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cell response against the respective invasive strain after oral *S. Typhi* challenge (Figure 6a–c), suggesting that *S. Typhi* challenge expands baseline reactive  $CD8^+$  T cells as part of the response to infection. Baseline  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cell reactivity to ST also correlated with the peak magnitude of the  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cell response against PA (Figure 6d). Notably, however, there was no correlation between baseline  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cell responses to ST and the peak magnitude of the  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cell iNTSTy response (Figure 6e) after *S. Typhi* challenge, indicating that volunteers with low or high  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cell baseline frequencies against ST did not have a predictable  $IFN-\gamma^+$   $CD8^+$   $T_{EM}$  cell peak response against iNTSTy. This pattern suggests that a proportion of the cross-reactive responses that are elicited after human oral *S. Typhi* challenge and that are reactive against iNTSTy are not expanded from the pre-existing repertoire of  $CD8^+$   $T_{EM}$  cells reactive to ST, underscoring differences in the properties of the cross-reactive T-cell responses to iNTSTy and PA.

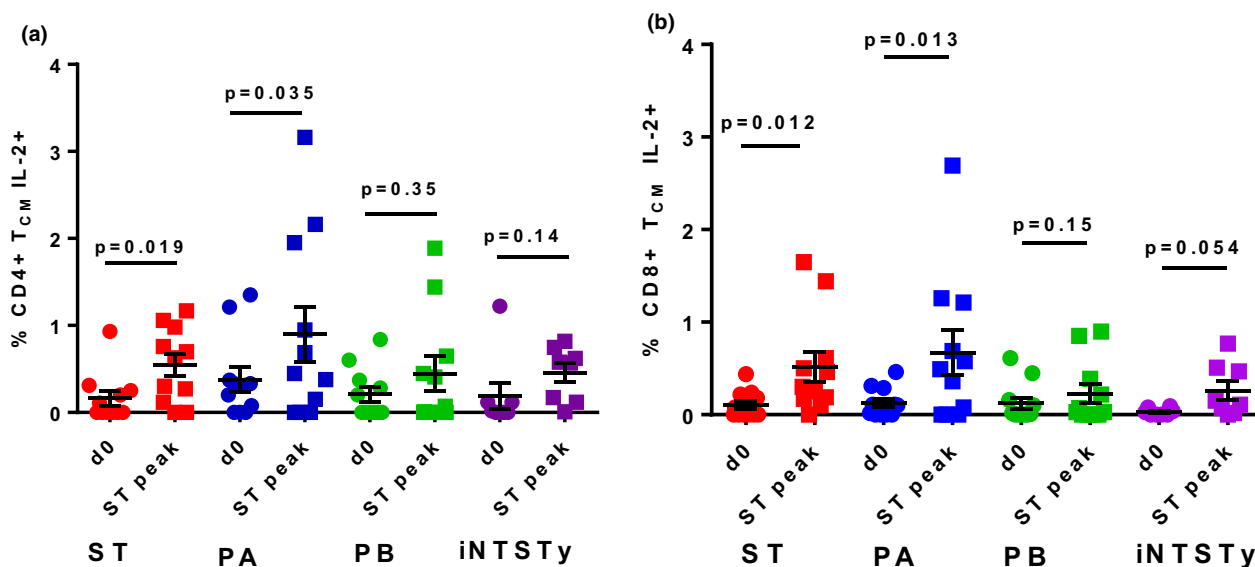
We evaluated whether expression of the gut mucosal-associated homing integrin  $\alpha 4\beta 7$  differed among the multifunctional *Salmonella* cross-reactive peripheral  $CD8^+$   $T_{EM}$  subsets observed after oral *S. Typhi* challenge, as evaluated at the peak of the blood  $CD8^+$   $T_{EM}$  cell  $IFN-\gamma$  response

### IFN- $\gamma^+$ /TNF- $\alpha^+$ /CD107a+/ MIP-1 $\beta$ (+/-)



**Figure 4.** Induction of multifunctional, serovar cross-reactive blood  $CD8^+$   $T_{EM}$  cells, but not  $CD8^+$   $T_{EMRA}$  cells that simultaneously produce  $IFN-\gamma$  and  $TNF-\alpha$  and express  $CD107a$ , with or without  $MIP-1\beta$  production, after *Salmonella Typhi* challenge. PBMCs prior to infection (d0) and at the timepoint of peak total peripheral  $CD69^+$   $CD8^+$   $T_{EM}$  cell  $IFN-\gamma$  response after *S. Typhi* challenge in each volunteer (ST peak), were stimulated with autologous targets infected with either ST, PA, PB or iNTSTy.  $CD69^+$   $CD8^+$   $T_{EM}$  cells (a) or  $CD69^+$   $CD8^+$   $T_{EMRA}$  cells (b) were analysed for simultaneous expression of 6 effector functions (production of  $TNF-\alpha$ , IL-17,  $IFN-\gamma$ ,  $MIP-1\beta$ , IL-2 and/or expression of  $CD107a$ ). Mean  $\pm$  SEM and individual responses are shown.  $n = 11$  volunteers for ST, PA and PB reactivity,  $n = 8$  volunteers for iNTSTy reactivity. One measurement was performed per volunteer. Data were analysed by a paired Student's  $t$ -test;  $P$ -values are displayed.

against ST. We observed no differences in the frequencies of integrin  $\alpha 4\beta 7$  expression among  $CD8^+$   $T_{EM}$  cells expressing two or more, or three or more concomitant functions, among the different



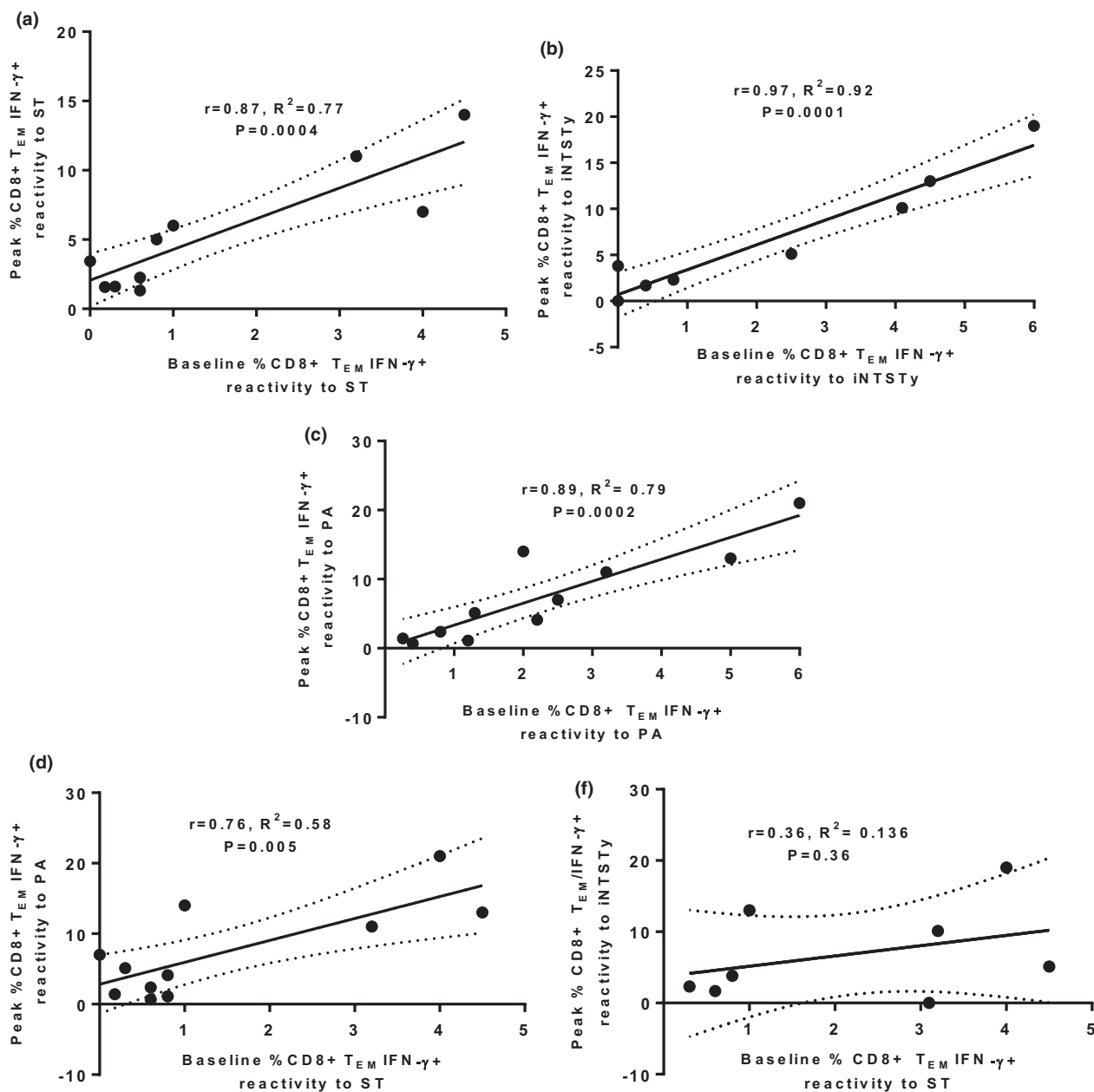
**Figure 5.** Increased frequencies of  $T_{CM}$  cells expressing IL-2 that are cross-reactive with *Salmonella* Paratyphi A after *Salmonella* Typhi challenge. PBMCs prior to infection (d0) or at the timepoint of peak total peripheral  $CD8^+ T_{EM}$  or  $CD4^+ T_{EM}$  IFN- $\gamma$  responses after *S. Typhi* challenge per individual volunteer (ST peak) were stimulated with targets infected with either ST, PA, PB, or iNTSTy.  $CD69^+ CD4^+ T_{CM}$  (a) and  $CD69^+ CD8^+ T_{CM}$  (b) cells were assessed for intracellular IL-2 expression.  $n = 11$  volunteers assessed for ST, PA and PB reactivity,  $n = 8$  volunteers assessed for iNTSTy reactivity. One measurement was performed per volunteer. Mean  $\pm$  SEM and individual responses are shown. Data were analysed by a paired Student's  $t$ -test;  $P$ -values are displayed.

invasive *Salmonella* (Supplementary figure 2). Additionally, there were no differences in cross-reactive frequencies of  $CD8^+ T_{EM}$  cells containing simultaneous effector functions of IFN- $\gamma^+$ /TNF- $\alpha^+$ /CD107a $^+$ /MIP-1 $\beta^{+/-}$  that also expressed integrin  $\alpha_4\beta_7$  (Supplementary figure 2). Hence, while the expression of  $\alpha_4\beta_7$  was noted in only a subset of the reactive multifunctional  $CD8^+ T_{EM}$  cells, there was no difference in frequencies among multifunctional subsets induced after human oral *S. Typhi* challenge, and no significant differences in frequencies of  $\alpha_4\beta_7$  multifunctional  $CD8^+ T_{EM}$  cell cross-reactive with PA, PB or iNTSTy.

We also assessed whether serovar cross-reactive multifunctional  $CD4^+ T$  cells secreting IFN- $\gamma$  (Th1) or IL-17A (Th17) cells are elicited after human oral *S. Typhi* challenge. High frequencies of multifunctional  $CD4^+ T_{EM}$  cells, concomitantly expressing at least IFN- $\gamma$  and one or more additional effector functions, were elicited after oral *wt S. Typhi* challenge, as assessed at the peak of the total IFN- $\gamma$   $CD4^+ T_{EM}$  cell response against ST, and these responses were similarly cross-reactive against PA, PB and iNTSTy (Figure 7a, c, e and g). Notably, at the peak of the  $CD4^+ T_{EM}$  cell IFN- $\gamma$  response there were no significantly induced multifunctional Th17 responses against any of the

invasive *Salmonella* strains (Figure 7b, d, f and h). More individuals had a peak peripheral blood  $CD4^+ T_{EM}$  cell IL-17A response at earlier timepoints after oral *S. Typhi* challenge than those observed for the peak peripheral blood  $CD4^+ T_{EM}$  cell IFN- $\gamma$  response (Figure 1b and e). When we measured multifunctional Th17 induction at the peak of the Th17 response against ST (ST IL-17A peak), we observed that significant frequencies of multifunctional Th17 with reactivity against ST were induced (Figure 7i). Interestingly, these lower magnitude peripheral multifunctional Th17 responses elicited after oral *wt S. Typhi* challenge were not significantly cross-reactive against PA, PB or iNTSTy (Figure 7j-l).

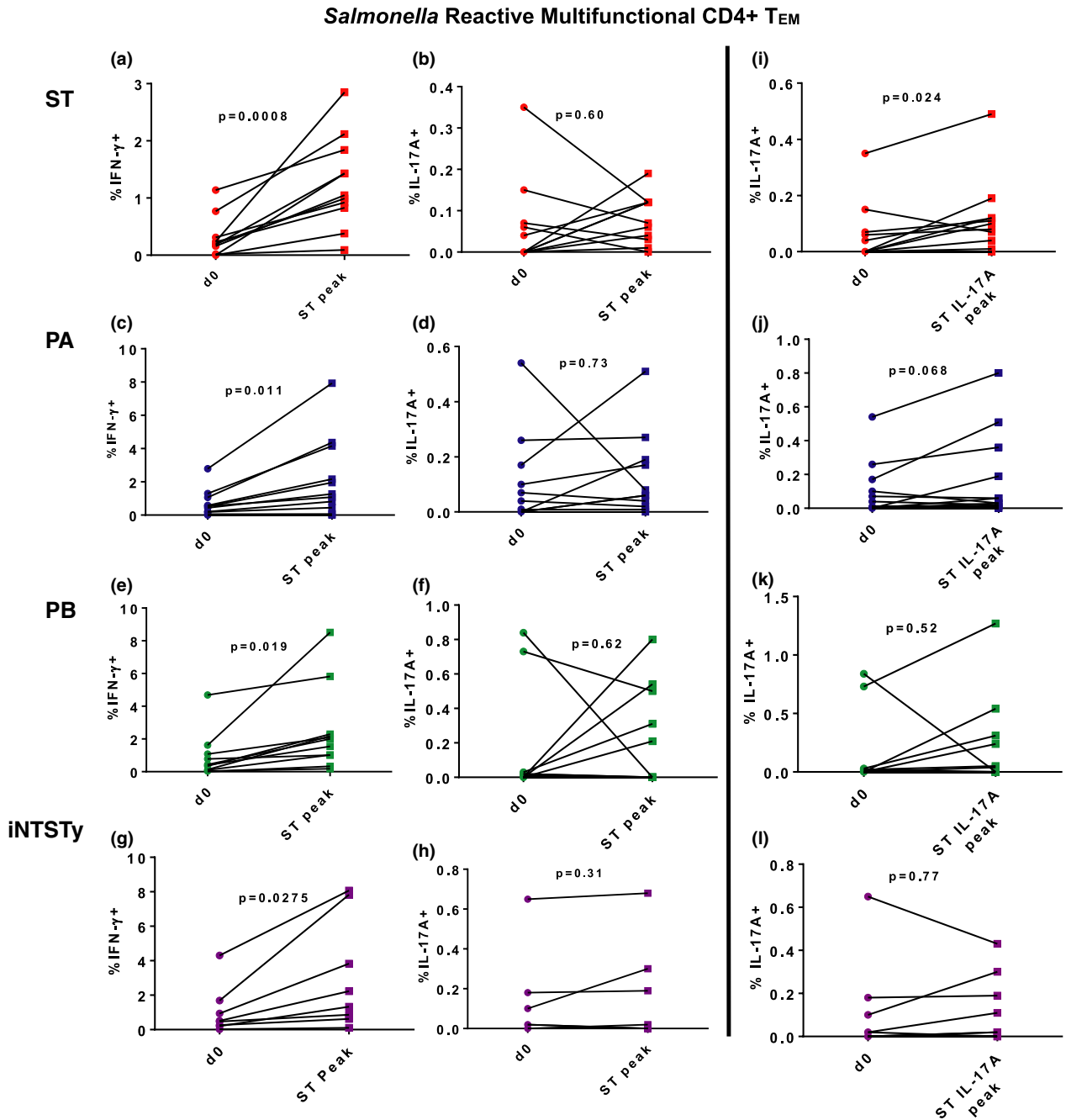
Multiple studies have demonstrated that  $CD4^+ T$  cells are required for the generation of optimal  $CD8^+ T$ -cell memory cell responses and that repeated antigenic stimulation does not substitute for  $CD4^+ T$ -cell help when priming  $CD8^+ T$ -cell memory responses.<sup>32</sup> Given the sizable frequencies of multifunctional serovar cross-reactive Th1 elicited after human oral *S. Typhi* challenge in the peripheral blood, we examined the hypothesis that robust induced multifunctional  $CD4^+ Th1$  responses against ST correlates with peak multifunctional  $CD8^+ T_{EM}$  responses against ST and



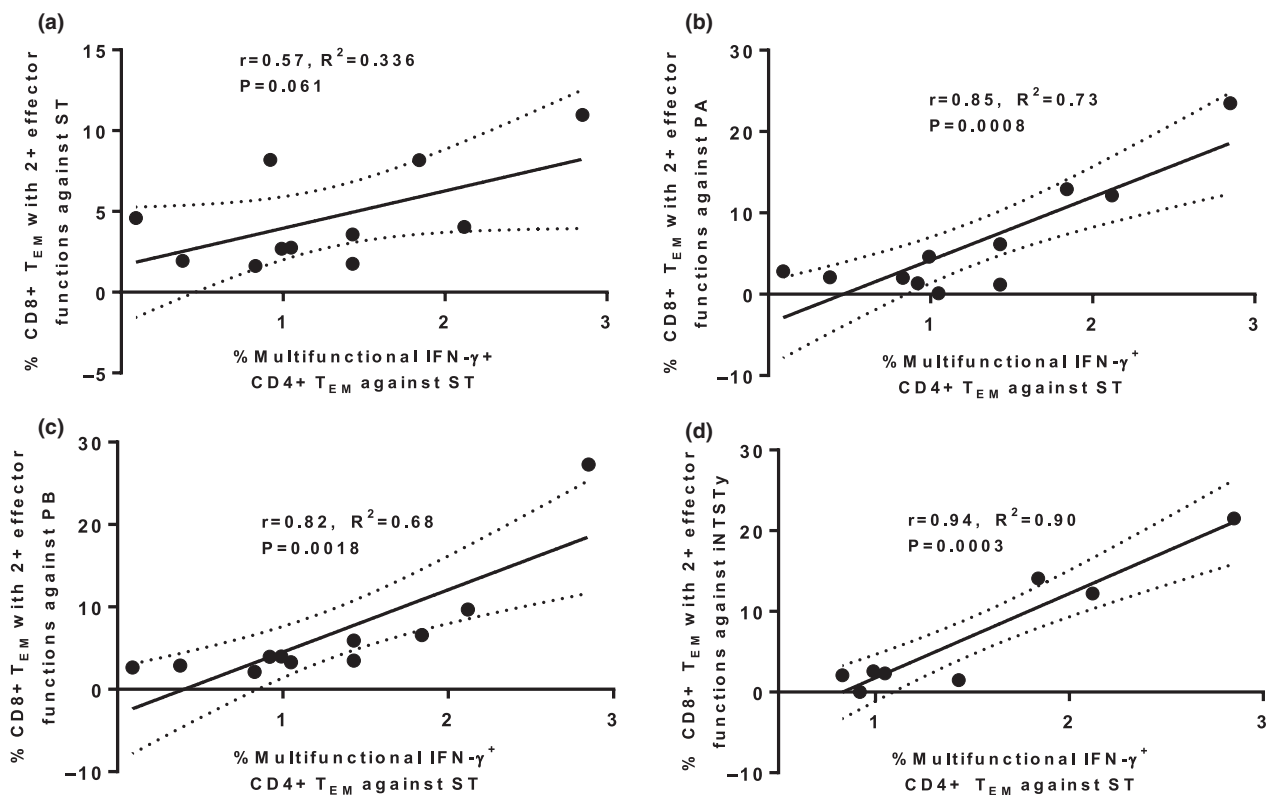
**Figure 6.** The magnitude of *Salmonella* Typhi baseline CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ responses correlates with the magnitude of peak CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ responses against *S. Typhi* and *Salmonella* Paratyphi A but not iNTSTy. PBMCs obtained prior to and after oral *S. Typhi* challenge at days 14, 21, 28 or 60 were stimulated with targets infected with either ST, PA, PB or iNTSTy. Individual peak CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ responses postchallenge were determined for each serovar and are plotted against the corresponding baseline CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ reactivity. Shown are baseline to peak CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ reactivity for ST (a), iNTSTy (b) and *S. Paratyphi* A (c). Individual volunteer paired reactivity measurements are plotted,  $n = 11$  for a and c, and  $n = 8$  for b. (d) Correlation of baseline ST CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ responses to peak CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ responses to PA (paired responses,  $n = 11$ ) (e) Correlation of baseline ST CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ to peak CD8<sup>+</sup> T<sub>EM</sub> cell IFN-γ responses to iNTSTy (paired responses,  $n = 8$ ).  $r$ ,  $R^2$ , and  $P$ -values are presented.

other invasive *Salmonella*. We observed that while there was a moderate positive relationship between the peak IFN-γ<sup>+</sup> CD4<sup>+</sup> T<sub>EM</sub> cell response against ST and the multifunctional CD8<sup>+</sup> T<sub>EM</sub> cell

response ( $P = 0.061$ ), this relation was not statistically significant (Figure 8a). However, we observed that the induced CD4<sup>+</sup> T<sub>EM</sub> cell response against ST correlated strongly with the



**Figure 7.** Differential kinetics, magnitude and frequencies of multifunctional, serovar cross-reactive CD4<sup>+</sup> Th1 and CD4<sup>+</sup> Th17 effector memory cells in peripheral blood after human oral *wt Salmonella* Typhi challenge. PBMCs collected from volunteers prior to *S. Typhi* challenge (d0) or at the timepoint of peak blood total CD4<sup>+</sup> T<sub>EM</sub> IFN- $\gamma$  responses after *S. Typhi* challenge (ST peak) or peak peripheral CD4<sup>+</sup> T<sub>EM</sub> IL-17A responses (ST peak IL-17A) were stimulated with targets infected with either ST (a, b, i), PA (c, d, j), PB (e, f, k) or iNTSTy (g-i). CD69<sup>+</sup> CD4<sup>+</sup> T<sub>EM</sub> cells were analysed for simultaneous expression of six effector functions (production of TNF- $\alpha$ , IL-17A, IFN- $\gamma$ , MIP-1 $\beta$ , IL-2 and/or expression of CD107a). Multifunctional Th1 or Th17 cells were defined as expressing IFN- $\gamma$  (Th1) or IL-17A (Th17) and at least one other additional effector function. Multifunctional CD69<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T<sub>EM</sub> (a, c, e, g) or CD69<sup>+</sup>/IL-17A<sup>+</sup> CD4<sup>+</sup> T<sub>EM</sub> (b, d, f, h) cells were evaluated at the peak of the total CD4<sup>+</sup> T<sub>EM</sub> cell IFN- $\gamma$  response. Multifunctional CD69<sup>+</sup>/IL-17A CD4<sup>+</sup> T<sub>EM</sub> cells were also evaluated at the peak of the total CD4<sup>+</sup> T<sub>EM</sub> cell IL-17A response (i-l).  $n = 11$  volunteers assessed for ST, PA and PB reactivity,  $n = 8$  volunteers assessed for iNTSTy reactivity. Paired responses are indicated by lines. One measurement per volunteer was made per timepoint. Data were analysed by a paired Student's *t*-test; *P*-values are displayed.



**Figure 8.** Induction of multifunctional Th1 against *Salmonella* Typhi after human oral challenge correlates with the induction of multifunctional serovar cross-reactive CD8 $^+$  T $_{EM}$  cells. PBMCs from individual volunteers at the peak of the total peripheral CD4 $^+$  T $_{EM}$  IFN- $\gamma$  response were analysed for the frequencies of IFN- $\gamma^+$ CD4 $^+$  T $_{EM}$  cells with at least one or more concomitant additional effector functions (either CD107a expression or TNF- $\alpha$ , MIP-1 $\beta$ , IL-17A or IL-2 production) against ST and plotted against the frequencies of CD8 $^+$  T $_{EM}$  cells, as evaluated at the peak of total peripheral CD8 $^+$  T $_{EM}$  cell IFN- $\gamma$  response per volunteer, with at least 2 or more effector functions (production of TNF- $\alpha$ , MIP-1 $\beta$ , IL-2, IL-17A and/or expression of CD107a) against either ST (a), PA (b), PB (c) or iNTSty (d). Eleven volunteers were studied in a–c and 8 volunteers in d. Single paired measurements were made for each volunteer and plotted. Responses of individual volunteers are shown with correlation coefficient ( $r$ ), coefficient of determination ( $R^2$ ) and  $P$ -value.

cross-reactive multifunctional CD8 $^+$  T $_{EM}$  cell response against PA, PB and iNTSty (Figure 8b–d). These data suggest a relationship between the induction of robust Th1 responses after *S. Typhi* challenge and the induction of high frequencies of cross-reactive multifunctional CD8 $^+$  T $_{EM}$  cells against invasive *Salmonella* strains in humans.

## DISCUSSION

There are no vaccines in clinical use against *S. Paratyphi* or iNTS infection, despite significant disease burden and mortality. Phylogenetic studies show *S. Typhi* more closely related to PA than to iNTS *S. Typhimurium* strains.<sup>7</sup> Biologic differences between iNTS *S. Typhimurium* and NTS *S. Typhimurium* impact antigen-presenting cell migration in murine infection and human

macrophage inflammatory and antibacterial cytotoxic responses.<sup>33,34</sup> Virulence factors, such as the Vi capsule, are present in *S. Typhi* but absent in PA and iNTS strains, and attachment pili differ considerably among serovars.<sup>35</sup> In a murine model of invasive *Salmonella* vaccination, effective host memory defence responses were serovar-dependent and LPS-independent after oral challenge with different nontyphoidal *Salmonella* serovars.<sup>36</sup> Recent studies have shown that very minor serovar differences in *Salmonella* immunodominant antigens PhoN and CdtB impact CD4 $^+$  T-cell cross-reactivity from T-cell clones expanded after human *wt S. Typhi* challenge.<sup>37</sup> Given genetic and phenotypic differences between *Salmonella* strains, the present study evaluates the development and functional specificity of adaptive heterologous T-cell memory

responses in humans against genetically distinct *Salmonella* sharing capacity for mucosal invasion and dissemination. Our methods emphasise assessment of T-cell functionality upon exposure to antigen-presenting cells infected with ST, PA, PB or iNTSTy, without selective expansion of memory cell clones, and on the characterisation of both human CD4<sup>+</sup> and CD8<sup>+</sup> T-cell memory compartments induced after experimental oral *wt S. Typhi* challenge.

Among individuals orally challenged with *wt S. Typhi* and developing typhoid disease, we observed that the peripheral blood T-cell response continues to evolve in most volunteers long after antibiotic treatment, with generation of peak peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>EM</sub> cells with different effector functions by 1 month or later postchallenge in most volunteers. The observation of peak peripheral human T memory cell responses occurring several weeks after clinical symptoms of typhoid disease contrasts with studies of different human viral mucosal infections, where elicitation of peripheral blood T cells with an effector memory phenotype peaks 1 week after development of symptoms in hantavirus infection<sup>38</sup> or 1 week after intranasal challenge with influenza.<sup>39</sup> In contrast to a variety of human viral infections, where systemic T-cell responses appear much earlier in the clinical course of infection, the evolution of peripheral blood T-cell effector memory responses against *S. Typhi* has a delayed kinetics.<sup>40,41</sup> One explanation for the observed delayed T<sub>EM</sub> presence in the blood after *S. Typhi* challenge may be the result of T<sub>EM</sub> cells migrating to mucosal sites initially during early infection and therefore present at reduced levels in circulation soon after infection. Alternately, the delayed peripheral blood T memory cell response may be consistent with early immune evasion mechanisms operative in invasive *S. Typhi* infection, whereby particular *S. Typhi* gene products impair antigen presentation to T cells<sup>42,43</sup> or impair recognition or inflammatory pathways by antigen-presenting cells or mucosal tissues.<sup>44,45</sup> Attenuated *S. Typhi* vaccine Ty21a is currently administered as multiple doses of vaccine over a 1-week period. Our observation of a delayed T-cell effector response after *S. Typhi* challenge may support the benefit of a late timepoint booster immunisation for next-generation live-attenuated *S. Typhi* vaccination, after the peripheral memory T-cell pool has matured in response to infection in

humans, to potentially extend the duration of long-lived immunity.

Multifunctional T-cell responses are associated with superior immune protection in human infection with viruses<sup>46–49</sup> and intracellular bacteria and parasites.<sup>50–52</sup> We have previously shown that individuals who resist development of typhoid disease after *S. Typhi* challenge have significantly increased frequencies of *S. Typhi*-reactive multifunctional CD8<sup>+</sup> T cells in peripheral blood prior to challenge relative to those who develop typhoid disease.<sup>24</sup> These baseline T-cell memory frequencies observed in individuals with no prior history of prior *S. Typhi* exposure or vaccination may be the result of exposure to other cross-reactive Enterobacteriaceae or cross-reactive environmental antigens.<sup>53</sup> Here, we observed that high frequencies of multifunctional CD8<sup>+</sup> T effector memory cells with two or more concomitant effector functions are induced after *S. Typhi* challenge and are highly cross-reactive with PA, PB, as well as iNTSTy-infected targets. Significant induction of highly multifunctional CD8<sup>+</sup> T effector memory cells with three or more effector functions, however, was only observed against PB-infected targets. This observation suggests that the heterologous CD8<sup>+</sup> T-cell responses induced in humans after *S. Typhi* challenge may involve a signal threshold for multifunctional responses that are not achieved from exposure to PA or iNTSTy-infected targets. One hypothesis for why this may occur is that antigens presented by PA or iNTSTy-infected targets may not interact as strongly with CD8<sup>+</sup> T-cell receptors, subsequently impacting downstream signalling and cytokine production, resulting in cross-reactive T cells with fewer measured effector functions. Alternately, the immunodominant antigens or their peptide sequences may significantly differ between PA, PB and iNTSTy, leading to different frequencies of multifunctional CD8<sup>+</sup> T-cell responses. These studies are the first to demonstrate that multifunctional CD8<sup>+</sup> T<sub>EM</sub> cells produced after human *wt S. Typhi* exposure produces significantly induced cross-reactive responses against PA, PB and iNTSTy, yet the functional characteristics of the CD8<sup>+</sup> T<sub>EM</sub> cell response are impacted, at least in part, by serovar.

Human CD8<sup>+</sup> T-cell cytolytic activity against *S. Typhi*-infected targets, induced after Ty21a vaccination, is MHC I-dependent<sup>25</sup> and is very highly correlated with frequencies of IFN- $\gamma$  producing

*S. Typhi*-specific CD8<sup>+</sup> T cells.<sup>26</sup> In a murine model of vaccination against invasive *Salmonella*, CD8<sup>+</sup> T cells and IFN- $\gamma$  signalling were important in recall host defence mechanisms on challenge with *S. Typhimurium*, impacting survival.<sup>54</sup> Here, we demonstrate that the IFN- $\gamma$  producing CD8<sup>+</sup> T<sub>EM</sub> cells induced after human *S. Typhi* infection is primarily multifunctional, as we observed no significant induction of CD8<sup>+</sup> T<sub>EM</sub> IFN- $\gamma$  monofunctional cells. Additionally, there was no observed induced response by cross-reactive CD8<sup>+</sup> T effector memory single positive IFN- $\gamma$ -producing cells against other invasive *Salmonella* strains. Hence, human challenge with *S. Typhi* leads to the induction of multifunctional serovar cross-reactive IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T effector memory cells, given our observation of significantly induced CD8<sup>+</sup> T<sub>EM</sub> cells with the IFN- $\gamma$ <sup>+</sup>/TNF- $\alpha$ <sup>+</sup>/CD107a<sup>+</sup>/MIP-1 $\beta$ <sup>+/-</sup> phenotype. Since IFN- $\gamma$  monofunctional T cells contrast significantly at the level of transcription from IFN- $\gamma$  multifunctional cells as observed in different human infections,<sup>55</sup> our data suggest after human infection with *S. Typhi*, there is preferential induction of multifunctional CD8<sup>+</sup> T<sub>EM</sub> cells within the memory compartment, and only multifunctional IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T<sub>EM</sub> cell responses are significantly cross-reactive.

A specific population of CD8<sup>+</sup> T<sub>EM</sub> cells with simultaneous evidence of degranulation (as measured by CD107a expression) and production of IFN- $\gamma$  and TNF- $\alpha$ , with or without production of MIP-1 $\beta$ , was highly induced after human *S. Typhi* challenge. This multifunctional population with 3 or more specific effectors was highly cross-reactive with PA and PB but not iNTSTy. In contrast, human Ty21a vaccination led to the development of multifunctional CD8<sup>+</sup> T<sub>EM</sub> cells expressing IFN- $\gamma$  and CD107a without TNF- $\alpha$  expression that were cross-reactive against PB but not against PA.<sup>31</sup> These data suggest that genetic changes in Ty21a, which lead to phenotypic changes including its attenuation and absence of Vi capsule for example, impact aspects of human antigen presentation and CD8<sup>+</sup> T-cell memory programming and that *wt S. Typhi* infection promotes a more robust cross-reactive CD8<sup>+</sup> T-cell response against PA. In contrast to cross-reactivity observed against PA after human *S. Typhi* challenge, this cross-reactivity is markedly decreased when assessed against targets infected with the more genetically distant iNTSTy. Genetic and phenotypic differences between Ty21a and *wt S. Typhi* may account for the more limited CD8<sup>+</sup> T-cell cross-reactive responses against PA

observed after Ty21a vaccination. Our findings underscore the potential for reengineering *S. Typhi*-based oral vaccines to obtain optimal cross-reactive CD8<sup>+</sup> T-cell responses against PA in vaccination.

We observed strong correlations between frequencies of *S. Typhi*-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cells prior to challenge and the magnitude of the peak *S. Typhi*-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cell response after challenge. This observation suggests that part of the immune response to infection involves expansion of baseline T-cell memory responses from the pre-existing repertoire in individuals with no history of prior *S. Typhi* infection or vaccination. We also observed a correlation in PA and iNTSTy baseline IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cell responses to peak responses, suggesting that the pre-existing IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cell repertoire reactive to these genetically distant invasive *Salmonella* are expanded by *S. Typhi* challenge. Interestingly, the relationship between baseline *S. Typhi*-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cell responses and peak PA IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cell responses also highly correlates, while the relationship to peak iNTS IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T<sub>EM</sub> cell responses does not. These data suggest that baseline CD8<sup>+</sup> T-cell reactivity with iNTS corresponds predominantly to different CD8<sup>+</sup> T-cell antigen specificities, which are expanded after *S. Typhi* challenge, and that dominant CD8<sup>+</sup> T-cell antigens are different between iNTSTy and *S. Typhi*. These findings support observations by Napolitani et al., where more cross-reactive T-cell clones and the antigens they react with were shared between *S. Typhi* and PA rather than iNTS *S. Typhimurium*.<sup>37</sup> Alternately, IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells induced by *S. Typhi* challenge may have differential activation to iNTS antigens compared to typhoidal serovars, secondary to potential differences in antigen presentation.

CD8<sup>+</sup> T<sub>EM</sub> cells induced after *S. Typhi* challenge expressing two or more effector functions, three or more effector functions, or the CD107a<sup>+</sup>/TNF- $\alpha$ <sup>+</sup>/IFN- $\gamma$ <sup>+</sup>/MIP-1 $\beta$ <sup>+/-</sup> phenotype, did not differ in expression of gut-homing molecule integrin  $\alpha$ 4 $\beta$ 7 upon exposure to antigen-presenting cells infected with either *S. Typhi*, PA, PB or iNTSTy. These data suggest that multifunctional, serovar cross-reactive CD8<sup>+</sup> T<sub>EM</sub> cells generated after human *S. Typhi* challenge have similar potential for homing to gut mucosal tissues. That a high proportion of these induced multifunctional CD8<sup>+</sup> T cells do not express integrin  $\alpha$ 4 $\beta$ 7 supports

similar data we have previously reported in volunteers immunised with attenuated *S. Typhi* vaccines.<sup>31,56,57</sup> These results underscore the increased presence in circulation of memory T cells elicited in *S. Typhi*-challenged adults with the ability to home to mucosal and other systemic tissues, which may prevent dissemination during future *Salmonella* infections.

We observed significant differences among memory T-cell compartments with regard to T-cell serovar cross-reactivity. *Salmonella Typhi* challenge induced CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>CM</sub> producing IL-2, but only, surprisingly, demonstrating significant cross-reactivity against PA and not against iNTSTy- or PB-infected targets. In some models of infection with intracellular bacteria or parasites, the T<sub>CM</sub> population plays an essential role in host defence.<sup>58,59</sup> Thus, the absence of cross-reactivity against iNTSTy or PB may impact vaccine design. In addition, after *wt S. Typhi* challenge, multifunctional CD8<sup>+</sup> T<sub>EMRA</sub> cells, terminally differentiated effector memory cells implicated in protection mechanisms in viral infections such as HIV and influenza,<sup>60,61</sup> were not significantly cross-reactive against PA, PB or iNTSTy. The clinical significance of the absence of heterologous T-cell responses within these memory compartments is unclear, but demonstrates that T-cell memory subset reactivation is specifically and differentially impacted by serovar, potentially through differences in bacterial phenotype during infection of antigen-presenting cells, or through specific differences in antigens between serovars, leading to differential T-cell subset cross-reactivity.

We show that the predominant multifunctional CD4<sup>+</sup> T<sub>EM</sub> cell response in human blood after *S. Typhi* challenge is a Th1 response, rather than a Th17 response. The induced multifunctional Th1 response is notably cross-reactive against PA, PB and iNTSTy. In contrast, while a multifunctional induced Th17 response occurs against *S. Typhi* after challenge, this response is generally of lower magnitude than the Th1 response and is notably not cross-reactive against iNTSTy, PA or PB. The role of Th17 immunity specifically in human host defence against *S. Typhi* or other typical invasive *Salmonella* strains is unclear. Studies with a nontyphoidal *S. Typhimurium* strain in a SIV-infected macaque model of infection showed importance of Th17 responses in limiting bacterial dissemination from the gut mucosa.<sup>62</sup> In a cohort of Bangladeshi patients with natural *S. Typhi*

infection, T cells reactive with *S. Typhi* antigens were found to express IFN- $\gamma$  early after developing *S. Typhi* bacteraemia, while T cells producing IL-17 reactive with *S. Typhi* antigens were only observed in significant numbers 2–4 weeks after bacteraemia development.<sup>63</sup> Human immunisation with Ty21a generates IL-17A-producing CD4<sup>+</sup> T cells at the terminal ileum mucosa, as well as CD8<sup>+</sup> T cells producing IL-17A at the mucosa and in the blood.<sup>64–66</sup> The dominance of multifunctional CD4<sup>+</sup> Th1 effector memory elicited in the blood in humans after oral *S. Typhi* challenge is consistent with findings demonstrating a prominent IFN transcriptional response in humans infected with *S. Typhi*<sup>67,68</sup> and may impact on the diversity of IFN- $\gamma$ -dependent cytolytic mechanisms against *Salmonella*.<sup>69</sup>

We demonstrate that the magnitude of multifunctional IFN- $\gamma$  CD4<sup>+</sup> T<sub>EM</sub> cell response to *S. Typhi* correlates with induction of cross-reactive multifunctional CD8<sup>+</sup> T<sub>EM</sub> cells in humans. Expansion and maintenance of memory CD8<sup>+</sup> T cells are CD4<sup>+</sup> T-cell-dependent in numerous infection models,<sup>32,70–73</sup> with data supporting an essential role of CD4<sup>+</sup> T cells in aspects of initial memory CD8<sup>+</sup> T-cell priming through factors such as CD27 and CD40 signalling.<sup>74,75</sup> We have previously shown that humans challenged with *S. Typhi* exhibited enhanced CD8<sup>+</sup> T<sub>EM</sub> cell responses *in vitro* when T regulatory cells are depleted during the process of stimulation with *S. Typhi*-infected antigen-presenting cells.<sup>76</sup> Given that a major risk factor in humans for invasive *Salmonella* infection, particularly with the nontyphoidal serovars, is HIV infection and CD4<sup>+</sup> T-cell deficiency, these data underscore one mechanism whereby CD4<sup>+</sup> T-cell responses could impact a critical effector mechanism against invasive *Salmonella* infection in humans.

These data further the hypothesis that CD4<sup>+</sup> T cells enhance immunity to invasive *Salmonella* in humans, at least in part, through priming memory CD8<sup>+</sup> T-cell responses and/or enhancing their activation upon secondary exposure to invasive *Salmonella* antigens. We show here that heightened multifunctional CD4<sup>+</sup> T-cell IFN- $\gamma$  responses after *S. Typhi* challenge correlate specifically with heterologous, serovar-independent CD8<sup>+</sup> T-cell multifunctional responses upon secondary stimulation with invasive *Salmonella* antigens, pointing to the capability of *S. Typhi* in generating heterologous

multifunctional CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in humans. Collectively, these data suggest that *S. Typhi*, as a parent strain for a vaccine for iNTS or *S. Paratyphi* strains, induces cross-reactive multifunctional effector memory CD4<sup>+</sup> T and CD8<sup>+</sup> T-cell responses. However, significant gaps in cross-reactive immune responses are noted in T-cell memory compartments and by specific cellular functionalities, dependent on invasive *Salmonella* strain, demonstrating that ultimately genetic differences between strains impact memory T-cell recognition, activation and signalling against invasive *Salmonella*-infected cells. Particularly for iNTS, these data underscore the importance of strain-specific vaccination to prevent invasive *Salmonella* infection in humans.

## METHODS

### Participants and human challenge model

Data were collected from clinical samples obtained from a human challenge model of typhoid fever (UKCRN ID 9297; approval: Oxfordshire Research Ethics Committee A [10/H604/53], as previously described by Waddington et al.<sup>77</sup> Male or female volunteers, healthy, between ages 18 and 60, were recruited by the Oxford Vaccine Group, and written informed consent was obtained for participation. Individuals were excluded from the study if they had a history of prior *S. Typhi* vaccination and prior typhoid disease, or had resided in an *S. Typhi* endemic area for more than 6 months. Volunteers underwent challenge with a single oral dose of  $\sim 1.98 \times 10^4$  CFU [range ( $1.5 \times 10^4$ – $2.69 \times 10^4$ )] wild-type *S. Typhi* (Quailes strain), suspended in sodium bicarbonate solution, administered after 90 min of fasting. The challenge study was conducted in an ambulatory setting with daily blood culture and daily surveillance for safety, clinical measurements and laboratory measurements. Participants received a two-week course of antibiotics (oral ciprofloxacin) starting immediately at time of diagnosis of typhoid disease (TD) or by 14 days after challenge. TD was defined as either (1) one or more positive blood cultures collected after day 5 postchallenge or (2) sustained fever observed after challenge (temperature above 38°C lasting 12 or more hours). PBMCs from 11 participants who developed TD were randomly selected for further characterisation of memory T-cell immune responses. A summary of demographic and clinical parameters of participants whose samples were used in this study is provided in Supplementary table 1; these parameters are similar to those observed in the broader study cohort as previously described.<sup>77</sup>

### Preparation of effector cells

Peripheral blood mononuclear cells were separated by gradient centrifugation and cryopreserved in liquid nitrogen within 4 h of blood draw. On thawing, cell

viability was assessed by trypan blue exclusion. Cells were rested overnight at 37°C, 5% CO<sub>2</sub> in RPMI containing 100 U mL<sup>-1</sup> penicillin, 100 µg mL<sup>-1</sup> streptomycin, 50 µg mL<sup>-1</sup> gentamicin, 1 mM L-glutamine, 10 mM HEPES buffer and 10% heat-inactivated foetal bovine serum, prior to presentation to infected stimulator cells.

### Preparation of stimulator cells and *Salmonella* infection of stimulator cells

Autologous Epstein–Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCL) were generated from PBMCs from individual volunteers, to ultimately serve as stimulator cells, as previously described.<sup>78</sup> *Salmonella* strains were obtained from University of Maryland-CVD reference stocks: *S. Typhi* strain ISP-1820 (a clinical isolate from Chile, Vi+), *S. Paratyphi* A strain CV 223 (ATCC #9150, Manassas, VA, USA) and *S. Paratyphi* B strain CV 23 (a clinical isolate from Chile), and the *S. Typhimurium* invasive nontyphoidal strain D65, with the ST313 genotype (a clinical isolate from a bacteraemic child in Mali<sup>79</sup>). B-LCL cells were infected with individual *Salmonella* strains (at a 10:1 bacteria:B-LCL cell ratio) in RPMI without antibiotics. Infection proceeded for 3 h, after which time cells were washed in cRPMI, and incubated overnight in the presence of 150 µg mL<sup>-1</sup> gentamicin. To confirm B-LCL infection with *Salmonella* strains, infected B-LCL were stained with FITC-conjugated antibody against *Salmonella* common structural Ag (CSA-1, Kierkegaard & Perry, Gaithersburg, MD, USA) and analysed by flow cytometry.

### Ex vivo stimulation of effector cells

B-LCL-infected stimulator cells were gamma-irradiated (6000 rad) and co-cultured with PBMCs. PBMCs cultured without target cells and with uninfected target cells were used as negative controls, and PBMCs cultured in the presence of Staphylococcal enterotoxin B (SEB) at 10 µg mL<sup>-1</sup> served as a positive control. *Ex vivo* stimulation proceeded in the presence of FITC-conjugated anti-CD107a (BD Biosciences, San Jose, CA, USA). After 2 h, monensin (1 µg mL<sup>-1</sup>) and Brefeldin A (2 µg mL<sup>-1</sup>) were added to cocultures. Cells were incubated overnight and harvested for immunostaining.

### Surface and Intracellular staining, and gating protocol

Peripheral blood mononuclear cells were stained for live/dead discrimination using yellow fluorescent viability dye (YEVID, Invitrogen, Carlsbad, CA, USA) and then washed with buffer (PBS with 2% FCS), and nonspecific Fc receptor binding was blocked by incubation with human immunoglobulin (3 µg mL<sup>-1</sup>; Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature. Cells were then stained extracellularly and intracellularly with a panel of fluorochrome-conjugated monoclonal antibodies as previously described.<sup>24</sup> Cells were fixed in 1% paraformaldehyde and stored at 4°C until analysis. Flow cytometry was performed using an LSRII flow cytometer

(BD), and during sample acquisition, between 300 000 and 500 000 events were collected per sample. Data were analysed using WinList version 9.0 (Verity Software House, Topsham, ME, USA). Singlet CD3<sup>+</sup> CD4<sup>+</sup> T cells or CD3<sup>+</sup> CD8<sup>+</sup> T cells and T memory subsets were evaluated for expression of CD45RA and CD62L for determination of T central memory (T<sub>CM</sub>; CD62L<sup>+</sup> CD45RA<sup>-</sup>), T effector memory (T<sub>EM</sub>; CD62L<sup>-</sup> CD45RA<sup>-</sup>) and T effector memory CD45 RA<sup>+</sup> (T<sub>EMRA</sub>; CD62L<sup>-</sup> CD45 RA<sup>+</sup>) subsets. Naïve T cells (T<sub>N</sub>) were defined as CD62L<sup>+</sup> CD45RA<sup>+</sup>. The FCOM analysis tool was used to characterise events based on selected gate combinations in multidimensional space to determine multifunctional subsets. Flow cytometry experiments were performed at the Flow Cytometry and Mass Cytometry Core Facility of the University of Maryland School of Medicine Center for Innovative Biomedical Resources (CIBR).

## Statistics

All statistical tests were performed with GraphPad Prism 6.0 software (GraphPad Prism, La Jolla, CA, USA). Comparisons between two groups were performed with a paired Student's *t*-test. Pearson's correlations were calculated assuming bivariate Gaussian distributions among variables, and linear regression lines were fit to the data with 95% confidence bands. Statistical significance was accepted for  $P < 0.05$ .

## Study approval

In obtaining the clinical samples used in this study, written informed consent was obtained, and the clinical protocol was approved by National Research Ethics Service, Oxfordshire Research Ethics Committee A (10/H0604/53). The clinical study proceeded in accordance with the International Conference on Harmonisation Good Clinical Practice Guidelines.

## ACKNOWLEDGMENTS

We express our gratitude to the volunteers who participated in the challenge study. We thank Paula Bernal, Regina Harley and Cathy Storrer for technical assistance. This work was supported, in part, by NIAID, NIH, DHHS grants R01-AI036525 and U19-AI082655 (Cooperative Center for Human Immunology [CCHI]) to MBS, U19-AI109776 (Center of Excellence for Translational Research [CETR]) and U19-AI142725 to MML and MBS; NIH K08-AI143923 and the Passano Foundation Clinician-Scientist Award to RRR; fellowship support to SF and RRR through NIH T32-AI007524; a Strategic Translation Award from the Wellcome Trust [grant number 092661] to AJP; and support from the NIHR Oxford Biomedical Research Centre, the Jenner Institute and the Oxford Martin School. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, the National Health Service, the National Institute for Health Research (NIHR) or the UK Department of Health.

## AUTHOR CONTRIBUTIONS

RRR, RW and MBS: Conceptualization; design of the experiments. RRR and SF: Conduction of the experiments. RRR, RW and MBS: Data analysis. SF, JB, TCD, CJ, CSW, MML, AJP and MBS: Contribution to reagents/materials/analysis/tools. RRR, RW, TCD, AJP, MML and MBS: Writing of the paper. TCD, CJ, CSW, MML and AJP: Set up of challenge model; clinical data generation; collection and processing of the PBMCs specimens used in this study.

## CONFLICTS OF INTEREST

AJP is chair of the UK Department of Health's Joint Committee on Vaccination and Immunisation and the European Medicine Agency Scientific Advisory Group on Vaccines, and a member of WHO's Strategic Advisory Group of Experts and an NIHR Senior Investigator. The other authors declare no conflicts of interest.

## REFERENCES

1. Ao TT, Feasey NA, Gordon MA, Keddy KH, Angulo FJ, Crump JA. Global burden of invasive nontyphoidal *Salmonella* disease, 2010(1). *Emerg Infect Dis* 2015; **21**:941–949.
2. Mogasale V, Maskery B, Ochiai RL et al. Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment. *Lancet Glob Health* 2014; **2**: e570–e580.
3. Collaborators GBDN-TSID, Stanaway JD, Parisi A et al. The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect Dis* 2019; **19**: 1312–1324.
4. Feasey NA, Hadfield J, Keddy KH et al. Distinct *Salmonella* Enteritidis lineages associated with enterocolitis in high-income settings and invasive disease in low-income settings. *Nat Genet* 2016; **48**: 1211–1217.
5. Okoro CK, Kingsley RA, Connor TR et al. Intracontinental spread of human invasive *Salmonella* Typhimurium pathovariants in sub-Saharan Africa. *Nat Genet* 2012; **44**: 1215–1221.
6. Kingsley RA, Msefula CL, Thomson NR et al. Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res* 2009; **19**: 2279–2287.
7. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. *Lancet* 2012; **379**: 2489–2499.
8. Haselbeck AH, Panzner U, Im J, Baker S, Meyer CG, Marks F. Current perspectives on invasive nontyphoidal *Salmonella* disease. *Curr Opin Infect Dis* 2017; **30**: 498–503.
9. Holt KE, Thomson NR, Wain J et al. Pseudogene accumulation in the evolutionary histories of *Salmonella enterica* serovars Paratyphi A and Typhi. *BMC Genom* 2009; **10**: 36.

10. Chattopadhyay S, Paul S, Kisiela DI, Linardopoulou EV, Sokurenko EV. Convergent molecular evolution of genomic cores in *Salmonella enterica* and *Escherichia coli*. *J Bacteriol* 2012; **194**: 5002–5011.
11. Prager R, Rabsch W, Streckel W, Voigt W, Tietze E, Tschape H. Molecular properties of *Salmonella enterica* serotype paratyphi B distinguish between its systemic and its enteric pathovars. *J Clin Microbiol* 2003; **41**: 4270–4278.
12. Klemm EJ, Shakoor S, Page AJ et al. Emergence of an Extensively Drug-resistant *Salmonella enterica* Serovar Typhi clone harboring a promiscuous plasmid encoding resistance to fluoroquinolones and third-generation cephalosporins. *MBio* 2018; **9**: e00105-18.
13. Levine MM, Simon R. The gathering storm: is untreatable typhoid fever on the way? *MBio* 2018; **9**: e00482-18.
14. Crump JA, Mintz ED. Global trends in typhoid and paratyphoid Fever. *Clin Infect Dis* 2010; **50**: 241–246.
15. Kariuki S, Gordon MA, Feasey N, Parry CM. Antimicrobial resistance and management of invasive *Salmonella* disease. *Vaccine* 2015; **33**(Suppl 3): C21–C29.
16. Kariuki S, Onsare RS. Epidemiology and genomics of invasive nontyphoidal *Salmonella* Infections in Kenya. *Clin Infect Dis* 2015; **61**(Suppl 4): S317–S324.
17. Holt KE, Thomson NR, Wain J et al. Multidrug-resistant *Salmonella enterica* serovar paratyphi A harbors IncHI1 plasmids similar to those found in serovar typhi. *J Bacteriol* 2007; **189**: 4257–4264.
18. Feasey NA, Masesa C, Jassi C et al. Three epidemics of invasive multidrug-resistant *Salmonella* bloodstream infection in Blantyre, Malawi, 1998–2014. *Clin Infect Dis* 2015; **61**(Suppl 4): S363–S371.
19. MacLennan CA, Gondwe EN, Msefula CL et al. The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children. *J Clin Invest* 2008; **118**: 1553–1562.
20. Gilchrist JJ, Rautanen A, Fairfax BP et al. Risk of nontyphoidal *Salmonella* bacteraemia in African children is modified by STAT4. *Nat Commun* 2018; **9**: 1014.
21. Dunstan SJ, Hue NT, Han B et al. Variation at HLA-DRB1 is associated with resistance to enteric fever. *Nat Genet* 2014; **46**: 1333–1336.
22. MacLennan C, Fieschi C, Lammas DA et al. Interleukin (IL)-12 and IL-23 are key cytokines for immunity against *Salmonella* in humans. *J Infect Dis* 2004; **190**: 1755–1757.
23. Pham OH, McSorley SJ. Protective host immune responses to *Salmonella* infection. *Future Microbiol* 2015; **10**: 101–110.
24. Fresnay S, McArthur MA, Magder L et al. *Salmonella* Typhi-specific multifunctional CD8<sup>+</sup> T cells play a dominant role in protection from typhoid fever in humans. *J Transl Med* 2016; **14**: 62.
25. Szein MB, Tanner MK, Polotsky Y, Orenstein JM, Levine MM. Cytotoxic T lymphocytes after oral immunization with attenuated vaccine strains of *Salmonella* typhi in humans. *J Immunol* 1995; **155**: 3987–3993.
26. Salerno-Goncalves R, Pasetti MF, Szein MB. Characterization of CD8<sup>+</sup> effector T cell responses in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. *J Immunol* 2002; **169**: 2196–2203.
27. Szein MB. Is a Human CD8 T-cell vaccine possible, and if so, what would it take? CD8 T-cell-mediated protective immunity and vaccination against enteric bacteria. *Cold Spring Harb Perspect Biol* 2018; **10**: a029546.
28. Crump JA, Ramadhani HO, Morrissey AB et al. Invasive bacterial and fungal infections among hospitalized HIV-infected and HIV-uninfected adults and adolescents in northern Tanzania. *Clin Infect Dis* 2011; **52**: 341–348.
29. Simanjuntak CH, Paleologo FP, Punjabi NH et al. Oral immunisation against typhoid fever in Indonesia with Ty21a vaccine. *Lancet* 1991; **338**: 1055–1059.
30. Levine MM, Ferreccio C, Black RE, Lagos R, San Martin O, Blackwelder WC. Ty21a live oral typhoid vaccine and prevention of paratyphoid fever caused by *Salmonella enterica* Serovar Paratyphi B. *Clin Infect Dis* 2007; **45** (Suppl 1): S24–S28.
31. Wahid R, Fresnay S, Levine MM, Szein MB. Immunization with Ty21a live oral typhoid vaccine elicits crossreactive multifunctional CD8<sup>+</sup> T-cell responses against *Salmonella enterica* serovar Typhi, S. Paratyphi A, and S. Paratyphi B in humans. *Mucosal Immunol* 2015; **8**: 1349–1359.
32. Rocha B, Tanchot C. Towards a cellular definition of CD8<sup>+</sup> T-cell memory: the role of CD4<sup>+</sup> T-cell help in CD8<sup>+</sup> T-cell responses. *Curr Opin Immunol* 2004; **16**: 259–263.
33. Ramachandran G, Perkins DJ, Schmidlein PJ, Tulapurkar ME, Tennant SM. Invasive *Salmonella Typhimurium* ST313 with naturally attenuated flagellin elicits reduced inflammation and replicates within macrophages. *PLoS Negl Trop Dis* 2015; **9**: e3394.
34. Carden SE, Walker GT, Honeycutt J et al. Pseudogenization of the secreted effector gene ssel confers rapid systemic dissemination of *S. Typhimurium* ST313 within migratory dendritic cells. *Cell Host Microbe* 2017; **21**: 182–194.
35. Sabbagh SC, Forest CG, Lepage C, Leclerc JM, Daigle F. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS Microbiol Lett* 2010; **305**: 1–13.
36. Hormaeche CE, Mastroeni P, Harrison JA, Demarco de Hormaeche R, Svenson S, Stocker BA. Protection against oral challenge three months after i.v. immunization of BALB/c mice with live Aro *Salmonella typhimurium* and *Salmonella enteritidis* vaccines is serotype (species)-dependent and only partially determined by the main LPS O antigen. *Vaccine* 1996; **14**: 251–259.
37. Napolitani G, Kurupati P, Teng KWW et al. Clonal analysis of *Salmonella*-specific effector T cells reveals serovar-specific and cross-reactive T cell responses. *Nat Immunol* 2018; **19**: 742–754.
38. Lindgren T, Ahlm C, Mohamed N, Evander M, Ljunggren HG, Bjorkstrom NK. Longitudinal analysis of the human T cell response during acute hantavirus infection. *J Virol* 2011; **85**: 10252–10260.
39. Wilkinson TM, Li CK, Chui CS et al. Preexisting influenza-specific CD4<sup>+</sup> T cells correlate with disease protection against influenza challenge in humans. *Nat Med* 2012; **18**: 274–280.

40. Heidema J, Rossen JW, Lukens MV et al. Dynamics of human respiratory virus-specific CD8<sup>+</sup> T cell responses in blood and airways during episodes of common cold. *J Immunol* 2008; **181**: 5551–5559.
41. Hillaire ML, van Trierum SE, Bodewes R et al. Characterization of the human CD8<sup>+</sup> T cell response following infection with 2009 pandemic influenza H1N1 virus. *J Virol* 2011; **85**: 12057–12061.
42. Atif SM, Winter SE, Winter MG, McSorley SJ, Baumler AJ. *Salmonella enterica* serovar Typhi impairs CD4 T cell responses by reducing antigen availability. *Infect Immun* 2014; **82**: 2247–2254.
43. Nickerson KP, Senger S, Zhang Y et al. *Salmonella* Typhi colonization provokes extensive transcriptional changes aimed at evading host mucosal immune defense during early infection of human intestinal tissue. *EBioMedicine* 2018; **31**: 92–109.
44. Wilson RP, Raffatellu M, Chessa D, Winter SE, Tukel C, Baumler AJ. The Vi-capsule prevents Toll-like receptor 4 recognition of *Salmonella*. *Cell Microbiol* 2008; **10**: 876–890.
45. Raffatellu M, Chessa D, Wilson RP, Dusold R, Rubino S, Baumler AJ. The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. *Infect Immun* 2005; **73**: 3367–3374.
46. Snyder LD, Chan C, Kwon D et al. Polyfunctional T-cell signatures to predict protection from cytomegalovirus after lung transplantation. *Am J Respir Crit Care Med* 2016; **193**: 78–85.
47. Betts MR, Nason MC, West SM et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8<sup>+</sup> T cells. *Blood* 2006; **107**: 4781–4789.
48. Harari A, Dutoit V, Cellerai C, Bart PA, Du Pasquier RA, Pantaleo G. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol Rev* 2006; **211**: 236–254.
49. Hernandez DM, Valderrama S, Gualtero S et al. Loss of T-cell multifunctionality and TCR-V B repertoire against EPSTEIN-Barr virus is associated with worse prognosis and clinical parameters in HIV<sup>+</sup> patients. *Front Immunol* 2018; **9**: 2291.
50. Arroyo L, Rojas M, Franken KL, Ottenhoff TH, Barrera LF. Multifunctional T cell response to DosR and Rpf antigens is associated with protection in long-term *Mycobacterium tuberculosis*-infected individuals in Colombia. *Clin Vaccine Immunol* 2016; **23**: 813–824.
51. Egui A, Ledesma D, Perez-Anton E et al. Phenotypic and functional profiles of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells associated with infection control in patients with cutaneous leishmaniasis. *Front Cell Infect Microbiol* 2018; **8**: 393.
52. George PJ, Anuradha R, Kumar NP et al. Helminth infections coincident with active pulmonary tuberculosis inhibit mono- and multifunctional CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in a process dependent on IL-10. *PLoS Pathog* 2014; **10**: e1004375.
53. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM. Virus-specific CD4<sup>+</sup> memory-phenotype T cells are abundant in unexposed adults. *Immunity* 2013; **38**: 373–383.
54. Mastroeni P, Villarreal-Ramos B, Hormaeche CE. 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* 1992; **13**: 477–491.
55. Burel JG, Apte SH, Groves PL, McCarthy JS, Doolan DL. Polyfunctional and IFN- $\gamma$  monofunctional human CD4<sup>+</sup> T cell populations are molecularly distinct. *JCI Insight* 2017; **2**: e87499.
56. Wahid R, Salerno-Goncalves R, Tacket CO, Levine MM, Sztein MB. 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. *Mucosal Immunol* 2008; **1**: 389–398.
57. Wahid R, Fresnay S, Levine MM, Sztein MB. Cross-reactive multifunctional CD4<sup>+</sup> T cell responses against *Salmonella enterica* serovars Typhi, Paratyphi A and Paratyphi B in humans following immunization with live oral typhoid vaccine Ty21a. *Clin Immunol* 2016; **173**: 87–95.
58. Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. *Nat Med* 2004; **10**: 1104–1110.
59. Vogelzang A, Perdomo C, Zedler U et al. Central memory CD4<sup>+</sup> T cells are responsible for the recombinant Bacillus Calmette-Guerin  $\Delta$ ureC:hly vaccine's superior protection against tuberculosis. *J Infect Dis* 2014; **210**: 1928–1937.
60. Northfield JW, Loo CP, Barbour JD et al. Human immunodeficiency virus type 1 (HIV-1)-specific CD8<sup>+</sup> T (EMRA) cells in early infection are linked to control of HIV-1 viremia and predict the subsequent viral load set point. *J Virol* 2007; **81**: 5759–5765.
61. Dunne PJ, Faint JM, Gudgeon NH et al. Epstein-Barr virus-specific CD8<sup>+</sup> T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. *Blood* 2002; **100**: 933–940.
62. Raffatellu M, Santos RL, Verhoeven DE et al. Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes *Salmonella* dissemination from the gut. *Nat Med* 2008; **14**: 421–428.
63. Bhuiyan S, Sayeed A, Khanam F et al. Cellular and cytokine responses to *Salmonella enterica* serotype Typhi proteins in patients with typhoid fever in Bangladesh. *Am J Trop Med Hyg* 2014; **90**: 1024–1030.
64. McArthur MA, Sztein MB. Heterogeneity of multifunctional IL-17A producing S. Typhi-specific CD8<sup>+</sup> T cells in volunteers following Ty21a typhoid immunization. *PLoS One* 2012; **7**: e38408.
65. Booth JS, Goldberg E, Patil SA, Barnes RS, Greenwald BD, Sztein MB. Effect of the live oral attenuated typhoid vaccine, Ty21a, on systemic and terminal ileum mucosal CD4<sup>+</sup> T memory responses in humans. *Int Immunol* 2019; **31**: 101–116.
66. Booth JS, Goldberg E, Patil SA, Greenwald BD, Sztein MB. Association between S. Typhi-specific memory CD4<sup>+</sup> and CD8<sup>+</sup> T responses in the terminal ileum mucosa and in peripheral blood elicited by the live oral typhoid vaccine Ty21a in humans. *Hum Vaccin Immunother* 2019; **15**: 1409–1420.
67. Blohmke CJ, Darton TC, Jones C et al. Interferon-driven alterations of the host's amino acid metabolism in the pathogenesis of typhoid fever. *J Exp Med* 2016; **213**: 1061–1077.

68. Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova JL. IL-12 and IFN- $\gamma$  in host defense against mycobacteria and salmonella in mice and men. *Curr Opin Immunol* 1999; **11**: 346–351.
69. Ingram JP, Tursi S, Zhang T et al. A nonpyroptotic IFN- $\gamma$ -triggered cell death mechanism in nonphagocytic cells promotes *Salmonella* clearance *in vivo*. *J Immunol* 2018; **200**: 3626–3634.
70. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 2003; **300**: 339–342.
71. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4<sup>+</sup> T cells are required for secondary expansion and memory in CD8<sup>+</sup> T lymphocytes. *Nature* 2003; **421**: 852–856.
72. Ballesteros-Tato A, Leon B, Lee BO, Lund FE, Randall TD. Epitope-specific regulation of memory programming by differential duration of antigen presentation to influenza-specific CD8<sup>+</sup> T cells. *Immunity* 2014; **41**: 127–140.
73. Rapetti L, Meunier S, Pontoux C, Tanchot C. CD4 help regulates expression of crucial genes involved in CD8 T cell memory and sensitivity to regulatory elements. *J Immunol* 2008; **181**: 299–308.
74. Borrow P, Tishon A, Lee S et al. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8<sup>+</sup> CTL response. *J Exp Med* 1996; **183**: 2129–2142.
75. Hendriks J, Gravestien LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol* 2000; **1**: 433–440.
76. McArthur MA, Fresnay S, Magder LS et al. Activation of *Salmonella* Typhi-specific regulatory T cells in typhoid disease in a wild-type *S. Typhi* challenge model. *PLoS Pathog* 2015; **11**: e1004914.
77. Waddington CS, Darton TC, Jones C et al. An outpatient, ambulant-design, controlled human infection model using escalating doses of *Salmonella* Typhi challenge delivered in sodium bicarbonate solution. *Clin Infect Dis* 2014; **58**: 1230–1240.
78. Salerno-Goncalves R, Fernandez-Vina M, Lewinsohn DM, Sztein MB. Identification of a human HLA-E-restricted CD8<sup>+</sup> T cell subset in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. *J Immunol* 2004; **173**: 5852–5862.
79. Tennant SM, Diallo S, Levy H et al. Identification by PCR of non-typhoidal *Salmonella enterica* serovars associated with invasive infections among febrile patients in Mali. *PLoS Negl Trop Dis* 2010; **4**: e621.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.



This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.