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Mucosal-activated Invariant T cells do not exhibit significant lung recruitment and proliferation profiles in macaques in response to infection with *Mycobacterium tuberculosis* CDC1551

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Abstract

TB is a catastrophic infectious disease, affecting roughly one third of the world's population. Mucosal-associated invariant T (MAIT) cells are innate-like T cells that recognize vitamin B metabolites produced by bacteria, possess effector memory phenotype, and express tissue-homing markers driving migration to sites of infection. Previous research in both *Mtb* and HIV infections has shown that MAIT cells are depleted in the human periphery, possibly migrating to the tissue sites of infection. We investigated this hypothesis using rhesus macaques (RMs) with active TB, latent TB (LTBI), and SIV-coinfection to explore the effects of different disease states on the MAIT cell populations *in vivo*. Early in infection, we observed that MAIT cells increased in the blood and bronchoalveolar lavage fluid (BAL) of all infected RMs, irrespective of clinical outcome. However, the frequency of MAIT cells rapidly normalized such that they had returned to baseline levels prior to endpoint. Furthermore, following infection, the chemokines expressed on MAIT cells reflected a strong shift towards a Th1 phenotype from a shared Th1/Th17 phenotype. In conclusion, MAIT cells with enhanced Th1 functions migrating to the site of *Mtb*-infection. The anti-mycobacterial effector functions of MAIT cells, particularly during the early stages of

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Author contributions

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Competing interests

The authors declare no financial conflicts of interest.

Ethics statement

The Tulane National Primate Research Center (TNPRC) facilities are accredited by the American Association for Accreditation of Laboratory Animal Care and licensed by the US Department of Agriculture. All animals were routinely cared for according to the guidelines prescribed by the NIH Guide to Laboratory Animal Care. Humane endpoints were pre-defined in this protocol and applied as a measure of reduction of discomfort. The TNPRC Institutional Animal Care and Use Committee approved all animal-related procedures and activities. The Tulane Institutional Biosafety Committee approved all procedures involving *Mtb*.

Mtb infection, had been of interest in promoting protective long-term TB immunity. Our research shows, however, that they have relatively short-acting responses in the host.

Keywords

Mycobacterium tuberculosis; Tuberculosis; Mucosal-activated Invariant T cells

1. Introduction

Tuberculosis (TB) caused by the gram-positive *Mycobacterium tuberculosis* (*Mtb*) is a catastrophic infectious diseases of mankind (1). It leads to ~10 million new cases and ~2 million deaths every year (2). The number of people infected with *Mtb* is much higher, as >90% of such individuals remain asymptomatic (latent TB infection, LTBI). AIDS due to HIV infection annually causes ~3 million deaths, a quarter of which involve co-infection with *Mtb*. HIV co-infection potentiates reactivation of LTBI (3). *Mtb* infection can have a spectrum of different outcomes in exposed humans, ranging from a life-long asymptomatic infection termed LTBI, a late stage reactivation of LTBI, usually due to HIV co-infection or other confounding factors like, slowly progressing chronic pulmonary TB, rapidly fulminating pulmonary TB, as well as extra-pulmonary TB (3). In each of these instances, the hallmark of the disease, i.e. the levels of bacterial burden, the extent of the granulomatous pathology as well as the immune responses differ significantly. This has made the identification of definitive immune correlates of protection from TB difficult.

It is well-accepted however, that components of the cellular adaptive immune response, in particular, CD4⁺ T cells, are required for protection from TB (4–6). Destruction of CD4⁺ T cells, including *Mtb*-specific responder cells is currently considered a primary cause of reactivation of LTBI in HIV-1-infected humans as well as in simian immunodeficiency virus (SIV)-infected macaques. However, data has accumulated over time indicating that other aspects of immunity may also play important and sometimes defining roles in protecting against TB (7, 8). Thus, it has been shown in model systems, including the macaque model, which mimics several aspects of the human TB syndrome (9, 10), that CD8⁺ T cells play a critical role in protection (11). A significant advantage of using macaques as models of TB is the ability to co-infect with simian immunodeficiency virus (SIV), which results in a HIV-like infection in these macaques, and which routinely results in a significant reactivation of chronic or asymptomatic infection (12, 13). Our co-infection model has demonstrated that both *Mtb* (14) and SIV (Bucsan et al in review) must be virulent for pathogenic co-infection to progress. In the co-infection model, animals which failed to progress to disease despite being infected with *Mtb* and while carrying productive peripheral viremia, exhibited signatures of strong CD8⁺ T-cell functions and higher accumulation of B cells in granuloma-associated lymphoid follicles (13). These animals were also characterized by reduced peripheral monocyte and tissue macrophage turnover (15).

The role of innate-like lymphoid cells in immunity to pathogens is now being increasingly recognized (16). While innate-like in many of their properties, these mirror the role of T cells. Thus, while natural killer (NK) cells are the innate counterparts of CD8⁺ T cells,

ILC1s, ILC2s, and ILC3s represent the innate counterparts of CD4⁺ T helper 1 (TH1), TH2, and TH17 cells, respectively (16). Another cell type to garner recent interest is the mucosal-associated invariant T (MAIT) cell, which is a type of innate-like T cell family that recognizes vitamin B metabolites produced by bacteria, possesses effector memory phenotype, and expresses tissue-homing markers driving their migration to sites of infection (17). MAIT cells are the most abundant T cell subset reacting against bacteria in humans (18). In patients with TB disease, MAIT frequencies decrease in the peripheral blood following *Mtb* infection (19–22). These data have led to the assumption that following infection MAIT cells traffic from circulation to the infected lung mucosa to potentially control infection (22). MAIT cells were also reported to proliferate in BCG-vaccinated as well as *Mtb*-infected RMs (23). We have previously employed the Indian RM model of inhalation TB to study various aspects of the host immune function (13, 24–35). Here, we used the same model to investigate if MAIT cells are activated upon infection of RM lungs with the low-virulence *Mtb* strain CDC1551.

2. Materials and methods

2.1 Animals

Tulane National Primate Research Center Institutional Animal Care and Use Committee (IACUC) and Tulane University Institutional Biosafety Committee (IBC) approved all activities on this study. Naïve mycobacteria-free Indian rhesus macaques (RMs) (9, 12, 29, 34–37) were exposed to physiologically relevant doses of (5–10 CFU) *Mtb* CDC1551 via aerosol. Clinical procedures have been previously described (9, 12, 30, 34–37). TST was performed before (week –2) and after (week 3, 7) infection. Blood was drawn weekly for CBC and chemistry while BAL and CXR were obtained at week 3 and every four weeks thereafter (34, 38). Radiological principles have been described in detail earlier (9, 12, 26, 30, 32, 34–37, 39). At 9 weeks post-infection, based on their clinical outcomes, a subset of these RMs (n=7) were co-infected with 300 TCID₅₀ SIVmac239 intravenously, as described (12, 13). Animals were euthanized due to signs of TB or as time-matched controls. At necropsy, lung, spleen, liver, bronchial lymph nodes, and kidney were collected and processed, as previously described, using two sections of pulmonary tissue that represented every lung lobe in at least one sample. CFU were determined per gram of tissue. Lung pathology at necropsy was determined as described previously (9, 12, 30, 34–37).

2.2 Experimental procedures

Clinical and bacterial measures of TB and LTBI; and the extent of lung pathology were determined over time as described earlier (9, 12, 26, 30, 32, 34–37, 39). SIV loads were determined as described earlier (12, 13). Flow-cytometry was performed using 2–3×10⁶ fresh PBMCs, BAL, lung, spleen, bronchial lymph node, colon, and liver cells (13, 24–26, 30, 31, 34, 35, 39). Stimulations were performed overnight on 1×10⁶ PBMCs, BAL, lung, spleen, bronchial lymph node, colon, and liver cells using 50 ng/mL of PMA (Sigma-Aldrich) and 1 µg/mL of ionomycin (Sigma-Aldrich) with brefeldin A (Biolegend). Stimulated samples were stained with MR1–5-OP-RU tetramer for 45 minutes, then stained with extracellular antibodies, and finally intracellular antibodies according to manufacturer's instructions.

Statistical tests were performed using JMP v10 (SAS Institute, Cary, NC USA). All other statistical comparisons used ANOVA with Tukey post-hoc tests.

3. Results

3.1 Disparate outcomes following *Mtb* infection and SIV co-infection.

Between the 2015–2017, 13 pathogen-free, mycobacteria-naïve Indian RMs were exposed to 5–10 CFUs of aerosolized *Mtb* CDC1551 (12–14, 24, 26, 29–31, 34–36, 39–41). Three animals developed signs of disease within four weeks of infection, resulting in their classification as animals with active TB (ATB). 10 animals did not exhibit signs of TB disease despite skin-test positivity, leading to their classification as animals with latent TB infection (LTBI). Of these, six were co-infected nine weeks later with SIVmac239 (intravenous 300TCID₅₀), as described earlier (12–14). Of these, three animals each were classified as reactivators and nonreactivators based on reactivation of LTBI due to coinfection (13). Four LTBI animals did not develop clinical signs of TB over the period of observation, a minimum of 20 weeks post-infection. Serum CRP levels were used to predict clinical development. Our prior data clearly shows that the levels of serum C-reactive protein (CRP) correlate significantly with pulmonary TB in RMs (26). The use of CRP to detect active TB has been extensively proposed in humans as well (42). As is our experience with previous cohorts, the expression of serum CRP correlated with the extent of TB and lung *Mtb* burdens (Fig 1A). Terminal serum CRP levels in animals with ATB were significantly higher than those with LTBI, whether with ($P<0.05$) or without ($P<0.01$) lentiviral co-infection (Fig 1A). This was also true for animals that exhibited reactivation, where the absolute terminal serum CRP values were highest, although not significantly different from animals with ATB (Fig 1A). As we have described earlier, the magnitude of serum CRP levels also correlated strongly with the extent of lung pathology (Fig 1B). Again, the levels of lung pathology were statistically indistinguishable between reactivators and animals with ATB, but the values in either instant were significantly higher than animals with LTBI or nonreactivators (Fig 1B). We also measured the time to humane euthanasia in these groups of animals (Fig 1C). As anticipated, animals in the ATB and the reactivation groups needed to be humanely euthanized due to TB, while those with LTBI and lack of reactivation were not euthanized until the 22nd week of the protocol. We also measured the body weight of the animals in the different groups and expressed it as percentage of their body weight at the time of *Mtb* infection (Fig 1D). Again, only animals with ATB and those with reactivation (after SIV co-infection) showed loss of weight and associated wasting symptoms. Animals with LTBI (with or without SIV co-infection) gained weight during the 22-week time-period (Fig 1D). A mirror image of this data was observed when we measured body temperature in these animals, with ATB and reactivation groups exhibiting pronounced pyrexia (Fig 1E).

3.2 Bacterial burdens following *Mtb* infection and SIV co-infection.

Having established that the clinical correlates of *Mtb* infection and *Mtb*/SIV co-infection were comparable to the previous cohorts that we have published, we then measured the extent of *Mtb* burden. As anticipated, viable *Mtb* bacilli could only be detected in the BAL of animals with ATB and reactivation following co-infection (Fig 2A). Of the eight animals with LTBI without or with SIV co-infection, only one had detectable CFUs in BAL. In

lungs, reactivators had higher CFUs relative to animals with ATB (Fig 2B). While this is different from our previously published results, these differences are likely a function of smaller group sizes. Like our previously published results however, animals with higher *Mtb* burdens in the bronchial lymph nodes (Fig 2C) and other extrathoracic organs belonged to the ATB and the reactivation group (Figs 2D–F).

3.3 Investigation into the dynamics of MAIT cells in *Mtb*-infected rhesus macaques.

We next measured the presence of MAIT cells in the different compartments of *Mtb*-infected Indian RMs. The percentage of MAIT cells (defined here by CD161 and TCR V α 7.2 double positivity as a fraction of CD3⁺ cells), increased somewhat following infection of Indian RMs with *Mtb* CDC1551, a low-virulence strain, both in PBMCs (Fig 3A) and BAL (Fig 3B), over the course of time. The magnitude of the increase in MAIT cell levels both in blood and BAL was greater in the case of animals with ATB, relative to those with LTBI. The differences were however not statistically significantly different relative to baseline, or within the two outcome groups. Furthermore, the levels of MAIT cells returned to baseline 15 weeks post-*Mtb* infection in both compartments. Similar results were obtained when the percentage of MAIT cells was analyzed as a fraction of CD3⁺CD8⁺ cells (Fig 3C, D). Individual dot plots for data derived from samples from every animal as a fraction of CD3⁺ and CD3⁺CD8⁺ cells respectively are also shown (Fig 3E, F). We also analyzed the presence of MAIT cells in various other compartments of *Mtb*-infected Indian RMs at the endpoint. The maximal presence of these cells was detected in colon where the levels of MAIT cells were significantly greater in animals with ATB relative to LTBI, as well as ATB relative to uninfected, naïve controls. The second highest frequency of MAIT cells was detected in liver. It is relevant to note here that these are not the major target organs affected during tuberculosis, but are the tissues normally enriched with MAIT cells (43).

3.4 Longitudinal analysis of the dynamics of MAIT cells in *Mtb*-infected versus *Mtb*/SIV co-infected RMs.

We first quantified MAIT (TCRV α 7.2⁺CD161⁺) cells as a proportion of CD3⁺ T cells in the BAL (Fig 4A) and in PBMCs (Fig 4B) of *Mtb*/SIV co-infected animals which showed reactivation, and the ones that didn't. These results were compared to those from *Mtb*-infected animals with no SIV coinfection (shown in Fig 3). SIV co-infection did not result in any significant increase in MAIT cell levels in the BAL, while some increase was observed in the blood of animals that reactivated, following SIV co-infection. The kinetics of MAIT cells in either compartment were similar to those of *Mtb*(only)-infected animals, with MAIT cell numbers in blood and BAL reducing to background levels by week 15. Next, we quantified absolute numbers of TCRV α 7.2⁺CD161⁺ cells in the BAL (Fig 4C) and in PBMCs (Fig 4D) of the four groups of RMs. This allowed us to measure the total presence of MAIT cells in these samples rather than as a percentage of either the total T cell compartment or the CD8⁺ T cell compartment. These results were very telling. In all RMs, very few (virtually none) MAIT cells were present at baseline, but their numbers increased in all samples in the lung compartment following *Mtb* infection (Fig 4C), declining to negligible levels by week 15. In the PBMCs however, we found that the absolute numbers of MAIT cells were highly variable at the baseline and no strong conclusions could be drawn

about their kinetics, except that the absolute number of these cells was the highest in animals with ATB and that the numbers declined precipitously in all groups by week 15.

We next studied the expression of various functional markers on these $\text{TCRV}\alpha 7.2^+ \text{CD161}^+$ cells. The expression of CCR5 is shown in the BAL (Fig 4E) and in PBMCs (Fig 4F). We found that virtually all (>70%) MAIT cells in the BAL of all *Mtb*-infected animals, irrespective of co-infection or disease status, expressed CCR5 (including baseline) (Fig 4E). On the other hand, CCR5 was expressed in about 50% of MAIT cells in the PBMCs derived from all animals (Fig 4F). When MAIT cells were similarly quantified for CCR6, we found far fewer positives. Thus, at baseline in BAL, about 60% of all $\text{TCRV}\alpha 7.2^+ \text{CD161}^+$ cells were also positive for CCR6, but these numbers declined post-*Mtb* infection in most instances (Fig 4G). The extent of CCR6 expression however appeared to increase in *Mtb*/SIV co-infected animals that exhibited reactivation disease due to co-infection, again emphasizing that MAIT cells are recruited to the lung in response to increased *Mtb* replication. At baseline in PBMCs, about 40% of all $\text{TCRV}\alpha 7.2^+ \text{CD161}^+$ cells were also positive for CCR6, and these numbers initially declined, but eventually increased to an average of 60% in all groups (Fig 4H). Finally, we quantified the expression of granzyme B⁺ on $\text{TCRV}\alpha 7.2^+ \text{CD161}^+$ cells in the BAL (Fig 4I) and in PBMCs (Fig 4J). On an average about 30% of these cells were positive for granzyme B at baseline in BAL. In groups with disease and higher bacterial burdens (ATB and reactivation), we observed significant increase in granzyme B positivity in MAIT cells post-infection (Fig 4I). Interestingly, in animals that did not reactivate, granzyme B expression was not enhanced. Once again, a high degree of heterogeneity was observed in PBMCs (Fig 4J), making it impossible to draw meaningful conclusions.

3.5 Investigation into the dynamics of MAIT cells in *Mtb*-infected RMs using the tetramer detection approach.

We validated our results taking advantage of the recent availability of the RM MR-1 tetramer by the NIH tetramer core. Towards this end, we tested the overlap between the classification of the $\text{TCRV}\alpha 7.2^+ \text{CD161}^+$ cells and MR-1 tetramer positivity. In both BAL (Fig 5A) and PBMCs (Fig 5B), there was significant overlap in MAIT cells detected by the $\text{TCRV}\alpha 7.2^+ \text{CD161}^+$ dual staining approach and the MR-1 tetramer. We also analyzed the polyfunctionality of the $\text{MR1-5-OP-RU}^+ \text{CD161}^+$ MAIT cells, comparing different outcomes following *Mtb* infection (ATB versus LTBI) (Figs 5C, D). We specifically focused on the critical 3-week post *Mtb*-infection time point and tested the polyfunctionality of $\text{MR1-5-OP-RU}^+ \text{CD161}^+$ MAIT expressing IFN- γ , TNF- α , IL-17 and granzyme B in BAL (Fig 5C) or PBMCs (Fig 5D). We observed that quadruple-positive ($\text{GrB}^+ \text{IFN-}\gamma^+ \text{IL-17}^+ \text{TNF-}\alpha^+$) polyfunctional tetramer-positive ($\text{MR1-5-OP-RU}^+ \text{CD161}^+$) MAIT were recruited to significantly higher levels in the BAL of animals that eventually controlled *Mtb* infection in a latent form, relative to animals that developed disease (Fig 5C). Single positive GrB^+ and double positive $\text{GrB}^+ \text{TNF-}\alpha^+$ cells were recruited to significantly higher levels in the BAL of animals that developed ATB. This effect was also observed in PBMCs (Fig 5D).

4. Discussion

The discovery that HIV-infected individuals are highly susceptible to TB led to the conclusion that CD4⁺ T cells of the adaptive arm of immunity are critical for the control of TB. Of late however, it has become clear that additional immune events regulate protection from TB. It has been postulated that innate-like lymphoid cells, particularly MAIT cells, may be important for optimal immune responses to TB. Using a well-characterized RM model of *Mtb*-infection via the aerosol route, we tested this hypothesis. We also studied the role of SIV (as a surrogate for HIV) co-infection with *Mtb* in the recruitment of MAIT cells to RM tissues and to explore the effects of different disease states on the MAIT cell populations *in vivo*. Towards this end we employed both a flow cytometric approach to detect TCRV α 7.2⁺CD161⁺ MAIT cells and a tetramer-based approach to detect MR1⁺ MAIT cells.

We find that either approach detects an overwhelmingly overlapping class of cells in RMs. Our results show that MAIT cells reside in the liver and the gut compartment in naïve primates, but following *Mtb*-infection, the number of MAIT cells increase in the blood and BAL. This happens in all infected RMs, irrespective of clinical outcome. However, the frequency of MAIT cells rapidly normalized such that they had returned to baseline levels prior to endpoint.

Furthermore, following infection, the chemokines expressed on MAIT cells reflected a strong shift towards a Th1/Tc1 (helper and cytotoxic) phenotype from a shared Th1/Th17 phenotype. In other words, these MAIT cells appeared to upregulate their effector function, shedding a more quiescent effector phenotype. Thus, infected animals with greater disease (ATB) had more granzyme B production, as well as higher CCR5 but progressively lower CCR6 expression. MAIT cells are specifically empowered by induction of granzyme B expression to contain bacterial infections (17). CCR5 is a classical marker of Th1 type function on lymphocytes (44). CCR6 is strongly believed to be mediator of immunity in the lung and gut (45) that affects migration of Th17 cells and regulation of effector T cells (46). Furthermore, in animals with SIV co-infection, the levels of granzyme B expression increased, indicating that in the absence of CD4⁺ T cells, MAIT cells may attempt to control co-infection via this cytotoxic pathway. In conclusion, MAIT cells with enhanced Th1/Tc1 functions migrated to the site of *Mtb*-infection. The anti-mycobacterial effector functions of MAIT cells, particularly during the early stages of *Mtb* infection, had been of interest in promoting protective long-term TB immunity. Our research shows, however, that they have relatively mild and short-acting responses in the host, at least in the model system we employed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Clinical information on *Mtb*/SIV co-infection.

(A) Serum C-reactive protein (CRP) levels at necropsy (in $\mu\text{g/mL}$); (B) Quantitative analysis of overall TB lung pathology as a percentage of lung involvement; (C) Survival curves reported as weeks of *Mtb*/SIV co-infection; (D) Percentage change in weight (in kg); (E) Percentage change in temperature (in $^{\circ}\text{C}$). Each group is classified as latent (blue, $n = 6$), active (orange, $n = 3$), non-reactivator (teal, $n = 3$), and reactivator (pink, $n = 3$). (A–B) * $P < 0.0332$, ** $P < 0.0021$, *** $P < 0.0002$, **** $P < 0.0001$, one-way ANOVA with Tukey's multiple testing correction.

Fig. 2. Bacterial burdens in *Mtb*/SIV co-infected lungs and extra-pulmonary tissues.

TB colony-forming units (CFUs) in total BAL samples (A) and per gram of plated tissue in the lungs (B), bronchial lymph node (C), kidney (D), spleen (E), and liver (F). Each group is classified as latent (blue, n = 6), active (orange, n = 3), non-reactivator (teal, n = 3), and reactivator (pink, n = 3). (A–E) *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001, one-way ANOVA with Tukey's multiple testing correction.

Fig. 3. TCRVα7.2⁺CD161⁺ quantification in latent and active TB infection.

Quantification of TCRVα7.2⁺CD161⁺ cells as a proportion of CD3⁺ T cells in the BAL (A) and in PBMCs (B). Quantification of TCRVα7.2⁺CD161⁺ cells as a proportion of CD8⁺ T cells in the BAL (C) and in PBMCs (D). Distribution of MAIT cells in the PBMCs and BAL at pre-infection as a proportion of CD3⁺ T cells (E) and CD8⁺ T cells (F). Quantification of TCRVα7.2⁺CD161⁺ cells in different tissues at necropsy as a proportion of CD3⁺ T cells in latent (blue, n = 6), active (orange, n = 3), and naive (teal, n = 3) rhesus macaques. (A–F) *P

< 0.0332 , $**P < 0.0021$, $***P < 0.0002$, $****P < 0.0001$, two-way ANOVA with Tukey's multiple testing correction.

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Fig. 4. TCRV α 7.2⁺CD161⁺ quantification and characterization in *Mtb*/SIV co-infection. Quantification of TCRV α 7.2⁺CD161⁺ cells as a proportion of CD3⁺ T cells in the BAL (A) and in PBMCs (B). Quantification of total TCRV α 7.2⁺CD161⁺ cells in the BAL (C) and in PBMCs (D). Quantification of CCR5⁺ TCRV α 7.2⁺CD161⁺ cells as a proportion of MAIT cells in the BAL (E) and in PBMCs (F). Quantification of CCR6⁺ TCRV α 7.2⁺CD161⁺ cells as a proportion of MAIT cells in the BAL (G) and in PBMCs (H). Quantification of granzyme B⁺ TCRV α 7.2⁺CD161⁺ cells as a proportion of MAIT cells in the BAL (I) and in PBMCs (J). Each group is classified as latent (blue, n = 6), active (orange, n = 3), non-reactivator (teal, n = 3), and reactivator (pink, n = 3). *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001, two-way ANOVA with Tukey's multiple testing correction.

Fig. 5. Validation of TCRV α 7.2⁺CD161⁺ classification and polyfunctionality of MR1-5-OP-RU⁺CD161⁺ MAIT cells.

Comparison of total MAIT cell populations using TCRV α 7.2⁺CD161⁺ cells with MR1-5-OP-RU⁺CD161⁺ in the BAL (A) and in PBMCs (B) using paired samples (n=20).

Polyfunctionality of MR1-5-OPRU⁺CD161⁺ MAIT cells in the BAL (C) and PBMCs (D) at week 3 post-TB infection as a proportion of total MR1-5-OP-RU⁺CD161⁺ T cells in active (blue, n = 5) and latent (orange, n = 5) rhesus macaques. (A–B) Linear regression analysis.

*P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001. (C–D) *P < 0.01, **P < 0.001 multiple t tests using the Holm-Sidak method.