Diversity of *Salmonella* Typhi-responsive CD4 and CD8 T cells before and after Ty21a typhoid vaccination in children and adults

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Abstract

Typhoid fever is a life-threatening disease caused by the human-restricted pathogen *Salmonella enterica* serovar Typhi (S. Typhi). The oral live attenuated Ty21a typhoid vaccine protects against this severe disease by eliciting robust, multifunctional cell-mediated immunity (CMI), shown to be associated with protection in wild-type S. Typhi challenge studies. Ty21a induces S. Typhi-responsive CD8⁺ and CD4⁺ T cells but little is known about the response to this vaccine in children. To address this important gap in knowledge, we have used mass cytometry to analyze pediatric and adult pre- and post-Ty21a vaccination CMI in an autologous S. Typhi antigen presentation model. Here, using conventional supervised analytical tools, we show adult T cells are more multifunctional at baseline than those obtained from children. Moreover, pediatric and adult T cells respond similarly to Ty21a vaccination, but adult responders remain more multifunctional. The use of the unsupervised dimensionality reduction tool tSNE (t-distributed Stochastic Neighbor Embedding) allowed us to confirm these findings, as well as to identify increases and decreases in well-defined specific CD4⁺ and CD8⁺ T-cell populations that were not possible to uncover using the conventional gating strategies. These findings evidenced age-associated maturation of multifunctional S. Typhi-responsive T-cell populations, including those which we have previously shown to be associated with protection from, and/or delayed onset of, typhoid disease. These findings are likely to play an important role in improving pediatric vaccination strategies against S. Typhi and other enteric pathogens.

Keywords: dimensionality reduction, multifunctionality, pediatric immunology, *Salmonella* Typhi, T-cell response

Introduction

Typhoid fever, caused by the human-restricted intracellular pathogen *Salmonella enterica* serovar Typhi (S. Typhi), leads to ~21 million illnesses and >200 000 deaths per year (1–3). S. Typhi is spread via the oral–fecal route, so the global burden of disease predominately lies among regions of the world with limited access to clean sources of water. School-aged children are particularly impacted by typhoid-associated morbidity (1–5). The baseline presence of robust, multifunctional S. Typhi antigen-responsive CD8⁺ T memory cells in the periphery is associated with protection from, and/or delayed onset of typhoid disease following challenge with wild-type (wt) S. Typhi (6, 7). Successful vaccination against the pathogen should therefore aim to induce robust

multifunctional cytotoxic T lymphocyte (CTL) responses. Previous studies have shown that oral live attenuated Ty21a vaccination induces both CTL and CD4⁺ cell-mediated immunity (CMI) responses to stimulation with a variety of S. Typhi-specific antigens in approximately two-thirds of adult recipients (8–10). Ty21a vaccination has also been shown to induce expression of gut- and mucosal-homing markers on circulating CD4⁺ and CD8⁺ T cells (10, 11), although little is known on the effects that this homing potential have on protection from development of typhoid disease. Taken together, these data suggest that successful vaccination against S. Typhi should induce robust, multifunctional CD4 and CD8 CMI in recipients, regardless of age.

Successful global health strategies require protective vaccination against *S. Typhi* among school-aged children in endemic regions. However, due to the difficulties associated with obtaining pediatric samples, and the relatively limited scope of previous generations of analytical instruments to study CMI in depth, there is little information comparing circulating pediatric and adult T-cell populations, in particular following immunization with oral, live attenuated vaccines. Studies analyzing peripheral blood mononuclear cells (PBMCs) from healthy participants show that the percentages of total T cells (CD3⁺) and CD8⁺ T cells remain relatively constant from childhood through adulthood, while the percentages of CD4⁺ T cells increase with age (12–14). Furthermore, among both CD4⁺ and CD8⁺ peripheral T cells, naive populations are present as a higher percentage in children than in adults, and the inverse is true for effector memory populations (12–16). Work from our group has shown that both at baseline and following mitogenic stimulation, the percentages of circulating activated (as defined by CD69 expression) CD8⁺ T cells increase throughout childhood, reaching adult levels by ~15 years of age (12) (M. Rudolph *et al.*, *Frontiers in Immunology*, *In Press*). Pediatric studies following killed oral cholera vaccination (17) and varicella vaccination (18) have both suggested that younger children may be less likely to

develop long-lasting protective CMI than older children or adults. Further, we have recently shown that children aged between 6 and 15 years are less likely to have baseline or Ty21a-induced multifunctional gut-homing CD8⁺ T effector memory (T_{EM}) and T effector memory CD45RA⁺ (T_{EMRA}) responses to human leukocyte antigen-E-restricted *S. Typhi* antigen presentation than either adults or 16- to 17-year-old pediatric vaccinees (M. Rudolph *et al.*, *Frontiers in Immunology*, *In press*). Together, these observations lend a possible explanation to Ty21a field trial data showing lower efficacy in vaccinees aged 9–14 than those 15 years and older (3, 19, 20).

To assess whether there are differences in *S. Typhi*-responsive CD4⁺ and CD8⁺ CMI, and the homing potential of the responsive cells, between children and adults following Ty21a oral immunization, in the present study, we utilized *S. Typhi*-infected autologous Epstein–Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCLs) as an antigen presentation model (6, 7, 21). To explore these responses in considerable depth, despite the scarcity of pediatric PBMCs, we utilized a comprehensive mass cytometry panel which encompasses not only a wide range of diverse CMI effector and homing molecules, but also T-cell memory, activation and proliferation markers (Table 1). Further, we utilized

Table 1. Mass cytometry panel

Target	Stable metal isotope	Clone	Description	Type
CD14	114 Cd (Qdot)	TüK4	Monocyte marker	Surface
CD19	114 Cd (Qdot)	SJ25-C1	B-cell marker	Surface
IL-4	142 Nd	MP425D2	Tc/h2 effector cytokine	Intracellular
<i>CXCR5</i>	<i>Biotin</i>	<i>RF8B2</i>	Follicular-homing chemokine receptor	Surface
Biotin	143 Nd	1D4-C5		Secondary
$\alpha 4\beta 7$	<i>FITC</i>	<i>ACT-1</i>	Gut-homing integrin	Surface
FITC	144 Nd	FIT22		Secondary
CD8	146 Nd	RPA-T8	CTL marker	Surface
IL-6	147 Sm	MQ213A5	Pro-inflammatory proliferation-associated cytokine	Intracellular
CCR4	149 Sm	L291H4	Chemokine homing to the skin	Surface
MIP-1 β	150 Nd	D21-1351	NK and monocyte recruiting chemokine	Surface
CD107a	151 Eu	H4A3	Degranulation marker	With Stimulation
TNF α	152 Sm	Mab11	Pro-inflammatory cytokine	Intracellular
CD62L	153 Eu	DREG-56	Lymphoid tissue-homing selectin	Surface
CD45	154 Sm	HI30	Pan-leukocyte barcoding marker	Barcode
CD27	155 Gd	L128	TNF superfamily—co-stimulatory molecule	Surface
CD45	156 Gd	HI30	Pan-leukocyte barcoding marker	Barcode
IL-2	158 Gd	MQ1-17H12	Induction of T-cell differentiation and proliferation	Intracellular
Ki67	161 Dy	B56	Intracellular marker of cell proliferation	Intracellular
CD69	162 Dy	FN50	Activation marker	Intracellular
CXCR3	163 Dy	G025H7	Chemokine homing to sites of inflammation	Surface
IL-17A	164 Dy	N49-653	Tc/h17 effector cytokine	Intracellular
IFN γ	165 Ho	B27	Pro-inflammatory cytokine	Intracellular
IL-10	166 Er	JES3-9D7	Anti-inflammatory cytokine	Intracellular
CD154 (CD40L)	168 Er	24-31	Co-stimulatory molecule; Tfh induction of B-cell maturation	Intracellular
CD45RA	169 Tm	HI-100	T cell memory marker	Surface
CD3	170 Er	UCHT1	TCR co-receptor (T cell marker)	Surface
Granzyme B	171 Yb	GB11	Secreted cytotoxic effector molecule	Intracellular
IL-21	172 Yb	3A3-N2	Tfh effector cytokine (germinal center formation)	Intracellular
ICOS (CD278)	173 Yb	C398.4A	Tfh co-stimulatory marker (B-cell help)	Surface
CD4	174 Yb	SK3	Helper T lymphocyte marker	Surface
PD-1	175 Lu	EH12.2H7	T-cell exhaustion/activation marker	Surface/intracellular
CCR6	176 Yb	G034E3	Chemokine homing to mucosal surfaces	Surface
Cell I.D. (DNA)	191/193 Ir	n/a	DNA intercalator	DNA
Viability	194/195 Pt	n/a	Viability stain	Viability

Italicized antibodies are not metal-conjugated and require a secondary, metal-conjugated detection antibody for use in mass cytometry. Shown are the antibody targets, stable metal isotope (or other label), antibody clone, a brief description of the target function, and whether the antibody was surface, secondary, intracellular, added with stimulation, or used for barcoding.

unbiased dimensionality reduction tools to study whether CD8⁺ subsets which we have previously associated with protection (6, 7), as well as other multifunctional T-cell responses, differ between Ty21a vaccinated children and adults. We describe age-associated differences among multifunctional baseline *S. Typhi* antigen-responsive T cells and identified novel CD4⁺ and CD8⁺ T-cell populations that are significantly impacted by Ty21a vaccination which only partially overlap between children and adults. These data point to significant differences in CMI responses between children and adults which should be taken into consideration to better inform future *S. Typhi* vaccine design and vaccination strategies.

Methods

Participants and isolation of PBMCs

PBMCs were collected from pediatric and adult participants prior to and between 14 and 42 days following Ty21a vaccination. The 15 healthy pediatric participants ranged in age from 6 to 17 years at the time of enrollment and received the Ty21a vaccination for medically indicated reasons (i.e. travel to endemic regions). The 14 healthy adult participants ranged in age from 20 to 65 years at the time of enrollment. The demographics of the volunteers are listed in Table 2. These participants were recruited from the Baltimore–Washington area and the University of Maryland at Baltimore campus. These studies were approved by the University of Maryland at Baltimore Institutional

Table 2. Participant demographics

Age (years)	Sex	Race
6	Male	African American
10	Female	Hispanic
10	Male	Caucasian
11	Male	Caucasian
11	Female	Caucasian
13	Female	African American
13	Female	African American
13	Male	Caucasian
13	Male	Caucasian
14	Male	Caucasian
14	Male	Caucasian
14	Female	Caucasian
15	Female	Caucasian
16	Male	Caucasian
17	Female	Caucasian
20	Female	African American
25	Female	African American
27	Female	African American
31	Female	African American
33	Female	Caucasian
36	Male	Caucasian
41	Female	Caucasian
43	Male	African American
43	Male	African American
44	Female	African American
51	Male	African American
62	Male	n/a
65	Male	n/a
65	Male	n/a

Ty21a vaccinees are listed by age (years), sex, race. n/a, not available.

Review Board (IRB) and were carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all adult participants, including written informed consent from the parents of any participant under the age of 18 years, as well as assent from the pediatric participants, prior to the conduct of the study procedures. Following blood collection, PBMCs were immediately isolated by density gradient centrifugation and stored in liquid nitrogen following standard cryopreservation techniques (22, 23) until they were thawed for use in the assays.

Preparation of autologous B-LCLs

PBMCs from each of our participants were thawed in complete 1640 RPMI media (Gibco, Carlsbad, CA, USA), supplemented with 100 U ml⁻¹ penicillin (Sigma), 100 µg ml⁻¹ streptomycin (Sigma, St Louis, MO, USA), 50 µg ml⁻¹ gentamicin (Gibco), 2 mM L-glutamine (Gibco), 2.5 mM sodium pyruvate (Gibco), 10 mM HEPES buffer (Gibco), non-essential amino acids (Lonza, Basel, Switzerland) and 10% fetal bovine serum (Gemini Bioproducts, West Sacramento, CA, USA), transformed with EBV and expanded to generate autologous EBV-transformed B-LCLs. Autologous B-LCLs were infected with wt *S. Typhi* strain ISP1820 (wt *S. Typhi*) in RPMI without antibiotics for 3 h at 37°C at a multiplicity of infection of 7:1 (3.5×10^7 wt *S. Typhi* as determined by OD600: 5.0×10^6 B-LCL cells). Uninfected target cells acted as controls. After the 3-h incubation, the cells were washed twice and incubated at 37°C, 5% CO₂ overnight in cRPMI supplemented with 0.3% gentamicin. Conventional flow cytometry was used to detect expression of wt *S. Typhi* antigens on live, infected targets cells (and not on uninfected cells) with LIVE/DEAD™ yellow cell stain (ThermoFisher Scientific, Waltham, MA, USA) and BacTrace® FITC-labeled anti-*Salmonella* CSA-1 antibody (SeraCare, Milford, MA, USA). Cells were then gamma-irradiated (6000 rad), washed and re-suspended in fresh cRPMI before being used as co-culture targets for effector T cells.

In vitro stimulation of effector cells

PBMCs were thawed and rested overnight at 37°C, 5% CO₂ in cRPMI prior to stimulation with B-LCL targets. Following the overnight rest, cells were washed and re-suspended in cRPMI at a concentration of 1×10^6 cells 500 µl⁻¹ in 5 ml cell culture tubes. Gamma-irradiated B-LCL targets were incubated with PBMCs at an effector-to-stimulator ratio of 5:1, along with 3 µl ml⁻¹ anti-CD107a monoclonal antibody (mAb) conjugated to 151Eu (Fluidigm, South San Francisco, CA, USA), for 2 h at 37°C in 5% CO₂. Following the 2-h incubation, 0.5 µl ml⁻¹ each of GolgiStop (containing monensin) and GolgiPlug (containing brefeldin A) from BD (San Jose, CA, USA) were added at to all tubes and cultures were maintained at 37°C in 5% CO₂ overnight.

Surface and intracellular immune labeling and mass cytometry analysis

Co-cultured PBMCs were centrifuged and barcoded with anti-CD45 (Fluidigm) mAbs, with pediatric samples labeled with

Fig. 1. Baseline CD8⁺ T effector memory responses in children and adults. Scatter plots showing pre-Ty21a vaccination *S. Typhi*-responsive activated (CD69⁺) CD8⁺ T effector memory cell (CD45RA⁺/CD62L⁺) production/expression of (A) MIP1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ and Granzyme B (GzmB); (B) observed populations with mono-, bi-, tri- and >3-functions, as defined by production/expression of one or more of the seven effector functions; (C) defined multifunctional populations shown in a previous *S. Typhi* challenge study to be associated with protection from developing typhoid disease following wt challenge; and (D) those same multifunctional populations also including Granzyme B expression (not explored in the challenge study). Bars represent medians with whiskers indicating interquartile ranges. Results are shown for the following populations: 6- to 15-year-old pediatric ($n = 13$), 16- to 17-year-old pediatric ($n = 2$) and adult ($n = 14$) participants. Scatter plot statistics were analyzed by unpaired *t*-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Significant differences between children aged 6–15 years are shown with blue numbers (trends) or asterisks (significant). Significant differences between children aged 6–17 years are shown with orange numbers (trends) or with asterisks (significant).

CD45-154Sm and adult samples labeled with CD45-156Gd for 30 min at 4°C. Cells were then washed twice: once with flow cytometry buffer (1 $\times$ phosphate-buffered saline; Quality Biological, Gaithersburg, MD, USA), 0.1% sodium azide (Sigma), 2% fetal bovine serum (Gemini Bioproducts) and once with serum-free RPMI (Gibco), and were then combined into their barcoded layout, with like-stimulated adult (CD45-156Gd), and pediatric (CD45-154Sm) PBMCs combined into a single tube for downstream staining. Mass cytometry staining was performed using the protocol described in the 'Materials and Methods' section titled, 'Mass Cytometry Measurements' from McArthur *et al.* (24) using the mAb panel described in Table 1. In short, cells were labeled with lanthanide metal-tagged antibodies against a number of surface and intracellular targets. The viability marker cisplatin and DNA intercalator iridium were utilized for cell identification. Cells were then prepared and run in the University of Maryland Flow and Mass Cytometry Core Laboratory on a CyTOF instrument (Fluidigm). Mass cytometry data were debarcoded using Premium Cytobank (Cytobank, Inc., Santa Clara, CA, USA) and analyzed using WinList version 9.0.1 (Verity Software House, Topsham, ME, USA).

Representative cytograms are shown in Supplementary Figure S1. tSNE (t-distributed Stochastic Neighbor Embedding) analysis was run utilizing the Cytokit package in biocLite on R (25). tSNE analysis was run on sub-populations of T cells as analysis of less distinct populations (i.e. total T cells) led to clustering biases towards phenotypic markers (CD4, CD8, CD62L, CD45RA) and a marked increase in the numbers of clusters; these effects likely mask differences found when analyzing downstream effector, activation and/or homing molecules. WinList version 9.0.1 was used for downstream gating of individual tSNE clusters.

Statistical analyses

Unpaired *t*-tests, paired *t*-tests and correlation analyses were performed using GraphPad Prism version 7.0c (GraphPad Software, La Jolla, CA, USA). *P* values of <0.05 were considered significant. Because relatively low numbers of children were available for these translational immunological studies, *P* values of <0.1 are also presented in figures as possible trends. This approach follows the recommendation of the American Statistical

Fig. 2. Baseline CD4⁺ T effector memory responses in children and adults. Scatter plots showing pre-Ty21a vaccination *S. Typhi*-responsive activated (CD69⁺) CD4⁺ T effector memory cell (CD45RA⁺/CD62L⁻) production/secretion of (A) MIP1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ and Granzyme B (GzmB); (B) observed populations with mono-, bi-, tri- and >3-functions, as defined by production/secretion of one or more of the seven effector functions; (C) defined multifunctional populations shown in a previous *S. Typhi* challenge study to be associated with protection from developing typhoid disease following wt challenge; and (D) those same multifunctional populations also including Granzyme B expression (not explored in the challenge study). Bars represent medians with whiskers indicating interquartile ranges. Results are shown for the following populations: 6- to 15-year-old pediatric ($n = 13$), 16- to 17-year-old pediatric ($n = 2$) and adult ($n = 14$) participants. Scatter plot statistics were analyzed by unpaired *t*-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Significant differences between children aged 6–15 years are shown with blue numbers (trends) or asterisks (significant). Significant differences between children aged 6–17 years are shown with orange numbers (trends) or with asterisks (significant).

Fig. 3. Baseline activated T effector memory cell correlation of CD107a and Granzyme B. Scatter plots showing correlation of pre-Ty21a vaccination *S. Typhi*-responsive activated (CD69⁺) CD8⁺ (A) and CD4⁺ (B) T effector memory cell (CD45RA⁺/CD62L⁻) production/secretion of CD107a and Granzyme B across all participants. Pearson correlation coefficients were calculated for each analysis with two-tailed *P* values.

Fig. 4. Pre- and post-Ty21a CD8⁺ T effector memory responses in children and adults. Scatter plots showing pre- and post-Ty21a vaccination S. Typhi-responsive (A) CD69 expression within CD8⁺ T effector memory cells (CD45RA⁺/CD62L⁺); (B) percent activated (CD69⁺) responders over baseline based on a 0.1% cut-off; and production/expression within activated CD8⁺ T_{EM} of: (C) MIP1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ and Granzyme B (GzmB); (D) observed mono-, bi-, tri- and >3-functional populations, as defined by production/expression of one or more of the seven effector molecules. Results are shown for the following populations: 6- to 15-year-old pediatric ($n = 13$), 16- to 17-year-old pediatric ($n = 2$) and adult ($n = 14$) participants. Statistics are non-parametric Wilcoxon matched-pairs signed rank paired t -tests between pre- and post-vaccinated participants. * $P < 0.05$.

Table 3. Post-Ty21a CD8⁺ and CD4⁺ T Effector Memory Responses Differences as related to Baseline Values.

(+/-) 0.1% Cutoff		Increase Over Baseline						Decrease Under Baseline					
		Ages 6-15 (n=13)		Ages 6-17 (n=15)		Adults (n=14)		Ages 6-15 (n=13)		Ages 6-17 (n=15)		Adults (n=14)	
		Fraction	Percent	Fraction	Percent	Fraction	Percent	Fraction	Percent	Fraction	Percent	Fraction	Percent
CD8 Activated Tem	MIP-1β	(5/13)	38.5%	(7/15)	46.7%	(9/14)	64.3%	(7/13)	53.8%	(7/15)	46.7%	(4/14)	28.6%
	CD107a	(7/13)	53.8%	(8/15)	53.3%	(7/14)	50.0%	(6/13)	46.2%	(7/15)	46.7%	(7/14)	50.0%
	TNFα	(3/13)	23.1%	(5/15)	33.3%	(8/14)	57.1%	(8/13)	61.5%	(8/15)	53.3%	(4/14)	28.6%
	IL-2	(4/13)	30.8%	(4/15)	26.7%	(5/14)	35.7%	(6/13)	46.2%	(8/15)	53.3%	(6/14)	42.9%
	IL-17A	(4/13)	30.8%	(5/15)	33.3%	(5/14)	35.7%	(6/13)	46.2%	(7/15)	46.7%	(9/14)	64.3%
	IFNγ	(6/13)	46.2%	(7/15)	46.7%	(9/14)	64.3%	(5/13)	38.5%	(6/15)	40.0%	(5/14)	35.7%
	GzmB	(5/13)	38.5%	(6/15)	40.0%	(9/14)	64.3%	(7/13)	53.8%	(8/15)	53.3%	(3/14)	21.4%
CD4 Activated Tem	MIP-1β	(4/13)	30.8%	(4/15)	26.7%	(2/14)	14.3%	(8/13)	61.5%	(9/15)	60.0%	(11/14)	78.6%
	CD107a	(2/13)	15.4%	(3/15)	20.0%	(3/14)	21.4%	(11/13)	84.6%	(12/15)	80.0%	(9/14)	64.3%
	TNFα	(3/13)	23.1%	(5/15)	33.3%	(4/14)	28.6%	(5/13)	38.5%	(5/15)	33.3%	(5/14)	35.7%
	IL-2	(6/13)	46.2%	(7/15)	46.7%	(5/14)	35.7%	(5/13)	38.5%	(5/15)	33.3%	(7/14)	50.0%
	IL-17A	(5/13)	38.5%	(5/15)	33.3%	(3/14)	21.4%	(5/13)	38.5%	(7/15)	46.7%	(9/14)	64.3%
	IFNγ	(7/13)	53.8%	(8/15)	53.3%	(5/14)	35.7%	(3/13)	23.1%	(4/15)	26.7%	(6/14)	42.9%
	GzmB	(8/13)	61.5%	(9/15)	60.0%	(5/14)	35.7%	(5/13)	38.5%	(6/15)	40.0%	(8/14)	57.1%

MIP-1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ , and Granzyme B production/expression response differences from baseline greater than (+/-) 0.1% for activated CD8⁺ and CD4⁺ effector memory T cells among pediatric participants from ages 6-15 years-old ($n = 13$), pediatric participants aged 6-17 years-old ($n = 15$), and adults aged 20-65 ($n = 14$). Differences over (grey) or under (black) baseline of over 50% are highlighted.

Fig. 5. Response-over-baseline CD8⁺ T effector memory responses in children and adults. Scatter plots showing responses over baseline of *S. Typhi*-responsive activated (CD69⁺) CD8⁺ T effector memory cell (CD45RA⁺/CD62L⁻) production/expression of (A) MIP1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ and Granzyme B (GzmB); (B) observed populations with mono-, bi-, tri- and >3-functions, as defined by production/expression of one or more of the seven effector functions; (C) defined multifunctional populations shown in a previous *S. Typhi* challenge study to be associated with protection from developing typhoid disease following wt challenge; and (D) those same multifunctional populations also including Granzyme B expression (not explored in the challenge study). Bars represent medians with whiskers indicating interquartile ranges. Results are shown for the following populations: 6- to 15-year-old pediatric ($n = 13$), 16- to 17-year-old pediatric ($n = 2$) and adult ($n = 14$) participants. Scatter plot statistics were analyzed by unpaired *t*-test ($*P < 0.05$). Significant differences between children aged 6–15 years are shown with blue numbers (trends) or asterisks (significant). Significant differences between children aged 6–17 years are shown with orange numbers (trends) or with asterisks (significant).

Association's statement regarding the context and purpose of *P* values, particularly in data with low *n*-numbers (26, 27).

Results

S. Typhi-responsive CD8⁺ T_{EM} in children and adults prior to Ty21a vaccination

Pre-Ty21a vaccinated (baseline) PBMCs were co-cultured with wt *S. Typhi* strain 1820-infected participant-specific (autologous) B-LCLs, and then labeled with metal-conjugated mAbs before being analyzed by mass cytometry. Uninfected B-LCL co-cultures were used to subtract non-*S. Typhi*-associated effector responses. We identified *S. Typhi*-associated baseline activated CD8⁺ T_{EM} (CD62L⁻ CD45RA⁺) effector responses among most pediatric (6–17 years old; $n = 15$) and adult (20–65 years old; $n = 14$) participants, with adults being more likely to express IL-2, and trending towards greater baseline expression of CD107a and IFN γ , particularly when comparing them to children under 16 years (Fig. 1A). Indeed, when exploring multifunctionality based

on expression or production of MIP-1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ and/or Granzyme B, adults show significantly higher percentages of baseline trifunctional and greater-than-trifunctional CD8⁺ T_{EM} than children, particularly when compared to younger pediatric participants (Fig. 1B). Further, we analyzed particular multifunctional populations that, when present at baseline, have been shown in wt *S. Typhi* challenge studies to be associated with protection from, and/or delayed onset of, the development of typhoid disease (6, 7). While MIP-1 β /TNF α double-positive populations are higher in pediatric participants at baseline, more multifunctional populations such as MIP-1 β /CD107a/TNF α /IL-2/IFN γ quintuple positive cells trend to be higher in adults (Fig. 1C). Further, when Granzyme B, which was not included in the panel used in the aforementioned challenge studies, is included in the protection-associated populations, highly multifunctional MIP-1 β /CD107a/TNF α /IFN γ /Granzyme B expressing T_{EM} cells are significantly higher in adult participants at baseline (Fig. 1D). Differences among baseline activated CD8 T_{EM} *S. Typhi*-responsive effectors are not observed when dividing

Fig. 6. Pre- and post-Ty21a CD4⁺ T effector memory responses in children and adults. Scatter plots showing pre- and post-Ty21a vaccination *S. Typhi*-responsive (A) CD69 expression within CD4⁺ T effector memory cells (CD45RA⁺/CD62L⁺); (B) percent activated (CD69⁺) responders over baseline based on a 0.1% cut-off; and production/secretion within activated CD4⁺ T_{EM} of: (C) MIP1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ and Granzyme B (GzmB); (D) observed mono-, bi-, tri- and >3-functional populations, as defined by production/secretion of one or more of the seven effector molecules. Results are shown for the following populations: 6- to 15-year-old pediatric (*n* = 13), 16- to 17-year-old pediatric (*n* = 2) and adult (*n* = 14) participants. Statistics are non-parametric Wilcoxon matched-pairs signed rank paired *t*-tests between pre- and post-vaccinated participants. **P* < 0.05.

Fig. 7. Response-over-baseline CD4⁺ T effector memory responses in children and adults. Scatter plots showing responses over baseline of *S. Typhi*-responsive activated (CD69⁺) CD4⁺ T effector memory cell (CD45RA⁺/CD62L⁺) production/secretion of (A) MIP1 β , CD107a, TNF α , IL-2, IL-17A, IFN γ and Granzyme B (GzmB); (B) observed populations with mono-, bi-, tri- and >3-functions, as defined by production/secretion of one or more of the seven effector functions. Bars represent medians with whiskers indicating interquartile ranges. Results are shown for the following populations: 6- to 15-year-old pediatric (*n* = 13), 16- to 17-year-old pediatric (*n* = 2) and adult (*n* = 14) participants. Scatter plot statistics were analyzed by unpaired *t*-test.

Fig. 8. tSNE analysis of activated CD8⁺ T effector memory populations. Percentage of cells per significant clusters showing differences between pediatric and adults divided among pre- (A) and post- (B) Ty21a vaccinated pediatric (blue) and adult (black) participants. (C) Percentage change of cells per significant cluster as in (A) following vaccination among pediatric (blue) and adult (black) participants. (D) tSNE maps of significant clusters as in (A–C) divided among pre- and post-Ty21a vaccinated pediatric and adult participants. (E–J) Functions of individual significant tSNE clusters, divided into: median expression of (E) Tc1 effectors MIP1 β , CD107a, TNF α , IL-2, IFN γ and Granzyme B (GzmB), (F) homing molecules CCR6, CXCR3, CCR4, α 4 β 7 and CXCR5, (G) IL-21, IL-10, IL-17A, IL-6 and IL-4, and (H) activation/exhaustion/additional molecules PD-1, ICOS, CD154, Ki67 and CD27. (I, J) Expression percentages of WinList FCOM analysis determined (I) Tc1 mono- and multifunctional cytokine expression and (J) multifunctional populations associated with protection from developing typhoid disease following *S. Typhi* challenge studies. Statistics were analyzed by unpaired *t*-test (A, B) and non-parametric (C) paired *t*-test. Shown are mean $\pm$ SE error bars. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

our participants parsed by sex (male: *n* = 15; female: *n* = 14) (Supplementary Figure S2A–D).

S. Typhi-responsive CD4⁺ T_{EM} in children and adults prior to Ty21a vaccination

We also explored baseline *S. Typhi*-responsive activated CD4⁺ T_{EM} among pediatric and adult participants. As observed in CD8⁺ T_{EM}, adults produce significantly more IL-2 than children (Fig. 2A). Interestingly, adults also produce significantly more IFN γ than children aged between 6 and 15 years, but the significance is lost when the two 16- to 17-year-old participants are included in the analysis (Fig. 2A). Additionally, *S. Typhi*-responsive baseline multifunctional CD4⁺ T_{EM} are significantly higher in adults than in children, particularly among younger pediatric participants (Fig. 2B). Despite the fact that the aforementioned protective populations (6, 7) were originally identified in CD8⁺ T cells, we also studied whether these subsets of *S. Typhi*-responsive baseline multifunctional

effector responses in CD4⁺ T_{EM} were differentially represented in children and adults. Interestingly, the magnitude of these responses was lower than we previously showed in CD8⁺ T_{EM}, and very few differences were observed between pediatric and adult participants (Fig. 2C and D). Interestingly, when dividing our participant pool by sex, females seem to show stronger baseline CD4⁺ T_{EM} responses than their male counterparts. This is particularly true with respect to expression of MIP-1 β , and is also seen among observed mono- and bi-functional CD4⁺ T_{EM}, as well as in two defined multifunctional populations which for CD8⁺ cells were associated with protection (Supplementary Figure S3A–D).

Baseline correlation of CD107a and Granzyme B-expressing T_{EM}

Because Granzyme B and CD107a expression are two of the best characterized indicators of CTL activity (28, 29), we then explored the correlation of degranulation marker

Fig. 8. continued

CD107a and the cytotoxic secreted serine protease Granzyme B among baseline circulating activated CD8⁺ and CD4⁺ T_{EM} cells among all participants. We observed that Granzyme B and CD107a correlate strongly within CD8⁺ T_{EM} (Fig. 3A), but less strongly among CD4⁺ T_{EM} (Fig. 3B) on the basis of Pearson correlation coefficients with two-tailed *P* values. Interestingly, the correlation within the CD8⁺ T_{EM} seems driven by the pediatric and female participants, both of whom show strong correlations, to a greater extent than adult and male participants, which do not reach significance (Supplementary Figure S4A–D). Among CD4⁺ T_{EM}, only the pediatric group shows significant correlations between Granzyme B and CD107a expression (Supplementary Figure S4E–H).

S. Ty21a-induced CD8⁺ T_{EM} effector responses in children and adults

In order to explore the effects of Ty21a vaccination on *S. Typhi*-responsive CD8⁺ T_{EM}, we compared pediatric and adult CTL effector responses at baseline to those 14–42 days following vaccination. First, we explored overall activation as defined by CD69 expression in pre- and post-Ty21a immunization CD8⁺ T_{EM}. While there were no significant differences in activation levels above baseline in children, CD8⁺ T_{EM} exhibited significantly

increased levels of CD69 expression in adults following vaccination (Fig. 4A). By setting a 0.1% cut-off as responders over baseline (M. Rudolph *et al.*, *Frontiers in Immunology*, *In Press*), we observed that ~60% of adults exhibited increases in *S. Typhi*-responsive CD69⁺ CD8⁺ T_{EM} following Ty21a immunization, compared to only 40% among pediatric participants (Fig. 4B). We next analyzed effector functions and multifunctionality within activated CD8⁺ T_{EM} cells. Among the seven T_C1/T_C17 effector functions analyzed, only IL-2 expression in adults showed a significant difference by paired *t*-test, decreasing following vaccination (Fig. 4C). Both trifunctional and greater-than-trifunctional effector responses exhibited trends to show higher responses following vaccination, but neither reached a significant *P* value of <0.05 (Fig. 4D). By defining differences from baseline as being either above or below 0.1%, we show that greater than or equal to half of adult participants show increases in MIP-1 β , CD107a, TNF α , IFN γ and Granzyme B among circulating activated CD8⁺ T_{EM}, while pediatric participants tend to show decreases, particularly among TNF α and Granzyme B (Table 3; Supplementary Table S1). To further analyze CD8⁺ T_{EM} effector responses-over-baseline, we subtracted the responses at baseline from those post-Ty21a vaccination. Through this analysis, we show that adult TNF α responses are significantly higher, and the majority positive, compared to

Fig. 9. tSNE analysis of activated CD4⁺ T effector memory populations. Percentage of cells per significant clusters showing differences between pediatric and adults divided among pre- (A) and post- (B) Ty21a vaccinated pediatric (blue) and adult (black) participants. (C) Percentage change of cells per significant cluster as in (A) following vaccination among pediatric (blue) and adult (black) participants. (D) tSNE maps of significant clusters as in (A–C) divided among pre- and post-Ty21a vaccinated pediatric and adult participants. (E–I) Functions of individual significant tSNE clusters, divided into: median expression of (E) Tc1 effectors MIP1 β , CD107a, TNF α , IL-2, IFN γ and Granzyme B (GzmB), (F) homing molecules CCR6, CXCR3, CCR4, α 4 β 7 and CXCR5, (G) IL-21, IL-10, IL-17A, IL-6, and IL-4 and (H) activation/exhaustion/additional molecules PD-1, ICOS, CD154, Ki67 and CD27. Expression percentages of WinList FCOM analysis determined (I) Tc1 mono- and multifunctional cytokine expression. Statistics were analyzed by unpaired *t*-test (A, B) and non-parametric (C) paired *t*-test. Shown are mean $\pm$ SE error bars. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

those mostly below baseline responses seen in 6- to 15-year-old pediatric participants (Fig. 5A). As a group, multifunctional responses-over-baseline do not show significant differences between age groups, but adult greater-than-trifunctional effectors exhibited a trend to show higher responses than pediatric participants, regardless of whether or not the older children are included in unpaired *t*-tests (Fig. 5B). Concerning the CD8⁺ populations that we had previously shown to be associated with protection (6, 7), MIP-1 β /TNF α double-positive (with or without Granzyme B production) were significantly decreased with respect to baseline in younger pediatric participants than in adults, but that significance was lost when 16- to 17-year-old children are included in the pediatric group (Fig. 5C and D).

By dividing participants by sex instead of age, we observed that female participants showed significant increases in TNF α and greater-than-trifunctional *S. Typhi*-responsive CD8⁺ T_{EM} following Ty21a vaccination. Male participants, on the other hand, showed increases in Granzyme B and bifunctional responses following vaccination (Supplementary Figure S5A and B). Of note, by applying a 0.1% cut-off as above, we observed increases over baseline

of MIP-1 β , CD107a, IFN γ and Granzyme B in >50% of male participants—adult and pediatric—following vaccination. This is in contrast with the female vaccinees, who show decreases in CD107a, IL-2, IL-17A, IFN γ and Granzyme B, but strong increases in TNF α (Supplementary Tables S1 and S2). Despite these differences, responses-over-baseline analyses showed few significant differences between male and female participants among individual effectors or multifunctional populations (Supplementary Figure S6A and B). Females showed significantly greater percentages of TNF α /MIP-1 β double-positive and TNF α /MIP-1 β /CD107a/IFN γ quadruple-positive CD8⁺ T_{EM} responses, but these differences do not extend to Granzyme B-expressing populations (Supplementary Figure S6C and D).

S. Ty21a-induced CD4⁺ T_{EM} effector responses in children and adults

We then analyzed the effects of Ty21a vaccination on *S. Typhi*-responsive CD4⁺ T_{EM} by directly comparing pediatric and adult effector responses at baseline to responses 14–42 days after

Fig. 9. continued

vaccination. When analyzing the levels of overall activation using CD69 expression, we observed that both adults and children exhibited significant increases in CD69⁺ CD4⁺ T_{EM} following vaccination (Fig. 6A). Further, using a cut-off of 0.1% to define positive response over baseline, we observed that ~50% of Ty21a recipients show increases in circulating *S. Typhi*-responsive CD69⁺ CD4⁺ T_{EM} cells 14–42 days post-vaccination (Fig. 6B). Of the seven T_H1/T_H17 effector functions analyzed, MIP-1 β and CD107a expression in adults decreased compared to baseline levels following vaccination (Fig. 6C). Interestingly, pediatric bifunctional and greater-than-trifunctional CD4⁺ T_{EM} responses significantly increased following vaccination, although inclusion of the 16–17-year-old children eliminates the significance from the greater-than-trifunctional analysis (Fig. 6D). In contrast to the observations in CD8⁺ T_{EM}, a majority of adult participants show decreases with respect to baseline in MIP-1 β , CD107a, IL-2, IL-17A and Granzyme B production among circulating CD4⁺ T_{EM}. Further, most children also show decreases in MIP-1 β and CD107a but show increases in IFN γ and Granzyme B (Table 3; Supplementary Table S1). No significant CD4⁺ T_{EM} response-over-baseline differences were observed among individual or multifunctional effector responses (Fig. 7).

Female participants exhibited decreases in circulating CD107a, IL-17A and Granzyme B-producing *S. Typhi*-responsive CD4⁺ T_{EM} following Ty21a vaccination, whereas male participants show increases in Granzyme B effector responses (Supplementary Figure S7A). No significant sex-associated differences were observed among mono- or multifunctional effector responses following Ty21a vaccination (Supplementary Figure S7B). Interestingly, applying the 0.1% cut-off for positive increases/decreases with respect to baseline, most males have increased production of Granzyme B, even among CD4⁺ T_{EM}, and pediatric males also tend to show increased expression of IL-2, IL-17A and IFN γ . This is in contrast to female participants, either pediatric or adults, who showed decreases in expression or production of MIP-1 β , CD107a, IL-17A and Granzyme B (Supplementary Tables S1 and S2). As a group, sex-associated CD4⁺ T_{EM} response-over-baseline data show no significant differences except for the greater, more positive Granzyme B production in males than females (Supplementary Figure S8).

tSNE analysis of S. Typhi-responsive CD8⁺ T_{EM}
Pre- and post-Ty21a vaccinated *S. Typhi*-infected B-LCL co-cultured activated CD8⁺ T_{EM} cell data were exported and

Fig. 10. tSNE analysis of activated CD8⁺ T effector memory RA populations. **A.** Percentage of cells per significant clusters showing differences between pediatric and adults divided among pre- (**A**) and post- (**B**) Ty21a vaccinated pediatric (blue) and adult (black) participants. (**C**) Percentage change of cells per significant cluster as in (**A**) following vaccination among pediatric (blue) and adult (black) participants. (**D**) tSNE maps of significant clusters as in (**A–C**) divided among pre- and post-Ty21a vaccinated pediatric and adult participants. (**E–J**) Functions of individual significant tSNE clusters, divided into: median expression of (**E**) Tc1 effectors MIP1 β , CD107a, TNF α , IL-2, IFN γ and Granzyme B (GzmB), (**F**) homing molecules CCR6, CXCR3, CCR4, α 4 β 7 and CXCR5, (**G**) IL-21, IL-10, IL-17A, IL-6 and IL-4, and (**H**) activation/exhaustion/ additional molecules PD-1, ICOS, CD154, Ki67 and CD27. Expression percentages of WinList FCOM analysis determined (**I**) Tc1 mono- and multifunctional cytokine expression and (**J**) multifunctional populations associated with protection from developing typhoid disease following S. Typhi challenge studies. Statistics were analyzed by unpaired *t*-test (**A, B**) and non-parametric (**C**) paired *t*-test. Shown are mean $\pm$ SE error bars. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

tSNE was run utilizing the Cytofit package available in the R biocLite module. Thirty clusters were identified following visualization using the ClusterX unbiased clustering method on the following markers: IL-4, CXCR5, α 4 β 7, IL-6, CCR4, MIP-1 β , CD107a, TNF α , CD27, Ki67, IL-2, CXCR3, IL-17A, IFN γ , IL-10, CD154 (CD40L), Granzyme B, IL-21, ICOS, PD-1 and CCR6. Clusters of interest were identified based on significant differences between percentages of cells per cluster among pediatric and adult participants either pre-vaccination (**Fig. 8A**) or post-vaccination (**Fig. 8B**) (unpaired *t*-test), as well as significant increases or decreases within age groups (paired *t*-test; **Fig. 8C**). These significant clusters are shown on tSNE maps divided by adult and pediatric, pre- and post-vaccination groups in order to better highlight the differences between groups (**Fig. 8D**). Median expression of Tc1 effectors within clusters of interest identifies clusters 3, 9, 12, 22 and 26 as containing CD8⁺ T_{EM} producing a diverse array of effector molecules (**Fig. 8E**). Of note, a majority of the significant

clusters contain CXCR3-expressing cells, indicative of homing to sites of inflammation (**Fig. 8F**). Among non-Tc1-associated interleukins, there was little expression in any of the dominant clusters, but cluster 15 contained cells producing IL-21, and cluster 17 contained cells producing IL-4 and IL-10 (**Fig. 8G**). Interestingly, the low-abundance cluster 17 is also the only cluster with cells expressing integrin α 4 β 7 (a gut-homing molecule) or CXCR5 (present in circulating T follicular/helper cells) (**Fig. 8F**) and is notable in its separation from the rest of the tSNE map (**Supplementary Figures S8D and S9B**). Clusters containing cells expressing CD27, which are not as terminally differentiated (30–33), as expected exhibit fewer effector functions, while the cluster high in median expression of co-stimulatory marker CD154 (12) shows high levels of IL-2 and TNF α expression (**Fig. 8H**). We used the FCOM function in WinList to identify multifunctional clusters based on Tc1 effectors (**Fig. 8I**), and as expected they map closely to the more diverse clusters seen in **Fig. 8(E)**. Finally,

Fig. 10. continued

we analyzed the significant clusters based on multifunctional populations which we previously found associated with protection from challenge (6, 7), both with and without Granzyme B. These, too, map closely to the diverse clusters seen above, with cluster 3, 9 and 22 containing particularly robust multifunctional populations (Fig. 8J). Of note, clusters 3, 9 and 22 are all significantly more abundant in our adult participants than in our pediatric participants, though neither age group shows a significant change in cluster abundance following Ty21a vaccination (Fig. 8A–C). The complete data set for all CD8⁺ T_{EM} clusters is shown in Supplementary Figure S9.

tSNE analysis of *S. Typhi*-responsive CD4⁺ T_{EM}

We next performed similar tSNE analyses with identical parameters on isolated, pre- and post-Ty21a vaccinated *S. Typhi*-infected B-LCLs co-cultured activated CD4⁺ T_{EM} with ClusterX generating a total of 18 unique clusters. We again identified clusters of interest based on significant differences between pre-vaccinated (Fig. 9A) and post-vaccinated (Fig. 9B) adult and pediatric participants with paired (Fig. 9A and B) and unpaired (Fig. 9C) analyses and visualized the clusters on a divided set of tSNE maps (Fig. 9D). Within CD4⁺ T_{EM},

clusters 2, 4 and 16 contain cells with the most diverse array of effector functions (Fig. 9E). Clusters 2 and 4 show low median expression of the inflammation-homing marker CXCR3, while clusters 16 and 18 are dominated by the mucosa-homing marker CCR6 (Fig. 9F). Additionally, cluster 16 contains cells with high median expression of IL-17A (Fig. 9G). The more functional clusters contain cells with high median expression of CD154, whereas CD27-high cluster 7 has very few T_h1 effectors (Fig. 9H). Clusters 2, 4 and 16 are the most multifunctional as determined by FCOM analysis (Fig. 9I). Clusters 2, 4 and 16 are all significantly higher in adult participants than in pediatric participants, both pre- and post-Ty21a vaccination. However, vaccination does not significantly impact the cluster abundance of any of these clusters as observed in the increases in cluster 7 among both children and adults (Fig. 9C). The complete data set for all CD4⁺ T_{EM} clusters is shown in Supplementary Figure S10.

tSNE analysis of *S. Typhi*-responsive CD8⁺ T_{EMRA}

We next analyzed pre- and post-Ty21a vaccinated activated CD8⁺ T_{EMRA} (CD45RA⁺/CD62L⁺) with the same conditions and protocol as above. Of the 27 resulting distinct clusters, 15 were

Fig. 11. tSNE analysis of activated CD4⁺ T effector memory RA populations. (A) Percentage of cells per significant clusters showing differences between pediatric and adults divided among pre- (A) and post- (B) Ty21a vaccinated pediatric (blue) and adult (black) participants. (C) Percentage change of cells per significant cluster as in (A) following vaccination among pediatric (blue) and adult (black) participants. (D) tSNE maps of significant clusters as in (A–C) divided among pre- and post-Ty21a vaccinated pediatric and adult participants. (E–I) Functions of individual significant tSNE clusters, divided into: median expression of (E) Tc1 effectors MIP1 β , CD107a, TNF α , IL-2, IFN γ and Granzyme B (GzmB), (F) homing molecules CCR6, CXCR3, CCR4, α 4 β 7 and CXCR5, (G) IL-21, IL-10, IL-17A, IL-6 and IL-4, and (H) activation/exhaustion/additional molecules PD-1, ICOS, CD154, Ki67 and CD27. Expression percentages of WinList FCOM analysis determined (I) Tc1 mono- and multifunctional cytokine expression. Statistics were analyzed by unpaired *t*-test (A, B) and non-parametric (C) paired *t*-test. Shown are mean $\pm$ SE error bars. **P* < 0.05, ***P* < 0.01 ****P* < 0.001.

significantly different based on unpaired pre-Ty21a (Fig. 10A) and post-Ty21a (Fig. 10B) and/or paired (Fig. 10C) analysis. These differences are shown as a percentage of cells per cluster (Fig. 10A–C) and as tSNE maps divided by age and vaccination state (Fig. 10D). Clusters 5, 7, 11 and 18 represent the most diverse clusters based on median expression of Tc1 effector functions (Fig. 10E). As with CD8⁺ T_{EM}, the inflammation-homing chemokine CXCR3 is the most commonly expressed homing molecule, and cluster 10 shows high median expression of the lymph-homing chemokine CXCR5 (Fig. 10F). Non-Tc1-associated interleukins are present in very low-abundance clusters 21 (IL-21), 23 and 26 (IL-6) (Fig. 10G). Clusters 5, 7 and 11 are the most multifunction clusters based on FCOM analysis (Fig. 10I) with cluster 5 containing TNF α /IFN γ double-positive cells we found in previous studies associated with protection (Fig. 10J) (6, 7). As with CD8⁺ and CD4⁺ T_{EM}, the most multifunctional cluster (5) is higher in adults than in children. Interestingly, multifunctional cluster 7 increases significantly in

adults, and trends towards significant increases in children, following Ty21a vaccination (Fig. 10C). The complete data set for all CD8⁺ T_{EMRA} clusters is shown in Supplementary Figure S11.

tSNE analysis of *S. Typhi*-responsive CD4⁺ T_{EMRA}

Utilizing the same tSNE protocol with activated CD4⁺ T_{EMRA} results in 15 ClusterX generated unique populations, 5 of which show significant differences. While cluster 8 is consistently more populated by adult cells than pediatric cells (Fig. 11A and B), a majority of the differences within this subset are paired decreases from pre- to post-Ty21a vaccinated states among both pediatric and adult participants (Fig. 11C). tSNE maps that are parsed between age and vaccination states are shown to better visualize these differences (Fig. 11D). Cluster 8 is the most diverse cluster in terms of T_h1 effector functions, containing high median expression of IL-2 and TNF α (Fig. 11E). The CD4⁺ T_{EMRA} have

Fig. 11. continued

low expression of homing molecules throughout, with some inflammation-homing CXCR3 in clusters 8 and 11 (Fig. 11F). None of the clusters show any non-T_H1 interleukin expression (Fig. 11G). CD154 is high in cluster 8, and also present in cluster 1, and CD27 is present at low levels in all but cluster 8 (Fig. 11H). As expected, cluster 8 is the most multifunctional (Fig. 11I). As seen in the other tSNE analysis, the most multifunction cluster (8) is significantly higher in adults than in the pediatric participants but does not change significantly in either group following Ty21a vaccination (Fig. 11A and B). Interestingly, abundant multifunctional cluster 1 increases in children following Ty21a vaccination, but not in adults (Fig. 11C). The complete data set for all CD4⁺ T_{EM}RA clusters is shown in Supplementary Figure S12.

Discussion

The oral live attenuated Ty21a vaccine aims to protect from the development of typhoid disease, at least in part by inducing robust, *S. Typhi*-specific effector T-cell responses. The importance of multifunctional *S. Typhi*-responsive CD8⁺ T_{EM} has been shown in challenge studies with wt *S. Typhi*,

which demonstrated that the presence of CD8⁺ responses at baseline was associated with protection from and/or delaying the onset of the development of typhoid disease in adults (6, 7). However, there is little information on the presence or quality of these *S. Typhi*-responsive CD8⁺ T cells in children beyond our previous HLA-E-restricted antigen presentation study (M. Rudolph *et al.*, *Frontiers in Immunology*, *In press*). Multifunctional CD4⁺ T_{EM} are increased following Ty21a vaccination (11, 34), but little is known about whether this is the case in pediatric vaccine recipients, or whether circulating multifunctional CD4⁺ cells play a role in protection from the development of typhoid disease. In this study, we compared pediatric and adult T-cell responses to *S. Typhi* antigen presentation by self-MHC prior to, and 14–42 days following Ty21a vaccination. We show that adults are more likely than children to have baseline *S. Typhi*-responsive circulating peripheral multifunctional CD8⁺ and CD4⁺ T_{EM} cells. This may be due, in part, to more previous encounters with gram-negative gastrointestinal pathogens or normal gastrointestinal microbiota that share similar antigens to those present in *S. Typhi* (e.g. flagellar proteins, outer membrane proteins, chaperonins) (35–37). Taken together with previous data showing that children

have lower proportions of total CD4⁺ T cells and CD8⁺ T_{EM} than adults, even into adolescence, the present data identify additional important differences in age-associated baseline responsiveness to *S. Typhi* antigens which might impact the effectiveness of oral vaccination in children.

Interestingly, these differences are harder to identify following Ty21a vaccination. We have not observed major differences between pre- and post-vaccine effector responses in *S. Typhi*-responsive CD4⁺ and CD8⁺ T_{EM} between adult and pediatric participants. This is largely due to the heterogeneity of the responses, showing both increased and decreased responses with regard to baseline. This is likely the result of Ty21a immunization resulting in T-cell homing of *S. Typhi*-responsive cells into and out of circulation. We observed that ~50–70% of our participants show positive CD8⁺ T cells effector responses 28–42 days post-vaccination. This observation is supported by several previous publications from our group in Ty21a immunized adult volunteers, which also demonstrated a decrease 1–6 weeks after challenge with wt *S. Typhi* and vaccination below baseline levels in some participants which typically rebounds 6–12 weeks after vaccination/challenge (6, 7, 9, 10, 21, 38, 39). Of note, *S. Typhi*-responsive cells with particular characteristics (e.g. expression of CD107a, IFN γ production) appear to remain in circulation for variable periods of time, as evidenced by either decreasing or increasing over baseline at the time of blood collection. This important observation should be further explored in future studies. By setting response thresholds, we show that CD8⁺ T_{EM} seem to remain in circulation following Ty21a vaccination longer than CD4⁺ T_{EM}, particularly among adult participants. To look at this phenomenon in further detail, we divided the pediatric participants into those with time points collected 14–27 days post-vaccination ($n = 3$) versus 28–42 days post-vaccination ($n = 12$). No differences were seen among these pediatric subgroups, although the numbers of children in the 14- to 27-day group were limited. Taken together, these observations highlight the age-associated variability in circulating T cells following vaccination as an important parameter to explore in upcoming studies.

We believe that exploring sex-associated differences among our participants is vital to ensuring a more complete understanding of how heterogeneity may impact immune response to Ty21a vaccination (40–42). Our baseline analysis showed few differences between CD8⁺ or CD4⁺ T_{EM} effector responses or multifunctionality based on sex. Further, responses-over-baseline were similar between male and female participants, with the main difference being that male CD8⁺ and CD4⁺ T_{EM} showed a general propensity towards increased Granzyme B production following Ty21a vaccination. Of note, both pediatric and adult female CD8⁺ and CD4⁺ T_{EM} responses tend to decrease following vaccination, whereas male CD8⁺ T_{EM} responses (and pediatric male CD4⁺ T_{EM} responses) tend to increase following vaccination. This potential Ty21a-induced sex- and age-associated differential migration of functional T cells merits further investigation in future studies.

The variability of responses among participants, along with the breadth of coverage offered by our mass cytometry panel, necessitated analyses beyond conventional supervised gating of a few functional or phenotypic markers at

a time. To address this need, we utilized the unsupervised tSNE dimensionality reduction tool to best identify clusters of cells sharing phenotypes, homing potential, and functionality, and to assess differences between pediatric and adult pre- and post-Ty21a vaccinated participants. We performed tSNE on isolated CD8⁺ and CD4⁺ T_{EM} and T_{EMRA} and divided each cluster in order to determine whether they significantly increased or decreased following Ty21a vaccination and/or whether they were significantly different between age groups. Among the four cell types analyzed with tSNE (CD4⁺ and CD8⁺, T_{EM} and T_{EMRA}), it was remarkable that the most multifunctional clusters were significantly and consistently higher among adults than pediatric participants. These age-associated differences in multifunctional T-cell responses are consistent with our previous findings in mitogen-stimulated and HLA-E-restricted *S. Typhi* antigen presentation models (12) (M. Rudolph *et al.*, *Frontiers in Immunology*, *In press*). Interestingly, as observed with conventional gating analyses, none of these multifunctional populations changed significantly from baseline following Ty21a vaccination. This observation is almost certainly due to the fact that all data, including decreases and increases over baseline, were included in the calculations, as well as the wide range of responses typically observed in different individuals, leading to a wide dispersion in the responses leading to a lack of statistically significant differences pre- and post-vaccination in these data set. Of note, many of the clusters identified based on phenotypic markers (CD8⁺ and CD4⁺ T_{EM} and T_{EMRA} clusters) showed no cytokine production, suggesting that these cells produced cytokines/chemokines not included in the mass cytometry panel. This should be further explored in future studies by expanding mass cytometry panels and using transcriptomics to assess concomitantly the expression of large numbers of genes.

Regarding homing, in nearly all of the analyses, but particularly among the CD8⁺ T_{EM} and T_{EMRA} clusters showing significant differences among adults and children, the inflammation-homing molecule CXCR3 was the dominant chemokine receptor, with few clusters expressing the gut-homing molecule integrin- $\alpha 4\beta 7$. This is likely due to the use of median expression to represent the data, which is typically used to best evidence unique features of each tSNE cluster. However, when looking at these data shown as mean expression of integrin- $\alpha 4\beta 7$ in CD8⁺ T_{EM}, for example (Supplementary Figure S13), virtually all of the clusters express at least some levels of this gut-homing molecule, which in aggregate likely accounts for the 20–25% of our group has previously reported among *S. Typhi*-responsive CD8⁺ T_{EM} (6, 7, 9, 21, 34). This is in contrast, however, with HLA-E-restricted CD8⁺ T_{EM} and T_{EMRA} tSNE analyses in a previous study which showed integrin- $\alpha 4\beta 7$ as the dominant homing molecule (M. Rudolph *et al.*, *Frontiers in Immunology*, *In press*). Further, while the decrease of these clusters following vaccination suggests homing out of circulation, they are likely to express homing molecules not included in the mass cytometry panel evaluated in this report, and therefore, their homing potential remains unclear at this time.

Several of the clusters that were found to increase significantly in circulation following Ty21a vaccination tended to

generally being less terminally differentiated, as evidenced by higher median CD27 expression, particularly in CD4⁺ T_{EM} (32, 33, 43). Interestingly, we observed that few *S. Typhi*-responsive cells expressed Ki67, suggesting that these cells in circulation were not proliferating (44). Also of note, many clusters, particularly of CD4⁺ T_{EM} and T_{EMRA}, showed expression of CD154, a marker closely associated with activation (45, 46).

Taken together, conventional and tSNE analyses identified adults as more likely to contain highly multifunctional CD8⁺ and CD4⁺ effector T cells to *S. Typhi*-infected autologous B-LCLs at baseline. These results are concordant with the results we previously reported following stimulation of PBMCs from both children and adults with staphylococcal enterotoxin B (12) and with the *S. Typhi*-infected 721.221 AEH cell line, which expresses HLA-E molecules in the absence of class I MHC molecules (M. Rudolph *et al.*, *Frontiers in Immunology*, *In press*). In both children and adults, we observed that Ty21a induced similar magnitudes of *S. Typhi*-responsive circulating CD8⁺ multifunctional T-cell subsets which we have previously reported to be associated with protection in individuals challenged with wt *S. Typhi* (6, 7). Of note, we observed a limited number of increases or decreases as a group in some of these subsets, likely the result, as discussed above, of cells homing in an out of blood.

In sum, the data presented in this study represent an important milestone in our understanding of CD4⁺ and CD8⁺ CMI responses elicited by oral immunization with the typhoid Ty21a vaccine. These studies not only uncovered the complexity of the responses to *S. Typhi* immunization, but also identified similarities and differences between children and adults. These observations could prove critically important in the development of novel, improved oral live attenuated vaccines targeting vulnerable pediatric populations.

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