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# **Clonal analysis of Typhoidal *Salmonella* specific effector CD4<sup>+</sup> T cells in challenged human volunteers reveals immunodominant Serovar specific and cross-reactive T cell responses**

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## **Abstract**

The development of optimal T cell vaccines against bacteria is hampered by the complexity of dissecting cross-reactive and pathogen specific T cell responses against related bacterial species. To address this challenge, we used a controlled human infection model of *Salmonella enterica* serovar Typhi (*S. Typhi*) or *S. Paratyphi A* to undertake the first characterization at the single cell level of the bacterial specific and cross-reactive repertoire of human effector CD4<sup>+</sup> T cells elicited in response to a bacterial pathogen.

By using mass cytometry, unbiased single cell cloning, live fluorescent barcoding, and TCR sequencing, we reconstructed the *Salmonella* specific repertoire of effector CD4<sup>+</sup> T cells, isolated from the peripheral blood of challenged volunteers.

Along with antigen specific responses broadly cross-reactive against distantly related *Salmonella* serovars, we described the expansion of clonotypes selectively recognizing *S. Typhi* or *S. Paratyphi*. A large proportion of these clonotypes were focused against two immunodominant antigens solely expressed by *S. Typhi* or *S. Paratyphi A*: hemolysin E (HlyE) and the catalytic component of the typhoid toxin cytolethal distending toxin (CdtB). Furthermore, we identified an additional immunodominant antigen, the non specific acid-phosphatase PhoN, recognized by effector T cells cross-reactive against typhoidal and non typhoidal serovars such as *S. Typhimurium* and *S. Enteritidis*. Finally, we found that single amino acid variations in CdtB and PhoN, lead to the accumulation of T cells failing to cross-react against the different serovars, demonstrating how minor sequence variations in a complex microorganism can shape the pathogen specific T cell repertoire. Our results identify immunodominant serovar specific and cross-reactive T cell antigens, which will help the design of T cell vaccination strategies against *Salmonella*.

## Introduction

Optimal design of pathogen specific T cell vaccines requires in depth characterization of the frequency, phenotype and specificity of human T cells responding to invading pathogens, as this knowledge underscores our understanding of immunodominant T cell responses and identification of correlates of protection and immunogenicity. Dissecting the repertoire of antigen specific T cell responses to bacteria has been hampered by the relatively large number of proteins expressed by bacteria, and by the presence of cross-reactive T cell responses as a consequence of the large degree of sequence homology between related bacterial species.

An example of human disease caused by closely related bacteria is enteric fever (also known as typhoid fever), a relatively common disease in developing countries, causing over 20 million cases and 150,000 deaths annually <sup>1, 2, 3</sup>. The main causative agents of enteric fever are *Salmonella* Typhi (*S. Typhi*) and *Salmonella* Paratyphi A (*S. Paratyphi A*), collectively referred to as typhoidal *Salmonella* serovars of the gastrointestinal pathogen *Salmonella enterica*. *S. Typhi* and *S. Paratyphi A* are human restricted, and have evolved the capacity to spread systemically in apparently uncompromised hosts, causing life threatening infections <sup>4</sup>. In comparison, non-typhoidal *Salmonella* serovars (NTS), such as *Salmonella* Typhimurium (*S. Typhimurium*) and *Salmonella* Enteritidis (*S. Enteritidis*), typically cause a localized gastrointestinal infection in immunocompetent hosts <sup>5</sup>. *S. Typhi* and *Paratyphi A* share an estimated ~95% of their proteome and ~85% with *S. Typhimurium* <sup>6, 7</sup>. The genetic differences distinguishing the typhoidal serovars from *S. Typhimurium* involve both the loss of function of a large number of genes, alongside the acquisition of novel genes through horizontal gene transfer <sup>8, 4</sup>. Currently there are no vaccines capable of conferring protection against both *S. Typhi* and *S. Paratyphi A*. Licensed vaccines against enteric fever include the live attenuated *S. Typhi*-strain Ty21a, and plain polysaccharide or protein-polysaccharide conjugated vaccines eliciting antibody responses against the Vi polysaccharide capsule, a virulence factor present on *S. Typhi*, but not *S. Paratyphi A* <sup>9, 10</sup>.

The design of vaccination strategies capable of protecting against different Serovars requires a better understanding of the impact of pathogen genetic variation/divergence on pathogen specific responses elicited during infection with the different *Salmonella* serovars, and the identification of serovar specific antigens and epitopes, which could guarantee appropriate coverage.

Both B and T cell responses are known to contribute to protection in mouse models of *Salmonella* infection and vaccination. While B cells can confer the first line of protection against mucosal invasion and systemic dissemination, T cells are needed for the efficient clearance of *Salmonella*<sup>11</sup>. In particular, CD4<sup>+</sup> T cells play a major protective role in mouse models of *S. Typhimurium* infection<sup>12</sup>, with CD8<sup>+</sup> T cells not essential for acquired immunity to this pathogen<sup>13</sup>. Additionally, genome wide association studies have shown that expression of specific MHC class II alleles confer resistance to enteric fever, suggesting a protective function of CD4<sup>+</sup> T cells in the control of *S. Typhi* and *S. Paratyphi A* infection in humans<sup>14</sup>. The characterization of T cell responses specific to typhoidal *Salmonella* is challenging, as humans are the only known natural host of *S. Typhi* and *Paratyphi A*.

To address this issue, we have recently established a human infection model of enteric fever in healthy adult volunteers based on earlier challenge studies<sup>15,16</sup>.

In this study, we aimed to characterize *Salmonella* specific CD4<sup>+</sup> T cells based on their capacity to cross-react or not against distinct *Salmonella* Serovars, and to identify antigens capable of mediating this cross-reactivity or lack thereof.

This requires: *i*) precise knowledge of the primary sequence of immunogenic proteins and pathogen's proteome; *ii*) samples collected at the peak of the immune response from infected individuals with no other concurrent infections; *iii*) accurate identification of effector T cells responding to immunization/infection; *iv*) the possibility to test individual T cells for their capacity of recognizing target cells infected with distinct bacteria.

In order to meet the first and second requirements, we used samples collected from volunteers experimentally challenged with *Salmonella Typhi* and *Paratyphi A*. Next, we used mass cytometry to identify a population of effector of CD38<sup>+</sup>CCR7<sup>-</sup>IFN- $\gamma$ <sup>+</sup> Mip1 $\beta$ <sup>+</sup> CD4<sup>+</sup> T cell differentiating in the peripheral blood of volunteers with enteric fever, and finally we performed unbiased single T cell cloning to reconstruct *in-vitro* the antigen specific repertoire of this population of effector T cells.

With this approach, we were capable of determining the proportion of individual effector T cell developing during *Salmonella* infection, which were recognizing distinct serovars and specific bacterial antigens. We showed in great depth that the antigen specific repertoire of CD4<sup>+</sup> T cells activated during *Salmonella* infection includes both broadly cross-reactive as well as serovar-specific T cell clones. Furthermore, we could demonstrate that, in the face of the remarkable antigenic complexity of *Salmonella*, a few immuno-dominant antigens, as well as single amino acid differences in shared antigens account for a major proportion of the serovar

specific CD4<sup>+</sup> T cell clones. These findings pave the way for the design of vaccination strategies capable of eliciting serovar specific and cross-reactive T cell responses, and for the development of diagnostic tools to assess T cell immunity induced upon natural immunity or vaccination against distinct *Salmonella* serovars.

## Results

### **Phenotypic and functional characterization of CD4<sup>+</sup> T cell responding to enteric fever.**

T cell responses against acute infections are usually associated with the emergence of a population of proliferating effector cells in peripheral blood. To identify circulating CD4<sup>+</sup> effector T cells during *Salmonella* infection, we utilized mass cytometry to perform deep phenotypic profiling of CD4<sup>+</sup> T cells in frozen peripheral blood mononuclear cells (PBMCs) collected from human volunteers with a confirmed diagnosis of enteric fever, initially focusing upon experimental infection with live *S. Paratyphi A*.

PBMCs were collected and frozen at baseline (D0) and after challenge at multiple time points covering the different phases of infection: incubation (four days after challenge), enteric fever (2-4 days after diagnosis), resolution of infection (28 and 90 days after challenge), (**Fig. 1a**). Frozen PBMCs were thawed and either stained with a panel of metal conjugated antibodies directed against a wide range of phenotypic, proliferation and homing markers (**Supplementary Methods Table S1-Proliferation and Trafficking Panel**), or stimulated with Phorbol 12-myristate 13-acetate (PMA) and Ionomycin, and stained with a second mass cytometry panel of antibodies capable of detecting the expression of different cytokines and activation markers (**Supplementary Methods Table S1-Function and Trafficking Panel**).

To identify T cells proliferating during the different phases of infection, we measured the frequency of CD4<sup>+</sup> T cells expressing the intracellular marker of proliferation Ki67 in samples stained with the Proliferation and Trafficking antibody panel. At baseline, all volunteers showed a low, but detectable, frequency of Ki67<sup>+</sup>CD4<sup>+</sup> T cells (median=0.52%, max=0.82%, min=0.17%, **Supplementary Methods Fig. 1 and Fig. 1b**). Ki67<sup>+</sup> T cells remained rare 4 days after challenge, but their frequency transiently increased in samples

collected after diagnosis of enteric fever (median=1.51%, max=1.76%, min=0.94%), suggestive of an accumulation of proliferating T cells upon infection.

The observed accumulation of proliferating T cells might be consequence of either a general enhanced proliferation of T cells due to the systemic inflammatory response, or of a specific T cell response against antigens released during *Salmonella* infection. To discriminate between these two possibilities, we focused our analysis on the phenotype of Ki67<sup>+</sup> T cells, and asked whether their expansion was associated to the selective accumulation of a phenotypically distinct subset of effector T cells, or to an overall increase in T cell proliferation. To answer this question, we used Phenograph<sup>17</sup>, an algorithm developed to define phenotypes in high-dimensional single-cell data, to perform unsupervised clustering of Ki67<sup>+</sup> T cells identified in all different volunteers across the distinct phases of *S. Paratyphi A* infection (**Fig. 1c**). PhenoGraph assigned Ki67<sup>+</sup> T cells to 20 different subsets characterized by distinct phenotypic profiles, which frequency differed among volunteers and across the different sampling points. Non-hierarchical clustering of Ki67<sup>+</sup> cells identified in the different samples, revealed that samples collected after enteric fever diagnosis showed an increase in the proportion of cells within subset 19, which frequency increased from a median of 4.1% of Ki67<sup>+</sup> T cells (and of 0.02% of total CD4<sup>+</sup> T cells) at time of challenge to a median of 36.2 % of Ki67<sup>+</sup> T cells (0.47% of CD4<sup>+</sup> T cells) at the peak of disease (**Fig. 1d and Supplementary Fig. 1a and 1b**). T cells within subset 19 were characterized by a relatively higher expression of the activation markers CD38 and ICOS, of CD127 and of homing receptors capable of driving migration to the site of Th1 inflammatory responses (CXCR3 and CCR5) and mucosal tissues (Integrin  $\beta$ 7 and CD49d). In parallel, these T cells expressed lower amount of skin and Th2 homing markers such as CLA, and CCR4, respectively, of the lymph-node homing marker CCR7 and of the survival factor BCL2 (**Fig. 1e and Supplementary Fig. 1c**).

Next, we searched for surface markers, which could identify cells from subset 19 within total CD4<sup>+</sup> T cells. We noticed that cells within this subset were characterized by elevated expression of CD38 and low expression of CCR7 (**Fig. 1e and Supplementary Fig. 2a**) and could be defined as CD38<sup>+</sup>CCR7<sup>-</sup>. Three lines of evidence suggest that CD38<sup>+</sup>CCR7<sup>-</sup> cells might recapitulate subset 19 within total CD4<sup>+</sup> cells, and identify a subset highly enriched in bona fide effector T cells responding to *Salmonella* infection: 1) within total CD4<sup>+</sup> T cells, CD38<sup>+</sup>CCR7<sup>-</sup> were rare at baseline and after resolution of the infection, but their frequency increased after the onset of clinical symptoms and bacteremia (**Fig. 1f-g**), 2) although cluster 7, 8, 15 and 20 also contained CD38<sup>+</sup>CCR7<sup>-</sup> T cells, their frequency accounted only for a minor proportion of Ki67<sup>+</sup> T cells in enteric fever samples (**Supplementary Fig. 2b**); 3) while the frequency of Ki67<sup>+</sup>CCR7<sup>-</sup>CD38<sup>+</sup> cells within total CD4<sup>+</sup> T cells significantly increased after diagnosis, the frequency of Ki67<sup>+</sup> T cells, not included within this subset, only marginally increased (**Supplementary Fig. 2c**).

We next aimed to confirm these findings using multicolor flow cytometry on fresh blood samples collected from volunteers challenged with *S. Typhi* and *S. Paratyphi A*. As expected, a marked accumulation of CCR7<sup>-</sup>CD38<sup>+</sup> T cells was observed in the peripheral blood of challenged volunteers who developed enteric fever upon challenge with *S. Typhi* or *S. Paratyphi A* (**Supplementary Fig. 3a,b**).

### **Functional characterization of CD4<sup>+</sup>CCR7<sup>-</sup>CD38<sup>+</sup> T cells induced during enteric fever**

In order to characterize the functional profile of CD4<sup>+</sup> T cells responding to *Salmonella* infection, we used CCR7 and CD38 to identify effector T cells in PBMC stimulated with the polyclonal stimuli PMA and Ionomycin, and stained with the Function and Trafficking mass cytometry panel (**Table S1-Function and Trafficking Panel**). Although activation with

PMA/Ionomycin reduces cell viability and thus the possibility of detecting rare and “unfit” subsets such as BCL2<sup>-</sup> T cells, we were able to detect sufficient numbers of CD38<sup>+</sup>CCR7<sup>-</sup> T cells to evaluate their functional profile in 4 out of the 6 tested volunteers.

CCR7<sup>-</sup>CD38<sup>+</sup> T cells were enriched in cells capable of producing IFN- $\gamma$ , MIP-1 $\beta$  and TNF, and expressing CD40L and CTLA4 (**Fig. 2a-b**). To understand whether CCR7<sup>-</sup>CD38<sup>+</sup> effector T cells represent a functionally homogeneous subset of CD4<sup>+</sup> T cells endowed with specific effector functions (compared to the total pool of non-naïve CD4<sup>+</sup> T cells), we performed t-distributed stochastic neighbor embedding (tSNE) to map in a bidimensional plot the heterogeneity of antigen experienced CD45RA<sup>-</sup> T cells based on their capacity to express different cytokines and functional markers (**Fig. 2c**). In contrast to the total pool of non-naïve CD45RA<sup>-</sup>CD4<sup>+</sup> T cells, a large proportion of CCR7<sup>-</sup>CD38<sup>+</sup> T cells was concentrated in a small region of this bi-dimensional map enriched in cells capable of producing MIP1- $\beta$  and IFN- $\gamma$  (**Fig. 2d-e**).

Thus, this Mass Cytometry analysis revealed that *Salmonella* infection is associated with the release in peripheral blood of a functionally homogeneous population of IFN- $\gamma$  Mip-1 $\beta$  producing CCR7<sup>-</sup>CD38<sup>+</sup> CD4<sup>+</sup> effector T cells, endowed with the capacity to migrate to mucosal tissues, potentially through the expression of the integrins CD49d and Integrin  $\beta$ 7 and to sites of Th1 inflammatory responses, attracted by agonists of CCR5 and CXCR3.

### **Cloning of CCR7<sup>-</sup> CD38<sup>+</sup> effector CD4<sup>+</sup> T cells to characterize the repertoire of *Salmonella* specific T cells.**

We next developed a single cell approach to dissect the antigen specific repertoire of effector CCR7<sup>-</sup>CD38<sup>+</sup> CD4<sup>+</sup> T cells (**Fig. 3**), and determine the proportion of individual effector T cell responding to distinct serovars. Briefly, CCR7<sup>-</sup>CD38<sup>+</sup> CD4<sup>+</sup> T cells, isolated from volunteers

after diagnosis of enteric fever, were sorted as single cells, expanded *in vitro* in the presence of phytohemagglutinin (PHA) and IL-2 for at least 3 weeks, and tested for their capacity of recognizing autologous EBV transformed lymphoblastoid cell lines (EBV-LCL) infected with live *Salmonella* (**Fig. 3a**). To minimize experimental variability when comparing different T cell clones and enable high throughput screening of T cell clone specificity, we developed a fluorescence cellular barcoding approach<sup>18</sup> to perform short-term antigen specific stimulation of distinct T cell clones in a single tube (**Fig. 3b and Supplementary Methods Fig. 2**). CD4<sup>+</sup> T cell clones generated from a single volunteer were labelled with distinct dilutions of up to four different fluorescent dyes, pooled, and stimulated with autologous EBV-LCL pulsed with different *Salmonella* serovars, strains or antigens. Then, we used multicolor flow cytometry to discriminate T cell clones by their specific fluorescent barcoding, and IFN- $\gamma$  intracellular cytokine staining to identify the pathogen specific T cell clones.

Analysis of libraries of T cell clones generated from CCR7<sup>+</sup>CD38<sup>+</sup> and CCR7<sup>-</sup>CD38<sup>-</sup> T cells (**Fig. 3c**) isolated from two volunteers infected with *S. Typhi* (T1 and T2), showed that libraries generated from CCR7<sup>-</sup>CD38<sup>+</sup> T cells were enriched in T cell clones capable of producing IFN- $\gamma$  in the presence of autologous EBV LCL infected with *S. Typhi* (Quailes, and Ty21a vaccine strain), as compared with CCR7<sup>-</sup>CD38<sup>-</sup> T cells, (**Fig. 3c,d**), consistent with the notion that CCR7<sup>-</sup>CD38<sup>+</sup> circulating T cells during enteric fever are enriched in *Salmonella* specific CD4<sup>+</sup> T cells.

Libraries of CCR7<sup>-</sup>CD38<sup>+</sup> T cell clones were generated from 4 further volunteers challenged with *S. Typhi* (T3, T4, T5 and T6) and 2 volunteers challenged with *S. Paratyphi A* (P1 and P2), and were tested for the presence *S. Typhi* or *S. Paratyphi A* specific T cells, respectively. These new libraries from CCR7<sup>-</sup>CD38<sup>+</sup> T cells were found to contain variable amounts of T cell clones specific for the pathogen used for the *in vivo* challenge (min=20%, max=86%) (**Fig. 3e,f**).

Next, we compared these T cell clones for their capacity of recognizing target cells infected with the two typhoidal serovars *S. Typhi* and *S. Paratyphi A*, and with the non-typhoidal serovar *S. Typhimurium*. Between 35 and 71% of the T cell clones generated from all volunteers were cross-reactive against autologous EBV-LCL infected with either *S. Typhi* (Ty21a, Quailles or BRD948 strains), *S. Paratyphi A* (NVGH308 strain) or *S. Typhimurium* (D25380, LT2 strains) (**Fig. 4a,b-d**). However, in each volunteer we identified T cell clones not cross-reactive against *S. Typhimurium*, but capable of recognizing autologous EBV-LCL infected with *S. Typhi*, *S. Paratyphi A* or both (**Fig. 4a and e**).

In conclusion, the analysis of CD38<sup>+</sup>CCR7<sup>-</sup> T cells clones isolated and expanded from volunteers with enteric fever revealed that the antigen specific repertoire of CD38<sup>+</sup>CCR7<sup>-</sup> T cells in response to *S. Typhi* and *Paratyphi* infection was enriched in *Salmonella* specific T cells, and comprised both serovar specific and cross-reactive T cells.

### **HlyE and CdtB specific CD4<sup>+</sup> T cells account for a large fraction of the typhoidal specific T cell clones.**

The results of the previous experiments underscored how the analysis of effector CD38<sup>+</sup>CCR7<sup>-</sup> CD4<sup>+</sup> T cell clones gives the opportunity of reconstructing *in-vitro* the heterogeneity of the CD4<sup>+</sup> T cell response, discriminating individual T cells based on their ability to cross-react or not with cells infected with distinct serovars. We reasoned that the same approach could be used to identify antigens capable of mediating this cross-reactivity or lack thereof, and determine the proportion of effector T cells recognizing these antigens within the total of the *Salmonella* specific T cells. Hemolysin E (HlyE) and cytolethal distending toxin B (CdtB), a component of typhoid toxin<sup>8, 19</sup>, have been characterized as toxins expressed by the typhoidal serovars *S. Typhi* and *S. Paratyphi A*, but predominantly not by non-typhoidal serovars<sup>20, 21</sup>. In particular, CdtB/typhoid toxin has been proposed as key virulence factor responsible for some

of the symptoms associated with enteric fever<sup>8, 22</sup>. Elevated antibody titers against these two toxins have been detected in patients with enteric fever, but not in healthy controls from non-endemic areas<sup>23</sup>. We designed two peptide pools encompassing the entire protein sequences of HlyE and CdtB in *S. Typhi* and *S. Paratyphi A* and asked whether these two antigens might represent targets for the typhoid and paratyphoid specific fraction of CCR7<sup>-</sup>CD38<sup>+</sup>CD4<sup>+</sup> T cell clones. We detected HlyE specific CD4<sup>+</sup> T cell clones in 6 and CdtB specific CD4<sup>+</sup> T cell clones in 5 out of the 8 volunteers analysed. As expected, T cell clones reactive against HlyE and CdtB were not capable of recognizing cells infected with *S. Typhimurium* (**Fig. 5a,b**) and, where present, CdtB specific T cell clones comprised from 9% to 40%, while HlyE specific T cell clones from 11% to 80% of the typhoidal specific T cell fraction (**Fig. 5c**). ELISPOT analysis on fresh PBMC collected after completion of the antibiotic treatment in challenged volunteers (28 days after challenge) confirmed an increased frequency of IFN- $\gamma$  producing T cells specific for HlyE and CdtB in challenged volunteers compared with healthy controls, with a greater response in volunteers who developed enteric fever (Diagnosed) after challenge, compared with challenged volunteers who did not develop disease (Not Diagnosed) (**Supplementary Fig. 4**).

Furthermore, we observed that HlyE specific T cell clones differed in their capacity to recognize distinct typhoidal strains: HlyE specific CD4<sup>+</sup> T cell clones recognized cells infected with the live attenuated vaccine *Salmonella Typhi* strain Ty21a more efficiently than cells infected with the challenge strains *S. Typhi* Quailles and *S. Paratyphi A* NVGH308 (**Fig. 5d,e**). The presence of HlyE specific T cell clones in infected volunteers indicates that this toxin is highly expressed *in vivo* by the challenge strains. However the different response of HlyE specific T cell clones to distinct typhoidal strains *in vitro* supports the notion that the expression of HlyE is tightly regulated, and is consistent with the observation that Ty21a has increased HlyE dependent hemolytic activity *in vitro*, as compared with other typhoidal strains<sup>24</sup>.

**Salmonella specific CD4<sup>+</sup> T cells recognize both constitutively expressed and inducible bacterial antigens.**

The expression of CdtB, as well as that of several virulence factors involved in *Salmonella* pathogenesis, is induced only upon infection of target cells <sup>19</sup> (Fig. 5f). Thus, we asked whether we could discriminate T cell clones recognizing antigens expressed only upon infection from T cell clones recognizing antigens constitutively expressed by the bacteria. To answer this question, we compared the capacity of different *Salmonella* specific CD4<sup>+</sup> T cell clones to recognize either autologous EBV-LCL pulsed with a lysate of a liquid culture of *S. Typhi* strain BRD948 or infected with live bacteria. A large number of *Salmonella* specific CD4<sup>+</sup> T cell clones, including those specific for CdtB, were capable of recognizing only cells infected with live bacteria, suggesting that a significant proportion of the CD4<sup>+</sup> T cell response against *Salmonella* targets antigens expressed only upon infection (Fig. 5g).

These results show how the antigen specific repertoire of *Salmonella* specific CD4<sup>+</sup> T cells is shaped by the transcriptional plasticity of *Salmonella* and highlight the importance of characterizing pathogen specific T cell responses directed against antigen expressed in infected tissues.

**Identification of CdtB specific CD4<sup>+</sup> T cell clones non cross-reactive against *S. Typhi* and *S. Paratyphi A*.**

The above data provide the first evidence that CdtB, a toxin involved in the pathogenic mechanisms of the typhoidal serovars, is a major T cell target of CD4<sup>+</sup> T cell responses against typhoidal *Salmonella*. The role of CD4<sup>+</sup> T cells in protection against intracellular bacteria such as *Salmonella* is largely dependent on their capacity to secrete cytokines capable to help macrophages in controlling bacterial growth. Indeed, CdtB specific T cell clones were capable

to limit bacterial spread *in vitro* (**Supplementary Fig. 5**), suggesting that these T cell clones might be able to exert a similar role *in vivo*.

However, although CdtB is expressed by both *Salmonella* Typhi and Paratyphi A, some of the isolated CdtB specific T cell clones failed to recognize cells infected with both bacteria. In particular, in *S. Typhi* challenged volunteer T4, 6 of the CdtB specific T cell clones recognized autologous EBV-LCL infected with the *S. Typhi* strains (**Fig. 6a**), but not cells infected with *S. Paratyphi A*.

The CdtB protein sequence is highly conserved in *S. Typhi* and *S. Paratyphi A*, with only two variations across the 185aa protein sequence: at position 110, with a Tyrosine in *S. Paratyphi A* and Histidine in *S. Typhi* (H110Y); and at position 228, with a Serine in *S. Paratyphi A* and Glutamine in *S. Typhi* (S228Q) (**Fig. 6b**). Thus, we performed epitope mapping of the CdtB specific T cell clones using overlapping peptides, spanning the full length CdtB sequence, and including also the peptide variants containing the polymorphic residues at position 110 and 228. All CdtB specific CD4<sup>+</sup> T clones isolated from volunteer T4 recognized a region of the *S. Typhi* CdtB sequence spanning across the Histidine in position 110. However, while all CdtB specific cross-reactive T cell clones were activated by CdtB peptides containing either Histidine (*S. Typhi* variant) or Tyrosine (*S. Paratyphi A*) at position 110, *S. Typhi* CdtB specific T cell clones were activated exclusively by CdtB peptides containing the *S. Typhi* Histidine variant (**Fig. 6d**).

Remarkably, a similar lack of cross-reactivity was also observed in a volunteer (P1) challenged with *S. Paratyphi A* (**Fig. 6e-f**), where two CdtB specific CD4<sup>+</sup> T cell clones were characterized: the first (clone 99) recognized an epitope present within a conserved region of CdtB (25-45) and reacted to both *S. Paratyphi A* and *S. Typhi* infected cells; the second (clone 106) reacted only to *S. Paratyphi A* infected cells (**Fig. 6e**), recognized peptides spanning the region across position 110, but only those containing the *S. Paratyphi A* variant 110Y.

Using a panel of partially matched EBV transformed B cell lines, we demonstrated that the CdtB (105-125) specific T cell clones isolated from volunteers T4, T5 and P1 were HLA-DRB4 restricted. ELISPOT analysis on frozen PBMC collected before challenge and after completion of the antibiotic treatment (28 days after challenge) showed that the increased frequency of CdtB specific IFN- $\gamma$  producing T cells was more pronounced in HLA-DRB4<sup>+</sup> compared to HLA-DRB4<sup>-</sup> individuals (**Supplementary Fig. 6a and b**), suggesting that CdtB (105-125) might be a major determinant in the CdtB CD4 T cell response to typhoidal *Salmonella*.

The distinct specificity of CdtB(105-125) specific T cell clones against *S. Typhi* and *S. Paratyphi A* was confirmed by using HLA-DRB4 tetramers loaded with either the ST (110H) or the SP (110Y) CdtB peptide, demonstrating differential tetramer staining of cross-reactive, Paratyphi specific and Typhi specific CD4<sup>+</sup> T cell clones (**Fig. 6f**). In order to extend these results further, HLA-DRB4 class II tetramers loaded with the two CdtB (105-125) peptides were used to measure the *ex-vivo* frequency of CdtB (105-125) specific HLA-DRB4 restricted CD4<sup>+</sup> T cells in frozen PBMC from HLA-DRB4<sup>+</sup> volunteers collected before and after challenge with *S. Typhi* and *S. Paratyphi A* (**Fig. 6h**). The frequency of CD4<sup>+</sup> T cells stained by either of the two HLA-DRB4 tetramers increased after challenge (**Fig. 6g**), but notably, volunteers challenged with *S. Typhi* showed an accumulation of effector T cells preferentially stained by the HLA-DRB4 tetramers loaded with the CdtB(105-125) ST110H peptide, while volunteers challenged with *S. Paratyphi A* showed a preferential accumulation of clones stained by the HLA-DRB4 tetramers loaded with the CdtB(105-125) ST110Y peptide (**Fig. 6h,i**).

These data show that a single aminoacid difference in a shared immunodominant antigen can elicit divergent serovar specific non cross-reactive responses, and is a major target of T cells non-cross-reactive against the two typhoidal serovars.

## **Identification of antigens recognized by non-typhoidal *Salmonella* cross-reactive T cell clones.**

The identification of T cell antigens shared across different typhoidal and non typhoidal *Salmonella* serovars may provide important targets for vaccination therapies capable of cross-reacting against different *Salmonella* serovars causing disease in humans. We screened CD4<sup>+</sup> T cell clones cross-reactive against non typhoidal serovars, for their ability to recognize autologous EBV-LCL pulsed with 30 different recombinant *S. Typhi* proteins conserved in *S. Typhimurium*<sup>25</sup> (**Supplementary Methods Table 2**). We tested 184 clones from five volunteers, and identified T cell clones specific for three different proteins, namely PhoN, YiaD, PgcL. PhoN specific CD4<sup>+</sup> T cell clones were identified in 5 out of the 7 volunteers tested (**Fig. 7a**). The prevalence of PhoN specific T cell clones across the different volunteers suggests that this protein might represent an immunodominant protein recognized during *Salmonella* Typhi and Paratyphi A infection. This was confirmed by ELISPOT analysis on frozen PBMC collected from challenged volunteers (**Supplementary Figure 6c**)

To determine to what extent T cell clones cross-reactive against *S. Typhi*, *S. Paratyphi A* and *S. Typhimurium* were capable of recognizing more distantly related *Salmonellae*, 167 T cell clones were tested against autologous EBV cells infected with either a different non typhoidal serovar, *S. Enteritidis*, or with a more distantly related species of *Salmonella*, *Salmonella bongori* (*S. bongori*). All but 12 of the tested T cell clones recognized *S. Enteritidis*, consistent with the close phylogenetic relatedness of these two serovars (**Fig. 7b, d**). Furthermore, 42 of the cross-reactive CD4<sup>+</sup> T cell clones also recognized target cells infected with *S. bongori*, indicating that a fraction of the cross-reactive response recognizes epitopes highly conserved across *Salmonella* species. None of the clones specific for PhoN, YiaD and PgcL recognized cells infected with *S. bongori*, consistent with the absence of these proteins in this bacterium (**Fig. 7b,c**). Interestingly, in volunteer T6 we also identified 4 clones

recognizing *S. bongori* and *S. Typhi* and *S. Paratyphi A*, but not *S. Typhimurium* and *S. Enteritidis* (**Supplementary Table 2**).

In addition, we noticed that in volunteer T5, 9 PhoN specific T cell clones reactive against *S. Typhi* and *S. Paratyphi A* infected cells (**Fig.7e**) failed to recognize cells infected with *S. Typhimurium* (**Fig. 7f**) or *S. Enteritidis* (**Fig. 7g**). We performed epitope mapping of 22 PhoN specific clones from this volunteer, and found that these recognized peptides spanning either of three distinct regions between aminoacid 45 to 70 (PhoN(45-70)), 95 to 120 (PhoN(95-120)) and 115 to 145 (PhoN(115-145)) (**Fig. 7h,i**). PhoN(45-70) and PhoN(115-145) specific clones recognized cells infected with all four serovars; in contrast, PhoN(95-120) specific clones failed to recognize *S. Typhimurium* and (with the exception of clone 101) *S. Enteritidis* (**Fig. 7h,i**). The PhoN(95-120) region is polymorphic across the distinct serovars: *Salmonella Typhi* and *S. Paratyphi A* differ at position 115 with a Glycine in *S. Typhi* and a Serine in *S. Paratyphi* (G115S). In contrast, the non-typhoidal serovars *S. Enteritidis* and *S. Typhimurium* differ from *S. Typhi* and *S. Paratyphi* at position 112 with a Lysine in *S. Typhi/S. Paratyphi* and a Threonine in the non-typhoidal serovars (K112T), while *S. Typhimurium* contained an additional polymorphic lysine at position 108 (Q108K) (**Fig 7j**). Consistent with their pathogen specificity, PhoN (95-120) specific T cell clones, recognized equally well peptides containing both the *S. Typhi* and *Paratyphi* PhoN(95-120) sequences, demonstrating that they can tolerate the G115S variant, recognized with lower affinity peptides containing the K112T variant (*S. Enteritidis*), and failed to respond to peptides containing both the K112T and Q108K variants (*S. Typhimurium*) (**Fig. 7j, k**). Furthermore, consistent with its unique ability among PhoN(95-120) specific clones to recognize cells infected with *S. Enteritidis*, clone 101 showed an increased reactivity to the K112T variant compared with the other PhoN(95-120) specific T cell clones.

### **Analysis of TCR CDR3 $\alpha$ and CDR3 $\beta$ of *Salmonella* specific CD4<sup>+</sup> T cell clones**

Finally, we investigated whether effector responses to *Salmonella* infection were associated with the oligoclonal expansion of pathogen specific TCR clonotypes, whether these were enriched in the CD38<sup>+</sup>CCR7<sup>-</sup> effector T cell subset, and whether specific CDR3 motifs were associated with the recognition of particular peptides.

Polyclonal analysis of the TCR $\beta$  repertoire of CCR7<sup>-</sup>CD38<sup>+</sup> cells and CCR7<sup>-</sup>CD38<sup>-</sup> T cells in volunteers T4 and P1, showed that the CCR7<sup>-</sup>CD38<sup>+</sup> subset was much less diverse than the CCR7<sup>-</sup>CD38<sup>-</sup> subset (**Supplementary Fig. 7a**), suggestive that the former subset is populated by clonally expanded effector T cells.

We determined the HLA-II restriction and CDR3 sequence of the TCR $\alpha$  and TCR $\beta$  chains of 33 T cell clones from participant T4 (**Fig. 8a**), and of 24 clones from participant P1 (**Supplementary Fig. 7b**). Among the analyzed T cell clones, similar to observations in a mouse model of *S. Typhimurium* infection<sup>26</sup>, we found evidence of clonotype expansion, and of T cell clones harboring similar CDR3 $\beta$  chains. As expected, all T cell clones sharing identical or nearly identical CDR3 $\beta$  had the same specificity in term of pathogen discrimination, fine specificity (if determined) and MHC Class II restriction.

In donor T4, we identified seven expanding clonotypes. One of these clonotypes gave rise to three out of the five sequenced T cell clones selective for the Typhi CdtB(105-125:100H) peptide, and two clonotypes gave rise to four out of the five clones cross-reactive against the Typhi and Paratyphi CdtB(105-125:H100Y). Six of the seven expanded clonotypes, and 7 additional clonotypes were also detected within the polyclonal TCR $\beta$  library generated from CCR7<sup>-</sup>CD38<sup>+</sup> T cells (**Fig. 8b**), and were virtually absent in the library generated from CCR7<sup>-</sup>CD38<sup>-</sup> T cells (**Fig. 8c**), consistent with the segregation of pathogen specific effector cells at the peak of the immune response within the CCR7<sup>-</sup>CD38<sup>+</sup> subset.

Expanded clonotypes were also observed in T cell clones isolated from donor P1. In this case only 6 of the 22 clonotypes characterized were identified within the CCR7<sup>+</sup>CD38<sup>+</sup> polyclonal library, which correlates with a lower frequency of pathogen specific T cell clones identified in donor P2 compared to donor T4 (**Fig. 3e,f** and **Supplementary Fig. 7b**).

We extended the results generated in volunteer T4 and P1 to the clonotype analysis of 140 clones isolated from volunteer T5 and 79 clones isolated from volunteer T6 (Supplementary Tables 1 and 2). Expanded clonotypes with as many as 7 T cell clones harboring the same TCR sequence were identified in both volunteers (**Supplementary Tables 1 and 2, Fig. 8f**), some of them targeting PhoN and HlyE. Furthermore, distinct specificity groups, with similar clonotypes recognizing specific HLADR-epitope combinations were also identified (**Fig. 8e-f** and **Supplementary Fig. 8a and b**).

The ability to match pathogen and antigen specificity with TCR sequencing data gave us the opportunity to draw snapshots of the effector response to *Salmonella* (**Fig. 8f**), partitioning the effector T cells based on their pathogen reactivity, protein and epitope specificity, HLA restriction and clonotype size. This analysis shows how, despite the large number of potential protein antigens, bacteria-specific T cell responses are largely associated with clonotype expansion and selection of effector T cells recognizing a limited number of specificities.

## Discussion

The large number of potential T cell epitopes in bacterial proteomes and the high degree of TCR cross-reactivity for different peptides raise the possibility that a large proportion of bacteria specific T cell responses may fail to discriminate between related bacterial species or distinct pathogenic variants. This is particularly relevant for T cell immune responses specific to typhoidal *Salmonella*, which have a very high degree of homology to common *Salmonella* serovars as well as other enteric bacteria, making it very likely that effector T cells responding to typhoidal *Salmonella* infection might have been already primed by the gut microbiota.

Different studies have attempted to dissect the complexity of T cell immunity to bacteria either by high through-put screening for bacterial antigens recognized by polyclonal memory T cell responses<sup>27 28</sup>, or by performing single cell sequencing to define the number of effector T cell clones activated during infection<sup>26</sup>. Both approaches have important limitations: the first approach falls short of discriminating between recently activated and memory T cells; while the latter approach fails to provide information on the specificity of effector T cells. Furthermore, single cell TCR sequencing approaches do not allow to dissect and quantify cross-reactive T cell responses, which requires testing individual T cells for their capacity to respond to distinct bacterial antigens and bacterial strains.

In this study, we overcome some of the limitations of the two above approaches by focusing our analysis on effector T cells expanded as a direct result of *Salmonella* infection in human, and by applying a novel approach based on the *in vitro* clonal expansion of effector T cells to define their specificity and cross-reactivity at single cell level.

In order to precisely identify effector CD4<sup>+</sup> T cells responding to *Salmonella* infection, we performed mass cytometry analysis on PBMC from challenged volunteers. Focusing our analysis on changes in the heterogeneity of proliferating Ki67<sup>+</sup> T cells over the course of infection, we revealed the accumulation of a CCR7<sup>-</sup>CD38<sup>+</sup> population of proliferating effector T cells during the inflammatory response associated with bacteremia in *Salmonella* challenged volunteers.

Next, we expanded *in vitro* individual effector CD4<sup>+</sup>CCR7<sup>-</sup>CD38<sup>+</sup> T cells isolated from human volunteers with enteric fever, and used fluorescent barcoding to define their pathogen specificity and cross-reactivity. This approach allowed us to dissect the cross-reactive repertoire of *Salmonella* specific CD4<sup>+</sup> T cells, and showed that the degree of pathogen selectivity of CD4<sup>+</sup> T cell responses to *Salmonella* spans from broadly cross-reactive T cells, recognizing a wide range of *Salmonella*, to T cells capable of discriminating between closely related serovars based on minor amino acid differences in a shared antigen.

*S. Typhi* and *S. Paratyphi A* specific T cell responses, which were not cross-reactive against other serovars, were mostly focused against a restricted number of immuno-dominant secreted toxins, such as CdtB and HlyE. In addition, *Salmonella* CD4<sup>+</sup> T cell response targets proteins constitutively expressed by the bacterium, but also proteins induced only upon infection, demonstrating how the antigen specific repertoire of CD4<sup>+</sup> T cells is shaped by the transcriptional plasticity of *Salmonella*, and how antigen expressed only in the infected tissue are a major target of the CD4<sup>+</sup> T cell response to *Salmonella*.

Despite the large number of proteins differentially expressed by the distinct serovars, single aminoacid variations in CdtB and PhoN were responsible for non cross-reactive T cell responses between *S. Typhi* and *S. Paratyphi A* (CdtB), and between the Typhoidal and non Typhoidal serovars (PhoN). Although these non cross-reactive responses represent only a fraction of the pathogen specific repertoire, the bacterial persistence associated with natural

enteric fever, as well as recurrent infections with *Salmonella* serovars, might further skew the pathogen specific reservoir in favour of immunodominant antigens, increasing the relevance of possible “escape” variants.

Several knowledge gaps have previously been identified, which hamper the development of T cell inducing vaccines against *Salmonella*<sup>29</sup>: *i*) the identity of immunogenic antigens expressed during infection, which might be harnessed to elicit broadly protective or serovars specific immune responses; *ii*) the identification of sequence variations in these antigens which might lead to lack of cross-reactivity; *iii*) the absence of tools to monitor *Salmonella* specific T cell responses capable to discriminate whether vaccination and infection elicit primary T cell responses against typhoidal antigens as opposed to boosting pre-existing cross-reactive T cell responses.

Previous studies identified CD4<sup>+</sup> T cell responses to *Salmonella* antigens in individuals with enteric fever<sup>30, 31</sup>, as well as increased *ex-vivo* responses to *S. Paratyphi A* following vaccination with the *S. Typhi* attenuated strain Ty21a<sup>32</sup>, suggestive of potential cross-reactivity between the different *Salmonella* serovars. However, none of these studies were capable of dissecting cross-reactive vs non cross-reactive components of the CD4<sup>+</sup> T cells responses, or of identifying T cells responses targeting selectively *S. Typhi* or *S. Paratyphi A* specific antigens compared with antigens shared by multiple *Salmonella* serovars.

Our results have addressed such knowledge gaps by identifying and characterizing T cell antigens based on their capacity to elicit cross-reactive and serovar specific T cell responses *in vivo*, and by providing a methodological framework to dissect at the single cell level cross-reactive and serovars specific cell responses induced during infection.

In this study, we have identified frequent antigen specific T cell responses to three distinct *Salmonella* antigens: HlyE, CdtB and PhoN. Assessment of the frequency of responses against

these antigens in patients with natural infection, and in field studies on population with diverse haplotypes will be required to better assess the translational potential of these findings. However, to the best of our knowledge HlyE and CdtB are the first proteins primarily expressed by *S. Typhi* and *S. Paratyphi A* to be identified as target of CD4<sup>+</sup> T cell responses during enteric fever. Recently, a peptide from a secreted virulence factor has been shown to elicit CD4<sup>+</sup> T cells mediated protective immunity in a mouse model of *S. Typhimurium* infection<sup>33</sup>, suggesting that secreted bacterial proteins might represent viable target for vaccination therapies against *Salmonella*. HlyE is a hemolysin, whose role in the pathogenicity of *Salmonella* is currently unclear<sup>34</sup>. Several bacteria have been described to encode toxins with haemolytic activity, and their role has been proposed to range from controlling the amount of iron available to the bacteria by hemolysis, to damaging phagosomal membranes<sup>35</sup>.

CdtB is the catalytic component of the Typhoid toxin which has been proposed as a key virulence factor in the pathogenicity of *S. Typhi* and *Paratyphi A*<sup>8</sup> due to its ability to block cell proliferation. Indeed, CdtB is considered a major target for vaccination strategies against enteric fever aimed at blocking the symptoms caused by the bacteria<sup>22</sup>. The finding that CdtB is a target of CD4<sup>+</sup> T cell responses suggests that this protein could be put forward as a candidate vaccine to elicit both antibody responses capable of neutralizing the toxin activity, as well as T cell responses targeting infected cells. In addition, our data also indicate that, despite the close similarities of the CdtB sequences in *Salmonella Typhi* and *Paratyphi A*, this toxin fails to elicit fully cross-reactive responses against the two pathogens. This suggests that both *Salmonella Typhi* and *Paratyphi A* CdtB proteins should be used to generate optimal CD4<sup>+</sup> T cell responses targeting both serovars.

PhoN is an acid phosphatase induced upon activation of the two-component regulatory system PhoP-PhoQ<sup>36</sup>, one of the molecular rheostats controlling the transcriptional switch associated with invasion of target cells. Although its role in pathogenicity is unknown, PhoN encodes a

secretion signal, and is found associated with outer membrane vesicles released from the bacteria, from which it is translocated in the host cytoplasm<sup>37</sup>.

The broad cross-reactivity of PhoN specific T cell responses against both pathogenic typhoidal and non-typhoidal serovars makes this antigen of interest not only for the development of broadly cross-reactive candidate vaccines, but also as a tool to measure broadly cross-reactive immunity to *Salmonella* upon infection and vaccination.

Transcriptional analysis of peripheral blood from volunteers challenged with *Salmonella* Typhi showed that enteric fever is associated with a potent Th1 type immune response<sup>38</sup>. Consistent with these findings effector CD4<sup>+</sup>CCR7<sup>-</sup>CD38<sup>+</sup> T cells had several hallmarks of Th1 effector cells: elevated expression of Ki67<sup>+</sup> and down-regulation of BCL2, expression of Th1 associated markers CXCR3, CCR5 and the capacity to secrete IFN- $\gamma$  and MIP-1 $\beta$  upon ex-vivo restimulation. *Salmonella* Typhi and Paratyphi A invade systemically through the gut mucosa. Many of the CD4<sup>+</sup>CCR7<sup>-</sup>CD38<sup>+</sup> T cells expressed the gut homing markers CD49d and Integrin- $\beta$ 7, suggesting that circulating effector T cells might have the capacity to migrate to the site of bacteria colonization, and join the pool of tissue resident CD4<sup>+</sup> T cell which patrol the gut mucosa. Gut resident CD4<sup>+</sup> T cells are likely to play an important role in protection against reinfection, and future studies will be needed to investigate whether infection shapes the antigen specific repertoire as well as the frequency of *Salmonella* specific tissue resident CD4<sup>+</sup> T cells. In particular, it will be important to understand whether tissue resident CD4<sup>+</sup> T cells have the same antigen specific repertoire of circulating CD4<sup>+</sup> T cells, and whether the frequency of tissue resident or/and circulating antigen specific responses might correlate with protection after infection or vaccination.

CCR7<sup>-</sup>CD38<sup>+</sup> effector T cells showed a remarkable functional and phenotypic homogeneity, however, within the CCR7<sup>-</sup>CD38<sup>+</sup> T cell population we could also identify a minor fraction of cells expressing a lower level of CD49d and Integrin-β7 and higher expression of other homing markers. Whether this heterogeneity reflects a distinct tissue restriction of these cells, and is associated with a distinct antigen specific repertoire remains to be determined.

In conclusion, in this study we have described a novel approach to interrogate the specificity and clonality of bacterial specific T cells, and demonstrated with unprecedented details that the antigen specific repertoire of CD4<sup>+</sup> T cells activated during *Salmonella* infection includes both largely cross-reactive and serovar specific T cell clonotypes. Furthermore, we have shown how individual variations in immunodominant T cell antigens (such as CdtB and PhoN) are major drivers in the immunological divergence against closely related bacterial species. Our findings highlight the importance of dissecting the target discrimination potential of effector T cell clones to identify cross-reactive and non cross-reactive responses to infection. By characterizing novel immunodominant T cells antigens, these findings pave the way for the design of vaccination strategies capable of eliciting serovar specific and cross-reactive T cell responses. In addition, the development of new tools to identify typhoidal specific immune responses provides new diagnostic tools to assess T cell immunity induced upon natural immunity or vaccination against distinct *Salmonella* serovars.

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## **Material and Methods**

### **Human challenge model**

Samples were collected from healthy community adult volunteers in two controlled human infection studies conducted at the Centre for Clinical Vaccinology and Tropical Medicine, Oxford, United Kingdom (ClinicalTrials.gov identifiers NCT02100397 and NCT02192008). Details of study protocols and enrolment criteria were as described elsewhere<sup>15, 39</sup> Briefly, participants underwent oral challenge with *S. Typhi* Quail's strain ( $10^4$  CFU) or *S. Paratyphi* A NVGH308 ( $10^3$  CFU) suspended in a NaHCO<sub>3</sub> solution (0.53g/30ml) following neutralization of stomach acid.

Participants were monitored in an outpatient setting with daily clinical review and collection of blood and stool cultures. A diagnosis of enteric fever was defined as *S. Typhi* or *S. Paratyphi* bacteremia identified between Day 3 to Day 14 post-challenge and/or a persistent fever  $\geq 38^\circ\text{C}$  for  $\geq 12$  hours. Individuals not meeting the pre-specified diagnostic criteria were commenced on antibiotics at Day 14 and were defined as not-diagnosed. The studies were approved by Oxfordshire Research Ethics Committee A (14/SC/0004 and 14/SC/1204) and performed according to the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

### **Stimulation, Staining, and CyTOF Data Acquisition**

CyTOF analysis was performed in order to identify cellular signatures in the CD4<sup>+</sup> T cell response associated with enteric fever. For this reason, samples were selected based on sample availability and high bacterial load at diagnosis. No sample selection was performed for subsequent analysis (whole blood staining, ELISPOT, Tetramer staining, and clone isolation). Cryopreserved samples were thawed and washed with pre-warmed RPMI supplemented with 10% FBS (GIBCO, Life Technologies), 1X Penicillin/Streptomycin/L-glutamine (GIBCO, Life Technologies), 1% 1M HEPES (GIBCO, Life Technologies) and 1X  $\beta$ -mercaptoethanol (GIBCO, Life Technologies). Cells from each sample were split into two wells, followed by staining with the indicated antibodies (Supplementary Table 1) in 96-well round bottom plates for 30 minutes at  $37^\circ\text{C}$  prior to stimulation. Cells were left untreated or stimulated at  $37^\circ\text{C}$  for 4 hours with 150 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) and 1  $\mu\text{M}$  ionomycin (Sigma-Aldrich) in the presence of monensin and Brefeldin A (eBioscience).

After stimulation, cells were washed twice in cold PBS, followed by incubation on ice with 200  $\mu$ M cisplatin (Sigma-Aldrich) for 5 minutes. Cells were then washed with CyFACS buffer (PBS + 4% FBS + 0.05% sodium azide) and stained with streptavidin- $\alpha$  Galcer with 10  $\mu$ M free biotin for 30 minutes at room temperature. This was followed by 30 minutes incubation in primary antibody cocktail on ice. Cells were then washed twice in CyFACS and stained with metal-tagged surface antibodies. After 30 minutes, stimulated cells were washed twice with CyFACS, once with PBS, and then fixed in 2% paraformaldehyde (PFA; Electron Microscopy Sciences) at 4°C. The untreated cells were washed twice with CyFACS and incubated in Foxp3 Fixation/Permeabilization buffer (eBioscience) on ice for 30 minutes. Cells were then washed twice in 1 x Permeabilization (perm) Buffer (Biolegend) and stained with biotin anti-human Foxp3 and metal-tagged intracellular antibodies for 30 minutes on ice. After washing twice with perm buffer, cells were incubated on ice with metal-tagged streptavidin for 10 minutes. Cells were then washed twice in perm buffer, once in PBS, and then fixed in 2% PFA at 4°C. The next day, stimulated cells were washed twice with perm buffer and stained with intracellular antibodies on ice. After 30 minutes, all the untreated and stimulated cells were washed twice with perm buffer and once with PBS. Cells were then incubated with cellular barcodes for 30 minutes as previously described<sup>40</sup>.

Pt-102, Rh-103, Pd-104, -105, -106, -108 and -110 were used for this experiment. 113 was not used due to interference with CD57 on mass 115, based on our observations from previous experiments. A small degree of interference was observed between 110 and 112/114 but this did not affect de-barcoding as we could easily gate on 110 positive cells without including 112/114 positive cells. In addition, since CD14 positive cells (112 and 114 positive) were excluded for analysis, interference between 110 and 112/114 was not an issue. Cells were then washed once with perm buffer and incubated in CyFACS for 10 minutes on ice. Cellular DNA was labeled at room temperature with 250 nM iridium interchelator (Fluidigm) diluted in PBS with 2% PFA. After cellular DNA labeling cells were washed twice with CyFACS and kept in a 96 well U-bottom plate.

To accommodate the required numbers of samples, two barcoded batches were prepared, ensuring each batch included all time-points for a given volunteer. A single healthy donor's PBMCs, that was prepared and stained in parallel with volunteer samples, was included in each batch as a control for batch-to-batch variation.

For acquisition, small aliquots of cells from each batch were filtered through a 0.35  $\mu\text{m}$  cell strainer into a 5 mL polystyrene tube. The batch aliquot was then washed twice in water before being resuspended in water at 500,000 cells/mL. EQ Four Element Calibration Beads (Fluidigm) were added at a final concentration of 1% prior to sample acquisition. The same pooling steps were repeated each time the acquisition of an aliquoted batch was completed. This approach was taken to minimize the duration the stained cells were kept in water (1–2 hours maximum).

Both batches of samples were acquired on subsequent days to minimize batch effects which can occur if stained cells are stored in CyFACS long term. Batch effect was analyzed by performing a tSNE plot to compare the PBMCs from the healthy donor stained and acquired with the two distinct batches. Cells acquisition was performed on a CyTOF2 (Fluidigm) and the total number of live cells acquired for each sample was between 30,000–100,000.

#### **Data Analysis:**

After mass cytometry acquisition, data were exported in flow-cytometry file (FCS) format, normalized<sup>41</sup> and events with parameters having zero values were randomized using a uniform distribution of values between minus-one and zero. Each sample containing a unique combination of two metal barcodes was de-convoluted using manual gating in FlowJo to select cells stained with two and only two barcoding channels.

Heterogeneity of CD4<sup>+</sup>Ki67<sup>+</sup> cells was evaluated with the Phenograph algorithm<sup>17</sup> embedded in the Bioconductor CyTOFkit package<sup>42</sup>. A total of 1143546 CD4 T cells was analysed (Min=4037, Max=74061, Median=31045) from which a total of 6185 Ki67<sup>+</sup> cells (Min=15, Max=470, Median=148.5; for samples at the peak of Ki67<sup>+</sup> cells accumulation: Min=234, Max=470) was extracted using FlowJo and analysed by Phenograph.

tSNE analysis of CD4<sup>+</sup> T cells stimulated with PMA/Ionomycin was performed using Cytobank<sup>43</sup> on a total of 224091 (Min=2005, Max= 35706, Median=9840) CD4<sup>+</sup>CD45RA<sup>-</sup> T cells from volunteers 6, 22 49 and 72, at time points D0, ED and D28, based on the expression of CD40L, CTLA-4, MIP-1 $\beta$ , TNF, IFN- $\gamma$ , IL-2, GM-CSF, IL-17, IL-22, CD107a, IL-4, IL-8 and IL-10. At the peak of the response 347 (Donor 22), 219 (Donor 49), 346 (Donor 6) and 398 (Donor 72) CCR7<sup>-</sup>CD38<sup>+</sup> events were identified.

#### **Medium, reagents and strains**

The medium used throughout was RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 1% pen/strep,  $5 \times 10^{-5}$  2ME (all from Gibco) and serum: 10% FCS (Sigma) or 5% Human AB Serum (Sigma) for T cell clones.. T cell clones medium was supplemented with 1000U/ml recombinant human IL-2, produced in our laboratory as described <sup>44</sup>.

Purified Phytohemagglutinin (PHA) (Remel), Ionomycin (Sigma), Phorbol 12-myristate 13-acetate PMA (Sigma), Cyclosporin B (Sigma), Gentamycin (ThermoFisher Scientific), Brefeldin A (Biolegend).

Autologous Epstein Barr virus (EBV)-transformed lymphoblastoid cell lines (EBV-LCL) were generated from PBMC collected before challenge, by incubation with supernatant from B95-8 Marmoset cells in the presence of 2 $\mu$ g/ml of Cyclosporin A (Sigma).

Bacterial strains were *Salmonella* Typhimurium LT2 and D23580, *S. Typhi* BRD948 (Ty2  $\Delta$ aroC aroD htrA), Quail and Ty21a, *S. Paratyphi* A NVGH308, *S. bongori* (M07), *S. Enteritidis*

### **T cell cloning and live fluorescent barcoding**

Individual cells were sorted in 96 well round bottom plate containing 125000 feeders cell/well in 150ul of RPMI medium containing Pen/Strep, Glutamine, NNAA, Sodium Piruvate, beta mercaptoethanol, 1000u/ml of IL-2 supernatant and Human Serum (Blood bank). Clones were expanded for 4-5 weeks and then tested for their specificity as follows.

Clones were collected and incubated overnight in the absence of IL-2. The next morning clones were washed 3 times in PBS, and labelled for 10 minutes in the presence of distinct dilution and combination of CellTrace™ CFSE Cell Proliferation Kit, CellTrace™ Far Red Cell Proliferation Kit, CellTrace™ Violet Cell Proliferation Kit, CellTracker™ Orange CMTMR Dye (all from ThermoFisher Scientific). Labelling was quenched with an equal volume of Fetal Calf Serum (Gibco), clones were washed 5 times in RPMI 10% FCS, and left to rest at 37C for 1-2h. After resting clones were pooled, counted and then incubated at  $10^6$  cells/ml in the presence of 1ug/ml Brefeldin A with either  $3 \times 10^5$ /ml autologous EBV cells lines infected overnight with *Salmonella* or pulsed overnight with 10ug/ml recombinant proteins, or incubated with 5ug/ml peptide pool of CdtB and HlyE.

After 6h stimulation cells were fixed, permeabilized and stained with anti CD4 (RPA-T4) Pe-710 (Biolegend), CD3 PE-Cy7, and IFN- $\gamma$  APC-e780.

Samples were then acquired using a BD FACS CANTO II flow cytometer, or a X50 BD Symphony machine and analysed with Flowjo 10 (TreeStar). Viability was assessed with live/dead Aqua staining, according to the manufacturer's instructions (Life Technologies). T cell clones were considered positive with IFN- $\gamma$ + > 5% in the presence of bacteria infected autologous cells, and/or IFN- $\gamma$ + > 20% in the presence of cells pulsed with proteins or peptide pools. For two of the volunteers (T4 and P2) the threshold of IFN- $\gamma$  production in the presence of bacteria infected with autologous cells was increase to 10% because of higher background IFN-g production. While some T cell clones expanded extensively and were capable to withstand several restimulation cycles, other expanded only to provide enough cell for the intial preliminary screenings of antigen specificity (which included in all cases HlyE, CdtB, Ty21a, Quailles, NVGH308 and LT2 strains) and in volunteer T5 and T6 PhoN), but could not be fully characterized in term of protein specificity, epitope mapping, haplotype restriction, and broad cross-reactivity.

### **Infection**

Autologous EBV LCL were washed two time in RPMI 10% FCS in the absence of antibiotics, then plated at  $10^6$  per ml in the presence of different *Salmonella* strains at a multiplicity of infection between 90:1 and 10:1 varying from strain to strain to reach at least 30% of infected cells. After 2h of incubation, Gentamycin was added at 50ug/ml and cells were kept in culture overnight. Efficiency of infection was evaluated using anti-*Salmonella* CSA-1 antibody.

For the T cell macrophages transwell experiments Monocyte derived macrophages were differentiated for 5 days in the lower chamber of 24 well transwell plates ( $0.5 \times 10^6$  /well). At day 5 a pool of 3 CdtB specific clones for a total of 200000 cells/well was added to the upper well in the presence or absence of 5 $\mu$ g/ml CdtB peptide pool (CdtB). After O.N. incubation the upper well was removed, macrophages were washed, infected with *S. Typhimurium* (LT2 strain, Multiplicity of Infection=10) for 30m, washed again and incubated with Gentamicin (30mg/ml) to limit bacterial overgrowth.

### **Proteins**

Proteins listed in Supplementary Table 2 were purified as His6 tagged proteins. Antigens were PCR-amplified from *Salmonella enterica* serovar Typhi Ty2 cloned into pET22b vector as previously described<sup>25</sup>. Antigens were expressed in *E.coli* BL21 and purified from inclusion bodies. Briefly, inclusion bodies (IB) were washed 3X in cold triton wash buffer (Tris-HCL

50mM, NaCl 100mM, DTT 1mM, EDTA 1mM, Triton X100 0.5%, Azide 0.1%) using glass homogenizer to remove bacterial debris and twice without triton, then solubilised in 8M urea solution (Tris-HCL 10mM, NaH<sub>2</sub>PO<sub>4</sub> 100mM, DTT 0.1 mM, EDTA 0.1mM, Urea 8M). Further antigens were purified using Ni-NTA spin columns as per instructions (Qiagen, Cat.No. 31314) and stored at -80 in small aliquots.

HlyE and CdtB were PCR amplified from *Salmonella enterica* serovar Typhi Ty21a, cloned into pET21a vector and expressed in a LPS modified *E.coli* BL21 strain to produce endotoxin free proteins (Lucigen, ClearCoil BL21 cells). Soluble His-Tagged proteins were purified using HisTrap nickel affinity column (GE Healthcare) followed by desalting on a HiPrep 26/10 (GE Healthcare).

### **Flow Cytometry**

Whole blood was stained after ACK red blood cell lysis using a cocktail of antibodies directed against CD4 (OKT4, A700, Biolegend) CD8 (HIT8a, PerCPCy5.5, Biolegend), HLADR (L243, APC-Cy7, Biolegend), PD1 (eBioJ105, PE-eFluor610, eBioscience), CD3 (SK7, BV650, BD Bioscience), CD38 (HIT2, BB515, BD Bioscience), CD45RA (HT100, BV711, Biolegend), CCR7 (G043H7, PE/Cy7, Biolegend), Pan-TCR $\gamma\delta$  (B1, BV421, Biolegend), V $\alpha$ 7.2(3C10, BV605, Biolegend), CD161 (HP-3G10, APC, eBioscience),  $\alpha$ GalCer-CD1d-Tetramer (PE). Dead cells were excluded LIVE/DEAD<sup>®</sup> Fixable Aqua Dead Cell Stain Kit (Life Technologies Ltd). Samples were acquired on a BD LSRFortessa<sup>™</sup> X20.

For single cell FACS sorting, Whole blood was stained after ACK red blood cell lysis using a cocktail of antibodies directed against CD4 (OKT4, A700, Biolegend) CD8 (HIT8a, PerCPCy5.5, Biolegend), CD3 (SK7, BV650, BD Bioscience), CD38 (HIT2, BB515, BD Bioscience), CCR7 (G043H7, PE/Cy7, Biolegend). Cell sorting was performed on BD FACSAria<sup>™</sup> Fusion Cell Sorter or BD FACSAria<sup>™</sup> III. Data was analyzed using FlowJo<sup>™</sup> cell analysis software (FlowJo, LLC), Cytobank, and CyTOFkit.

HLADRB4\*0101 class II tetramers loaded with peptide CdtB(105-125) SP110Y RYIYYSAIDVGARRVNLAIV, and with peptide ST CdtB (105-125) ST110H RYIYHSAIDVGARRVNLAIV, were produced by the Benaroya Tetramer core Facility. PBMC were thawed in presence of Benzonase and rested for 1h at 37C. 10<sup>6</sup> PBMC were stained with 0.5 $\mu$ g of Tetramer in 50 $\mu$ l for 1h at RT. Dead cells were excluded with LIVE/DEAD<sup>®</sup> Fixable Aqua Dead Cell Stain Kit (Life Technologies Ltd), and cells were stained with CD4 (OKT4, A700, Biolegend) CD8 (HIT8a, PerCPCy5.5, Biolegend), CD14 (M5E2, Brilliant

Violet 510™, Biolegend), CD19 (HIB19, BV510, Biolegend), CD38 (HIT2, BB515, BD Bioscience), CD45RA (HT100, BV711, Biolegend), CCR7 (G043H7, PE/Cy7, Biolegend). Samples were acquired on a BD LSRFortessa™.

### **TCR sequencing and analysis**

RNA was extracted from individual clones or sorted cell populations using RNAqueous-Micro Total RNA Isolation Kit (Ambion), following the manufacturer's instructions. cDNA was generated by template switch reverse transcription using SMARTScribe Reverse Transcriptase (Clontech), using a template-switch oligo with a 6bp unique molecular identifier (TSO-UMI) and primers designed for the constant regions of Trac and Trbc genes. TCR amplification was achieved by performing two rounds of nested PCR using Phusion High-Fidelity PCR Master Mix (New England Biolabs). During the first PCR priming, indexes were included, to identify each sample. A last PCR was performed to add the Illumina adaptors. Primers' sequences are described in Supplementary Methods Table 4. TCR libraries were sequenced on Illumina Miseq using Miseq Reagent Kit V2 300-cycle (Illumina). FASTQ files were demultiplexed for each clone or cell population. Sequences from clones were analysed using MiXCR<sup>45</sup>. Sequences from cell populations were analysed using MIGEC<sup>46</sup>. Post analysis was performed using VDJtools<sup>47</sup>.

**Data availability.**

The data that support the findings of this manuscript are available from the corresponding authors upon request.

## Figure legends

**Figure 1. Mass cytometry identification of effector CD4<sup>+</sup> T cells responding to *Salmonella* infection:** (a) Human Challenge Model of typhoidal *Salmonella* infection and sample collection time points. Enteric fever (EF) time point corresponds to 2-4 days after clinical diagnosis of enteric fever. For the patients analyzed (n=6) in these experiments, diagnosis was between day 6 and day 12 (Median= 8 days); (b) Frequency of Ki67<sup>+</sup> cells within CD4<sup>+</sup> T cells in frozen PBMC thawed, stained with metal tagged antibodies and analyzed by Mass Cytometry. X axis indicates days (D) from infection. EF indicates samples collected 2-4 days after diagnosis of enteric fever. Friedman Test p=0.0342, Dunn's Multiple Comparison Test day 0 (D0) vs day 4 (D4), day 28 (D28), day 90 (D90) =ns, D0 vs EF =0.02247; (c) t-distributed stochastic neighbor embedding (tSNE) map describing the heterogeneity of Ki67<sup>+</sup>CD4<sup>+</sup> T cells identified in all volunteers. Cells are colored by cell-type assignments detected by PhenoGraph; (d) Heatmap showing the hierarchical clustering of samples based on the frequency of the distinct PhenoGraph Ki67<sup>+</sup> subsets at different time points. Each column represents one of the 30 samples analyzed. The legend indicates the color key use to describe the frequency of the different subsets within each sample (x-axis). (e) Heatmap describing the relative expression of each marker within cluster 19, compared with its expression in the other clusters. Median expressions of the distinct phenotypic markers were normalized, scaled across clusters and then ranked from top to bottom based on their z-score value in cluster 19; (f) Accumulation of CD38<sup>+</sup>CCR7<sup>-</sup>CD4<sup>+</sup> T cells in PBMC collected after diagnosis of enteric fever in a representative volunteer. Metal tagged antibodies are indicated on the X and Y axes. (g) frequency of CD38<sup>+</sup>CCR7<sup>-</sup>CD4<sup>+</sup> T cells across different time points in all volunteers analyzed by mass cytometry. Friedman Test p=0.0019, Dunn's Multiple Comparison Test D0 vs D4, D28, D90 =ns, D0 vs EF =0.0021.

**Figure 2. CCR7<sup>-</sup>CD38<sup>+</sup> CD4<sup>+</sup> T cells contain a homogenous population of IFN $\gamma$ <sup>+</sup> MIP-1 $\beta$ <sup>+</sup> producing cells:** (a) Bivariate plots showing expression of different cytokines and activation markers in CCR7<sup>-</sup>CD38<sup>+</sup> CD4<sup>+</sup> T cells compared to memory CD45RA<sup>-</sup>CD38<sup>-</sup> CD4<sup>+</sup> T cells in PBMC stimulated for 4h with PMA/Ionomycin in the presence of Brefeldin A and Monensin. Metal tagged antibodies are indicated on the X and Y axes.

(b) Frequency of T cells expressing the indicated cytokines and activation markers within CD4<sup>+</sup>CD45RA<sup>-</sup> and CD4<sup>+</sup>CCR7<sup>-</sup>CD38<sup>+</sup> T cells. Two-tailed paired t-test was applied to compare frequency of cytokine producing cells within the two distinct subsets: \* p<0.05, \*\* p<0.01. (c) tSNE plots generated based on the analysis of cytokines and activation markers, and color-coded according to the relative expression of the indicated markers. (d) Overlay of the memory and effector (CCR7<sup>-</sup>CD38<sup>+</sup>) T cell populations in one representative individual and gating of multifunctional (MF) effector cells (e) Frequency of MF effector cells within CD38<sup>+</sup>CCR7<sup>-</sup> T cells and total CD45RA<sup>-</sup> T cells in four volunteers (n=4) during enteric fever. Two-tailed paired t-test : \* p<0.05.

**Figure 3. Analysis of libraries of CCR7<sup>-</sup> CD38<sup>+</sup> T cell clones and fluorescent barcoding to dissect the antigen specific repertoire of effector CD4<sup>+</sup> T cells.**

(a) Effector CD4<sup>+</sup> T cells were FACS sorted as individual T cell, expanded with PHA and IL-2 in the presence of irradiated feeders and then tested for their capacity to recognize *Salmonella* infected autologous lymphoblastoid cell lines.

(b) Schematic example of fluorescent cell barcoding to test and compare the specificity of different T cell clones. Clones were labelled with 3 different fluorescent dyes, pooled and co-cultured for 6h with autologous EBV transformed B cell lines pulsed with a recombinant antigen, or infected with a *Salmonella* serovar. Brefeldin A was added for the last 4 hours of stimulation. Shown is the different response measured as intracellular cytokine staining of IFN- $\gamma$  by two clones in the presence of two distinct stimulatory conditions.

(c) T cell clones were generated from CCR7<sup>-</sup>CD38<sup>+</sup> or CCR7<sup>-</sup>CD38<sup>-</sup> CD4<sup>+</sup> T cells isolated 2 days after diagnosis from *S. Typhi* infected volunteers T1 and T2.

Shown are the proportion of *S. Typhi* specific T cell clones within the total of the expanding clones and (d) the IFN- $\gamma$  response (depicted as % of IFN- $\gamma$  positive cells in intracellular cytokine staining) of the different clones in the presence of autologous EBV-LCL infected with *Salmonella Typhi* (Quailes strain) (One-tailed Mann-Whitney test). (e) Pie charts show the proportion of CCR7<sup>-</sup>CD38<sup>+</sup> CD4<sup>+</sup> T cell clones recognizing EBV-LCL infected with *S. Typhi* (Ty21a strain) in six *S. Typhi* infected volunteers, and (f) EBV-LCL infected with *S. Paratyphi A* (NVGH308) in two *S. Paratyphi A* infected volunteers.

**Figure 4. Cross-reactivity of *Salmonella* specific CD4<sup>+</sup> T cell clones against different *Salmonella* Serovars.**

(a) IFN- $\gamma$  production by three clones isolated from a *S. Typhi* infected volunteer in the presence of only autologous EBV-LCL, or EBV-LCL infected with *S. Typhi* (Quailes), *S. Paratyphi A* (NVGH308) or *S. Typhimurium* (LT2). (b-d) Plots describing the cumulative response of CCR7<sup>-</sup> CD38<sup>+</sup> CD4<sup>+</sup> T cell clones isolated from *Salmonella Typhi* challenged volunteer T4 in the presence of EBV-LCL infected with different *Salmonella* serovars. Each dot represents IFN- $\gamma$  production measured by intracellular cytokine staining by an individual T cell clone in response to EBV-LCL infected with: (b) two distinct strains of *S. Typhi* (the challenge strain Quailes and the recombinant attenuated strain BRD948); (c) the *S. Typhi* challenge strain Quailes and the *Salmonella Paratyphi A* challenge strain NVGH308, (d) the Quailes strain of *S. Typhi* and the *S. Typhimurium* strain LT2 (e). Venn diagram describing the number of T cell clones isolated from the different *Salmonella* infected volunteers, recognizing cells infected with one or more of the different Serovars: *S. Typhi* (S.T.), *S. Paratyphi A* (S.P.) and *S. Typhimurium* (S. Tym.).

**Figure 5. HlyE and CdtB are *S. Typhi* and *S. Paratyphi A* specific immuno-dominant T cell antigens.**

(a) IFN- $\gamma$  production by T cell clones from volunteer T4 stimulated with HlyE and CdtB peptide pools. Red and blue dots represent HlyE and CdtB specific clones, respectively. (b) IFN- $\gamma$  production by CdtB (blue dots) and HlyE (red dots) specific T cell clones in response to EBV-LCL infected with *S. Typhi* (Quailes strain) or *S. Typhimurium* (LT2 strain). (c) Proportion of HlyE and CdtB specific T cell clones among T cell clones non cross-reactive against *S. Typhimurium*, but specific for typhoidal serovars across 8 different volunteers. (d) IFN- $\gamma$  production by T cell clones generated from volunteer T4 in response to *S. Typhi* strains Quailes and Ty21a, red dots represent HlyE specific clones. (e) IFN- $\gamma$  production by T cell clones generated from volunteer T4 in response to recombinant HlyE, and autologous EBV-LCL infected with *S. Paratyphi A* (NVGH308 strain), *S. Typhi* Quailes strain (S. Ty), *S. Typhi* Ty21a strain (Ty21a), and *S. Typhimurium* LT2 strain. Red dots represent HlyE specific T cell clones. (f) Kinetic of mRNA expression (plotted as Arbitrary Unit compared to 16S rRNA) of CdtB in *S. Typhi* infected EBV-LCL. (g) IFN- $\gamma$  production by T cell clones isolated from volunteer T4

in the presence of autologous EBV LCL infected with live *S. Typhi* (BRD948 strain) or pulsed with a lysate of *S. Typhi* (BRD948 strain). Red dots represent CdtB specific T cell clones.

**Figure 6. Sequence dependent recognition of different *Salmonella* serovars by CdtB specific T cell clones.**

(a) IFN- $\gamma$  production of CdtB specific T cell clones generated from volunteer T4 in the presence of autologous EBV-LCL infected with *S. Typhi* and *S. Paratyphi A*. Red dots represent CdtB specific clones recognizing only *S. Typhi* infected cells and blue dots represent CdtB specific clones recognizing EBV-LCL infected with both *S. Typhi* or *S. Paratyphi A*. (b) Aminoacid sequence of CdtB in the two strains used in the human challenge model: *S. Typhi* (Quailes) and *S. Paratyphi A* (NVGH308). (c) IFN- $\gamma$  production by CdtB specific T cell clones from volunteer T4 in the presence of the CdtB(105-125) peptide containing either the *S. Typhi* or the *S. Paratyphi A* sequence. Blue dots represent clones cross-reactive against *S. Typhi* and *S. Paratyphi A*, while red dots represent T cell clones specific for *S. Typhi*. Mann Witney two tailed t-test \*\*:  $p < 0.005$ . (d) IFN- $\gamma$  production from T cell clones from volunteer P1 in response to EBV-LCL infected with *S. Typhi*, *S. Paratyphi A*, or stimulated with CdtB peptide pool. Clone 99 is depicted in red, Clone 106 depicted in blue. (e) IFN- $\gamma$  production by Clone 99 (red) and Clone 106 (Blue) from volunteer P1 in the presence of different CdtB peptides. (f) Staining of a *S. Typhi* specific (red), *S. Paratyphi* specific (green) and cross-reactive (blue) CdtB(105-125) specific T cell clones with HLA DRB4 tetramers loaded with either the CdtB(105-125)SP110H (PE) or the CdtB(105-125) ST110Y (APC) peptide variants. (g) Increased *ex-vivo* frequency of combined HLA DRB4 tetramer positive T cells (PE<sup>+</sup>+APC<sup>+</sup>+PE<sup>+</sup>APC<sup>+</sup> cells) 28 days after challenge in volunteers challenged with *S. Typhi* (black dots) and *S. Paratyphi A* (red dots). (h) *Ex-vivo* HLA DRB4 tetramer staining of frozen PBMC collected 28 days after challenge with *S. Typhi* or *S. Paratyphi* from two representative volunteers identifying CD4 T cells decorated by the HLA DRB4/SP110H tetramer (lower right quadrant) or the DRB4/ST110Y (upper left quadrant) or both (upper right quadrant). Number within each quadrant represent the % of CD4<sup>+</sup> T cells labeled by the distinct tetramers. (i) Proportion of CD4<sup>+</sup> tetramer positive cells stained *ex-vivo* with DRB4\*SP110H, DRB4\*SP110H ST110Y or both DRB4 tetramers in frozen PBMC collected from different volunteers 28 days after challenge with *S. Typhi* or *S. Paratyphi A*. Color coding as indicated in panel f. Mann-Whitney Two Tailed Test for the difference in the proportion of ST110H<sup>+</sup>

cells in *S. Typhi* compared to *S. Paratyphi A* challenged volunteers  $p < 0.005$ , and in the proportion of SP110Y<sup>+</sup> in *S. Paratyphi A* compared to *S. Typhi*  $p < 0.005$ .

**Figure 7. Characterization of the specificity of T cell clones cross-reactive against non typhoidal *Salmonella*.**

(a) Antigenic proteins recognized by cross-reactive T cell clones. A total of 184 Typhoidal-non typhoidal cross-reactive T cell clones from 7 volunteers were tested. For each volunteer the number of clones tested, the protein recognized and, in brackets, the number of T cell clones specific for the indicated proteins are shown (b,c) Cross-reactivity of 167 *S. Typhimurium* specific CD4<sup>+</sup> T cell clones against *S. Enteritidis* and *S. bongori*. PhoN specific T cell clones are depicted as red dots, YiaD specific T cell clones as green dots, and PgcL specific T cell clones as blue dots. (d) Venn diagram depicting the number of cross-reactive T cell clones recognizing *S. Typhimurium* infected cells that can also recognize *S. Enteritidis* (113 clones), and both *S. Enteritidis* and *S. bongori* (43 clones). (e-f-g) IFN- $\gamma$  production from PhoN specific T cell clones from volunteer T5 in the presence of cells infected with *S. Typhi* vs *S. Paratyphi A* (e), *S. Typhi* vs *S. Typhimurium* (f), and *S. Typhi* vs *S. Enteritidis* (g). (h-j) IFN- $\gamma$  production from T cell clones specific for distinct PhoN epitopes (aa 45-70, 95-120, 115-145) in the presence of cells infected with *S. Typhi* vs *S. Typhimurium* (h) and *S. Typhi* vs *S. Enteritidis* (i) Arrows indicates clone 101. (j) Sequence of PhoN(95-120) in *S. Typhi*, *S. Paratyphi A*, *S. Typhimurium* and *S. Enteritidis*. (k) IFN- $\gamma$  production by PhoN (95-120) specific T cell clones in the presence of the 95-115 and 100-120 *S. Typhi* (Ty), *S. Paratyphi A* (P), *S. Enteritidis* (E) and *S. Typhimurium* (Tym) peptide variants. Red dots represent *S. Enteritidis* reactive clone 101. Friedman multiple comparison test ( $p < 0.0005$ ), and Dunn's multiple comparison test of IFN- $\gamma$  production in the presence of peptide containing the Typhi variant compared to the Paratyphi A, Typhimurium or Enteritidis variants.

**Figure 8. Clonal expansion of Salmonella specific effector T cells.**

(a) Pathogen selectivity, antigen specificity, HLA restriction, TCR CDR3 $\alpha$  and CDR3 $\beta$  sequence of T cell clones isolated from volunteer T4. Colored sequences were identified also in the TCR $\beta$  repertoire of CCR7<sup>+</sup>CD38<sup>+</sup> T cells from the same volunteer. (b) Pie Chart depicting the frequency (as fraction of total sequences identified) of CDR3 $\beta$  sequences within

the TCR $\beta$  repertoire of CCR7<sup>-</sup>CD38<sup>+</sup> T cells (9130 cells probed) from volunteer T4. Highlighted are the CDR3 $\beta$  sequences identified also in the isolated T cell clones. **(c)** Frequency of CDR3 $\beta$  sequences within the polyclonal repertoire of CCR7<sup>-</sup>CD38<sup>+</sup> T cells and CCR7<sup>-</sup>CD38<sup>-</sup> ( $1.3 \times 10^5$  cells probed) T cells in volunteer T4. Red circles indicated CDR3 $\beta$  sequences of clones highlighted in panel **b**. **(d)** Similar CDR3 $\alpha$  and CDR3 $\beta$  motifs in HlyE specific T cell clones in donor T6. 4 of the 5 clonotypes were characterized as HlyE(40-65) specific, the fine specificity of the fifth was not determined (HlyE?). Indicated is also the number (N) of clones with the same TCR sequence identified in our screening. **(e)** Similar CDR3 $\beta$  sequences in HLA DRB1\*0701 restricted HlyE(155-175) specific T cell clones from volunteer T6 and P1. **(f)** Diagram describing the pathogen specific T cell repertoire of the representative volunteer T6. Circles represent distinct clonotypes, with size proportional to the number of clones with that specific clonotype identified, and colors indicating the HLA-class II restriction (when determined). Clonotypes are grouped based on pathogen selectivity (continuous line) and protein specificity (dashed line).



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