Title: Postnatal expansion, maturation and functionality of MR1T cells in humans

Short title: Human MR1 T cell development

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Abstract

MR1-restricted T (MR1T) cells are a T cell subset that recognizes and mediates host immune responses to a broad array of microbial pathogens, including *Mycobacterium tuberculosis*. Here, we sought to characterize development of circulating human MR1T cells defined by MR1-5-OP-RU tetramer labelling and expression of TRAV1-2/CD26/CD161. We analysed postnatal expansion, maturation and functionality of peripheral blood MR1T cells in cohorts from three different geographic settings with different tuberculosis (TB) vaccination practices, levels of exposure to and infection with *M. tuberculosis.* Early after birth, frequencies of MR1-5-OP-RU tetramer-defined MR1T cells increased rapidly by several fold. This coincided with marked phenotypic changes, from a predominantly $CD4^+$ and TRAV1-2^- phenotype in neonates, to predominantly TRAV1-2^+CD8^+ MR1T cells that also expressed CD26 and CD161. We also observed that tetramer$^+$ MR1T cells that expressed TNF upon mycobacterial stimulation were very low in neonates, but increased ~10-fold in the first year of life. These functional MR1T cells in all age groups were MR1-5-OP-RU tetramer$^{+,}$ TRAV1-2^+ cells and expressed CD26 and CD161, markers that appeared to signal phenotypic and functional maturation of this cell subset. This age-associated maturation was also marked by the loss of naïve T cell markers on tetramer$^+$ TRAV1-2^+ MR1T cells more rapidly than tetramer$^+$TRAV1-2^- MR1T cells and non-MR1T cells. These data suggest that neonates have infrequent populations of MR1T cells with diverse phenotypic attributes; and that exposure to the environment rapidly and preferentially expands the MR1-5-OP-RU tetramer$^+$TRAV1-2^+ population of MR1T cells, which becomes the predominant population of functional MR1T cells early during childhood.

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Author Summary

MR1-restricted T (MR1T) cells defend against many microbial infections that cause illness and death in young children, including *Mycobacterium tuberculosis,* which causes tuberculosis (TB). Here, we characterized the development of human MR1T cells from after birth to adulthood. We analysed expansion, maturation and functionality of MR1T cells in populations from three different geographic settings with different tuberculosis vaccination practices and levels of exposure to and infection with *M. tuberculosis.* We found that early after birth, MR1T cells were rare but then expanded rapidly coinciding with marked phenotypic changes, from heterogeneous phenotypes in neonates, to one predominant type of MR1T cells in adults. We also observed that MR1T cells that defend against mycobacteria were rare in neonates but then increased ~10-fold in the first year of life. We also observed that MR1T cells matured more rapidly than other types of T cells. Our data suggest that neonates have rare populations of MR1T cells with diverse phenotypes and that exposure to the environment rapidly and preferentially expands a dominant population of MR1T cells early during childhood. Our data improves our understanding of the human development of MR1T cells relevant to important childhood pathogens, which could aid in the improvement of infant vaccines.

Introduction

MR1-restricted T (MR1T) cells, otherwise known as mucosal-associated invariant T (MAIT) cells, are a highly conserved population of T-cells that exhibit innate-like functions similar to those described for NKT cells. MR1T cells are defined by their recognition of metabolite antigens presented by the monomorphic MHC class 1-related molecule, MR1, the most highly

conserved MHC class I related molecule in mammalian species [1]. MAIT cells, the predominant subset of MR1T cells, are also defined by a highly restricted T-cell-receptor (TCR) repertoire, comprising an invariant TCR α-chain (TRAV1.2-TRAJ33/20/12 in humans; TRAV1-TRAJ33 in mice) with a CDR3 of constant length paired with a limited number of Vβ segments (Vβ2/13 in humans; Vβ6/8 in mice) [2-5].

MR1T cell development is a stepwise process, with an intra-thymic selection followed by peripheral expansion. Koay *et al* reported a three-stage development pathway for mouse and human MR1T cell populations, where three populations of human MR1T precursors were defined based on differential expression of CD27 and CD161. The initial two stages of MR1-5-OP-RU tetramer^{+}TRAV1-2+CD161^{-} cells, which were either CD27- or CD27+, occurred in the thymus and these cells were low in the periphery. The more mature MR1^{+}TRAV1-2+CD161^{+}CD27^{+} cells, designated as stage 3, have been shown to represent only a small fraction of T cells but form a more abundant population in adult blood [3, 6, 7]. Such studies indicate that MR1T cell thymopoeisis is complemented by an important postnatal peripheral expansion and maturation, which was recently shown to be dependent upon acquisition of the intestinal microbiome [8, 9].

MR1T cells recognize microbial derived riboflavin (Vitamin B2) biosynthesis intermediate derivatives, such as 5-OP-RU (5-(2-oxopropylideneamino)-6-d-ribitylaminouracil) and a broad spectrum of microbes that synthesize riboflavin, including *S. aureus, E. coli, and C. albicans* [10-12]. While 5-OP-RU was the first MR1 ligand defined, several other ligands have subsequently been defined, including additional riboflavin-derived ligands, including both stimulatory and inhibitory ligands [1, 12]. Upon recognition of microbial antigens or activation through cytokine signaling, MR1T cells display immediate effector function by secreting inflammatory cytokines and cytotoxic molecules, which can kill infected cells [6, 11, 13-15]. In a mouse model of

pulmonary legionella infection, MR1T cells exhibited enhanced protection in an MR1-dependent manner, mediated by the effector molecules, IFN-γ and GM-CSF [16]. MR1T cells have also been shown to facilitate control of *Klebsiella pneumonia, Mycobacterium bovis* BCG, and *Francicella tularensis* infections [17-20]. Many of the bacterial pathogens that are important causes of infant morbidity and mortality, including *S. aureus*, *S. pneumoniae*, and enteric pathogens (e.g. *E. coli*, *Klebsiella*, *Salmonella*), are recognized by MR1T cells. MR1T cells possess immediate innate-like, effector functions, and can therefore mediate immune functions before priming, clonal expansion and maturation of antigen-specific MHC-restricted T cells. Therefore, MR1T cells could play an important role in host defense to bacterial pathogens, especially in infancy.

MR1T cells are known to also recognize and respond to *Mycobacterium tuberculosis,* the leading global cause of mortality from a single infectious agent. Infants and young children suffer disproportionately from disseminated and severe forms of tuberculosis (TB) disease and are significantly more susceptible to disease than adults [21-23]. Here, we sought to characterize development of MR1T cells, defined by MR1-tetramer labeling and TRAV1-2/CD26/CD161 expression, by analyzing postnatal expansion, maturation and functionality in humans. We compared MR1T cells development in cohorts from three different settings with differing TB vaccination practices and levels of exposure and infection with *M. tuberculosis.*

Results

Human participants

We enrolled nine cohorts across three different countries to study MR1T cell development. The cohorts in South Africa (SA) were neonates (cord blood, n = 10), infants (aged 10 weeks, BCG vaccinated at birth, n = 10) and adolescents (aged 14-18 years, BCG vaccinated at birth,

QFT-, *n* = 10). The cohorts enrolled in the United States (US) were neonates (cord blood, *n* = 10), infants (aged 9-15 months, BCG unvaccinated, *n* = 10) and adults (aged 29-60 years, *n* = 10). The cohorts in Uganda (UG) were infants (2-24 months old, BCG vaccinated at birth +/-, TST +/-, *n* = 36), children (24 - 60 months, BCG vaccinated at birth +/-, TST +/-, *n* = 23) and adults (aged 18-60 years, BCG vaccinated at birth +/-, TST +/-, *n* = 91).

Frequencies of MR1T cells increase with age irrespective of BCG vaccination

We used two distinct definitions of MR1T cells. Tetramer-defined MR1T cells were defined as $CD3^+$ T cells positively labeled with the MR1-5-OP-RU tetramer, which also defined these cells as MR1-restricted T cells. 6FP is a MR1 ligand that is not stimulatory and MR1/6FP tetramers are used as a negative control. Phenotypically defined MR1T cells were defined as $CD3^+$ T cells that stained positive for antibodies against TRAV1-2, CD161 and CD26. This phenotypic definition of MR1T cells is concordant with tetramer-defined MR1T cells in adults [24]. Using these two definitions, we determined frequencies of MR1T cells in all cohorts (Fig 1). Because of limited availability of the MR1-5-OP-RU tetramer, determination of tetramer-defined MR1T cell frequencies in the Ugandan cohorts was performed on a subset of 10 donors (Fig 1B and S1 Fig). In all cohorts, using either definition, MR1T cell frequencies increased with age (Fig 1B).

MR1T definitions are concordant in adults, adolescents and infants but discordant in neonates

We next determined the degree of concordance between MR1T cell populations defined by phenotype or tetramer. The majority of phenotypically and tetramer-defined MR1T cells were common in infants, adolescents and adults and therefore these definitions were essentially concordant (Fig 1C). However, in cord blood from both SA and US neonates, these two

populations were discordant (Fig 1C). The expression of CD4 and CD8 across discordant, phenotypically defined MR1T cell populations varied between cohorts.

The MR1-5-OP-RU tetramer$^+$ subset of phenotypically-defined MR1T cells contain the functional MR1T cell population

To investigate whether tetramer-defined and phenotypically defined MR1T cells have different functional capacity, we measured TNF expression in the US cohort by intracellular cytokine staining (ICS) and flow cytometry following incubation with *M. smegmatis*-infected A549 cells. Among all phenotypically defined MR1T cells, only the MR1/5-OP-RU tetramer$^+$ subset expressed TNF; MR1-5-OP-RU tetramer-negative cells, by contrast, did not appear to respond to *M. smegmatis* stimulation (Fig 2). Because of this result we focused on the functional, tetramer-defined MR1T cells for subsequent experiments.

Neonates have higher proportions of CD4$^+$ and TRAV1-2$^-$ MR1T populations compared to infants, adolescents and adults

To explore the diversity of MR1T cells and how this may change during early development, we applied the dimensionality reduction method, t-distributed stochastic neighbor embedding (tSNE), on tetramer-defined MR1T cells. Five discrete phenotypic MR1T cell clusters were identified in both the SA and US cohorts (Fig 3A-D), the relative proportions of which varied with age (Fig 3B and D). Specifically, the CD4$^+$ (Clusters 3 and 4) and TRAV1-2$^-$CD26$^-$CD161$^-$ (Clusters 1, 2 and 3) MR1T cell clusters predominated in neonates, while CD4$^-$CD8$^{+/-}$TRAV1-2$^+$CD26$^+$CD161$^+$ cluster (Cluster 5) was predominant in infant, adolescent, and adult cohorts from both the US and SA (Fig 3B and D). Relative proportions of MR1T cell clusters in 10 week old (SA) and 12 month old (US) infants were more similar to those in adults and adolescents than to those in neonates, consistent with the hypothesis that MR1T cells mature rapidly upon exposure

to MR1T cell antigens (Fig 3E and F). It was also noteworthy that $CD4^{-}CD8^{+/-}$TRAV1-$2^{+}CD26^{+}CD161^{+}$ MR1T cells that fell into cluster 5 were virtually absent in cord blood, while forming the largest cluster in 12-month-old (US) infants. In the 10 week old (SA) infants, this cluster had started to appear but was not yet "fully developed", suggesting rapid development during the first months of life, from a predominantly TRAV 1-2 negative to a TRAV1-2 positive population with loss of expression of CD4 and acquisition of CD26 and CD161.

MR1T cells become more functional with age

Next, we explored how tetramer-defined MR1T cells developed functionally with age in the US cohort by tracking the TNF-expressing population following *M. smegmatis* or PMA/ionomycin stimulation. TNF-expressing $tetramer^{+}$ MR1T cells in neonates were present at very low frequencies (Fig 2B) and represented a much smaller proportion of the total $tetramer^{+}$ MR1T cell population than in infants and adults (Fig 4A and B). Proportions of $tetramer^{+}$ MR1T cells responding to either mycobacteria or PMA/ionomycin were higher in infants than adults or neonates. However, because $tetramer^{+}$ MR1T cell frequencies were higher in adults than infants (Fig 1B), overall frequencies of TNF-producing MR1T cells were highest in adults. To explore the functional capacity of MR1T cells relative to other T cell subsets within each cohort, we compared proportions of TNF-expressing MR1T cells ($CD3^{+}TCR\gamma\delta^{-}$MR1-5-OP-$RU^{+}$) with TCRγδ T cells ($CD3^{+}TCR\gamma\delta^{+}$ MR1-5OPRU^{-}), $CD4^{+}$ T cells ($CD3^{+}TCR\gamma\delta^{-}$MR1-5OP$RU^{-}CD4^{+}$) and $CD8^{+}$ T cells ($CD3^{+}TCR\gamma\delta^{-}$MR1-5OP$RU^{-}CD4^{+}$) after PMA/Ionomycin stimulation (Fig 4C). In neonates, proportions of TNF-expressing MR1T cells were significantly higher than proportions of TCRγδ T cells and $CD8^{+}$ T cells. In infants and adults, proportions of TNF-expressing MR1T cells were significantly higher than $TCR\gamma\delta^{+}$, $CD4^{+}$ and $CD8^{+}$ T cells (Fig 4C).

Functional CD3$^+$MR1-5-OP-RU$^+$ MR1T cells are TRAV1-2$^+$

Next, we investigated TRAV1-2 expression among functional, TNF-expressing CD3$^+$MR1-5-OP-RU$^+$ MR1T cells. Only TRAV1-2$^+$ MR1 T cells produced TNF in response to mycobacterial stimulation while TRAV1-2$^-$ MR1 T cells were not functional (Fig 4D). This pattern was unique to mycobacterial stimulation since TNF-expressing TRAV1-2$^-$ MR1T cells were observed in response to PMA/Ionomycin stimulation, particularly in infants and adults (Fig 4E). Interestingly, the vast majority of these functional, TNF-expressing TRAV1-2$^+$ MR1T cells also expressed the phenotypic markers, CD26 and CD161 (Fig 4F-G). Thus, the CD3$^+$MR1-5-OP-RU$^+$TRAV1-2$^+$CD26$^+$CD161$^+$ MR1T cell population contains the functional MR1T cells that respond to mycobacteria. The frequency of this population increases very quickly with age.

TRAV1-2$^+$ MR1T cells lose naïve markers more rapidly with age than TRAV1-2$^-$ MR1T cells or non-MR1T cells

To characterize the development of MR1T cells further, we also measured expression of CD27 and CD161, markers previously described to indicate MR1T cell maturation, and CD45RA, CCR7, CD38 and CD56 on tetramer-defined MR1Ts (Figs 5-6) [25]. A population of immature MR1T cells, defined as CD27$^+$CD161$^-$ cells, was observed in neonates but not in infants and adults, as previously described [7]. This was also reflected by expression of the more traditional T cell maturation markers, CD45RA, CCR7 and CD38 (Fig 6 A, C, E). All tetramer-defined MR1T cells in neonates displayed a CD45RA$^+$CCR7$^+$CD38$^+$ "naïve" phenotype, but this phenotype was lost more rapidly with age on TRAV1-2$^+$ MR1Ts than on TRAV1-2$^-$ MR1T cells or non-MR1T cells (Fig 6A-F). The inverse was true for the activation marker, CD56, which was increased in neonatal, infant and adult TRAV1-2$^+$ tetramer-defined MR1T cells compared to TRAV1-2$^-$ tetramer-defined MR1T cells and non-MR1T cells (Figure 6G-H). These results are consistent with our finding that

TRAV1-2$^+$ tetramer-defined MR1T cells display functional capacity in all age groups, even though they represent the minority of neonatal MR1T cells.

Discussion

We characterized the development of MR1T cells during early childhood in three cohorts, two from Africa and one in the USA, to elucidate phenotypic and functional changes that occur after birth and during the first few years of life. A better understanding of MR1T cell development is critical given the likely role of MR1T cells as innate-like effectors that act as key mediators of immunity in newborns, who are at very high risk of infection, including *M. tuberculosis.* We found that neonates had relatively low frequencies of MR1T cells with high phenotypic diversity, which included considerable proportions of CD4$^+$ and TRAV1-2$^-$ cells. These patterns changed as early as 10 weeks after birth, and became more prominent at 1-2 years of age, adolescence and ultimately by adulthood. This included higher frequencies of MR1T cells in peripheral blood, characterized by a preferential expansion in TRAV1-2$^+$CD8$^+$ MR1T cells such that TRAV1-2$^+$CD8$^+$MR1T cells constituted the vast majority of the tetramer$^+$ MR1T cell population. The development also included changes in expression of maturation markers and MR1T cell effector function, since MR1T cells from older individuals had lower expression of the naïve T cell markers, CD45RA, CCR7 and CD38, and an increased ability to express TNF.

Our finding that MR1T cells are found at low frequency in cord blood and increase in frequency with age confirms our previous observations as well as those of others, who have utilized either the MR1-5-OP-RU tetramer or phenotypic characterization of MR1T cells to demonstrate the expansion of MR1T cells from birth through adulthood [3, 7, 10, 26-28]. The very rapid changes in MR1T cells seen after birth are striking and were clearly seen in both African and

American settings. This finding rules out the possibility that BCG substantially contributes to these changes, since BCG is not routinely administered at birth in the USA. Rather, it is more likely that changes that occur upon the transition from the limited microbial exposure in the womb to the postnatal environment, accompanied by the rapid colonization of the gut microbiome. MR1T cells are absent in germ-free mice and depend on the colonization of the gut microbiome with normal intestinal flora, which produce riboflavin [1, 8, 14]. We suggest that this colonization of the gut microbiome is the driving force that accounts for the increase and phenotypic changes in MR1T cells in blood after birth and the gain in functional capacity.

While we confirm our previous work that phenotypic definition of MR1T cells accurately identifies functional MR1T cells in adults, we find that there is a high level of discordance in neonates in part due to MR1-5-OP-RU tetramer^{+} cells that don't express TRAV1-2, CD26 or CD161. These cells are by definition MR1-restricted T cells as they bind the MR1-5-OP-RU tetramer. However, these cells are poorly reactive to mycobacteria, suggesting that they either recognize a different antigen or represent immature MR1T cells. The poorer PMA/Ionomycin-induced TNF secretion of these cells supports the latter.

We also show that neonates possess MR1-5-OP-RU tetramer^{-} cells that phenotypically resemble MR1T cells (the discordant MR1-5-OP-RU tetramer^{-} phenotypically defined (CD26^{+}, CD161^{+}, and TRAV1-2^{+}) MR1T cell population). As Fergusson *et al* suggested that distinct T subsets that express CD161 share a common transcriptional profile characteristic of typical MR1T cells [29], it is certainly possible that these CD161^{+} cells are MR1-restricted or share common attributes with MR1T cells. Further characterization of this neonatal T cell population is required to determine whether or not these cells are indeed MR1-restricted and if so, if these cells are immature or recognize distinct microbes, or produce a different cytokine not detected in our

functional assay. Given that, in the mouse model, 5-OP-RU produced by the microbiome is necessary and sufficient to drive the expansion of typical MR1T cells [8], it is perhaps expected that with age tetramer-positive MR1T TRAV1-2^{+} cells would quickly become the predominant MR1T cell population, while tetramer negative MR1T cells might persist as a minority population of naïve T cells.

Consistent with our prior findings [10] and those of Youssef *et al*. [28], our phenotypic analysis of MR1-5-OP-RU tetramer^{+} cells in neonates demonstrates that MR1T cells expressed a naïve cell surface phenotype (CD45RA^{+}CCR7^{+}CD38^{+}) and included a prominent subset of CD4^{+} cells. It is noteworthy that these MR1T cells rapidly lost expression of CD45RA, CCR7 and CD38 and acquired a phenotype consistent with those observed in antigen-experienced memory T cells much more rapidly than other types of T cells [13, 30, 31].

While Koay *et al* showed the functional potential of neonatal MR1T cells that produced TNF following stimulation with PMA/ionomycin, we show for the first time the presence of functional microbe-reactive MR1T cells in neonates, albeit at low frequency. Virtually all of the mycobacteria-reactive TNF-producing MR1T cells were contained within the TRAV1-2^{+}CD26^{+} CD161^{+} subset of 5-OP-RU tetramer^{+} cells, which are also concordant with "Stage 3" MR1T cells defined by Koay *et al*. Although the immature CD4^{+}, CD4-CD8- and/or TRAV1-2- MR1T cells were maintained through to adulthood, we observed that CD8^{+}TRAV1-2^{+} MR1T cells expanded preferentially over TRAV1-2^{-} MR1T cells. This expansion coincided with the acquisition of CD26 and CD161 as markers of mature functional MR1 T cells. In addition, we show that the frequency of these functional mycobacterial-reactive MR1T cells increases ~10-fold in the first year of life. Interestingly, we demonstrated that relative to other T cell subsets, MR1T cells had the highest capacity to secrete pro-inflammatory cytokines, such as TNF-α, in infants. Thus, MR1T cells may

represent an important source of pro-inflammatory effectors in infants, who are more vulnerable to serious bacterial infections in the first two years of life, relative to older children and adults.

Regarding newborns, it is not known to what degree the naïve phenotype and low functionality of MR1T cells contributes to the very high susceptibility to bacterial infection in the first two months of life [32]. Since MR1T cells recognize important childhood bacterial pathogens, including *S. aureus, S. pneumoniae and enteric pathogens (e.g. E. coli, Klebsiella, Salmonella species*, it is plausible that the low frequency of functional MR1T cells during the neonatal period contributes to neonatal susceptibility to infection. At the very least, our findings add to the well-described differences in adaptive and innate immunity between neonates and adults [32].

With regard to *M. tuberculosis*, there is mounting evidence that supports an important host defense role for MR1T cells, especially during early infection. Upon encounter with mycobacterial-infected cells, MR1T cells produce pro-inflammatory cytokines such as IFN-γ and TNF [11], known to be essential mediators to host defense against *M. tuberculosis* [30]. MR1-deficient mice control BCG less well than wild type mice [31]. In humans, MR1 polymorphisms are associated with susceptibility to TB meningitis [33]. It is tempting to speculate, in light of the high susceptibility of infants to TB, and the phenotypic and functional attributes of MR1T cells we observed, that MR1T cells contribute to control of early infection with *M. tuberculosis* through their early and robust anti-mycobacterial response, particularly in the lung.

Methods

Study participants

University of Cape Town, Western Cape, South Africa. All participants were enrolled either at the South African Tuberculosis Vaccine Initiative Field Site, Well-Baby Clinics or Maternity

Wards in the Worcester region, near Cape Town, South Africa. Pregnant mothers were enrolled for collection of cord blood immediately after childbirth by Cesarean section. Mothers who may have been exposed to *M. tuberculosis*, HIV infected, had any other acute or chronic disease, and receiving medication other than anesthetic given during delivery were excluded. Infants born to HIV negative mothers, and who had received BCG at birth, had no perinatal complications, or household contact with any person with TB, and did not have any acute or chronic disease were enrolled for collection of blood. Healthy adolescents of 12-18 years of age without evidence of latent Mtb infection as determined by positivity negativity for the Tuberculin Skin Test (<10mm) or QuantiFERON (<0.35IU/mL) were enrolled for blood collection. Heparinized blood was collected for cord blood mononuclear cell (CBMC) and peripheral blood mononuclear cells (PBMC) isolation by means of density gradient centrifugation.

Oregon Health and Science University, Portland, OR, USA. Umbilical cord blood was obtained from the placenta of uncomplicated term pregnancies after delivery into citrate CPT tubes (BD). Blood was processed per manufacturer's instructions within 24hr of collection, and resulting CBMC cryopreserved. Infants aged 1-24 months undergoing elective surgery at OHSU Doernbecher Children's Hospital were enrolled. Infants had not received BCG vaccination, which is not part of the childhood immunization series in the United States. Blood was drawn into citrate CPT tubes (BD) and processed per manufacturer's instructions to isolate PBMC within 24hr of collection, and PBMC subsequently cryopreserved. Adult PBMC were obtained by leukapheresis from healthy adult donors and cryopreserved. Adult participants lacked any detectable responses to *Mycobacterium tuberculosis* (Mtb) immunodominant antigens CFP-10 and ESAT-6 and therefore were assumed to be Mtb uninfected.

Makerere University, Kampala, Uganda. Children from age 2-60 months hospitalized with lower respiratory infection (LRTI) from etiologies other than TB were recruited from Mulago Hospital Paediatric Wards. All children were HIV-negative and had no known recent TB contacts. Children were reassessed two months following enrollment to establish vital status and ensure clinical improvement. Following completion of the study, data from all participating children was reviewed by study investigators (DAL, CLL, SK) to confirm diagnosis of non-TB LRTI. Blood was drawn into citrate CPT tubes (BD) and processed per manufacturer's instructions to isolate PBMC within 24hr of collection, and PBMC subsequently cryopreserved. Adult participants were recruited from the National TB Treatment Center at Mulago Hospital, hospital staff, and the community surrounding Kampala, Uganda, between 2001 and 2014. Participants were HIV uninfected and included individuals with (tuberculin skin test, TST ≥ 10) or without (TST < 10 mm) evidence of Mtb infection. All had the absence of signs or symptoms of TB (prolonged cough, hemoptysis, fever, weight loss, and night sweats). Heparinized blood was collected for PBMC isolation by means of density gradient centrifugation.

Ethics approvals

The studies of all participants were conducted in accordance with the World's Medical Association's Declaration of Helsinki guidelines. The studies from the Western Cape, South Africa were approved by the Human Research Ethics Committee (HREC) of the University of Cape Town. Written informed consent was obtained from parents or legal guardians of adolescents and infants. Adolescents also provided written informed assent. The Institutional Review Board of Oregon Health & Science University (Portland, Oregon) approved this study of participants from Portland, OR. Written, informed consent was obtained from all adult participants and from a parent or legal guardian of infants before enrollment. For participants from Kampala, Uganda, the

National Council for Science and Technology, HIV/AIDS Research Committee in Uganda, the Institutional Review Board of Oregon Health & Sciences University (Portland, OR) and the Institutional Review Board of Makerere University (Kampala, Uganda) approved this study. Written, informed consent was obtained from all adult participants and from a parent or legal guardian of infants and children before enrollment.

Infection of antigen presenting cells

A549 cells (ATCC CCL-185) were used as stimulators for direct ex vivo determination of mycobacterial-reactive T cells. *Mycobacterium smegmatis* (strain mc^2155, ATCC) was grown to late log phase and functionally titered in this assay to find the optimal volume of *M. smegmatis* that elicited a strong response in an adult control donor but did not reduce the viability of the antigen presenting cells. A549 cells were seeded in T25 flasks at 1.25e6 in 7.5ml of antibiotic free media. After allowing cells to adhere for 3 hours, the flasks were infected with 31µl of *M. smegmatis*. After 18 h, the cells were harvested and washed twice in RPMI/10% human serum containing antibiotic (gentamicin) before being resuspended in RPMI/10% human serum containing antibiotic (gentamicin).

Flow cytometry assays

To perform ICS, cryopreserved PBMC were thawed in the presence of DNAse, resuspended, in 10% heat inactivated human serum with RPMI (Lonza) at a concentration of 2 x 10^7/ml. PBMC (2 x 10^6 cells/well) were stimulated with anti-CD28 (1 µg/ml) and anti-CD49d (1 µg/ml) (FastImmune, BD Biosciences) and 1e5 *M. smegmatis*-infected or uninfected A549 cells in RPMI/10% Human Serum for 18 h at 37°C with 5% $C0_2$. During the last 12 h of this incubation, Brefeldin-A (5 µg/ml) was added. PBMC incubated with uninfected A549 cells were used to assess

for background cytokine production. To determine the total functional capacity of T cells, PBMC were incubated with uninfected A549 cells and PMA/Ionomycin (PMA, 20ng/ml, Sigma; Ionomycin, 1μM, Sigma) for 3 hr. PBMC were then harvested and stained with MR1-5-OP-RU or MR1-6FP tetramers [34] (NIH Tetramer Core) at a 1:500 dilution for 45 minutes at room temperature. All tetramers used were conjugated to R-phycoerythrin (PE) or Brilliant Violet (BV) 421. After 45 minutes, an antibody cocktail containing LIVE/DEAD Fixable Dead Cell Stain Kit (Thermo Fisher), and the surface antibodies listed in S1 Table were added for 30 minutes at 4°C. After washing, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) per manufacturer's instructions. Cells were then stained for CD3 and TNF, then fixed in 1% PFA. Acquisition was performed using a (Beckman Coulter) Cytoflex flow cytometer with CytExpert software (BC). All flow cytometry data was analyzed using FlowJo software (TreeStar) and Prism (Graphpad).

To perform cell surface staining for phenotyping MR1T cells, cryopreserved PBMC were thawed as described above, and 1-2 x 10^6 PBMC were stained with MR1/5-OP-RU or MR1/6FP tetramers at 1:500 for 45 minutes at room temperature. Cells were then subsequently stained with an antibody cocktail containing LIVE/DEAD Fixable Dead Cell Stain Kit (Thermo Fisher), and surface stained with the antibodies listed in S2-S4 Tables for 30 minutes at 4°C. Samples were washed and fixed with 1% PFA. Acquisition of the US samples was performed using a Cytoflex flow cytometer (Beckman) with CytExpert software (BC). For experiments with the Ugandan samples, acquisition was performed on a BD LSRFortessa. For experiments performed at SATVI, samples were acquired on a BD LSR-II flow cytometer configured with 4 lasers: solid state blue (488nm; 100mW; 3 detectors), solid state violet (405nm; 25mW; 8 detectors), HeNe gas red (635nm; 70mW; 3 detectors), and diode-pumped Coherent Compass yellow-green (532nm;

150mW; 8 detectors). Flow cytometric analysis was performed using FlowJo version 10.5.3 (Treestar, Ashland, OR, USA).

Data analysis

Adjusted frequency (raw frequency of cytokine production following stimulation minus the frequency of cytokine production in resting/unstimulated condition), was used to account for the potential presence of background, or non-specific, responses. A positive cytokine response was defined as the detection of at least 0.05% cytokine-positive T cells (following background subtraction). Prism version 7 (GraphPad, La Jolla, CA, USA) was used for data analysis. A p-value < 0.05 was considered as statistically significant.

To characterize phenotypic diversity within MR1T cell populations at the different ages we performed t-Stochastic Neighbor Embedding (tSNE) on MR1-5-OP-RU tetramer$^+$ defined CD3$^+$ T cells. Data from MR1-5-OP-RU tetramer$^+$ cells from all individuals across the age groups were concatenated into a single FCS file to perform tSNE analysis in FlowJo. Equal event sampling was not performed due to low events per individual in the cord blood and infant samples. Clustering analysis was performed based on the expression of five surface markers (CD4, CD8, TRAV1-2, CD26, and CD161). Parameter settings were fixed at 2000 iterations with a learning rate (Eta) of 10 and perplexity 30, respectively [35].

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Figure Legends

Fig 1. Frequencies of MR1T cells in peripheral blood from individuals of different ages. PBMC or CBMC were stained with a live/dead discriminator, antibodies to CD3, CD4, CD8, TRAV1-2, CD26, CD161, and either the MR1-5-OP-RU or MR1-6FP tetramers. Live, $CD3^+$ lymphocytes were gated and the frequencies of MR1-5-OP-RU^+ or TRAV1-$2^+CD26^+CD161^+$ cells as a percentage of $CD3^+$ lymphocytes were determined. **A**. Flow cytometry plots showing $CD3^+$ T cell staining with MR1-5-OP-RU tetramer, MR1-6FP tetramer, TRAV1-2 or CD26/CD161 of samples from a representative US adult, infant and neonate. **B.** Frequencies of tetramer-defined ($CD3^+$MR1-5-OP-RU^+) and phenotypically-defined ($CD3^+$TRAV1-$2^+CD26^+CD161^+$) T cells in neonates, 10 week-old infants and adolescents from South Africa, neonates, 12 month-old infants and adults from the United States and infants (0-2 years old), children (2-5 years old) and adults

from Uganda (all cohorts, *n*=10). Mann-Whitney t-tests were used to test differences between groups. Horizontal lines depict the median and the error bars the 95% confidence interval. **C.** Relative proportions of median phenotypically-defined MR1T cells that are MR1-5-OP-RU$^+$ (Blue) and MR1-5-OP-RU$^-$ (Red shades) for each age group at each site. In each doughnut chart the MR1-5-OP-RU$^-$ population is further divided into CD8$^+$, CD8$^+$CD4$^+$, CD4$^+$, and CD8$^-$CD4$^-$ populations.

Fig 2. Functional analysis of MR1T cells in individuals of different ages. PBMC or CBMC from the US cohort were incubated overnight with *M. smegmatis*-infected A549 cells or uninfected A549 cells and then stained with the MR1-5-OP-RU or MR1/6FP tetramers, followed by staining with a live/dead discriminator and antibodies to TCRγδ, CD3, CD4, CD8, TRAV1-2, CD26 and CD161. ICS was then performed and the cells stained for TNF. **A.** Dot plots showing representative co-staining of live, TCRγδ$^-$CD3$^+$TRAV1-2$^+$CD26$^+$CD161$^+$ cells with MR1-5-OP-RU and TNF in *M. smegmatis* stimulated or unstimulated samples. Examples of the TNF response in a neonate, infant and adult are shown. **B.** Frequencies of functional, phenotypically-defined MR1T cells that are MR1-5-OP-RU$^+$ or MR1-5-OP-RU$^-$ as a proportion of CD3$^+$ TCRγδ$^-$ T cells. Horizontal lines depict the median and the error bars the 95% confidence interval. Wilcoxon-rank sum was used to test differences within the same cohort.

Fig 3. Phenotypic diversity of MR1T cells in individuals of different ages. t-Distributed stochastic neighbor embedding (tSNE) plots showing MR1-5-OP-RU tetramer-defined MR1T cells and the phenotypic clusters of MR1T cells identified in the South African (SA) or United States (US) cohorts. Plots represent cells from 10 samples from each age group. **A.** and **C.** The five clusters identified in both sites are shown in **A** (SA) and **C** (US). **B.** and **D.** Dot plots colored by the relative expression level of each marker (CD4, CD8, TRAV1-2, CD161 or CD26) in

tetramer-defined MR1T cells for each age group in **B** (SA) and **D** (US). Red = highest expression, blue = lowest expression. **E.** and **F.** The proportion of each cluster of tetramer-defined MR1T cells by age group is shown in **E** (SA) and **F** (US). Horizontal lines depict the median and the error bars the 95% confidence interval. Mann-Whitney t tests were used to test differences between groups.

Fig 4. Phenotypic analysis of functional MR1T cells at different ages. PBMC or CBMC from the US cohort were incubated overnight with *M. smegmatis*-infected A549 cells, uninfected A549 cells, or uninfected A549 cells and PMA/ionomycin. All cells were then stained with the MR1-5-OP-RU or MR1-6FP tetramers, followed by a live/dead discriminator and antibodies to TCRγδ, CD3, CD4, CD8, TRAV1-2, CD26 and CD161. ICS was then performed and the cells stained for TNF. Live, TCRγδ$^-$CD3$^+$MR1-5-OP-RU$^+$ cells were gated and the TNF$^+$ percentage determined in both the *M. smegmatis* stimulated and unstimulated conditions. **A.** Dot plots showing TNF expression by live, TCRγδ$^-$MR1-5-OP-RU$^+$ CD3$^+$ cells in a representative neonate, infant and adult. **B.** Background subtracted frequencies of TNF$^+$ MR1-5-OP-RU$^+$ CD3$^+$ cells in response to *M. smegmatis*-infected A549 cells as a percentage of total MR1/5-OP-RU$^+$ CD3$^+$ cells are shown. Mann-Whitney t-tests were used to test differences between groups. **C.** Background subtracted frequencies of TNF$^+$ cells to PMA/ionomycin among different T cell subpopulations, 1) MR1T cells (CD3$^+$TCRγδ$^-$MR1-5-OP-RU$^+$ cells); 2) γδ T cells (CD3$^+$TCRγδ$^+$MR1-5-OP-RU$^-$ cells); 3) CD8$^+$ T cells (CD3$^+$TCRγδ$^-$MR1-5-OP-RU$^-$CD8$^+$ cells); and 4) CD4$^+$ T cells (CD3$^+$TCRγδ$^-$MR1-5-OP-RU$^-$CD4$^+$ cells). Wilcoxon-rank sum was used to test differences within the same cohort. **D.** and **E.** Frequencies of TNF$^+$TRAV1-2$^+$ MR1-5-OP-RU$^+$ CD3$^+$ and TNF$^+$TRAV1-2$^-$ MR1-5-OP-RU$^+$ CD3$^+$ cells in response to *M. smegmatis* infected A549 cells (**D**) or PMA/ionomycin

stimulation (**E**) minus the background frequencies of TNF$^+$TRAV1-2$^+$/- MR1-5-OP-RU$^+$ CD3$^+$ cells. Wilcoxon-rank sum was used to test differences within the same cohort.

Fig 5. Analysis of MR1T cell development by measuring maturation markers. PBMC or CBMC from the US were stained as in Fig 1 with the addition of an antibody to CD27. Live, CD3$^+$MR1-5-OP-RU$^+$ cells were gated for the maturation pattern of S1, S2 and S3 described in [7]. S1 = CD161$^-$CD27$^-$, S2=CD161$^-$CD27$^+$, S3=CD161$^+$CD27$^{+/-}$. **A.** Gating of MR1T cells for S1, S2 and S3 in a representative neonate, infant and adult. **B.** Frequencies of S1, S2, and S3 MR1T cells as a percentage of CD3$^+$ MR1-5-OP-RU$^+$ cells are shown for U.S. neonates, infants and adults. Bars represent the median and the error bars the 95% confidence interval.

Fig 6. Comparative phenotypic analysis of MR1T and non-MR1T cells or TRAV1-2$^+$ and TRAV1-2$^-$ MR1T cells at different ages. PBMC or CBMC from the US were stained as in Figure 1 with the addition of antibodies to either CD45RA (**A, B**), CCR7 (**C, D**), CD38 (**E, F**) or CD56 (**G, H**). Frequencies of cells expressing each marker in non-MR1T cells (CD3$^+$MR1-5-OP-RU$^-$) or MR1T cells (CD3$^+$MR1-5-OP-RU$^+$) in each age group (**A, C, E, G**). The frequency of each marker expressed in TRAV1-2$^+$ or TRAV1-2$^-$ MR1T cells is shown in each age group (**B, D, F, H**). The median and 95% confidence intervals are shown in each graph. When the 95% confidence intervals do not overlap between conditions, those conditions are considered statistically significant.

Supporting Information

S1 Fig. Frequencies of MR1T cells in peripheral blood from the full sample size of Ugandan individuals of different ages. PBMC were stained with a live/dead discriminator, and antibodies to CD3, CD4, CD8, TRAV1-2, CD26 and CD161. Live, $CD3^+$ lymphocytes were gated and the percentage of $TRAV1\text{-}2^+CD26^+CD161^+$ cells were determined. Horizontal lines depict the median and the error bars the 95% confidence interval. Mann-Whitney t-tests were used to test differences between groups.

S1 Table. Antibodies used for ICS assay (US cohorts).

S2 Table. Antibodies used for flow cytometry (US cohorts).

S3 Table. Antibodies used for flow cytometry (South African Cohorts).

S4 Table. Antibodies used for flow cytometry (Ugandan Cohorts).

Competing Interests

No competing interests.

Contributions

GMS, AG, DML, TJS and DAL contributed to the conception and/or design of the work. GMS and AG contributed equally to the work. TJS and DAL jointly supervised the work. MN, HMK, SK, JK, CL, EN, MS, WAH, MH and TJS contributed to clinical activities. EN, WAH, MH, DML, DAL and TJS raised grants to fund the research. GMS, AG, MEC, MDN, RBD, DML, TJS and DAL substantially contributed to the acquisition, analysis or interpretation of data and drafting of the manuscript. All authors substantially contributed to revising and critically reviewing the manuscript for important intellectual content. All authors approved the final version of this manuscript to be published and agree to be accountable for all aspects of the work.

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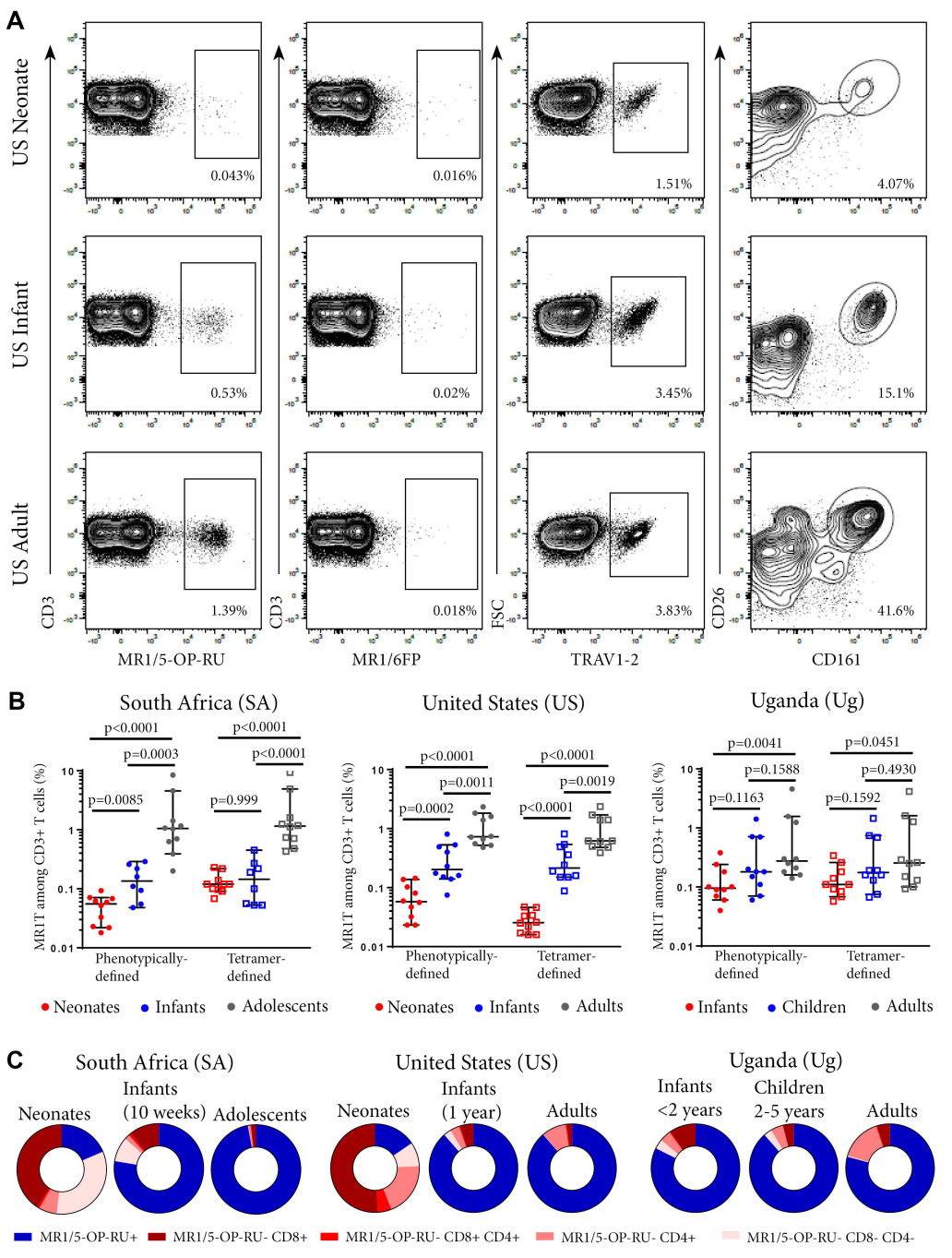
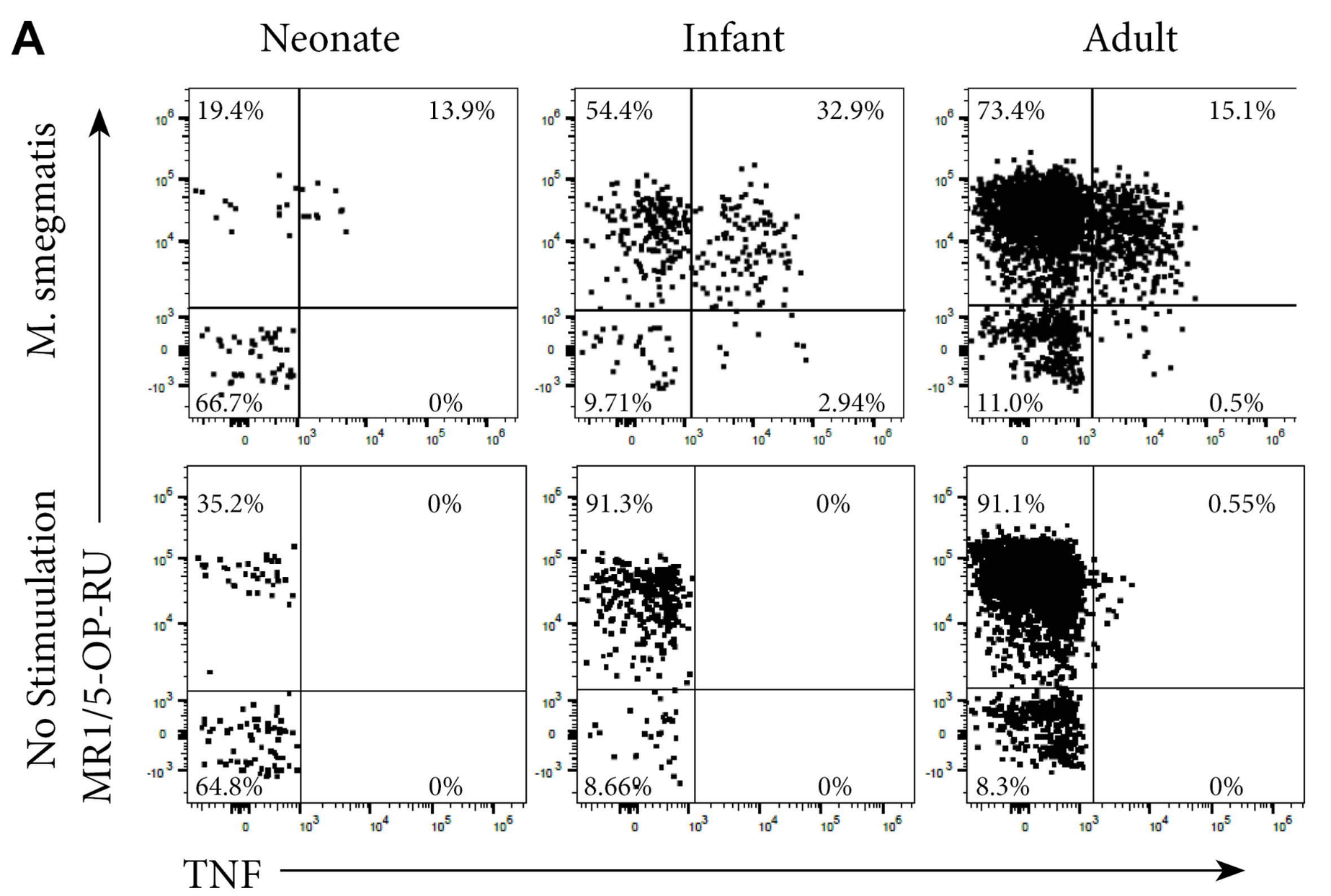
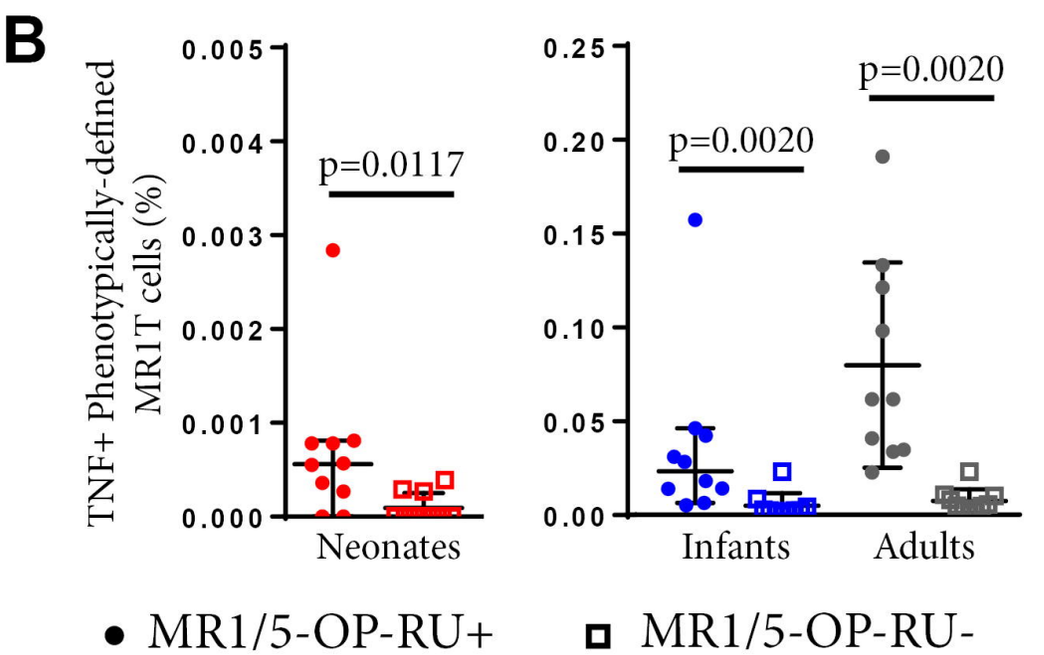
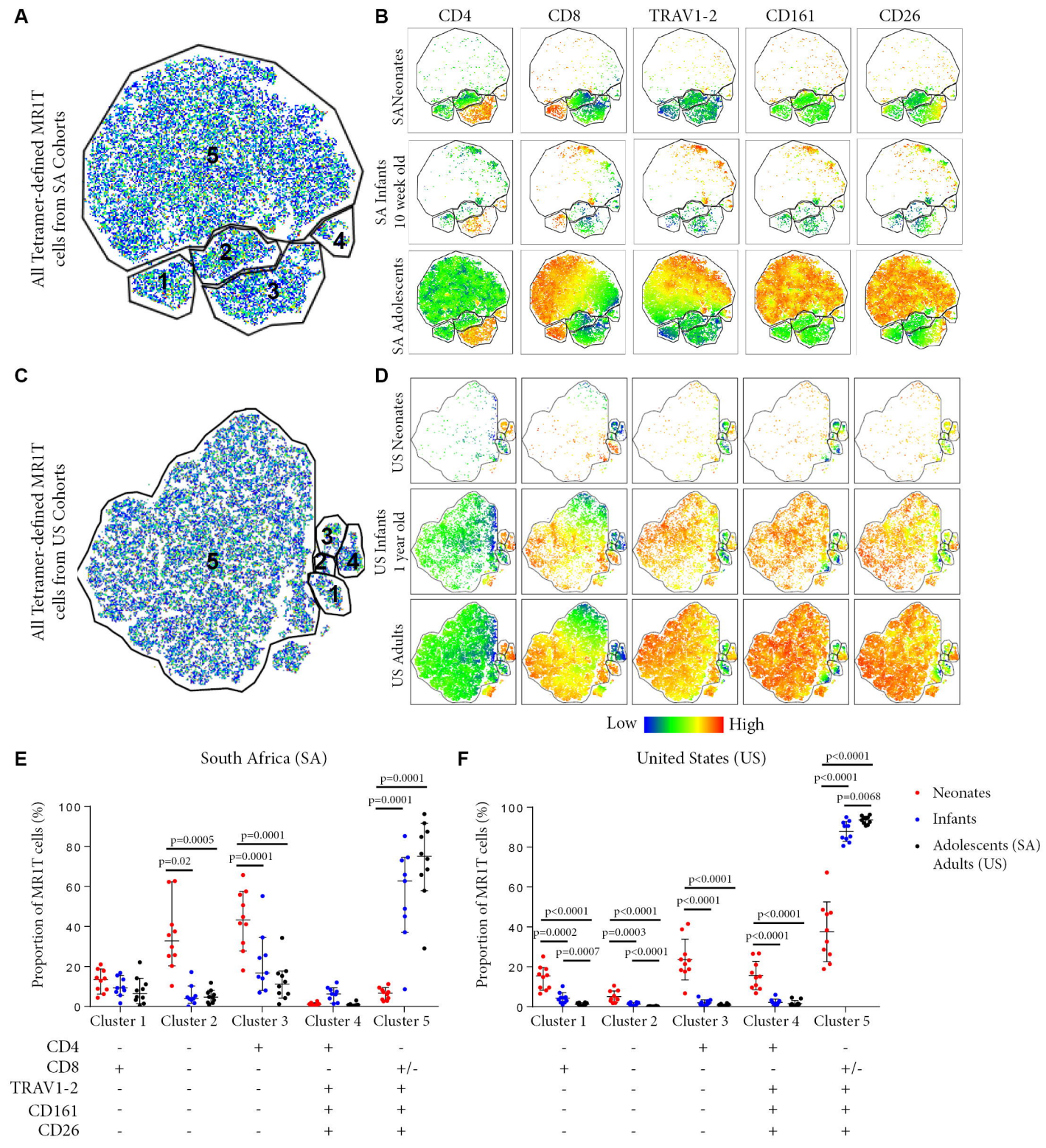
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.


A
US Neonate
US Infant
US Adult
0.043%
0.016%
1.51%
4.07%
0.53%
0.02%
3.45%
15.1%
1.39%
0.018%
3.83%
41.6%
CD3
MR1/5-OP-RU
CD3
MR1/6FP
FSC
TRAV1-2
CD26
CD161
B
South Africa (SA)
United States (US)
Uganda (Ug)
MR1T among CD3+ T cells (%)
p<0.0001
p=0.0003
p=0.0085
p<0.0001
p<0.0001
p=0.999
p<0.0001
p<0.0001
p=0.0011
p=0.0002
p<0.0001
p<0.0001
p=0.0019
p=0.0041
p=0.1588
p=0.1163
p=0.0451
p=0.4930
p=0.1592
Phenotypically-defined
Tetramer-defined
Neonates
Infants
Adolescents
Adults
Children
C
South Africa (SA)
Neonates
Infants (10 weeks)
Adolescents
United States (US)
Neonates
Infants (1 year)
Adults
Uganda (Ug)
Infants <2 years
Children 2-5 years
Adults
MR1/5-OP-RU+
MR1/5-OP-RU- CD8+
MR1/5-OP-RU- CD8+ CD4+
MR1/5-OP-RU- CD4+
MR1/5-OP-RU- CD8- CD4-


A
Neonate
Infant
Adult
M. smegmatis
No Stimulation
MR1/5-OP-RU
19.4%
13.9%
66.7%
0%
54.4%
32.9%
9.71%
2.94%
73.4%
15.1%
11.0%
0.5%
35.2%
0%
64.8%
0%
91.3%
0%
8.66%
0%
91.1%
0.55%
8.3%
0%
TNF


B
TNF+ Phenotypically-defined MR1T cells (%)
p=0.0117
p=0.0020
p=0.0020
Neonates
Infants
Adults
MR1/5-OP-RU+
MR1/5-OP-RU-


A
All Tetramer-defined MR1T cells from SA Cohorts
1
2
3
4
5
B
CD4
CD8
TRAV1-2
CD161
CD26
SANeonates
SA Infants 10 week old
SA Adolescents
C
All Tetramer-defined MR1T cells from US Cohorts
1
2
3
4
5
D
US Neonates
US Infants 1 year old
US Adults
Low
High
E
South Africa (SA)
Proportion of MR1T cells (%)
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
p=0.02
p=0.0005
p=0.0001
p=0.0001
p=0.0001
p=0.0001
F
United States (US)
Proportion of MR1T cells (%)
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
p=0.0002
p=0.0007
p<0.0001
p=0.0003
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p=0.0068
Neonates
Infants
Adolescents (SA)
Adults (US)
CD4 - - + + -
CD8 + - - - +/-
TRAV1-2 - - - + +
CD161 - - - + +
CD26 - - - + +
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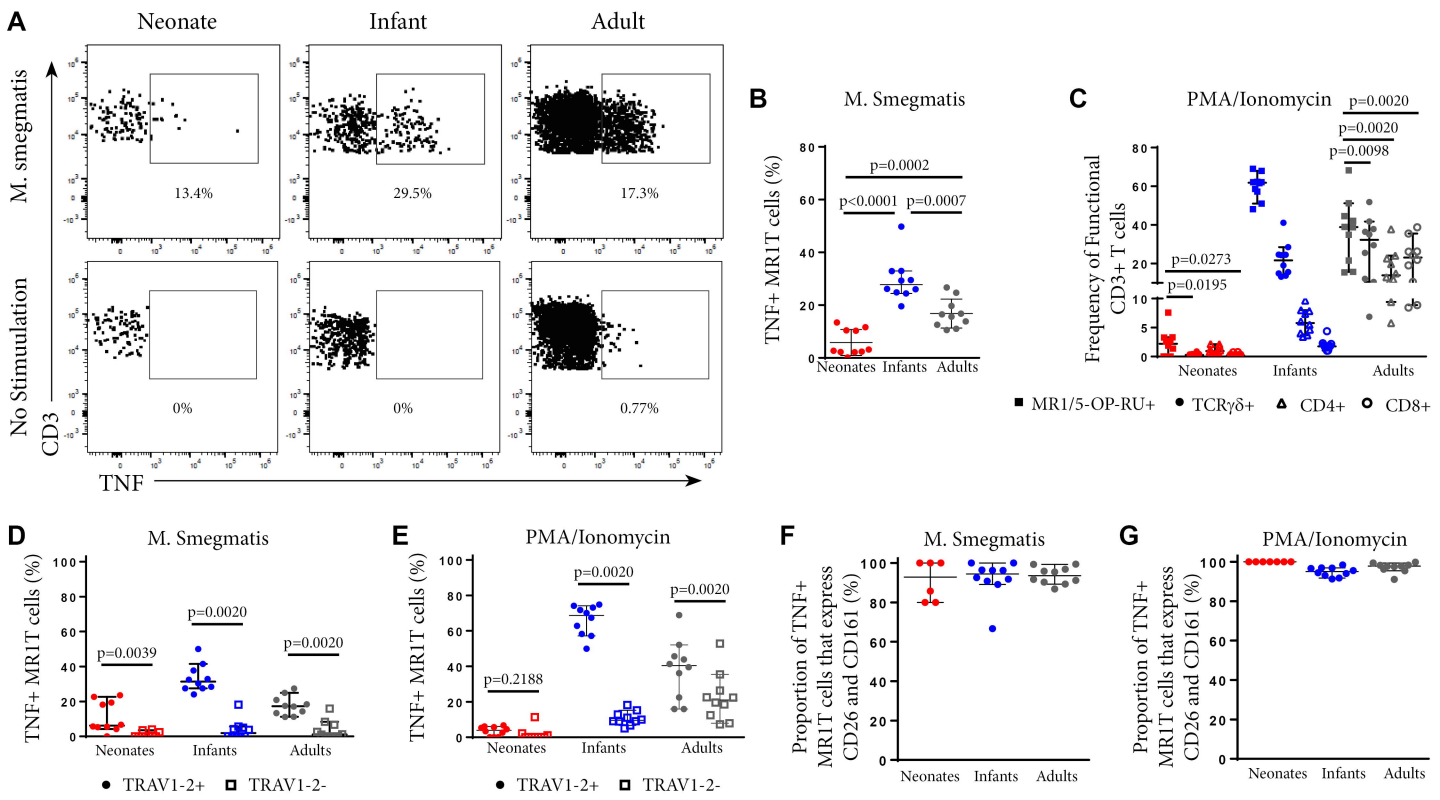
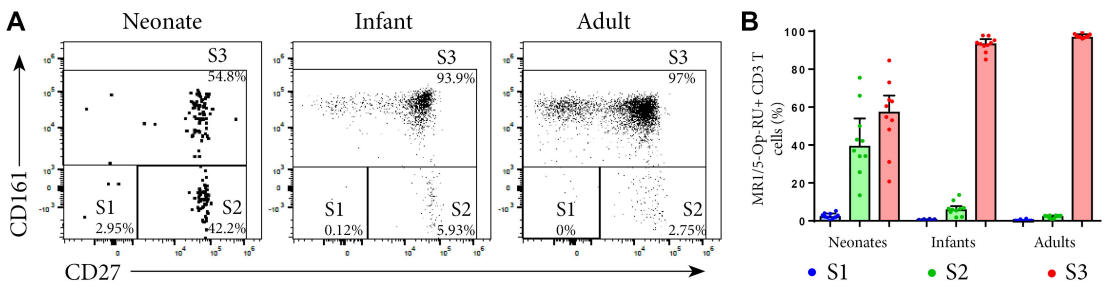
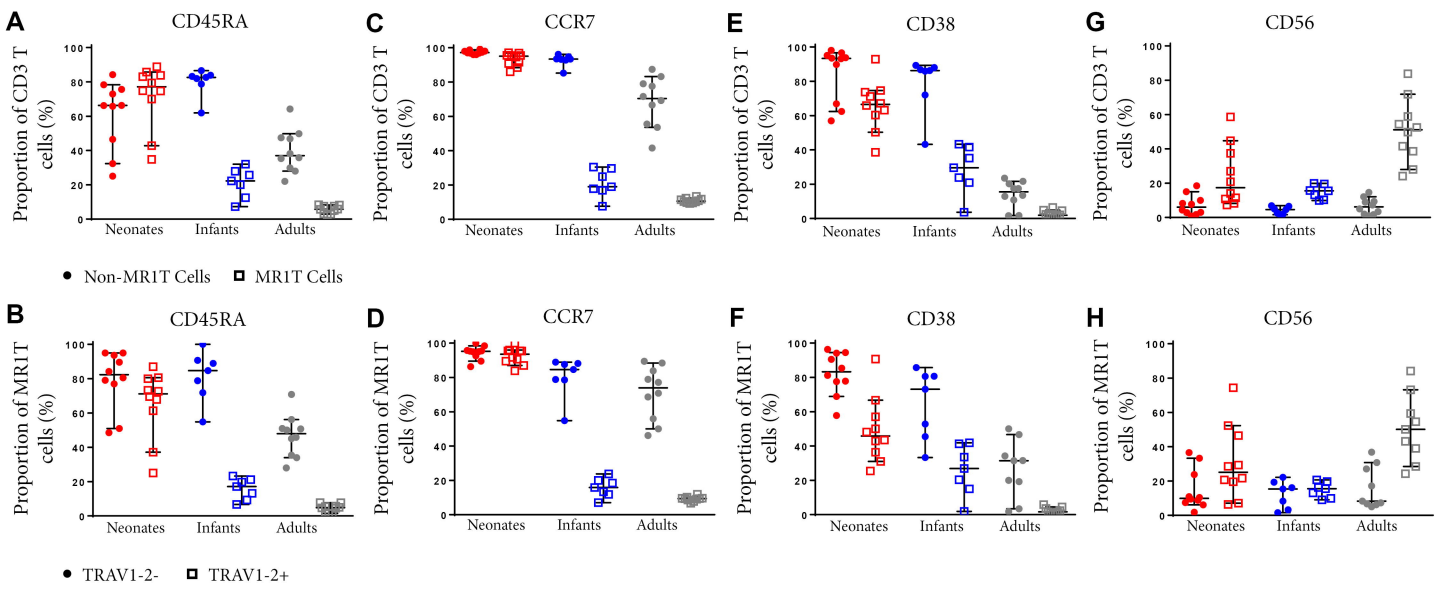
+ - - - +/-
- - - + +
- - - + +
- - - + +


A
Neonate
Infant
Adult
M. smegmatis
No Stimulation
CD3
TNF
13.4%
29.5%
17.3%
0%
0%
0.77%
B
M. Smegmatis
TNF+ MR1T cells (%)
p=0.0002
p<0.0001
p=0.0007
Neonates
Infants
Adults
C
PMA/Ionomycin
Frequency of Functional CD3+ T cells
p=0.0020
p=0.0020
p=0.0098
p=0.0273
p=0.0195
Neonates
Infants
Adults
MR1/5-OP-RU+
TCRγδ+
CD4+
CD8+
D
M. Smegmatis
TNF+ MR1T cells (%)
p=0.0039
p=0.0020
p=0.0020
Neonates
Infants
Adults
TRAV1-2+
TRAV1-2-
E
PMA/Ionomycin
TNF+ MR1T cells (%)
p=0.2188
p=0.0020
p=0.0020
Neonates
Infants
Adults
TRAV1-2+
TRAV1-2-
F
M. Smegmatis
Proportion of TNF+ MR1T cells that express CD26 and CD161 (%)
Neonates
Infants
Adults
G
PMA/Ionomycin
Proportion of TNF+ MR1T cells that express CD26 and CD161 (%)
Neonates
Infants
Adults


A
Neonate
Infant
Adult
S3
54.8%
S1
2.95%
S2
42.2%
S3
93.9%
S1
0.12%
S2
5.93%
S3
97%
S1
0%
S2
2.75%
CD161
CD27
B
MR1/5-Op-RU+ CD3 T cells (%)
Neonates
Infants
Adults
S1
S2
S3


A
CD45RA
Proportion of CD3 T cells (%)
Neonates
Infants
Adults
C
CCR7
E
CD38
G
CD56
Non-MR1T Cells
MR1T Cells
B
CD45RA
Proportion of MR1T cells (%)
D
CCR7
F
CD38
H
CD56
TRAV1-2-
TRAV1-2+