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

Ling Shen\textsuperscript{a,b,1}, James Frencher\textsuperscript{a,b,1}, Dan Huang\textsuperscript{a,b,1}, Wandang Wang\textsuperscript{a,1}, Enzhuo Yang\textsuperscript{1}, Crystal Y. Chen\textsuperscript{a}, Zhuanor Zhang\textsuperscript{a,b}, Richard Wang\textsuperscript{a}, Arwa Qaqish\textsuperscript{b}, Michelle H. Larsen\textsuperscript{c}, Hongbo Shen\textsuperscript{a,2}, Steven A. Porcelli\textsuperscript{c}, William R. Jacobs Jr.\textsuperscript{c,2}, and Zheng W. Chen\textsuperscript{a,2}

\textsuperscript{a}Department of Microbiology and Immunology, Center for Primate Biomedical Research, University of Illinois College of Medicine, Chicago, IL 60612; \textsuperscript{b}Department of Immuno-Oncology, Beckman Research Institute, City of Hope National Cancer Center, Duarte, CA 91010; \textsuperscript{c}Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461; \textsuperscript{1}Clinical and Research Center of Tuberculosis, Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai 200433, China; and \textsuperscript{2}Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461

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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 (Mtb) 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 primates 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\textsuperscript{*}) 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\textsuperscript{*} 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 Mtb growth. Furthermore, selective immunization of Vγ2Vδ2 T cells enabled CD4\textsuperscript{+} and CD8\textsuperscript{+} 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.

Tuberculosis (TB), caused by Mycobacterium tuberculosis (Mtb), is the leading killer among infectious diseases (1), largely due to the concurrent epidemic of HIV/AIDS and multidrug resistance (2–4). The current TB vaccine, bacillus Calmette–Guérin, protects young children from severe disseminated TB, but inconsistently protects against pulmonary TB in adults (5–11). Development of a better TB vaccine requires a deeper understanding of protective anti-TB components and mechanisms in humans (12). Recent clinical TB vaccine trials yielded both protective and unprotective results (13–15), while vaccine candidates against Mtb infection were actively tested in animal models (16–22). However, the protective components of the immune system and the mechanisms for enhanced vaccine protection remain poorly defined (23–26).

T cells expressing γδ T cell antigen receptors are a non-conventional T cell population (27–29). Studies carried out over several decades have addressed fundamental aspects of the major Mtb-reactive γδ T cell subset, Vγ2Vδ2 T cells, during TB and other infections (29–33). Vγ2Vδ2 T cells are the sole γδ T cell subset capable of recognizing the isoprenoid metabolites isopentenyl pyrophosphate (IPP) and microbial (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which are usually referred to as phosphoantigens (34, 35). HMBPP is produced only by the nonmevalonate pathway present in some selected microbes, including Mtb and Listeria, whereas IPP can be produced by the mevalonate pathway in host cells (34, 35). HMBPP-specific Vγ2Vδ2 T cells exist only in humans and nonhuman primates (NHPs). They constitute 65–90% of total circulating human γδ T cells, contribute to both innate and adaptive immune responses in infections (36–39), and mount major expansion and migration to sites of infection.


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1LS, J.F., D.H., and W.W. contributed equally to this work.
2To whom correspondence may be addressed. Email: hbshen@tongji.edu.cn, william.jacobs@einstein.yu.edu, or zchen@uic.edu.

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effectors during infections with Mtb and other pathogens (29, 30, 31, 33, 41). Recent seminal studies demonstrate that HMBPP plus IL-10 treatment of NHPs can specifically expand VT2V62 T cells in vivo; following expansion, they are multifunctional and protective against infection with high doses of Mtb infection and other pathogens (29–33). Consistently, adoptive transfer studies showed that VT2V62 T effector cells can traffic to and accumulate in the lungs as early as 6 h after transfer and attenuate Mtb infection in NHPs (42). Notably, rapid recall-like expansion of VT2V62 T cells correlates with detectable immunity against fatal TB after Mtb challenge of bacillus Calmette–Guérin-vaccinated young rhesus macaques (29).

Protective features of VT2V62 T cells raise the question of whether selective immunization of VT2V62 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 T cells in conjunction with other T cell subsets. To this end, we have employed an HMBPP-producing Listeria monocytogenes (Lm) vaccine vector for immunization of VT2V62 T cells. While attenuated forms of Lm have been used as delivery systems to vaccinate humans against a variety of cancers (43), we combined ΔactA and prfA* mutations to develop an attenuated but highly immunogenic vector (31, 44, 45). We have shown that Lm ΔactA prfA* itself or its recombinants expressing various immunogens are highly attenuated and safe, eliciting remarkable expansion of VT2V62 T effector cells after systemic or respiratory vaccination of NHPs (46–48). In addition, recent studies, including ours, have shown that respiratory vector vaccination of NHP is safe and immunogenic (18, 20, 22, 48, 50). We therefore conducted a proof-of-concept study to test the hypothesis that respiratory Lm ΔactA prfA* immunization of VT2V62 T cells without concurrent immunization against other Mtb antigens can elicit protective effector memory responses and reduce Mtb infection in macaques. Our results showed that substantial protection was achieved by this approach.

Results

Expansion of HMBPP-Specific γδ T Cells by Immunization with HMBPP-Producing LmΔactA prfA*. To target VT2V62 T cells for vaccine design, we have employed an attenuated live Lm strain (Lm ΔactA 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 ΔactA 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 VT2V62 T cells. The ΔgcpE deletion mutant of Lm ΔactA prfA* served as a vector control, as this mutant no longer produced HMBPP due to the disruption of the gene gcpE encoding HMBPP synthase (48).

Intratracheal or respiratory vaccination of rhesus macaques with Lm ΔactA prfA*, but not the ΔgcpE variant, elicited a prolonged expansion of HMBPP-specific VT2V62 T cells in the circulation and airway [bronchoalveolar lavage (BAL) fluid; Fig. 1]. At months 1–3 after vaccination, the VT2V62 T cell subset increased and sustained up to almost 30% and 60% of total CD3+ T cells in the blood (Fig. 1A) and airway (Fig. 1B), respectively.

Respiratory Lm ΔactA prfA* Vaccination Elicited Sustained Increases in Th1-Like VT2V62 T Cells in the Airway. IFN-γ plays a crucial role in anti-TB immunity, and also regulates multiple effector functions of VT2V62 T cells (30, 32, 40, 42). We used intracellular cytokine staining (ICS) and flow cytometry to measure IFN-γ-producing VT2V62 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 ΔactA prfA* immunization elicited prolonged expansion of VT2V62 T cells in the lungs and blood. (A, Left) Representative flow cytometry histograms show percentages of VT2+ T cells in total 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 ΔactA prfA* (Top) and ΔgcpE deletion mutant (Bottom; ΔgcpE) of Lm ΔactA prfA*, respectively. Panels were gated on CD3+ lymphocytes. Numbers in the upper right quadrant indicate the percentages of VT2+ T cells in the total CD3+ T cell population. Expanded VT2 T cells are mostly VT2-coexpressing after Lm vaccination or primary TB infection, and therefore are interpreted as VT2V62 T cells as described in previous publications (29–31). Ctrl, control. (A, Right) Dot plots with means ± SD representing expansion of VT2V62 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; ** <0.01; ***P < 0.001 when comparing groups using a paired t test or Mann–Whitney U test. No 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 ΔactA prfA* vaccination, about 10–20% of VγVδ T cells in BAL fluid samples were spontaneously producing IFN-γ without the need for HMBPF 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 ΔactA prfA*, but not the ΔgcpE control (SI Appendix, Fig. S1A).

Although direct ICS assay revealed much lower levels of IFN-γ* VγVδ 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% of IFN-γ* VγVδ T cells in the total blood CD3+ T cells at 1 and 3 mo after the vaccination with Lm ΔactA prfA*, but very low detection with the ΔgcpE control (SI Appendix, Fig. S1C).

Improved Control of Mtb Infection Following Vaccine-Induced Expansion VγVδ T Cells. We next sought to examine if the vaccine-elicited prolonged expansion of the VγVδ T effector subset led to detectable protection against Mtb challenge. To this end, macaques from groups immunized with Lm ΔactA 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 Vγ 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 Vγ 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 Vγ T cell-immunized animals also had limited extrapulmonary Mtb dissemination (Fig. 2B, Lower). Macaques in the Vγ 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 Vγ 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 ΔactA prfA* immunization of VγVδ 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 ΔactA prfA* Immunization of VγVδ 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 ~2 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. S3A). 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, that body weights at post time points are subtracted by values at pre-infection for each of individuals before data analysis. Ctrl., control. (B) Bacterial burdens (colony-forming unit counts) in homogenized tissues. Mean colony-forming unit counts shown were from 1-cm² samples of different lung lobes as indicated (upper) or from samples of extrapulmonary organs (lower) collected at the time of necropsy. Dot plots in graph data represent colony-forming unit counts for individual macaques in each group with means ± SD. *P < 0.05; **P < 0.01 (Mann–Whitney U test and ANOVA).
organisms 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 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.

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+ Vγδ2 T cells were detected in lung tissues from Lm ΔactA prfA*-vaccinated macaques but not control animals (Fig. 5 A 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 coexpression as well as Mtb inhibition 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 Vδ2 T cells were purified from spleens of the test or control group animals, we found that splenic Vδ2 T cells from the Lm ΔactA prfA*-vaccinated group inhibited intracellular Mtb growth more potently in MΦ 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 the right caudal lobe (Fig. 3). Most macaques in the γδ T cell-immunized group did not show detectable gross granulomas in the spleen, liver, or kidney (as reflected by the entire pathology score in Fig. 3B and also shown in SI Appendix, Fig. S2A).

Comparison of the entire TB pathology between groups using established quantitative scoring criteria (19, 30, 42, 56) confirmed that the γδ T cell-immunized macaques had significantly milder TB lesions or pathology than the vector and saline control groups (Fig. 3B; P < 0.05 and P < 0.01, respectively). Overall, the 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 exhibited 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.
cells and were maintained at 3 V. Th1-like cells in the lungs were significantly greater in the Lm ΔactA pf4A*-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 capacity 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 cytoytic effector molecules, including granulysin or granzyme A, can inhibit intracellular Mtb growth (61, 63).

Rapid recall expansion of IFN-γ Vγ2Vδ2 T cells after Mtb challenge of Lm ΔactA pf4A*-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 pf4A*-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 pf4A*-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

Fig. 4. Rapid and sustained increases in Th1-like Vγ2Vδ2 T cells in lungs after Mtb challenge of Lm ΔactA pf4A*-vaccinated macaques. (A) Representative flow cytometry histograms (Upper) and a graph (Lower) show percentages of IFN-γ+ Vγ2+ T cells in CD3+ T cells in BAL fluid samples collected after 80-cfu Mtb challenge of the three groups vaccinated with Lm ΔactA pf4A* (Top), control (Ctrl) ΔgcpE (Middle), or saline (Bottom). The graph data are dot plots representing values for individual macaques in each group, and are derived from direct ICS assay without HMBPP stimulation in culture. (B) Graph dot plots showing percentages of Vγ2+ T cells in CD3+ T cells in BAL fluid samples from individual macaques of three indicated groups. ***P < 0.01; ****P < 0.001 (Mann–Whitney U test and ANOVA).

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 pf4A*-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 pf4A*-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).
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 prfAΔ expression of Mtb in macrophages. We developed and reported the ΔgcpE deletion mutant of Lm ΔactA prfAΔ, which no longer produces HMBPP (31, 44, 48). The Mtb Erdman strain was used for bronchoscope-guided challenge or infection of macaques. The H37rv strain was used for in vitro intracellular inhibition of Mtb growth in macrophages.

**Vaccine Vector and Mtb Strains.** Attenuated Lm strain Lm ΔactA prfAΔ 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 prfAΔ, which no longer produces HMBPP (31, 44, 48). The Mtb Erdman strain was used for bronchoscope-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 10⁵ cfu of Lm ΔactA prfAΔ 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.

**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.
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 with antibodies against cell surface markers for 15 min. Cells were washed 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 V52 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 Middlebrook 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 Middlebrook 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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