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Cell mediated immune responses elicited in volunteers following immunization with candidate live oral *Salmonella enterica* serovar Paratyphi A attenuated vaccine strain CVD 1902

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ABSTRACT

The incidence of *Salmonella enterica* serovar Paratyphi A (PA) infection is on the rise and no licensed vaccines are available. We evaluated cell mediated immune (CMI) responses elicited in volunteers following immunization with a single dose (10^9 or 10^{10} cfu) of a novel attenuated live oral PA-vaccine strain (CVD 1902). Results showed increases in PA-lipopolysaccharide-specific IgG- and/or IgA B-memory cells and production of IFN-γ, TNF-α, IL-10, IL-23 and RANTES following stimulation with PA-antigens by peripheral blood mononuclear cells obtained 28 days post immunization. Flow cytometry assays revealed that vaccine elicited PA-specific CD8+ and/or CD4+ T effector/memory cells were predominantly multifunctional concomitantly expressing CD107a and/or producing IFN-γ, TNF-α and/or IL-2. Similar proportions of these MF cells expressed, or not, the gut homing marker integrin α4β7. The results suggest that immunization with CVD 1902 elicits CMI responses against PA supporting its further evaluation as a potential vaccine candidate against paratyphoid A fever.

1. Introduction

Currently, the two main causes of enteric (typhoid and paratyphoid) fever worldwide are the human-host restricted pathogens *Salmonella enterica* serovar Typhi (*S.* Typhi) and *S.* Paratyphi A [1]. Besides the substantial endemic Paratyphoid A disease burden recorded in Asia, *S.* Paratyphi is an increasing problem in U.S. and European travelers to endemic areas [2–5]. Among both endemic and travel-associated cases *S.* Paratyphi A strains carrying resistance to multiple clinically-relevant antibiotics are common, limiting treatment options [4].

Whereas the ultimate control of typhoid and paratyphoid fevers globally must derive from the universal provision of potable water and improved sanitation to all populations, in the interim the expanded use of safe, effective vaccines constitutes an important tool for preventing disease in both endemic populations and travelers [6]. Although well tolerated effective licensed vaccines are available to prevent *S.* Typhi disease, there are presently no licensed vaccines to prevent *S.* Paratyphi A disease; thus, the development of such vaccines is a priority [7].

Candidate vaccines against *S.* Paratyphi A in development include parenteral conjugate vaccines consisting of *S.* Paratyphi A O polysaccharide linked to different carrier proteins and attenuated strains of *S.* Paratyphi A such as CVD 1902 [8] and MGN10028 [9] used as live oral vaccines. The well-established efficacy of licensed live oral typhoid vaccine strain Ty21a (Vivotif®) [10] provided the impetus to develop attenuated *S.* Paratyphi A vaccine strain CVD 1902 by introducing into wild type *S.* Paratyphi A deletions in the *guaBA* chromosomal operon (which impairs the biosynthesis of guanine nucleotides) and *clpX* (which encodes a chaperone ATPase). Each of these mutations attenuates *Salmonella* [10,11]. The *clpX* mutation also increases expression of *S.* Paratyphi A flagellar antigens [11] which may enhance the induction of antibodies, cell-mediated immunity (CMI) and innate responses via engagement of TLR5 [12–16]. Pre-clinical studies in mice showed CVD 1902 to be safe, immunogenic and protective against intraperitoneal challenge with wild type *S.* Paratyphi A and paved the way for assessment of CVD 1902 in a dose-escalating phase 1 clinical trial in healthy adults in which single doses as high as 10^9 and 10^{10} colony forming units (CFU) were well tolerated.

CMI and humoral immune responses elicited by live oral typhoid vaccine Ty21a, including cross-reactive responses to *S.* Paratyphi A or B, have been extensively studied. The results indicate that, in addition to humoral responses, Ty21a induces robust CD8+ and CD4+ T effector/memory responses (T_{EM}) [17–27]. Interestingly, CD8+ T

Abbreviations: CMI, Cell-mediated immunity; MF, Multifunctional; PA, *S.* Paratyphi A; S+, Single positive cells.; T_{EM}, T effector/memory

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memory (T_M) responses predominantly mediated by the T_{EM} subset have been shown to be associated with protection against typhoid disease in a human challenge model with wild type *S.* Typhi [28,29], suggesting a significant role for these responses in protecting against typhoid fever [30–33]. Accordingly, we undertook to evaluate the ability of a single high (10^9 or 10^{10} CFU) dose of CVD 1902 to elicit *S.* Paratyphi A-specific B and T cell-mediated responses.

2. Materials and methods

2.1. Vaccine and phase 1 clinical study design

Attenuated *S.* Paratyphi A vaccine strain CVD 1902, which harbors deletions in *guaBA* and *clpX* [13], was administered to successive groups of healthy adult Maryland volunteers, who received vaccine ($n = 6$) or placebo ($n = 2$) in step-wise, dose-escalating fashion. A single dose of vaccine containing 10^6, 10^7, 10^8, 10^9 or 10^{10} CFU was administered with $NaHCO_3$ buffer (1.3% wt/vol); placebo consisted of PBS solution without vaccine strain with cornstarch (USP) to match the turbidity of the vaccine inoculum. At each dose step, participating volunteers were followed for 18 days after immunization in a Research Isolation Ward to monitor the clinical, microbiological, and immunological responses to the vaccine. The University of Maryland Baltimore Institutional Review Board approved the study and all volunteers gave informed consent prior to their enrollment ClinicalTrials.gov (Identifier: NCT01129453). Herein we report the B- and T-CMI responses of the groups that received a 10^9 or 10^{10} CFU dose of vaccine, or placebo.

2.2. Isolation of peripheral blood mononuclear cells (PBMC)

Whole blood samples were collected from volunteers before (day 0) and 28 days after immunization and PBMC were purified using density gradient centrifugation to perform B- and T-CMI assays for this study. Isolated cells were cryopreserved in liquid nitrogen until use [14].

2.3. Antigens

2.3.1. S. Paratyphi A LPS

S. Paratyphi A LPS was purified under the supervision of Dr. Raphael Simon in the CVD Antigen Purification Unit, as described [34].

2.3.2. S. Paratyphi A flagella H-antigen (PA-flagella)

Flagellar antigen was purified from a rough (lacking smooth O-polysaccharide) *S.* Paratyphi A strain, as described [13].

2.3.3. Heat-phenolyzed whole-cell S. Paratyphi A particles (PA-particles)

S. Paratyphi A particles antigen consisted of heat-killed, phenol-preserved whole cell *S.* Paratyphi A (strain ATCC®9150) maintained in the CVD as a reference stock). The PA-particles were washed several times to remove residual phenol and preserved at 4 °C re-suspended in PBS (equivalent to 2×10^9 inactivated organisms/mL).

2.4. B memory (B_M) assay

The B_M enzyme-linked immunosorbent spot (B_M ELISpot) assay was described previously using expanded PBMC expanded for 5–6 days culture in the presence of B cell mitogens (Expansion phase) [34]. Briefly, duplicate wells of nitrocellulose membrane plates (MAHAN, Millipore, Billerica, MA) were coated with 5 μg/mL in PBS of purified *S.* Paratyphi A lipopolysaccharide (PA-LPS), anti-IgG or -IgA antibodies (Jackson ImmunoResearch), as well as with complete media (no-antigen, negative control) only. PA-LPS coated and no-antigen control duplicate wells were seeded with 150,000 expanded cells/well, developed and visualized using HRP-labeled anti-human IgA or IgG secondary antibodies and 3-Amino-9-ethylcarbazole (AEC) (Calbiochem, Millipore Sigma) substrate. An automated Immunospot reader (Immunospot 3B, Cellular Technologies, OH) was used to read the plates and the quantification of spot-forming cells (SFC) was performed using Immunospot version 5.D software S2 (Cellular Technologies) (Fig. S1). B memory responses are reported as SFC/10^6 expanded cells. The frequencies of measured total IgG and/or IgA were used to assess the adequate expansion of B_M cells in each sample. Any specimen with fewer SFC than the 10th percentile of all the samples assayed in this study (e.g., 20,300 and 2,785 SFC/10^6 expanded PBMC for IgG and IgA, respectively) was excluded from analysis. Among all samples studied, one of 12 vaccinees (a recipient of 10^9 CFU) was excluded from the analysis based on these criteria. LPS-PA-specific IgA or IgG B memory spots were normalized by subtracting non-specific background (no antigen control) as net SFC/10^6 expanded cells. Volunteers showing a post-vaccination over pre-immunization level increase of LPS-PA specific B_M cells > 15 SFC/10^6 expanded cells (mean of IgA B_M responses in placebo controls + 3 SE) were considered vaccine responders.

2.5. Cytokine measurements in culture supernatants

PBMC samples collected pre-and post-vaccination from 6 vaccinees who ingested 10^{10} CFU and 2 who ingested placebo) were cultured (1×10^6 PBMC/mL of culture media, final volume 200 μL/well in 96 well plates) in the absence (media only control) or presence of PA-flagella (5 μg/mL) or PA-particles (1×10^6 particles/mL) for 3 days. Culture supernatants were collected and kept at −80 °C until tested for cytokines using the flow cytometry-based BD Cytometry Bead Array (CBA) human TH1/Th2 kit assay (BD biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's recommendations with some modifications described previously [35]. Net cytokine production was calculated by subtracting the cytokine levels (in pg/mL) of the "media alone" control wells from cytokine levels of antigen-stimulated wells for each individual volunteer on each day.

2.6. Target/stimulator cell preparation

Autologous Epstein Barr virus (EBV)-transformed B-LCL (EBV-B cells) were generated from PBMC obtained from each volunteer as previously described [36]. Wild-type *S.* Paratyphi A (wild-type *S.* Paratyphi A, ATCC®9150 strain maintained in the CVD, as a reference stock).

2.7. Ex-vivo PBMC stimulation

Thawed, overnight rested PBMC were stimulated with *S.* Paratyphi A-infected targets (Section 2.7) at a ratio of 10:1 (PBMC:target). After 2 h, the protein transport blockers Monensin (1 μg/mL, Sigma) and Brefeldin A (2 μg/mL; Sigma) were added to the PBMC cultures incubated overnight at 37 °C in 5% CO_2. Media alone and uninfected autologous EBV-B cells were used as negative controls. Staphylococcal enterotoxin B (SEB) (10 μg/mL; Sigma) was used as a positive control.

2.8. Surface and intracellular staining

Surface and intracellular staining (ICS) were performed as described [27,37]. Briefly, cells were surface stained with a panel of fluorochrome conjugated monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD45RA, CD62L, integrin α4β7, CD107a, CD14, CD19 and yellow Live/DEAD™ fixable dead-cell dye (Invitrogen). Surface stained cells were fixed and permeabilized for ICS with mAbs against IFN-γ, TNF-α, IL-2, and CD69. Stained PBMC were fixed in 1% paraformaldehyde and stored at 4 °C until analyzed. Flow cytometry was performed using a customized LSRII flow cytometer (BD) and data were analyzed using WinList version 9 (Verity Software House, Topsham, ME, USA).

2.9. Gating protocol

The dead (live/dead dye positive), CD14+ and CD19+ cells, as well as "doublets" were excluded from the analysis. Total CD8+ (live CD3+, CD8+ CD4−) and CD4+ (live CD3+, CD8−CD4+) T cells and different T memory (T_M) subsets were defined by their expression of CD45RA and CD62L, i.e., T central memory (T_{CM}; CD62L+ CD45RA−), T effector memory (T_{EM}; CD62L− CD45RA−) and T effector memory CD45RA+ (T_{EMRA}; CD62L− CD45RA+) and Naïve T cells (T_N; CD62L+ CD45RA+) (Fig. S2). The FCOM analysis tool (WinList version 9) was used to classify events based on combinations of gates in multidimensional space, i.e., whether cells express single (single positives; S+) or multiple intracellular cytokines and/or CD107a in all possible combinations for the enumeration of multifunctional cells (MF). Flow cytometry analyses were performed in at least 150,000 events in the live lymphocyte gate.

2.10. Statistical analyses

The statistical tests used to analyze each set of experiments are indicated in each Figure Legend. *P* values < 0.05 were considered significant.

3. Results

3.1. Induction of S. Paratyphi A-specific B memory responses

S. Paratyphi A-LPS specific B_M responses were evaluated in PBMC obtained from vaccinees ($n = 11$) before (D0) and 28 days (D28) following immunization with either 10^9 ($n = 5$) or 10^{10} ($n = 6$) CFU of CVD 1902 and in PBMC from placebo controls ($n = 4$).The data are shown as combined vaccinees from both dose levels, since no differences were observed in induction of B_M responses between the 10^9 CFU versus the 10^{10} CFU cohorts (Fig. 1A–D). The PA-LPS specific IgG (Fig. 1A, B) and IgA B_M (Fig. 1C, D) were detectable in both D0 and D28 samples; however, significant increases in the IgG and IgA B_M responses were observed in post-vaccination day 28 compared to the corresponding pre-vaccination (D0) levels among vaccinees (Fig. 1A, C), but not in placebo controls (Fig. 1B, D). The net post-vaccination increases (Mean ± SEM of SFC/10^6 expanded cells) in PA-LPS specific IgG B_M cells (23.0 ± 5.9) observed among CVD immunized volunteers were significantly higher ($p < 0.01$) than those observed in placebo controls (0 ± 0) (Fig. 1E). Similarly, the magnitudes of post vaccination increases in PA-LPS specific IgA B_M in vaccinees and placebo controls were 27.3 ± 10.28 and 5.0 ± 3.3 SFC/10^6 expanded cells, respectively ($p < 0.1$) (Fig. 1E). Using increases of > 15 SFC to define responders (Fig. 1E), 7 and 6 out of 11 vaccinees for IgG (64%) and IgA (55%), respectively, showed increases in B_M responses, while no such increases were observed in the placebo group (0 out of 4, $p < 0.05$) (Fig. 1F).

3.2. T cell mediated immune responses elicited by CVD 1902

3.2.1. Cytokine production in PBMC culture supernatants

To evaluate the T-CMI response, we first measured cytokine/chemokine production by PBMC collected before and 28 days after immunization, following in-vitro stimulation with PA-flagella or PA-bacterial particles preparation in recipients of 10^{10} CFU. Post-vaccination increases of > 2 fold over pre-vaccination levels in the production of IFN-γ (Fig. 2A), TNF-α (Fig. 2B), IL-10 (Fig. 2C), RANTES (regulated on activation, normal T cell expressed and secreted) (Fig. 2D) and IL-23 (Fig. 2E) were observed in most immunized volunteers, but not in placebo controls. Overall, 5 out of 6 (83%) immunized volunteers showed > 2 fold vaccination increases in at least two or more

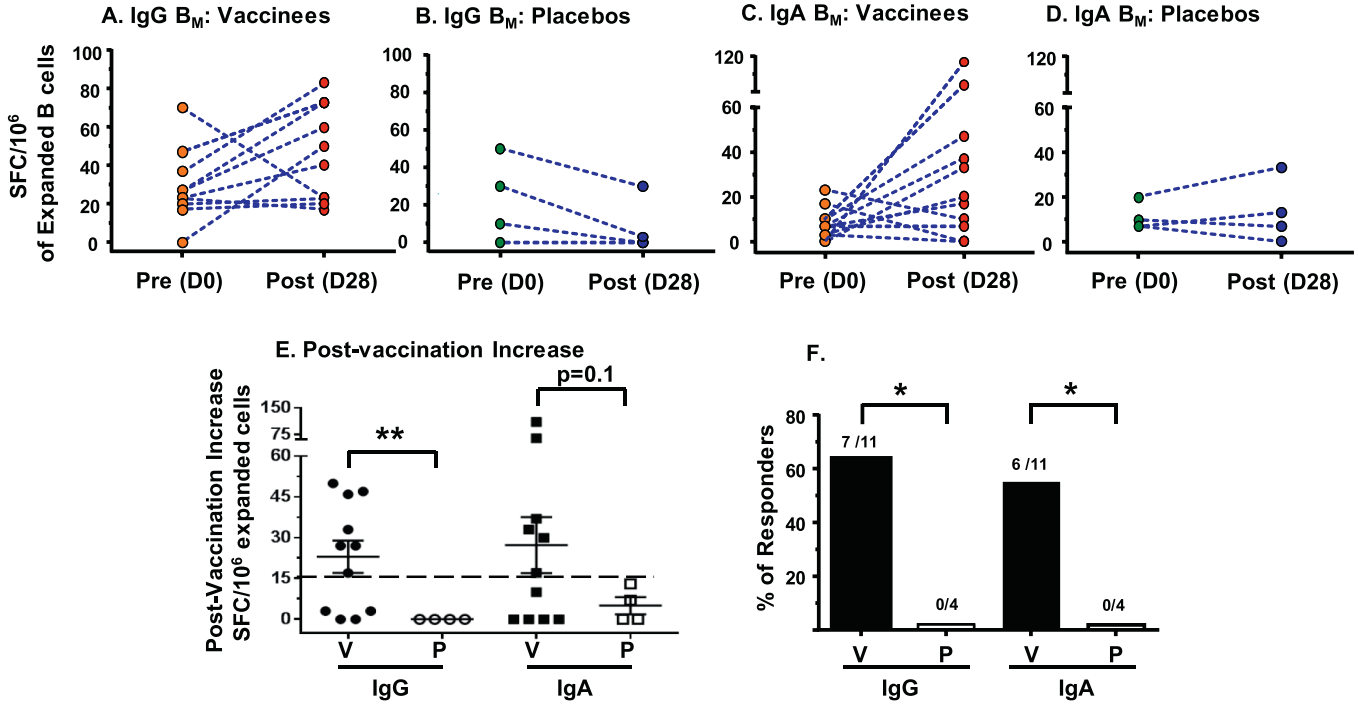

Fig. 1. Measurement of *S.* Paratyphi A Lipopolysaccharide (PA-LPS) specific memory B (B_M) cells in volunteers immunized with CVD 1902. PBMC were obtained from CVD 1902 vaccinees (V; $n = 11$) and placebo (P; $n = 4$) controls in Cohort 4 (10^9 CFU; V = 5, *P* = 2) and Cohort 5 (10^{10} CFU; V = 6, P = 2), before (D0) and 28 days (D28) after immunization and PA-LPS specific IgG (A,B) and IgA (C,D) PA-LPS specific B_M responses were measured. Net data were calculated as antigen coated wells minus non-antigen coated wells (A–D). Dotted lines connect the baseline and D28 values of the same individual (A–D). Post-vaccination increases were calculated as D28 minus D0 levels (E). Volunteers showing Post-vaccination increases > 15 SFC/10^6 (dotted line) were considered responders (E) and their percentages among vaccinees (V) or placebo (P) are shown in panel F. *P* values were calculated using 1-tail Mann-Whitney *t*-test (E) or Chi-square test (F). *$p < 0.05$, **$p < 0.01$.

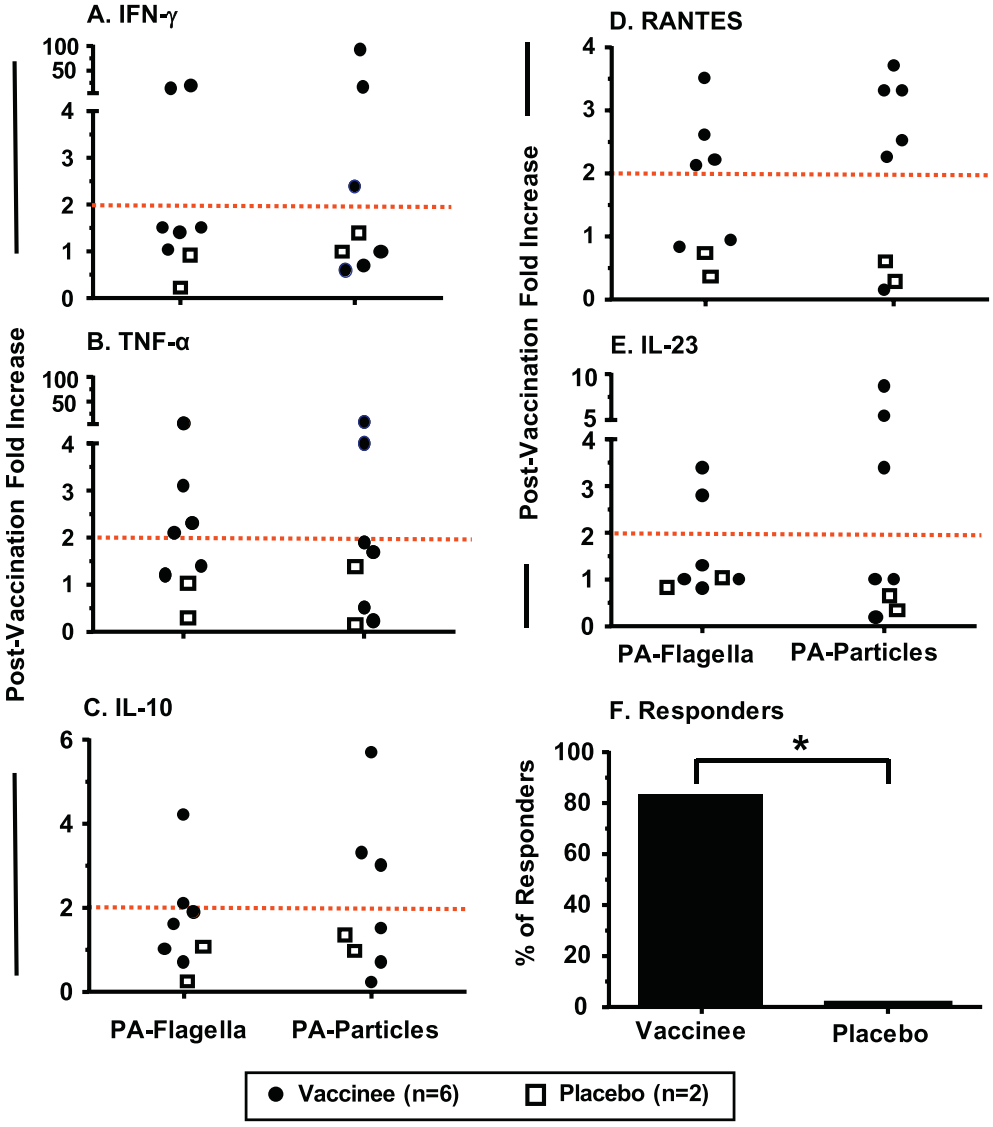

Fig. 2. Cytokine production in culture supernatants of CVD 1902 immunized volunteers. PBMC samples were obtained from volunteers participating in Cohort 5 (10^{10} CFU, 6 vaccinees, closed circles and 2 placebo controls, open squares). Cytokine/chemokine levels in *S.* Paratyphi A (PA) flagella- or bacterial particles (PA-Particles)-stimulated culture supernatants for IFN-γ (A), TNF-α (B), IL-10 (C), RANTES (D) and IL-23 (E). Net data were calculated as antigen stimulated minus non-antigen (media only) stimulated levels. Post-vaccination increases were calculated as post-vaccination / pre-vaccination levels (fold increases). Broken horizontal lines represent 2-fold post-vaccination increases. Vaccine responders were defined as subjects showing > 2-fold post-vaccination increases for two or more cytokines (F). *P* values were calculated using 1 tail chi-square tests. $^*p < 0.05$.

cytokines, while no increases were observed in placebo controls (Fig. 2F).

3.2.2. Induction of S. Paratyphi A-specific CD8+ T effector/memory (T_{EM}) cells

We next evaluated T cell immune responses elicited by CVD 1902 by characterizing the CD8+ T effector/memory (T_{EM}, live CD3+ CD4- CD8- + CD45-CD62L-) cells for their expression of CD107a or production of cytokines (IFN-γ, IL-2 and TNF-α) following ex-vivo culture with PA-infected autologous targets, as described in the Methods (Section 2.8). We used PBMC obtained before and 28 days after immunization from the vaccinees ($n = 12$), ingesting 10^9 ($n = 6$) and 10^{10} CFU (n = 6) of CVD 1902, as well as from placebo controls ($n = 4$). The magnitude of the post-vaccination increases in PA specific CD69 + CD8+ T_{EM} cells expressing CD107a or producing IFN-γ, IL-2 or TNF-α in vaccinees was higher than observed in placebo controls, but did not reach statistical significance (Fig. 3A). However, 7 out of these 12 immunized volunteers (58%), showed post-vaccination increases in two or more specific responses or "functions" (e.g., expression of CD107a, production of IFN-γ, IL-2 and/or TNF-α) and were considered CD8+ vaccine responders. No CD8+ vaccine responders were observed among the 4 placebo recipients (Fig. 3B). These CD8+ vaccine responders ($n = 7$) showed a significantly higher magnitude of post-vaccination increases in CD107a, IFN-γ, IL-2 and/or TNF-α + CD8+ T_{EM} cells compared to those observed among vaccinees who were not CD8+ vaccine responders (non-responders, 5 out of 12 vaccinees) or in placebo ($n = 4$) controls (Fig. 3C). Of note, 2 placebo controls showed post-vaccination increases only in CD107a + CD8+ T_{EM} cells but not with any of the other cytokines evaluated.

3.2.3. Multifunctional and gut-homing characteristics of CD8+ T_{EM} cells in CD8+ vaccine responders

We further categorized PA-infected target specific CD8 + T_{EM} cells as single positive cells (S+) expressing / producing only one of the functions (e.g., CD107a, IFN-γ, IL-2 or TNF-α) or those concomitantly producing two or more functions as multifunctional cells (MF) cells (Fig. 4). We observed that CD8+ T_{EM} cell-mediated PA-specific responses in CD8+ vaccine responders ($n = 7$, described earlier in Section 3.2.2) were mostly comprised of MF cells (Fig. 4A) compared to the corresponding S+ cells. Further characterization of these MF cells showed that vaccine elicited PA-specific induction of MF PA-specific cells that produced IFN-γ (Fig. 4C), IL-2 (Fig. 4D), or TNF-α (Fig. 4E) were observed at a significantly higher magnitude than the corresponding S+ cells. However, increases observed in total (Fig. 4A) and CD107a + (Fig. 4B) MF cells were not statistically significant. MF cells can also be categorized into double (2+), triple (3+) or quadruple

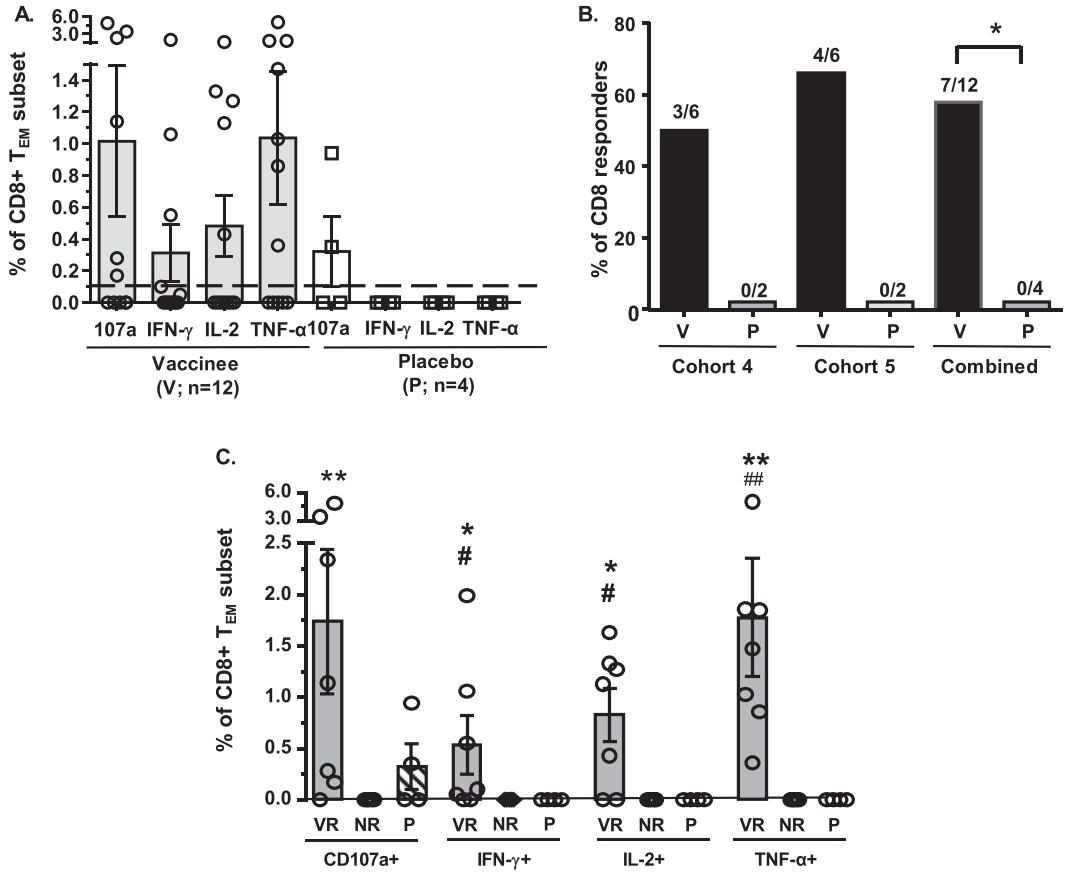

Fig. 3. CMI responses mediated by CD8+ T_{EM} cells following immunization with CVD 1902 or Placebo. PBMC were collected before (Day 0) and 28 days post-vaccination from vaccinees (V; open circles) immunized with 10^9 CFU (cohort 4) or 10^{10} CFU (cohort 5) of CVD 1902 or Placebo (P; buffer only; open squares). Post vaccination increases in the expression of CD107a (107a) or production of cytokines (IFN-γ, TNF-α, IL-2) in response to *S.* Paratyphi A-infected EBV-B (target) cells were measured in CD8+ CD69+ T effector/memory (T_{EM}) cells (A). Post-vaccination increases of ≥ 0.1% (dotted horizontal line in A) in PA target-specific CD8+ CD69+ T_{EM} cells producing/expressing at least 2 functions (IFN-γ, TNF-α, IL-2 and/or CD107a) were used to define CD8 vaccine responders (VR; B) in cohorts 4, 5 or combined. Comparisons of the magnitudes of post-vaccination increases in expression of CD107 or cytokine responses in VR, NR (non-responders) and Placebo control (P) volunteers are shown in C. Post-vaccination increases were calculated as Post-vaccination responses at day 28 minus pre-vaccination (day 0) levels. P values were obtained by comparing the VR to the corresponding NR (*p < 0.05; **p < 0.01) or P (#, < 0.05, ## p < 0.01) by Mann-Whitney test, one tail.

(4+) positive based on their concomitant expression of CD107a and/or production of IFN-γ, IL-2 and/or TNF-α. Such detailed subtyping of MF cells revealed that CVD 1902-elicited MF cells in CD8+ vaccine responders were comprised of similar proportions of 2+, 3+ and 4+ subsets (Fig. S3A). We further investigated the gut-homing potential of the PA specific MF cells by measuring their concomitant expression of the gut homing marker integrin α4β7 (Fig. 5) and observed that vaccine elicited total PA-specific CD8+ T_{EM} MF cells were equally represented by cells expressing integrin-α4β7 (α4β7+; 58.1% ± 11.8) or not (α4β7-; 56.6% ± 13.2) (Fig. 5). Integrin α4β7+ and α4β7- cells in PA-specific CD107a+, IFN-γ+, IL-2+ and TNF-α + MF cells were also elicited at similar magnitudes following immunization with CVD 1902 in CD8+ vaccine responders (Fig. 5).

3.2.4. Characterization of S. Paratyphi A–specific CD4+ T_{EM} cells by CVD 1902 immunization

Because PA-specific CD4+ T_{EM} cells are also an important component of the CMI response to *Salmonella*, we next investigated these responses in volunteers following immunization with CVD 1902 (Fig. 6). Post-vaccination increases of > 0.1% of PA-specific CD4+ T_{EM} cells in at least 2 or more functions (e.g., expression of CD107a and/or production of IFN-γ, IL-2 or TNF-α) were observed in 6 out of 12 (50%) vaccinees (CD4+ vaccine responders) (Fig. 6A). Similar to our observations with CD8+ vaccine responses, the magnitude of the post-vaccination increases in total PA-specific CD4+ T_{EM} MF cells among these CD4 vaccine responders ($n = 6$) were significantly higher ($p < 0.02$) than the corresponding S+ cells (Fig. 6B). The predominance of MF cells over S+ was also observed in PA-specific CD107a + (Fig. 5C, p < 0.02), IFN-γ + (Fig. 5D, p < 0.02), IL-2+ (Fig. 5E, p < 0.02) and TNF-α + (Fig. 5F, p < 0.02) CD4 + T_{EM} cells among CD4+ vaccine responders, but not among vaccinees who did not respond by CD4+ mediated T-CMI (non-responders, 6 out of 12; $p > 0.2$) or placebo controls ($n = 4$, p > 0.2). Similar to our observations with CD8+ responses, vaccine elicited CD4+ MF cells were also comprised of similar proportion of 2+, 3+ and 4+ subsets (Fig. S3B).

3.2.5. Comparison and multifunctional profiles of vaccine elicited CD8 and CD4 T cells responses

We next investigated whether individual volunteers responded to immunization by exhibiting CD4, CD8 or both responses following vaccination. We first examined the magnitude of post-vaccination increases observed in PA-specific T cell responses mediated by CD8+ and CD4+ T_{EM} subsets in CVD 1902 vaccinees ($n = 12$) to determine whether a particular T cell subset predominates. Although of different magnitude, not significant differences were observed in vaccine-elicited PA-specific CD4 and CD8 responses (Fig. S4A). We then examined whether the CD4 and CD8 responses were concordant in each individual. Out of 12 volunteers who were immunized with CVD 1902, 8 of them (67%) were either CD8+ and/or CD4+ vaccine responders

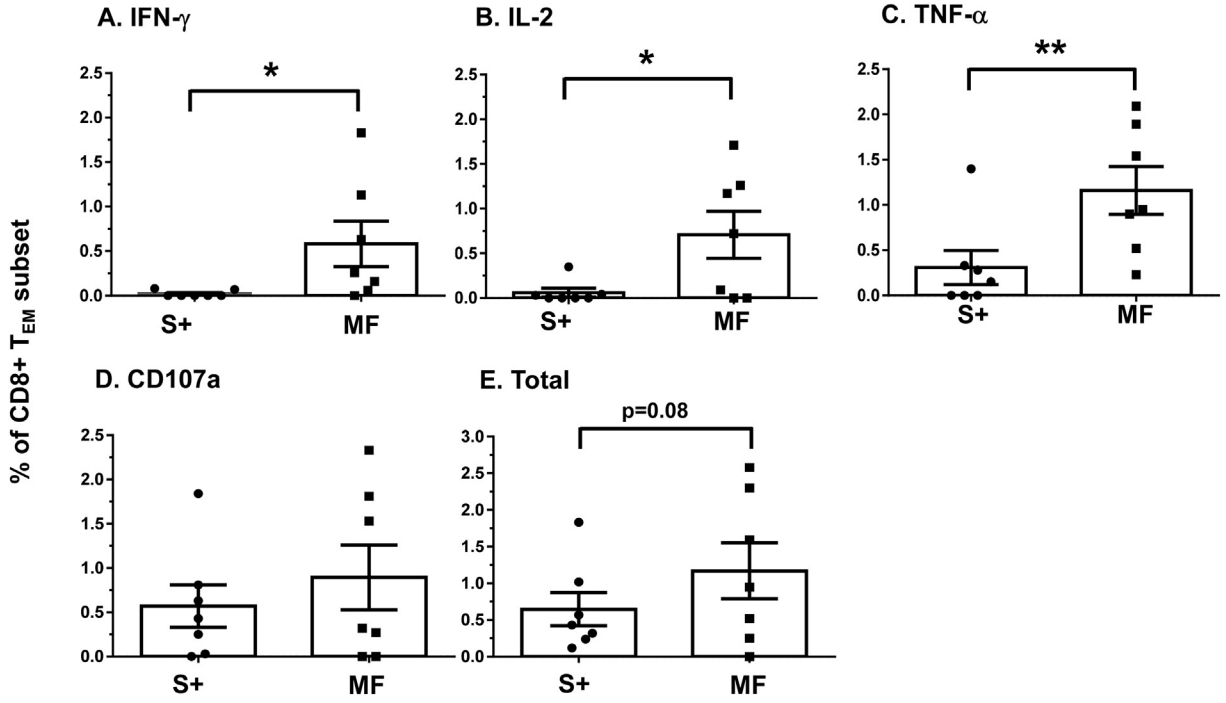

Fig. 4. Induction of multifunctional (MF) CD8+ T_{EM} cells following immunization with CVD 1902 in CD8 vaccine responders. *S.* Paratyphi A (PA)-responses in PBMC collected from CVD 1902 vaccinees ($n = 7$) who were considered "CD8 vaccine responders" as defined in the legend to Fig. 3 and the text. Shown are post-vaccination increases in IFN-γ + (A), IL-2 + (B), TNF-α + (C), CD107a + (D), and total PA-specific CD8+ CD69+ T_{EM} cells (any function) (E), with cells categorized as single positive (S+) or multifunctional (MF, > 2 functions). P values were obtained by comparing the corresponding S+ and MF cells by Wilcoxon signed rank test, one tail. * $p < 0.05$, ** $p < 0.01$.

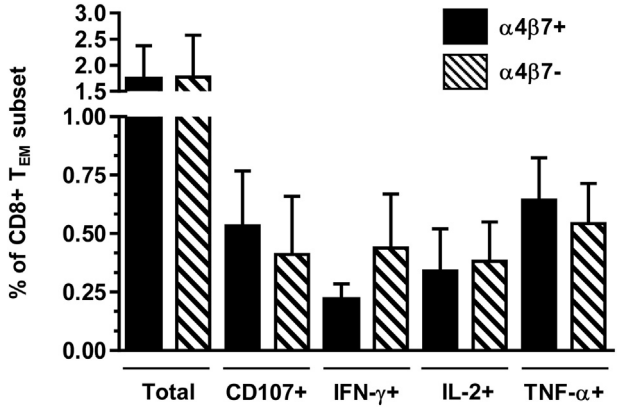

Fig. 5. Gut homing potential of CVD 1902 elicited multifunctional (MF) CD8+ T_{EM} cells in CD8 vaccine responders. Post-vaccination increases in *S.* Paratyphi A specific CD8 T_{EM} MF cells were categorized according to their concomitant expression of the gut homing marker integrin α4β7 in CD8 vaccine responders (n = 7) as defined in the legends to Figs. 3 and 4. Data are shown as mean ± SE. Statistical analyses between cells co-expressing integrin α4β7 or not were performed using non-parametric Mann-Whitney test.

(Sections 3.2.2 and 3.2.3, respectively). The majority (63%, 5 out of 8) of these CD8+ and/or CD4+ vaccine responders elicited both PA-specific CD8+ and CD4+ T_{EM} cells. A correlation analysis between the magnitude of post-vaccination increases in PA-specific CD8+ and CD4+ T_{EM} cells showed that significant correlations were only observed in IFN-γ responses (Fig. S4B) by CD4+ and CD8+ cells.

4. Discussion

CVD 1902 is the first live oral vaccine candidate against paratyphoid A fever, so far, to be evaluated in a Phase 1 clinical trial. Herein we describe in detail the induction of immune memory by evaluating PA-specific B- and T-cell-mediated effector/memory responses in PBMC from the two groups of volunteers who ingested, with buffer, a single dose of CVD 1902 containing either 10^9 or 10^{10} CFU, or placebo. Previous studies reported the induction of B memory cells (B_M) against LPS, O-polysaccharides and protein antigens in humans following natural infection or after immunization with oral vaccines against *S.* Typhi or other enteric bacteria (e.g., *Shigella*, *Vibrio cholerae* O1, *Escherichia coli*) [34,38–45]. In agreement with those earlier findings, in this study we also observed increases in the magnitudes of PA-LPS-specific IgG and IgA B_M following oral immunization with *S.* Paratyphi A CVD 1902.

IgG and IgA B_M responses against LPS or O-specific polysaccharides were shown to correlate with protection from natural *V. cholerae* clinical infection and with decreased disease severity in a human challenge model of shigellosis [38,43,46]. Data reported herein suggest that a single dose of CVD 1902 with either 10^9 or 10^{10} CFU primed the host-immune system to generate PA-specific B_M that could play a role in host-immunity against PA infection. Of note, albeit of lower magnitude, we also detected IgG or IgA B_M cells responding to the LPS, purified from *S.* Paratyphi A, in some pre-vaccination specimens. It is likely that B_M responses detected in the pre-vaccination samples or placebo controls resulted from cross-reactivity derived from natural exposure to other gram-negative or *Salmonella* responses (e.g., minor antigen 12 which is shared among Group A, Group B and Group D *Salmonella* serovars).

Thus, we demonstrate that a single oral dose of an attenuated PA vaccine elicits PA-LPS-responsive B_M cells. However, due to the lack of PBMC samples collected at later post-vaccination time-points, we could not evaluate the persistence or predominance of a specific isotype (e.g., IgG or IgA) of this vaccine induced PA specific B_M cells in circulation beyond 28 days after immunization.

Cytokine measurements in PBMC culture supernatants following in vitro stimulation with PA-specific antigens were performed in vaccinees receiving 10^{10} CFU of CVD 1902 due to limitations in the availability of

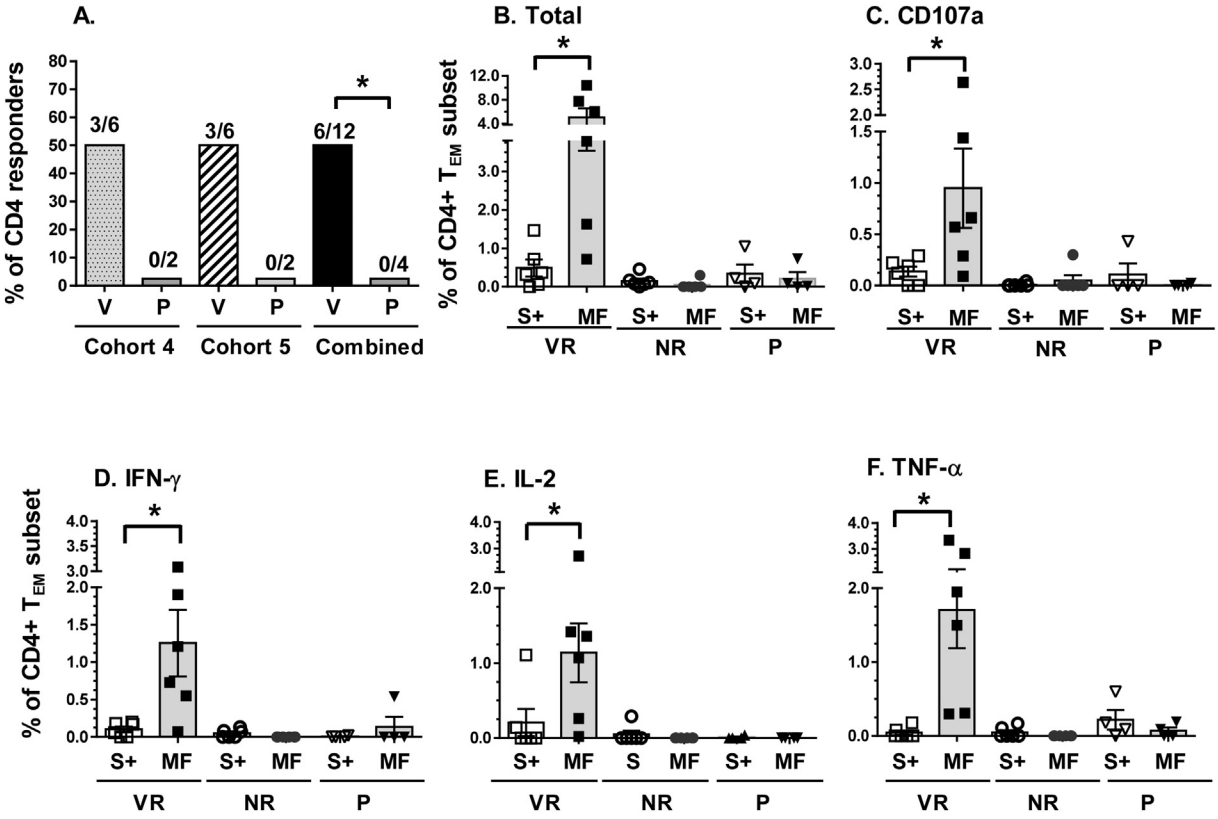

Fig. 6. Induction of multifunctional (MF) CD4+ T_{EM} cells following immunization with CVD 1902. PBMC collected before (Day 0) and 28 days post-vaccination from vaccinees (V) immunized with 10^9 CFU (cohort 4) or 10^{10} CFU (cohort 5) of CVD 1902 or Placebo (P) were used to measure the expression of CD107a (107a) or production of cytokines (IFN-γ, TNF-α, IL-2) in response to *S.* Paratyphi A-infected autologous EBV-B (target) in CD4+CD69+ T effector/memory (T_{EM}) cells. Post-vaccination increases of ≥ 0.1 in PA target-specific CD4+CD69+ T_{EM} cells producing/expressing at least 2 functions (IFN-γ, TNF-α, IL-2 and/or CD107a) was used to define CD4 vaccine responders (A). Post-vaccination increases in Total PA-specific CD4+CD69+ T_{EM} cells (any function) (B) and individual functions, including CD107a+ (C), IFN-γ + (D), IL-2+ (E) and TNF-α + (F) cells categorized as single positive (S+) and multifunctional (MF) are shown in CD4 vaccine responders (VR, $n = 6$), non-responders (NR, n = 6) and in Placebo control (P, $n = 4$). P values were obtained by comparing the corresponding S+ and MF+ cells by Wilcoxon signed rank test, one tail. *$p < 0.05$.

PBMC samples from other cohorts. The cytokines/chemokines that distinguished vaccine elicited responses (cytokine vaccine responders), from placebo controls included those predominantly produced by T cells (e.g., IFN-γ, TNF-α, IL-10, RANTES) and monocytes/type 1 macrophages (e.g., IL-23) [47,48]. Immunization of volunteers with live oral *S.* Typhi vaccines were also reported to show post-vaccination increases in production of IFN-γ, TNF-α and IL-10 following in vitro stimulation with *S.* Typhi antigens [10,32,35,49]. Moreover, similar responses showing increased production of various cytokines including IFN-γ, MIP-1β and TNF-α have also been reported in convalescent typhoid fever patients [50]. The critical role these cytokines (e.g., IFN-γ, TNF-α, IL-12, IL-23 or RANTES) have been implicated in the clearance of *Salmonella* infections and/or potentiating the host-immunity against *Salmonella* by mobilizing various immune cells to the sites of infection, including the gut mucosa [48]. Collectively, observations in this manuscript suggest that CVD 1902 induces a T-CMI response showing a Th1 cytokine profile, arguably an important element in protection against *Salmonella* infection.

To characterize the cell subsets that contribute to vaccine-elicited T-CMI responses, we profiled antigen-specific T_{EM} cells, a key subset of T_M cells that are elicited following immunization with live oral S. Typhi vaccines and which has been associated with protection from wt *S.* Typhi challenge [28,29]. We also evaluated their MF properties as well as gut-homing potential [51–53]. Antigen specific MF T cells are known to produce higher levels of individual cytokines, exhibit enhanced functions and their role in protection from disease has been recognized [51,52]. We have previously shown that immunization with a moderately effective licensed live oral attenuated typhoid vaccine (Ty21a) elicits MF CD8+ and CD4+ T_{EM} responses against *S.* Typhi which are cross reactive with PA [27,37]. Our current study with PA vaccine strain CVD 1902 also showed the induction of PA specific CD8+ T_{EM} cells expressing CD107a + (representing cytotoxic T cells) and/or producing other key Th1 cytokines (e.g., IFN-γ, TNF-α, IL-2), which may contribute to effective immunity against PA [32]. Of note, in this study, almost two-thirds of the CVD 1902 vaccinees were CD8+ vaccine responders, a similar proportion to CD8+ vaccine responders observed with Ty21a vaccination [27,32,33,54]. *Salmonella* including PA infects humans via the gut mucosa and therefore, in addition to systemic immunity, an effector/memory response at the gut level is thought to be a critical component of vaccine elicited immunity [31,32,55]. Previously we observed that *S.* Typhi vaccines (e.g., Ty21a, CVD 909) elicited *S.* Typhi-specific MF CD8+ T_{EM} cells which express, or not, integrin α4β7 [22,27,37,56] in peripheral blood, as well as in the terminal ileum lamina propria of the vaccinees [57]. Similar to those studies, we observed the induction of a substantial proportion of PA-specific T_{EM} CD8+ MF cells that co-expressed the gut homing molecule integrin α4β7 in the majority of CVD 1902 immunized volunteers. These observations are suggestive of an ability of CVD 1902 to induce a mucosal immune response mediated by PA specific MF CD8+ T_{EM}, which can be critical in preventing a PA infection.

In addition to the induction of CD8+ T_{EM} responses, *S.* Typhi immunized volunteers also demonstrated the induction of a *Salmonella* cross-reactive CD4+ T_{EM} responses to both *Salmonella*-infected targets and *S.* Typhi soluble antigens (e.g., flagella) [24,31,35,37]. Our current

observations demonstrated a similar induction of PA-specific MF CD4+ mediated T_{EM} cell responses in a significant proportion of CVD 1902 immunized volunteers. These PA specific MF cells produced IFN-γ, TNF-α and/or IL-2 MF cells with a smaller subset also showing cytotoxic potential by co-expressing CD107a [37]. The role of these PA-specific cytokine producing CD4+ T_{EM} cells in protection remains unknown; however, in a mouse model, it was shown that efficient induction of CD4+ T cell mediated Th1 response, e.g., production of IFN-γ, TNF-α and/or IL2 is needed for clearance or resistance to *Salmonella* infection [58,59]. Overall, these observations demonstrated that CVD 1902 is immunogenic in humans and elicits an orchestrated immune response favoring a Th1 response. However, further studies are required to evaluate whether these immune responses are correlated with protection against PA infection.

In previous studies with S. Typhi vaccine strains Ty21a, CVD 908-*htrA* or CVD 909 immunized volunteers, we showed different dominance of CD4 and CD8 responses depending on the time point after immunization (up 1 year post-vaccination) and the antigens used for in vitro stimulation [22–24,27,35,37,56,60]. Unfortunately, the current study was limited by the availability of a single time point post-immunization (28 days) and a relatively small number of volunteers. Thus, the interpretation of the current findings will need to be revisited in future studies in which a larger number of participants and multiple later times points become available.

5. Conclusions

Paratyphoid A fever caused by PA has emerged as a major health problem in enteric fever endemic areas of the world. Currently no vaccine against this disease is available. A novel live attenuated PA vaccine (CVD 1902) was developed to aid in the prevention of PA infection. In the current study we evaluated, for the first time, B and T cell mediated immune responses in volunteers immunized with the vaccine strain CVD 1902. Our observations showed that a single dose of either 10^9 CFU or 10^{10} CFU of CVD 1902 elicited PA specific B_M and T effector memory responses mediated by both CD8+ and CD4+ T cells in almost two third of the vaccinees. The vaccine induced T cell responses were predominately mediated by PA specific-MF cells with a cytokine production pattern suggestive of Th1 responses. These results suggest that this vaccine strain has the potential to protect against PA infection. Future challenge studies with wt PA and field studies will establish the importance of these B and T memory responses in protection and accelerate the development of attenuated vaccines, in single or multiple doses, for PA. Of note, the CVD 1902 vaccine has been licensed to Bharat Biotech International, Ltd. (Hyderabad, India), which has also licensed live oral *S*. Typhi vaccine strain CVD 909 [61], thereby paving the way for commercial development of a bivalent (CVD 909/CVD 1902) live oral vaccine against the two major agents that cause enteric fever.

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Disclosures

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.03.003.

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