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Characteristics of Regulatory T-Cell Populations Before and After Ty21a Typhoid Vaccination in Children and Adults

Mark E. Rudolph^{1,2}, Monica A McArthur^{1,3}, Laurence S. Magder⁴, Robin S. Barnes¹, Wilbur H. Chen^{1,5}, and Marcelo B. Sztein^{1,2,3,5}

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

²Molecular Microbiology and Immunology Department, University of Maryland Graduate Program in Life Sciences, Baltimore, MD, USA

³Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

⁴Epidemiology and Public Health, University of Maryland School of Medicine, Baltimore, MD, USA

⁵Department of Medicine, University of Maryland School of Medicine, Baltimore, MD, USA

Abstract

Typhoid fever, caused by the pathogen *Salmonella enterica* serovar Typhi (*S. Typhi*), is a serious global health concern. Challenge studies with wild type *S. Typhi* identified associations between gut-homing regulatory T cells (Treg) and development of typhoid disease. Whether oral live-attenuated Ty21a vaccination induces gut-homing Treg remains unclear. Here, we analyze pediatric and adult Treg pre- and post-Ty21a vaccination in an autologous *S. Typhi*-antigen presentation model to address this knowledge gap. We show that peripheral memory Treg populations change from childhood to adulthood, but not following Ty21a vaccination. Unsupervised dimensionality reduction with t-distributed stochastic neighbor embedding (tSNE) identifies homing, memory, and functional features which evidence age-associated maturation of multifunctional *S. Typhi*-responsive Treg, which were not impacted by Ty21a vaccination. These findings improve understanding of pediatric regulatory T cells, while identifying age-related

Corresponding Authors: Mark Rudolph: mark.rudolph@umaryland.edu, Marcelo Sztein: msztein@som.umaryland.edu.

Author Contributions

MER, MAM and MBS designed the study, analyzed the data and wrote the manuscript; MER performed the experiments; WHC and RSB contributed to the design, collected and processed the clinical samples and helped draft the manuscript. LSM provided statistical expertise.

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Ethics Statement

Peripheral blood mononuclear cells were collected from 15 healthy pediatric (6–17 years of age at the time of enrollment) and 14 healthy adult (20–65 years of age at the time of enrollment) volunteers, being 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 Review Board (IRB) and were carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all adult participants, as well as written informed consent from the parents of any participant under the age of 18 years old, as well as assent from the pediatric participants, prior to the conduct of any study procedures.

Disclosure

The authors declare no conflicts of interest.

differences in *S. Typhi*-responsive Treg, which may aid in the development of improved pediatric vaccination strategies against *S. Typhi*.

Keywords

Regulatory T cells; dimensionality reduction; Ty21a; pediatric immunology; Salmonella Typhi; typhoid

1. Introduction

Salmonella enterica serovar Typhi (*S. Typhi*) is a human restricted intracellular pathogen and the causative agent of typhoid fever, which leads to a global impact of nearly 21-million illnesses and 200,000 deaths annually [1, 2]. Most of the disease burden lies in regions with limited access to clean sources of water, driving the oral-fecal transmission of the pathogen. While typhoid fever mortality predominates in the very young, the elderly, and the immunocompromised, school-aged children are severely impacted by typhoid-associated morbidity, likely acting as carriers to more susceptible individuals [1, 3–7]. The oral, live-attenuated Ty21a vaccine aims to induce robust, multifunctional cell mediated immunological (CMI) responses against *S. Typhi* which, in challenge studies with wild-type *S. Typhi*, were associated with protection from and/or delayed onset of typhoid disease when present in the periphery prior to challenge [8, 9]. Interestingly, these responses are typically seen in approximately two-thirds of adult Ty21a recipients [2, 10, 11]. Further, large scale field trials of Ty21a vaccination have shown that vaccine efficacy is lower in younger children, and improves through adolescence [6, 7], despite T cell proliferation shown in Ty21a recipients as young as 2 years old [12]. Little is known about the impact of Ty21a immunization on circulating regulatory T cells (Treg), however the baseline presence of gut-homing integrin- $\alpha 4\beta 7$ on Treg prior to *S. Typhi* challenge has been shown to be associated with the development of typhoid disease [13].

Treg are a subset of CD4⁺ T cells defined by their expression of transcription factor Forkhead box protein (FoxP3) and interleukin (IL)-2 receptor α (CD25) [14–16]. In healthy individuals, Treg utilize a variety of immunosuppressive functions to maintain homeostasis with effector T cells. In addition to CD25^{hi} (IL-2 receptor) expression which out-competes IL-2 in the microenvironment from effector T cells, Treg also express CD39, an endonucleotidase that is capable of hydrolyzing ATP to AMP [17]. This is the first step in generating extracellular adenosine, an inhibitor of T effectors and an enhancer of Treg, through increasing the stability of Treg under inflammatory conditions [18, 19]. CD95, or Fas receptor, a member of the tumor necrosis factor (TNF) receptor superfamily, has been shown to be a Treg activation marker, which mediates a pro-apoptotic death pathway [20–22]. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; CD152) is a surface-expressed inhibitory immune checkpoint molecule that competes for binding of antigen presenting cell co-stimulatory molecules CD80 and CD86, is present on Treg and some activated T effectors [23]. Of note, several groups have shown that the percentages of total Treg are maintained throughout life [24–28], and that higher proportions of Tregs are of an effector memory (CD45RA⁻) than naïve phenotype from young adulthood throughout life [29, 30].

Further, studies have shown that the proportion of peripherally induced Tregs increases with age in adults, and that aging may be associated with localization and improved function in distinct peripheral tissues [31–33]. While several Treg studies included different age strata, there is virtually no information regarding the induction of naïve/effector FoxP3⁺ CD25⁺ Treg populations and their function in healthy children and adults following vaccination.

An interesting feature of Treg immunity is their reciprocal balance with IL-17 producing Th17 cells [34]. Indeed, CD39⁺ Treg have been shown to suppress Th17 responses in an inflammatory setting, and impaired Treg function is known to play a role in autoimmunity [35]. Of note, pro-inflammatory cytokine IL-17A is involved in protection against extracellular and intracellular bacterial pathogens at the mucosa and therefore may play an important role in protection from *S. Typhi* [36–38]. Indeed, Ty21a has been shown to induce IL-17A⁺ multifunctional CD8⁺ T cells in adult vaccinees [39]. The importance of IL-17 for mucosal immune responses to bacterial pathogens, as well as the unique repressive relationship between Treg and Th17, the increased typhoid-disease susceptibility of *S. Typhi* challenge participants with higher baseline levels of gut-homing *S. Typhi*-responsive Treg, lead us to postulate that functional Treg are a key immune response to explore in relation to Ty21a vaccination, particularly among pediatric vaccinees.

In this study, we utilized an established *S. Typhi*-infected autologous Epstein Barr virus (EBV) transformed B-lymphoblastoid cell lines (B-LCL) antigen presentation model (7, 8, 13) to explore regulatory T cell responses and their homing potential utilizing peripheral blood mononuclear cells (PBMC) obtained from healthy children and adults before and after immunization with the licensed Ty21a attenuated oral typhoid vaccine. We identified significant age-associated differences among unstimulated naïve and terminal effector peripheral Treg, while affirming previous findings that the percentages of total Treg remain relatively constant with increasing age. Importantly, we show that despite the heterogeneity of the expression of effector and homing molecules at baseline and following vaccination, unbiased dimensionality reduction tools identify age-associated differences in the homing potential, memory status, and functionality of Treg that may prove critical to our understanding of pediatric susceptibility to typhoid disease.

2. Materials and Methods

2.1 Participants and isolation of PBMC

We collected PBMC from pediatric and adult participants prior to, and between 14–42 days following Ty21a vaccination. The ages of the fourteen pediatric participants ranged from 6- to 17-years-old at the time of enrollment. All of the pediatric participants received the Ty21a vaccination for medically indicated reasons, namely travel to endemic regions. The ages of the eleven healthy adult participants ranged from 27- to 65-years-old at the time of enrollment (Table 1). The volunteers were recruited from the Baltimore-Washington area and the University of Maryland at Baltimore campus and these studies were approved by the University of Maryland at Baltimore Institutional Review Board (IRB) and were carried out in accordance with the Declaration of Helsinki. Prior to conducting study procedures, we obtained written and informed consent from all adult participants, as well as written informed consent from the parents of any participant under the age of 18 years old in

addition to assent from the pediatric participants. PBMC were immediately isolated by density gradient centrifugation following blood collection and stored in liquid nitrogen following standard cryopreservation techniques [40, 41] until use in the experiments.

2.2 Preparation of autologous B-lymphoblastoid cell lines (B-LCL)

Stored PBMC from each of the participants were thawed in complete 1640 RPMI (cRPMI) media (Gibco, Carlsbad, CA), supplemented with 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma, St. Louis, MO), 50 µg/mL 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). B cells were then transformed with Epstein-Barr virus and expanded, generating autologous EBV-transformed B-lymphoblastoid cell lines.

These autologous B-LCLs were infected with wild-type *S. Typhi* strain ISP1820 (wt *S. Typhi*) in RPMI without antibiotics for 3 hours at 37°C at a multiplicity of infection (MOI) of 7:1 (3.5×10^7 wt *S. Typhi* as determined by OD600: 5.0×10^6 B-LCL cells) with uninfected targets cells acted as controls. Following incubation, the cells were washed twice and incubated at 37°C, 5% CO₂ overnight in 0.3% gentamycin-supplemented cRPMI. The B-LCL were then gamma-irradiated (6000 rad), washed, and re-suspended in fresh cRPMI for use as co-culture targets for effector T cells. We used conventional flow cytometry to detect the presence of wt *S. Typhi* antigens on live, infected targets cells (and not on uninfected controls) with LIVE/DEAD™ yellow cell stain (ThermoFisher Scientific, Waltham, MA) and BacTrace® FITC-labelled anti-*Salmonella* CSA-1 antibody (SeraCare, Milford, MA) (Fig. S1A).

2.3 *In vitro* stimulation of effector cells

Prior to stimulation with matched B-LCL targets, PBMC were thawed and rested overnight at 37°C, 5% CO₂ in cRPMI. Rested cells were then washed and resuspended in cRPMI at a concentration of 1×10^6 cells/500µL in 5mL cell culture tubes. PBMC were then incubated with gamma-irradiated B-LCL targets (infected with the wt Ty2 strain of *S. Typhi* -or left uninfected- as described in section 2.2 and prepared the day before) at an effector-to-stimulator ratio of 5:1 for 2 hours at 37°C in 5% CO₂. 0.5 µL/mL each of GolgiStop (containing monensin) and GolgiPlug (containing brefeldin A) from BD (San Jose, CA) were added at to all tubes following the 2-hour incubation, and cultures were maintained at 37°C in 5% CO₂ overnight.

2.4 Surface and intracellular immune-labelling and mass cytometry analysis

Co-cultured and stimulated PBMC were centrifuged and barcoded with anti-CD45 (Fluidigm South San Francisco, CA) monoclonal antibodies (mAbs), with pediatric samples labelled with CD45–89Y and adult samples labelled with CD45–156Gd, as well as FITC-labelled integrin-α4β7 for 30 minutes at 4°C. Cells were then washed twice with Maxpar® Cell Staining Buffer (Fluidigm) before barcoded like-stimulated adult and pediatric PBMC were combined into a single tube for downstream staining as per the Maxpar® Nuclear Antigen Staining directions. Briefly, combined cells were washed once with PBS (Quality Biological, Gaithersburg, MD) before being incubated with Cell-ID™ Cisplatin (Fluidigm)

for 1 minute at room temperature. Cisplatin staining was then quenched and washed twice with Maxpar® Cell Staining Buffer (Fluidigm). Cells were then Fc-blocked with human IgG for 20 minutes at room temperature to prevent non-specific antibody binding. Fifty μ L surface monoclonal antibody (mAb) cocktail (Table 2) was added to each tube for 30 minutes at 4°C before being washed twice with Maxpar® Cell Staining Buffer (Fluidigm). One hundred μ L of Maxpar® Nuclear Antigen Staining Buffer (Fluidigm) was then added to each tube for 30 minutes at room temperature. Cells were then washed twice with Maxpar® Nuclear Antigen Staining Perm (Fluidigm) before adding 50 μ L of the intracellular antibody cocktail containing mAbs (Table 2) for 45 minutes at room temperature. Cells were then washed twice with Maxpar® Cell Staining Buffer (Fluidigm) before being incubated with 1 mL per tube of Cell-ID™ Intercalator-Ir for 1 hour at 4°C. Cells were washed once with Maxpar® Cell Staining Buffer (Fluidigm) and twice with Maxpar® Water (Fluidigm) before being prepared and run in the University of Maryland Mass and Flow Cytometry Core on a Helios instrument (Fluidigm). Debarcoding of mass cytometry files was done using Premium Cytobank (Cytobank, Inc, Santa Clara, CA) and the resulting data was analyzed using WinList version 9.0.1 (Verity Software House, Topsham, ME). The total number of Treg (CD3+ CD4+ CD25+ FoxP3+) collected from pediatric participants ranged from 938 to 5,719 events, while in adults the range was between 1,036 and 10,588 events. tSNE analysis was performed utilizing the Cytokit package [42] in biocLite on R with WinList version 9.0.1 used for downstream gating of individual tSNE clusters.

2.5 Statistical analyses

GraphPad Prism version 7.0c (GraphPad software, La Jolla, CA, USA) was used to run unpaired t-tests, paired t-tests, and correlation analyses. P values of < 0.05 were considered significant. Sixteen-17 year old children were grouped with the pediatric population, and statistics were run comparing the entire group (n = 14, ages 6–17; orange asterisks) and the younger group (n = 12, ages 6–15; blue asterisks) versus adults (n = 11) based on previous reporting from our group that T cells from older children are more similar to adults than younger children ([43] and Rudolph et al., *Frontiers in Immunology*, in press, 2019). P values of < 0.1 were also presented as possible trends as per the recommendation of the American Statistical Association's statement concerning the context and purpose of p-values, particularly in data with low n-numbers [44, 45].

3. Results

3.1 Age-associated heterogeneity in baseline regulatory T cell phenotypes

Unvaccinated (baseline) unstimulated PBMC were labeled with metal-conjugated mAbs before being analyzed with mass cytometry. Cells were gated to identify total, naïve, effector, and terminal effector Treg populations based on their expression of FoxP3, CD25, CD45RA, CD45RO, and HLA-DR as previously described [14]. The gating strategy for a representative unstimulated pediatric participant is shown in Figure S1. We observed no differences in the percentages of total Treg among CD4+ T cells between pediatric (6–17 years old; n = 14) and adult (27–65 years old; n = 11) participants (Figure 1A). Of note, we and others have previously shown that the percentage of CD4+ T cells within the periphery is higher in adults than in children [24–28, 43]. Thus, while the percentages of total Treg

may not differ by age, the total numbers of circulating Treg is likely to be higher in adults. Interestingly, children have a significantly higher percentage (median of 36.5%, interquartile range between 18–38%) of circulating naïve regulatory T cells than adults (median 20.1%, interquartile range of 14–26%), while adults have significantly higher percentages of terminal effector Treg (median of 25.8%, interquartile range between 24–31% compared to pediatric median of 18.7%, interquartile range of 14–27%) (Figure 1B). Additionally, these differences within naïve and terminal effector regulatory T cell percentages are significantly correlated with age (Figure 1C). There is no significant heterogeneity among pediatric and adult HLA-DR negative effector Treg (Figure 1B+C). Together, this data characterized age-associated differences in circulating Treg memory populations in healthy individuals.

3.2 Baseline characteristics of peripheral regulatory T cells

Next, we explored the baseline regulatory T cell characteristics among pediatric and adult participants after in vitro exposure to *S. Typhi*-infected autologous targets. Gut-homing integrin- $\alpha 4\beta 7$ expression by Treg have been shown to be important for the development of typhoid disease in a wt *S. Typhi* challenge model [13]. We observed low baseline expression levels and no age associated differences in regulatory T cells after in vitro exposure to *S. Typhi*-infected autologous targets over the levels in uninfected targets with respect to their expression of integrin- $\alpha 4\beta 7$ (Figure 2A), CD39 (Figure 2B) or CD95 (Figure 2C). In contrast, we show that at baseline nearly all participants have some level of CTLA-4-expressing Treg following stimulation with *S. Typhi*-infected B-LCL over their expression in co-cultures with uninfected B-LCL. However, pediatric and adult Treg, regardless of memory status, show no significant age-associated differences in their CTLA-4 baseline expression levels (Figure 2D).

3.3 Changes in regulatory T cell subset frequencies following Ty21a vaccination

We next explored whether Ty21a vaccination induced changes in circulating naïve, effector and terminal effector Treg populations. Although the percentages of total Treg, as well as their various subsets, increased or decreased in individual volunteers, as a group Treg did not significantly change in either pediatric or adult participants following Ty21a vaccination (Figure 3A+B). Indeed, a near-equal proportion of participants show increases and decreases among their percentages of memory Treg phenotypes (Figure 3A+B). This heterogeneity is likely to represent the dynamic, ongoing homing of T cells, including regulatory T cells, into and out from circulation following vaccination which is dependent on individual volunteers.

3.4 Changes in regulatory T cell functional phenotypes following Ty21a vaccination.

Next, we examined regulatory T cell functions following Ty21a vaccination by subtracting 14–42-day expression of integrin- $\alpha 4\beta 7$, CD39, CD95, and CTLA-4 responses from those present at baseline. No changes were observed in the expression of integrin- $\alpha 4\beta 7$ among circulating Treg, nor any significant differences were observed between age groups (Figure 4A). In contrast, adult Treg generally showed increased CD39 expression, particularly among the effector Treg populations, whereas pediatric Treg were more heterogeneous (Figure 4B). CD95 expression following Ty21a vaccination generally increased in both pediatric and adult terminal effectors (albeit not significantly), but the responses were more variable among naïve and pediatric effector Treg populations (Figure 4C). CTLA-4

responses compared to baseline were variable in adults, but generally showed decreases, albeit not significant, particularly in the naïve population (Figure 4D); however, expression of CTLA-4 generally decreased in pediatric naïve and effector Treg populations.

3.5 tSNE analysis of regulatory T cells pre- and post-Ty21a vaccination

Dimensionality reduction tools are invaluable for the examination of larger and more complex biological datasets, with tools such as tSNE allowing for unbiased exploration and discovery within multi-parametric cytometry data [46, 47]. Thus, to further characterize Treg responses we performed tSNE analysis utilizing the Cytokit package [42] available in the R biocLite module on pre- and post-Ty21a vaccinated *S. Typhi*-infected B-LCL co-cultured regulatory T cells from both children and adults. Twenty-two clusters were identified following visualization using the ClusterX unbiased clustering method on the following markers: CD45RA, CD45RO, HLA-DR, integrin- $\alpha 4\beta 7$, CCR4, CD39, CD95, and CTLA-4. Clusters of interest were identified based on significant differences among pre- or post-Ty21a vaccinated pediatric and adult participant percentages of cells per cluster (unpaired t-test). We also explored significant increases or decreases within age groups following vaccination (paired t-test) but found none (Figure 5A). tSNE maps showing the significant clusters are divided by adult and pediatric, pre- and post-vaccination groups to best highlight the differences between groups (Figure 5B). Of note, the adult participants preferentially clustered on the left of the map (clusters 2, 9, and 11) while the pediatric participants more densely populated the clusters on the right (4, 7, and 15). In order to determine the characteristics of these clusters showing significant differences between pediatric and adult participants, we determined the median expression levels of memory, homing, and functional Treg markers. Among clusters 2, 9, and 11 (higher in adults), there was a high median expression of CD45RO, indicative of effector Treg (Figure 5C). Clusters overrepresented in children (4, 7, and 15) were conversely comprised of cells containing high median expression of naïve cell marker CD45RA (Figure 5C). Interestingly, CCR4 (skin homing) expression dominated the effector-like clusters which were more populated by adults, whereas integrin- $\alpha 4\beta 7$ was found in the naïve-like clusters that dominated significantly in pediatric participants (Figure 5D). While all six clusters with significant differences showed CTLA-4 expression, all three adult-dominated clusters also had notable median expression levels of CD95 and CD39, which was not the case in the pediatric-dominated clusters (Figure 5E). Further, we utilized the FCOM feature in Winlist to analyze the multifunctionality of the clusters of interest based on expression of integrin- $\alpha 4\beta 7$, CCR4, CD39, CD95, and CTLA-4. As expected, and despite the very low percentages of CCR4/integrin- $\alpha 4\beta 7$ double-positive cells, the three clusters more populated by adult participants had approximately 50% or greater simultaneously expressing three or four functions (Figure 5F). In contrast, the predominant clusters in pediatric participants exhibited close to 60–70% observed mono- and bi-functional Treg (Figure 5F). Together, these data suggest that adult circulating Treg, present both at baseline and following Ty21a vaccination are more mature, more functional, and less likely to home to the gut than the naïve, less functional, gut-homing phenotypes more frequently found within pediatric Treg. The complete data set for all Treg clusters is shown in Fig. S2.

4. Discussion

Treg memory is as important a feature of adaptive T cell immunity, as is effector T cell memory. However, while several groups have shown [24–28], and we have confirmed, that total Treg percentages are maintained throughout life, little work has been done to explore differences in Treg memory phenotypes and their function between children and adults following vaccination. Previous work has shown that percentages of naïve CD4+ T cells are higher in children than in adults, and that the reverse is true for CD4+ effectors [43, 48–51]. Here, we show that among unstimulated circulating FoxP3+ CD25+ total Treg, naïve Treg decrease, and terminal effector Treg increase in the periphery as a function of age through adolescence and into adulthood. This finding suggests that adult circulating Treg are more mature and suppressive than those found even during the teenage years [52]. Ty21a vaccination did not lead to significant changes in either total circulating Treg or memory Treg population percentages among either pediatric or adult recipients 14–42 days following vaccination. Indeed, the observed heterogeneity among all recipients and Treg populations, with decreases in some individuals and increases in others with respect to baseline, may represent the dynamics of T cell homing into and out from circulation following Ty21a vaccination, which we've previously observed in effector T cell subsets (Rudolph et al., International Immunology, In press, 2019).

Homeostasis between regulatory and effector T cells is critical for a host to successfully fight infection while limiting the pathologies associated with excessive inflammation. In this study, we explored the presence of *S. Typhi*-responsive Treg functions and homing potential in children and adults before and after Ty21a vaccination. Previous work has shown that baseline *S. Typhi*-responsive circulating Treg expressing the gut homing molecule integrin- $\alpha 4\beta 7$ are associated with development of typhoid disease in a wt *S. Typhi* challenge model [13]. Conventional gating did not show age-associated differences regarding integrin- $\alpha 4\beta 7$ expression in total or memory Treg subsets before or after Ty21a vaccination. Indeed, conventional gating of functional Treg markers CD39, CD95, and CTLA-4 all showed great heterogeneity among children and adults at baseline and 14–42 days post-vaccination. Interestingly, utilization of unbiased clustering algorithms in tSNE identified significant populations of cells that were differentially populated based on age. Clusters dominated by adult cells were generally less naïve, more functional/multifunctional, and characterized by expression of CCR4, a skin homing molecule [53–55]. Conversely, clusters more populated in pediatric participants were more naïve, less functional, and preferentially expressed the gut homing integrin- $\alpha 4\beta 7$. These analyses also showed that Ty21a vaccination did not significantly affect the percentage of any cluster, suggesting that Ty21a does not greatly impact the characteristics of peripheral regulatory T cells.

In summary, we have identified age-associated differences in circulating regulatory T cell subsets among healthy children and adults that may inform susceptibility to typhoid disease. Namely, pediatric Treg, while less mature, are more likely to express integrin- $\alpha 4\beta 7$, previously shown to be associated with increased susceptibility to typhoid disease. Of note, even these more naïve Treg showed similar median expression levels of CTLA-4 when compared to the more functional/multifunctional adult Treg. Further, we observed an age-associated decrease in circulating naïve Treg percentages. Together with field trials showing

Ty21a efficacy improving as a function of age throughout childhood, these findings represent a possible explanation for this phenomenon, although this hypothesis requires further investigation. Importantly, Ty21a did not significantly impact circulating Treg populations or function, suggesting that age-intrinsic features rather than vaccine responsiveness play a dominant role. Further investigation into *S. Typhi*-responsive Treg, particularly in children, may provide important information to aid in the development of improved vaccines against typhoid fever, as well as other enteric infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Age-associated heterogeneity in baseline regulatory T cell phenotypes.

Scatter plots showing baseline unstimulated percentages of **A.** Total Treg (FoxP3+ CD25+) within CD4+ T cells and **B.** naïve (CD45RA+), effector (CD45RO+HLA-DR-) and terminal effector (CD45RO+ HLA-DR+) Treg within total Treg. Scatter plot bars represent medians with whiskers indicating interquartile ranges. **C.** Correlation analysis between percentages of baseline unstimulated Treg memory subsets and age of participants. Results are shown for the following populations: 6–15-year-old pediatric (n=12, blue circles), 16–17-year-old pediatric (n=2, orange circles) and adult (n=11, black squares) participants. Scatter plot statistics were analyzed by unpaired t-test, and correlation analysis were run with two-tailed Pearson correlation coefficients. Significant differences between children 6–15 yrs and adults are shown with blue asterisks. Significant differences between children 6–17 yrs and adults are shown with orange asterisks. (* $p < 0.05$; ** $p < 0.01$)

Fig 2. Age-associated differences in baseline regulatory T cell functions.

Scatter plots showing percentages of **A.** integrin $\alpha 4\beta 7$, **B.** CD39, **C.** CD95 (FasR), and **D.** CTLA-4 among baseline *S. Typhi*-infected B-LCL coculture minus uninfected B-LCL coculture total, naïve, effector, and terminal effector Treg. Scatter plot bars represent medians with whiskers indicating interquartile ranges. Results are shown for the following populations: 6–15-year-old pediatric (n=12, blue circles), 16–17-year-old pediatric (n=2, orange circles) and adult (n=11, black squares) participants. Scatter plot statistics were analyzed by unpaired t-test.

Fig 3. Age-associated changes in regulatory T cell phenotypes following Ty21a vaccination.

Before-and-after scatter plots showing unstimulated pre- (closed symbols) and post-Ty21a (open symbols) vaccination percentages of **A.** total Treg and **B.** naïve (CD45RA+), effector (CD45RO+ HLA-DR-) and terminal effector (CD45RO+HLA-DR+) Treg within total Treg. Results are shown for the following populations: 6–15-year-old pediatric (n=12, blue circles), 16–17-year-old pediatric (n=2, orange circles) and adult (n=11, black squares) participants. Scatter plot statistics were analyzed by paired t-test.

Fig 4. Age-associated differences in regulatory T cell functional responses compared to baseline. Scatter plots showing percentage responses as compared to baseline of **A.** integrin $\alpha 4\beta 7$, **B.** CD39, **C.** CD95 (FasR), and **D.** CTLA-4 among *S. Typhi*-infected B-LCL coculture minus uninfected B-LCL coculture total, naïve, effector, and terminal effector Treg. Scatter plot bars represent means with whiskers indicating standard errors. Results are shown for the following populations: 6–15-year-old pediatric (n=12, blue circles), 16–17-year-old pediatric (n=2, orange circles) and adult (n=12, black squares) participants. Scatter plot statistics were analyzed by unpaired t-tests of variance. A trend towards significant differences between children 6–15 yrs and adults is shown with a blue number. A significant difference between children 6–17 yrs and adults is shown with an orange asterisk. (* p<0.05)

Fig 5. tSNE Analysis of Total Treg.

A. Percentages of cells in clusters showing significant differences parsed among pre- (closed bars) and post- (open bars) Ty21a vaccinated *S. Typhi*-responsive pediatric (blue) and adult (black) participants with bars representing median and whiskers interquartile range. **B.** tSNE maps of significant differences among clusters parsed into pre- and post-Ty21a vaccinated pediatric and adult participants. Cluster numbers (over each cloud) are assigned arbitrarily. **(C-F)** Phenotypes and functionality of individual tSNE clusters with significant differences parsed into median expression based on the tSNE algorithm reduction analyses of **C.** memory markers (CD45RA, CD45RO, HLA-DR), **D.** homing molecules (integrin- $\alpha 4\beta 7$, CCR4), and **E.** functional markers (CD39, CD95, CTLA-4), as well as **F.** FCOM-calculated multifunctionality (based on expression of integrin- $\alpha 4\beta 7$, CCR4, CD39, CD95, and/or CTLA-4) divided by whether the clusters exhibit higher proportions of cells in adult or pediatric participants. Statistics were analyzed by unpaired t-test (blue). (* $p < 0.05$, ** $p < 0.01$)

Table 1.**Participant demographics.**

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

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
14	Female	Caucasian
14	Female	Caucasian
14	Male	Caucasian
15	Female	Caucasian
16	Male	Caucasian
17	Female	Caucasian
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

Table 2.**Mass cytometry panel.**

Shown are the antibody targets, stable metal isotope (or other label), antibody clone, and a brief description of the target function. Primary surface antibodies are filled with blue, secondary surface antibodies with green, and intracellular antibodies with orange (*anti-CTLA-4 is in both the surface and intracellular antibody mixes). Barcoding antibodies are shaded in yellow.

Target	Stable Metal Isotope	Clone	Description
CD45	89Y	HI30	Pan-leukocyte barcoding marker
CD49d	141Pr	9F10	α -chain of integrin VLA-4
<i>$\alpha 4\beta 7$</i>	<i>FITC</i>	<i>ACT-1</i>	Gut-homing integrin
FITC	144 Nd	FIT22	
CD4	145Nd	RPA-T4	Helper T lymphocyte marker
CCR4	149Sm	L291H4	Chemokine homing to the skin
CD45RA	153Eu	HI100	T cell memory marker
CD3	154Sm	UCHT1	TCR co-receptor (T cell marker)
CD45	156 Gd	HI30	Pan-leukocyte barcoding marker
CD39	160Gd	A1	Endonucleotidase
FoxP3	162Dy	PCH101	Regulatory T cell transcription factor
CD95 (Fas)	164Dy	DX2	Tumor necrosis factor receptor
CD45RO	165Ho	UCHL1	T cell memory marker
CD25	169Tm	2A3	High affinity IL-2 receptor α
CD152 (CTLA-4)*	170Er	14D3	Inhibitory immune checkpoint molecule
HLA-DR	174Yb	L243	Class II MHC associated with terminal effector Treg
CD127	176Yb	A019D5	IL-7 receptor α
Cell I.D. (DNA)	191/193 Ir	n/a	DNA intercalator
Viability	194/195 Pt	n/a	Viability stain