Immunization of Vγ2Vδ2 T cells programs sustained effector memory responses that control tuberculosis in nonhuman primates

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Tuberculosis (TB) remains a leading killer among infectious diseases, and a better TB vaccine is urgently needed. The critical components and mechanisms of vaccine-induced protection against Mycobacterium tuberculosis (Mtbb) remain incompletely defined. Our previous studies demonstrate that Vγ2Vδ2 T cells specific for (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) phosphoantigens are unique in primes as multifunctional effectors of immune protection against TB infection. Here, we selectively immunized Vγ2Vδ2 T cells and assessed the effect on infection in a rhesus TB model. A single respiratory vaccination of macaques with an HMBPP-producing attenuated Listeria monocytogenes (Lm ΔactA prfA*) caused prolonged expansion of HMBPP-specific Vγ2Vδ2 T cells in circulating and pulmonary compartments. This did not occur in animals similarly immunized with an Lm ΔgcpE strain, which did not produce HMBPP. Lm ΔactA prfA* vaccination elicited increases in Th1-like Vγ2Vδ2 T cells in the airway, and induced containment of TB infection after pulmonary challenge. The selective immunization of Vγ2Vδ2 T cells reduced lung pathology and mycobacterial dissemination to extrapulmonary organs. Vaccine effects coincided with the fast-acting memory-like response of Th1-like Vγ2Vδ2 T cells and tissue-resident Vγ2Vδ2 effector T cells that produced both IFN-γ and perforin and inhibited intracellular Mtbb growth. Furthermore, selective immunization of Vγ2Vδ2 T cells enabled CD4+ and CD8+ T cells to mount earlier pulmonary Th1 responses to TB challenge. Our findings show that selective immunization of Vγ2Vδ2 T cells can elicit fast-acting and durable memory-like responses that amplify responses of other T cell subsets, and provide an approach to creating more effective TB vaccines.

Significance

**Despite the urgent need for a better tuberculosis (TB) vaccine, relevant protective mechanisms remain unknown. We previously defined protective phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)–specific Vγ2Vδ2 T cells as a unique subset in primes, and, here, we immunized them selectively for protection against TB. A single respiratory vaccination of macaques with attenuated HMBPP-producing Listeria monocytogenes (Lm ΔactA prfA*), but not an HMBPP-lacking ΔgcpE Listeria strain, expanded Vγ2Vδ2 T cells, elicited Th1-like Vγ2Vδ2 T cell responses, and reduced TB infection/pathology after moderate-dose TB challenge. Such protection correlated with rapid memory-like, Th1-like Vγ2Vδ2 T cell responses, the presence of tissue-resident Vγ2Vδ2 T effector coproducing IFN-γ/perforin and inhibiting intracellular Mycobacterium tuberculosis growth, and enhanced CD4+/CD8+ T cell responses. These findings establish a concept incorporating immunization of human Vγ2Vδ2 T cells for TB vaccine development.**
Protective features of \( \gamma \delta \) T cells raise the question of whether selective immunization of \( \gamma \delta \) T cells can elicit protective responses and induce immunity against Mtb infection. Proving this concept would be valuable for advancing our understanding of the role of these cells in immunity to infections, and would also provide a foundation for the development of new TB vaccines that include approaches to recruit protective \( \gamma \delta \) T cells in conjunction with other T cell subsets. To this end, we have employed an HMBPP-producing \textit{Listeria monocytogenes} (Lm) vaccine vector for immunization of \( \gamma \delta \) T cells. While attenuated forms of Lm have been used as delivery systems to vaccinate humans against a variety of cancers (43), we therefore used this vector for respiratory immunization of \( \gamma \delta \) T cells in the first instance, to circumvent the issue of respiratory vector vaccination of NHPs.

Results

Expansion of HMBPP-Specific \( \gamma \delta \) T Cells by Immunization with HMBPP-Producing Lm \( \Delta \text{actA prfA}\). To target \( \gamma \delta \) T cells for vaccine design, we have employed an attenuated live Lm strain (Lm \( \Delta \text{actA prfA}\)) that shares with Mtb the ability to produce HMBPP via the nonvalonate pathway (44). We showed that respiratory or systemic immunization of macaques with this attenuated Lm \( \Delta \text{actA prfA}\) strain or derivatives of this strain expressing microbial immunogens exhibited excellent safety profiles, elicited robust immune responses, and protected against life-threatening simian HIV-related malaria in macaques (31, 44, 46–48). We therefore used this vector for respiratory immunization of \( \gamma \delta \) T cells. The \( \Delta \text{gcpE} \) deletion mutant of Lm \( \Delta \text{actA prfA}\) served as a vector control, as this mutant no longer produced HMBPP due to the disruption of the gene \( \text{gcpE} \) encoding HMBPP synthase (48).

Intratracheal or respiratory vaccination of rhesus macaques with Lm \( \Delta \text{actA prfA}\), but not the \( \Delta \text{gcpE} \) variant, elicited a prolonged expansion of HMBPP-specific \( \gamma \delta \) T cells in the circulation and airway [bronchoalveolar lavage (BAL) fluid; Fig. 1]. At months 1–3 after vaccination, the \( \gamma \delta \) T cell subset increased and sustained up to almost 30% and 60% of total \( \text{CD3}^+ \) T cells in the blood (Fig. 1A) and airway (Fig. 1B), respectively.

\textbf{Respiratory Lm \( \Delta \text{actA prfA}\) Vaccination Elicited Sustained Increases in Th1-Like \( \gamma \delta \) T Cells in the Airway.} IFN-\( \gamma \) plays a crucial role in anti-TB immunity, and also regulates multiple effectors of \( \gamma \delta \) T cells (30, 32, 40, 42). We used intracellular cytokine staining (ICS) and flow cytometry to measure IFN-\( \gamma \)-producing \( \gamma \delta \) T cells in peripheral blood mononuclear cells (PBMCs) and in BAL fluid cells. To circumvent the issue of limited numbers of BAL fluid cells available for conventional

Fig. 1. Respiratory Lm \( \Delta \text{actA prfA}\) immunization elicited prolonged expansion of \( \gamma \delta \) T cells in the lungs and blood. (A, Left) Representative flow cytometry histograms show percentages of \( \text{CD3}^+ \) T cells in total \( \text{CD3}^+ \) T cells in blood at ∼0.5 mo (Pre) and at months (M) 1, 2, and 3 after respiratory vaccination of macaques with Lm \( \Delta \text{actA prfA}\) (Top) and \( \Delta \text{gcpE} \) deletion mutant (Bottom; \( \Delta \text{gcpE} \)) of Lm \( \Delta \text{actA prfA}\), respectively. Panels were gated on \( \text{CD3}^+ \) lymphocytes. Numbers in the upper right quadrant indicate the percentages of \( \text{CD3}^+ \) T cells in the total \( \text{CD3}^+ \) T cell population. Expanded \( \gamma \delta \) T cells are mostly \( \text{CD8}^+\)coexpressing after Lm vaccination or primary TB infection, and therefore are interpreted as \( \text{CD8}^+\) T cells as described in previous publications (29–31). Ctrl, control. (A, Right) Dot plots with means ± SD representing expansions of \( \gamma \delta \) T cells for individual macaques per group before and 1–3 mo after the respiratory vaccination. (B) Representative flow cytometry histograms and graph of data as in A, except for cells from BAL fluid. Data in the graphs are dot plots with means ± SD of expansions for individual macaques per group. * \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \) when comparing groups using a paired \( t \) test or Mann–Whitney \( U \) test. No \textit{Listeria} could be isolated from the blood and BAL samples collected at indicated times from the vaccinated macaques as previously described (48).
ICS, we directly measured effector cells without prior antigen stimulation in culture using a direct ICS method that has been previously validated (31, 32, 49, 51–53). At 1 mo after respiratory Lm actA prfA* vaccination, about 10–20% of VγVδ2 T cells in BAL fluid samples were spontaneously producing IFN-γ without the need for HMBPP phosphoantigen stimulation in culture (SI Appendix, Fig. S1A). This high frequency of effector activity was maintained for at least 3 mo after the vaccination of macaques with Lm actA prfA*, but not the ΔgcpE control (SI Appendix, Fig. S1A).

Although direct ICS assay revealed much lower levels of IFN-γγ VγVδ2 T cells in the blood than we observed in the lungs (SI Appendix, Fig. S1B), the conventional ICS method with HMBPP stimulation in vitro allowed detection of ~18–20% IFN-γ γVγVδ2 T cells in the total blood CD3+ T cells at 1 and 3 mo after the vaccination with Lm actA prfA*, but very low detection with the ΔgcpE control (SI Appendix, Fig. S1C).

Improved Control of Mtb Infection Following Vaccine-Induced Expansion VγVδ2 T Cells. We next sought to examine if the vaccine-elicited prolonged expansion of the VγVδ2 T effector subset led to detectable protection against Mtb challenge. To this end, macaques from groups immunized with Lm actA prfA*, the ΔgcpE vector control, or saline were challenged with 80 cfu of Mtb Erdman through bronchoscope-guided spread into the right caudal lung lobe at 12 wk after vaccination. Eighty colony-forming units of Mtb was considered a moderate–high dose for Chinese rhesus macaques (54). We assessed weight loss for vaccine effect, as it is a consistent clinical marker during primary active Mtb infection of macaques (42, 55). The γδ T cell-immunized group did not show an apparent weight loss over time (Fig. 2A). In contrast, vector and saline control groups exhibited significant losses of body weight after Mtb challenge (Fig. 2A).

Consistently, the γδ T cell-immunized macaques showed significantly lower Mtb colony-forming unit counts in the right caudal lung lobe (infection site), right middle lung lobe, and left lung lobe than those in both the vector and saline control groups at ~2.5 mo after challenge (Fig. 2B, Upper, P < 0.05 and P < 0.01, respectively). Moreover, the γδ T cell-immunized animals also had limited extrapulmonary Mtb dissemination (Fig. 2B, Lower). Macaques in the γδ T cell-immunized group showed significant lower colony-forming unit counts in the spleen than those in the vector and saline control groups, respectively (Fig. 2B, Lower). Similarly, macaques in the γδ T cell-immunized group showed overall lower colony-forming unit counts in the liver or kidney tissues than animals in the vector and saline control groups (Fig. 2B, Lower). These results demonstrated that respiratory Lm actA prfA* immunization of VγVδ2 T cells conferred the ability to contain pulmonary Mtb infection and extrapulmonary dissemination after a pulmonary Mtb challenge.

Reduced Pathology in the Lung and Other Organs with Lm actA prfA* Immunization of VγVδ2 T Cells. We then evaluated TB pathology at ~2.5 mo after challenge, as published studies show that TB pathology in the lungs can be well established at ~12 mo after Mtb infection of NHPs (30, 42). Overall, vector and saline control groups exhibited similar severe TB pathology in lung, especially in the infection site in the right caudal lung lobe (Fig. 3A). Most of control animals (four or five in the vector or saline group) had TB pneumonia or miliary caseating lesions and extensive coalescing granulomas in the right caudal lobe and, to a lesser extent, in the right middle lobe (Fig. 3A). In addition, TB granulomas were often found in the opposite lung, mostly in the left caudal lobe (Fig. 3A; also reflected by the entire pathology scores in Fig. 3B). Notably, most control macaques exhibited disseminated TB granulomas in the spleen (as reflected by the entire scores in Fig. 3B and also shown in SI Appendix, Fig. S2A). Such TB dissemination was also seen in other extrapulmonary tissues.
macroscopic TB pathology lesions were consistent with the histopathological changes in lung sections derived from the right caudal lobe, middle lobes, and left caudal lobe (SI Appendix, Fig. S2B). Compared with the vector and saline control group macaques, the γδ T cell-immunized animals appeared to exhibit less necrotic and more lymphocytic granulomas, with fewer inflammatory macrophages, giant cells, or neutrophils infiltrating the granulomatous lesions (SI Appendix, Fig. S2B).

**Rapid Recall of Th1-Like Vγ2Vδ2 T Cell Responses in the Airway After Mtb Challenge of Lm ΔactA prfA*^-Vaccinated Macaques.** To establish immune correlates of protection against Mtb infection in Lm ΔactA prfA*^-vaccinated macaques, we investigated whether IFN-γ* Vγ2Vδ2 T cells coincided with protection against Mtb challenge. This was done using the direct ICS assay (as discussed above), which enabled us to use limited BAL fluid cells to assess how fast Vγ2Vδ2 T cell effector responses developed after pulmonary Mtb challenge. Surprisingly, as early as 10 d after Mtb challenge, IFN-γ* Vγ2Vδ2 T effector cells rapidly increased to the level of mean ~40% of CD3* T cells within the lungs of Lm ΔactA prfA*^-vaccinated macaques (Fig. 4A). Pulmonary IFN-γ* Vγ2Vδ2 T cells in this group were maintained at ~30% of total airway T cells on day 28 and, subsequently, at ~20–30% on days 45 and 56, respectively (Fig. 4A). The sustained IFN-γ* Vγ2Vδ2 T cell response was consistent with the high frequency of Vγ2Vδ2 T cells in the airway (Fig. 4B). Blood IFN-γ* Vγ2Vδ2 T effector cells did not increase like those in the airway following Mtb challenge (SI Appendix, Fig. S3), which may have reflected the pulmonary migration of these circulating γδ T cells.

**Inhibition of Intracellular Growth of Mtb by Vaccine-Induced Tissue-Resident Vγ2Vδ2 T Effector Cells.** Our previous mechanistic studies showed that Vγ2Vδ2 T cells inhibited intracellular Mtb growth in an IFN-γ* and perforin-dependent fashion (30, 42). To determine whether Vγ2Vδ2 T cells coproducing IFN-γ and perforin, and capable of inhibiting intracellular Mtb, were detectable in the airway, lung, or lymphoid tissues after Mtb infection of vaccinated macaques, we used in situ confocal microscopic immune staining and ICS assays. With the in situ approach, appreciable numbers of IFN-γ* and perforin Vγ2 T cells were detected in lung tissues from Lm ΔactA prfA*^-vaccinated macaques but not control animals (Fig. 5A and SI Appendix, Fig. S4). Consistently, the direct ICS assay revealed that the Lm ΔactA prfA*^-vaccinated rhesus macaque group showed approximately fivefold greater percentages of Vγ2Vδ2 T cells coproducing both IFN-γ and perforin in the airway compared with the vector control (Fig. 5B).

We then examined if greater numbers of IFN-γ* and perforin-coexpressing Vγ2 T cells in Lm ΔactA prfA*^-vaccinated animals were also associated with a stronger ability to inhibit Mtb growth in autologous macrophages (M4). Due to the limited availability of lymphocytes isolated from lungs, we evaluated IFN-γ* and perforin coproduction as well as Mtb infection by resident Vγ2Vδ2 T cells in the spleen, which harbors large numbers of γδ T cells in rhesus macaques (57). Similar to the lungs, the numbers of IFN-γ* and perforin-coexpressing Vγ2 T cells were higher in spleens of Lm ΔactA prfA*^-vaccinated macaques than in the control group, regardless of HMBPP stimulation (Fig. 5C, Left and Center). When Vδ T cells were purified from spleens of the test or control group animals, we found that splenic Vδ T cells from the Lm ΔactA prfA*^-vaccinated group inhibited intracellular Mtb growth more potently in M4 than did those from vector control animals (Fig. 5C, Right).

**Rapid Recruitment of Conventional CD4*CD8* T Cells by Immunization of Vγ2Vδ2 T Cells.** Given the multifunctional potential of Vγ2Vδ2 T cells (58), we examined whether Lm ΔactA prfA*^-induced Vγ2Vδ2 T cells could facilitate recruitment of αβ CD4* and CD8* T cells in organs as well as in the liver and kidney of most control macaques. In contrast, most macaques in the γδ T cell-immunized group did not show TB pneumonia or miliary TB caseating lesions or extensive coalescing granulomas, but generally exhibited less-coalescing or noncoalescing granulomas. Green arrows indicate caseating pneumonia or extensive coalescing granulomas. Green arrows demonstrate areas with fewer coalescing or noncoalescing granulomas. (Vertical and horizontal scale bars: 1 cm.) Overall, three representatives display relatively low, moderate, and high intensities of lesions as seen in each group. (B) Graph dot plots represent entire pathology scores for all individual macaques in each group. Pathology scores that we and other primate groups employ and publish actually include all of the subscores derived from each of the lung lobes and extrapulmonary organs. Pathology scoring of lungs and other organs was performed by a blinded pathologist. Data ranges for each group are shown as means ± SD. *P < 0.05, **P < 0.01 (Mann-Whitney U test and ANOVA). Microscopic pathology data are shown in SI Appendix, Fig. S2B.

**Fig. 3.** Effect of respiratory Lm ΔactA prfA*^ immunization on gross and microscopic pathology of the lungs. (A) Gross pathology of lungs from representatives of the test and control groups removed at necropsy ~2.5 mo after Mtb challenge. The right caudal lung lobe, the Mtb infection site, is displayed in the bottom right portion of each photograph. Black arrows indicate caseating pneumonia or extensive coalescing granulomas. Green arrows demonstrate areas with fewer coalescing or noncoalescing granulomas. (Vertical and horizontal scale bars: 1 cm.) Overall, three representatives display relatively low, moderate, and high intensities of lesions as seen in each group. (B) Graph dot plots represent entire pathology scores for all individual macaques in each group. Pathology scores that we and other primate groups employ and publish actually include all of the subscores derived from each of the lung lobes and extrapulmonary organs. Pathology scoring of lungs and other organs was performed by a blinded pathologist. Data ranges for each group are shown as means ± SD. *P < 0.05, **P < 0.01 (Mann-Whitney U test and ANOVA). Microscopic pathology data are shown in SI Appendix, Fig. S2B.
the lungs. At 10 d after Mtb challenge, CD4 Th1 cells in the airway increased to ~10% of total CD4+ cells and were maintained at 3–7% at later time points in Lm ΔactA prfA*-vaccinated animals (Fig. 6A and SI Appendix, Fig. S5). In contrast, vector and saline control rhesus macaque groups had <1% of CD4+ Th1 cells in the airway at most time points after the challenge (Fig. 6A and SI Appendix, Fig. S5). Concurrently, percentages of CD8+ Th1-like cells in the lungs were also significantly greater in the Lm ΔactA prfA*-vaccinated group compared with control groups (Fig. 6B). Of note, γδ T cell-associated increases in CD4+ and CD8+ Th1 cells after Mtb challenge were seen only in the airway, as there were no differences in frequencies of CD4+ or CD8+ Th1 cells in the blood between groups after Mtb challenge with or without in vitro restimulation with purified protein derivative (PPD).

**Discussion**

The current study reports that a single respiratory vaccination targeting the TB-reactive Vγ2Vδ2 T cell subset without concurrent immunization of Mtb-specific conventional γδ T cells can generate prolonged expansion of HMBPP-specific Vγ2Vδ2 T cells. This is associated with expression of their fast-acting capability to mount a rapid recall Th1-like effector response to Mtb challenge, and thereafter reduce Mtb infection. In previous studies, we employed two innovative “gain-of-function” manipulations, namely, HMBPP/IL-2 in vivo expansion and adoptive transfer of Vγ2Vδ2 T cells, and showed that Vγ2Vδ2 T cells can attenuate high-dose (500 cfu) Mtb infection in cynomolgus macaques (30, 33, 42). Here, in a proof-of-concept vaccine study, we showed that a single respiratory immunization of Vγ2Vδ2 T cells reduced Mtb infection and pathology after challenge with a moderate–high Mtb dose (80 cfu) in rhesus macaques. Vaccine effects also coincide with tissue-resident Vγ2Vδ2 effector T cells that can coproduce IFN-γ and perforin and inhibit intracellular Mtb growth. The ability of vaccine-elicited Vγ2Vδ2 T cells to coproduce IFN-γ and perforin is consistent with earlier reports that Vγ2Vδ2 T cells have the pleiotropic capability to produce multiple cytokines (30, 42, 59). The correlation between anti-TB immunity and coproduction by γδ T cells of IFN-γ and perforin was consistent with the earlier observation that both IFN-γ and perforin are involved in the ability of Vγ2Vδ2 T effector cells to inhibit intracellular Mtb growth (30, 42). It has also been reported that Vγ2Vδ2 T cells producing other cytolytic effector molecules, including granulysin or granzyme A, can inhibit intracellular Mtb growth (61, 62).

Rapid recall expansion of Vγ2Vδ2 T cells after Mtb challenge of Lm ΔactA prfA*-vaccinated macaques coincided with accelerated pulmonary CD4+ and CD8+ Th1-like effector responses. Although the mechanism for this remains to be established, we speculate that Lm ΔactA prfA*-elicited Vγ2Vδ2 T cells and the cytokines they produced during immunization might have primed or activated these antigen-specific CD4+ and CD8+ T cell subpopulations. In addition, the remarkable recall expansion of Vγ2Vδ2 T cells after Mtb infection likely provided further “helper” function enabling these activated CD4+ and CD8+ precursors to differentiate into IFN-γ Th1-like effectors. This notion explains why there was a lack of apparent CD4+ or CD8+ Th1 responses before Mtb challenge of Lm ΔactA prfA*-vaccinated macaques (Fig. 6 and SI Appendix, Fig. S5). Our findings suggest that rapid pulmonary Th1 responses of CD4+ and CD8+ T cells after respiratory immunization of Vγ2Vδ2 T cells may contribute to the vaccine-induced reduction of Mtb infection after challenge.

Establishing the concept of protective recall responses to Mtb by selective Vγ2Vδ2 T cell vaccines may help to open a new avenue for vaccine design. It is important to note that the HMBPP-specific Vγ2Vδ2 T cell subset exists only in primates, in which it constitutes 65–90% of total circulating γδ T cells in human adults. It is also noteworthy that in the 30 y that have elapsed since discovery of γδ T cells, the potential protective nature and vaccine utility of the human Vγ2Vδ2 T cell subset have not been defined. Further studies extending our findings in...
NHPs will provide an opportunity to close this long-standing knowledge gap. We previously demonstrated protective mechanisms by which CD4+ and CD8+ T cell populations protect against TB infection in primate models (56, 62, 63). The data presented in the current study support the view that TB vaccine design should include approaches to stimulate and expand the dominant Vγ2Vδ2 T cell subset, and support the feasibility and utility of inhaled Lm ΔactA prfA+ immunization as an approach to capture the potential of these cells for improving TB vaccines.

Materials and Methods

Macaque Animals and Institutional Animal Care and Use Committee Approval. Female and male rhesus macaques aged 4–8 y were used in the current study. All macaques had negative routine PPD TB test results. The use of macaques and all experimental procedures were approved by Institutional Animal Care and Use Committee and Biosafety Committees at University of Illinois at Chicago.

Vaccine Vector and Mtb Strains. Attenuated Lm strain Lm ΔactA prfA+ was originally obtained from Nancy Freitag, University of Illinois at Chicago, Chicago, as previously described (26). This strain carries the gcpE gene encoding the enzyme producing HMBPP. We developed and reported the ΔgcpE deletion mutant of Lm ΔactA prfA+, which no longer produces HMBPP (31, 44, 48). The Mtb Erdman strain was used for bronchoscopy-guided challenge or infection of macaques. The H37Rv strain was used for in vitro intracellular inhibition of Mtb growth in macrophages.

Respiratory Vaccination with Lm Strains. A total of 10^6 cfu of Lm ΔactA prfA+ or the ΔgcpE mutant was administered through intratracheal inoculation to Chinese-origin rhesus macaques (six per group), as previously described (48). Macaques were sedated with ketamine (10 mg/kg) and xylazine (1–2 mg/kg) by i.m. injection. An endotracheal tube was inserted through the larynx into the trachea and placed at the carina, and a 1-mL solution containing the inoculum was administered through the endotracheal tube. A 5-mL air bolus was administered through the tube following the inoculum to ensure the entire solution was given.
Fig. 6. Rapid pulmonary Th1 responses to Mtb challenge in Lm ΔactA prfA−/−-immunized macaques. Graphs show frequencies of IFN-γ+ CD4+ Th1-like (A) and IFN-γ+ CD8+ Th1-like (B) effector cells in BAL fluid (BALF) samples from individual macaques in each group in the period from vaccination through the indicated end points after Mtb challenge. Shown in graphs are dot plots representing values with means ± SD for individual macaques; data are derived from direct ICS assay without antigen stimulation in the culture. *p < 0.05; **p < 0.01 (Mann–Whitney U test and ANOVA). Ctrl, control; D, day; M, month.

BAL and Isolation of Lymphocytes and PBMCs. Following sedation of macaques with ketamine and xylazine, BAL and fluid collection were carried out using a pediatric bronchoscope as previously described (42, 48). The bronchoscope was inserted into the bronchial branches distributing to the infected right caudal and other lung lobes of the animals to allow for harvesting of cells, including lymphocytes, in the airway. Isolation of lymphocytes from BAL fluid or the spleen and PBMCs from EDTA blood was done as previously described (32).

Phenotyping of PBMCs and BAL Lymphocytes. Cell surface markers on PBMCs and BAL fluid cells were analyzed by flow cytometry using fluorochrome-conjugated antibodies as previously described (51). Cells were incubated and fixed with 2% formalin and analyzed on an LSR Fortessa flow cytometer (BD Biosciences).

ICS. Analysis of cytokine production following antigen restimulation ex vivo was done using previously described methods (51). We also used direct ICS to assess limited BAL cells or PBMCs for intracellular cytokines without prior in vitro Ag stimulation. Direct ICS was previously validated and described (32, 49, 51, 53, 52). Details are provided in SI Appendix.

Intracellular Mtb Growth Inhibition Assay. The extent of inhibition of Mtb growth in autologous monocyte-derived macrophages by V62 T cells was assessed using a modification of the previously described method (30, 42) (SI Appendix). Inhibition data were expressed as a growth index (colony-forming unit counts of monocytes plus effector cells/colony-forming unit counts of monocytes alone) as described (64).

Mtb Infection of Rhesus Macaques. Macaques were sedated with ketamine (10 mg/kg) and xylazine (1–2 mg/kg) by i.m. injection. A pediatric bronchoscope was inserted into the right caudal lung lobe of the animals, and 80 cfu of Mtb Erdman strain was injected in 3 mL of saline followed by a 3-mL bolus of air to ensure full dose administration. The colony-forming unit dose for infection was confirmed by careful postinoculation titration on a Middeldrop 7H11 plate (Becton Dickinson) as previously described (52).

Determination of Tissue Bacterial Loads. Tissues were harvested and processed for Mtb colony-forming unit determination as described previously (30, 42, 52) and in SI Appendix. Briefly, tissue homogenates were made using a homogenizer (PRO 200; PRO Scientific) and were diluted using sterile PBS + 0.05% Tween-80. Fivefold serial dilutions of samples were plated on Middeldrop 7H11 plates. The colony-forming unit counts on plates were measured after 3–4 wk of culture.

Macroscopic and Microscopic Pathological Analysis of TB Lesions. Details are described in previous studies (30, 42, 52) and SI Appendix. Multiple tissue specimens were collected from all organs whether or not they showed gross lesions. For organs with visible lesions, their number, location, size, distribution, and consistency were recorded. A standard scoring system was used to calculate gross pathology scores for TB lesions (30, 42, 52), and all scorings were performed in a blinded fashion. Microscopic pathological analysis was done essentially the same as described elsewhere (30, 42, 52).

Statistical Analysis. Statistical analysis was done using a paired t test or Mann–Whitney U test or ANOVA as indicated. P < 0.05 was considered significant. All statistical analyses were conducted using GraphPad software (Prism).

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