Antiretroviral therapy does not reduce tuberculosis reactivation in a tuberculosis-HIV coinfection model

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While the advent of combination antiretroviral therapy (ART) has significantly improved survival, tuberculosis (TB) remains the leading cause of death in the HIV-infected population. We used Mycobacterium tuberculosis/simian immunodeficiency virus–coinfected (M. tuberculosis/SIV–coinfected) macaques to model M. tuberculosis/HIV coinfection and study the impact of ART on TB reactivation due to HIV infection. Although ART significantly reduced viral loads and increased CD4+ T cell counts in blood and bronchoalveolar lavage (BAL) samples, it did not reduce the relative risk of SIV-induced TB reactivation in ART-treated macaques in the early phase of treatment. CD4+ T cells were poorly restored specifically in the lung interstitium, despite their significant restoration in the alveolar compartment of the lung as well as in the periphery. IDO1 induction in myeloid cells in the inducible bronchus-associated lymphoid tissue (IBALT) likely contributed to dysregulated T cell homing and impaired lung immunity. Thus, although ART was indispensable for controlling viral replication, restoring CD4+ T cells, and preventing opportunistic infection, it appeared inadequate in reversing the clinical signs of TB reactivation during the relatively short duration of ART administered in this study. This finding warrants the modeling of concurrent treatment of TB and HIV to potentially reduce the risk of reactivation of TB due to HIV to inform treatment strategies in patients with M. tuberculosis/HIV coinfection.

Introduction

The modern combination antiretroviral therapy (cART) regimen, which is known for its high efficacy and reduced toxicity, has significantly improved life expectancy in people living with HIV (PLHIV). Yet comorbidities such as tuberculosis (TB) (1) contribute significantly to all-cause mortalities in ART-adherent populations (2, 3), and the WHO reported 215,000 TB-associated deaths in HIV-infected populations in 2018. This suggests that ART fails to completely restore protective immunity to Mycobacterium tuberculosis. Despite scale-up efforts to facilitate ART accessibility to approximately 84% of notified PLHIV (4), an increased risk of mycobacterial infections with an associated risk of mortality (5) remains high within the first year of treatment in resource-limited settings (5, 6). Additionally, a paradoxical worsening of TB symptoms has been shown to occur soon after the initiation of ART in HIV-infected TB patients (7, 8). This is followed by a significant decline in reactivation risk with increased duration of adherence to ART and associated improvements in CD4+ T cell counts. Although ART is associated with a significant reduction in the incidence of TB irrespective of CD4+ T cell count status (9), the lifetime risk of TB reactivation in ART-adherent cases remains 4- to 7-fold higher than in HIV-uninfected populations (9, 10). CD4+ T cell dysfunction and chronic immune activation are reported alongside CD4+ T cell restoration in ART-adherent PLHIV (11–13). Lung pathology was reported in 75%–85% of HIV/AIDS autopsies (14), and the plethora of opportunistic infections that can arise in the lung of HIV-infected individuals suggests that their lung environments remain immunologically impaired. Thus, understanding the components of TB immunity that remain impaired following ART and chronic immune activation and TB reactivation. Our results indicate that while ART effectively and rapidly controlled SIV replication in coinfectcd macaques, leading to CD4+ T cell restoration, it did not decrease SIV-induced TB reactivation during the period studied.

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Results

Clinical correlates of TB reactivation in ART-treated NHPs with M. tuberculosis/SIV coinfection. Sixteen Indian-origin mycobacteria- and SPF-4–naïve rhesus macaques (Macaca mulatta) that were infected with approximately 10 CFU M. tuberculosis CDC1551 via the aerosol route (15, 21) converted to positive tuberculin skin tests (TSTs) after 3 weeks of M. tuberculosis exposure (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI136502DS1), confirming M. tuberculosis infection. The macaques (n = 16) were considered to have developed latent TB infection (LTBI), as they remained devoid of signs and symptoms of TB disease, and were enrolled for further perturbation studies (see Methods for additional details). A subset of the animals with established LTBI (n = 12) was challenged with 300 TCID_{50} (50% tissue culture infective dose) SIVmac251 via the intravenous route (15, 17, 22). Infection with SIV was confirmed by plasma viral load measurement performed at longitudinal time points, and 13 weeks after M. tuberculosis infection, 4 animals were initiated on the WHO-recommended ART regimen consisting of 2.5 mg/mL dolutegravir (DTG) and the 2 nucleoside reverse transcriptase inhibitors tenofovir disoproxil (PMPA) (20 mg/mL) and emtricitabine (FTC) (30 mg/mL) once daily through a single subcutaneous injection (23). The 3 experimental groups namely, LTBI, ART-naïve, and ART (Figure 1A), were studied longitudinally for their clinical and pathological features and immune responses.

The ART-treated animals showed a rapid and significant (-3-log; P < 0.00001) decline in plasma SIV viral loads compared with untreated controls within 2–9 weeks of ART initiation (Figure 1B). Despite the decline in SIV viral loads, however, we observed no improvement in survival, with a relative risk (RR) of TB reactivation in the ART-treated group (RR 1.23; 95% CI 0.97–1.5) compared with ART-naïve controls (Figure 1C). None of the animals in the LTBI group (n = 4) showed any clinical or pathological signs of TB at any point over the length of the study and were euthanized at week 24. Clinical parameters such as changes in body weight and temperature were monitored weekly (Supplemental Figure 1, A and B) and showed no significant differences between the groups. We have previously shown that elevated serum C-reactive protein (CRP) levels correlated with lung M. tuberculosis burden irrespective of SIV infection status (20). We did not detect serum CRP levels in the LTBI group, consistent with the low bacterial burdens in these animals. However, we found that CRP levels were significantly elevated (P = 0.0248) in both ART-naïve and ART-treated groups with M. tuberculosis/SIV coinfection at the necropsy endpoint relative to the pre–SIV infection time point (week 9). Furthermore, we detected no differences in serum CRP levels in the ART-naïve or ART-treated groups (P = 0.9073) at necropsy (Figure 1D). Viral loads in the acellular bronchoalveolar lavage (BAL) fluid, obtained at necropsy, were also significantly decreased (P = 0.0353) relative to viral loads in the controls (Figure 1E). We used the RNAscope ISH assay, which detects intracellular SIV RNA targets, and performed a semiquantitative analysis of the number of cells with discrete intracellular punctate red dots. The semiquantitative scores were as follows (mean ± SEM): 0.833 ± 0.16 and 0 ± 0 for lungs; 4 ± 0 and 2.4 ± 0.4 for spleens; and 2.83 ± 1.16 and 2.1 ± 0.29 for lymph nodes in the ART-naïve (n = 3) and ART-treated (n = 3) groups, respectively, and were not significantly different (Supplemental Table 2). We found that ART-naïve animals displayed a high density of SIV viral RNA (vRNA) cells in lymphocyte-rich structures such as germinal centers in spleen and bronchial lymph nodes (BrLNs) and periarterial lymphatic sheaths in the spleen (Figure 1F). However, there were few vRNA cells in the lung tissue, mostly localized in peribronchovascular bronchus–associated lymphoid tissue (BALT), and rarely in the lung parenchyma, consistent with findings from various studies reporting that lungs harbor few productively infected cells during acute SIV infection (24, 25). Besides, as early as 2 weeks after ART initiation, we detected substantially lower numbers of SIV vRNA cells in germinal centers of the spleen and BrLNs, with minimal change in the lungs.

M. tuberculosis burden and associated lung pathology during ART therapy in M. tuberculosis/SIV coinfection. The M. tuberculosis burden was assessed in LTBI, ART-naïve, and ART-treated animals by plating of BAL samples and lung, BrLN, and splenic tissues collected at necropsy. BAL samples from the LTBI group had no detectable tubercle bacilli, however, a significantly higher M. tuberculosis burden (P = 0.0014) was present in the SIV-coinfected animals irrespective of ART treatment. Similarly, SIV-coinfected animals from both ART-naïve and ART-treated groups had a higher M. tuberculosis burden in the lungs (P = 0.4644), BrLNs (P = 0.0067), and spleen (P = 0.0402) compared with burdens in the LTBI group, with no significant differences between the ART-naïve and ART-treated groups (P = 0.8568, P = 0.6577, P = 0.2437, respectively) (Figure 2, A–E). Although granulomas are a hallmark of TB pathology, lung granulomas are known to be heterogeneous in cellular composition, bacterial burden, and gross pathology (26). At necropsy, lungs resected from the macaques were manually dissected for macroscopic identification of granulomas and study of their gross, mycobacterial, and immune characteristics. We found a significantly higher bacterial burden (P = 0.0002) in granulomas from SIV-coinfected animals than in LTBI animals, with ART conferring no significant reduction (P = 0.1348) in granuloma M. tuberculosis CFU. Using the grid overlay technique described previously (27), we assessed serial lung sections for the number of lesions in each field to generate an arbitrary score corresponding to the percentage of lung involvement (Figure 2F). Animals with M. tuberculosis/SIV coinfection showed significantly higher numbers of TB lesions (P = 0.0240) compared with the LTBI-only group, whereas ART-treated animals had lesion scores comparable to those for ART-naïve controls (P = 0.99). Gross pathology (Figure 2G) showed that reactivated animals harbored numerous large granulomas, and H&E staining showed confluent granulomas with necrotic cores in both ART-naïve and ART-treated animals.

Differential CD4+ T cell restoration in alveolar and interstitial compartments after ART therapy in M. tuberculosis/SIV coinfection of NHPs. CD4+ T cell depletion in the setting of SIV infection and its role in TB reactivation has been actively studied (15, 21). We assessed the effect of ART on CD4+ T cells in various tissues using multiparameter flow cytometry. Compared with ART-naïve controls, ART-treated animals showed significantly higher CD4+ T cells in whole blood (P = 0.0427), BAL (P = 0.0009), BrLNs (P = 0.0229), and spleen (P = 0.0174) (Figure 3, A–E), indicating that ART was able to significantly restore CD4+ T cells in these compartments. In contrast, we observed minimal restoration of CD4+ T cells in the lung interstitium (supporting tissue that includes
Chronic immune activation is associated with HIV, and TB/HIV coinfection and can result in delayed functional recovery of the immune system and accelerate the progression to AIDS (29).

alveolar epithelium and pulmonary capillary endothelium, along with perivascular and perilymphatic tissues) following ART (Figure 3, C, F, and G). We have previously reported a role for interstitial CD4+ T cell depletion in the dissemination of TB (28). Thus, the lack of or delay in restoration of interstitial CD4+ T cells in the lungs of ART-treated animals in our study is consistent with the reactivation of TB that occurred despite ART.

Chronic immune activation is associated with HIV, and TB/HIV coinfection and can result in delayed functional recovery of the immune system and accelerate the progression to AIDS (29).
We noted a significant increase \((P < 0.0001)\) in the frequency of CXCR3+CCR6+CD4+ T cells in ART-treated animals (Supplemental Figure 4). CD4+ T cells coexpressing CXCR3 and CCR6 are reported to be preferentially enriched with HIV DNA in PLHIV on highly active ART (HAART) (31). We observed a selective expansion of CXCR3+CD4+ and CCR6+CD4+ T cell populations within 2 weeks of cART initiation (Supplemental Figure 4, A and B). Our findings are in line with a recent report suggesting that an increase

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**Figure 2. M. tuberculosis bacterial burden and lung pathology.** *M. tuberculosis* bacterial burden was determined by normalizing the CFU counts to log-transformed CFU per gram of tissue. (A) *M. tuberculosis* CFU in the total BAL sample (cellular plus the acellular components). (B) The *M. tuberculosis* CFU count was normalized per gram of lung tissue collected at necropsy. (C) Multiple granulomas \((n = 1-6)\) per animal were grouped according to the experimental classification of the animal; each granuloma was weighed, and its CFU count was normalized per gram of granuloma tissue. (D) BrLN *M. tuberculosis* burden. (E) Splenic *M. tuberculosis* burden. (F) The percentage of lung involvement was calculated by pathologists through extensive analysis of serially cut fresh lung samples and quantification of the number of lesions at low-power magnification. (G) Gross pathology and H&E staining show the large granulomatous and necrotic lesions in animals with SIV-induced *M. tuberculosis* reactivation, whereas samples from animals with LTBI show minimal pathology. Scale bars: 500 μm. The following 3 groups were studied: *M. tuberculosis* infection only, i.e., LTBI \((n = 4, \text{ green})\), *M. tuberculosis*/SIV coinfection, i.e., ART-naive \((n = 8, \text{ red})\), and *M. tuberculosis*/SIV coinfection with ART treatment, i.e., ART \((n = 4, \text{ blue})\). (A–F) Data represent the mean ± SEM; error bars indicate the SEM. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\), by 1-way ANOVA with Tukey’s multiple-comparisons test.
in expression of CXCR3/CCR6/CD4+ T cells is associated with the onset of TB–immune reconstitution inflammatory syndrome (TB-IRIS) in patients with HIV recently initiated on HAART (32).

We observed an increased presence of CD68+CD163+ macrophages in lung sections from M. tuberculosis/SIV–coinfected animals, though the differences between ART-naive and ART-treated groups were not significant (P = 0.2654) (Supplemental Figure 6). This increase in myeloid cell populations could have been due to type I IFN–mediated proliferation and trafficking of M. tuberculosis–permissive innate immune cells that may further contribute to exacerbation of TB disease (33).

Dysregulation in homing of CD4+ T cells to iBALT in lung interstitium of M. tuberculosis/SIV–coinfected macaques. The presence of effector lymphoid tissue bronchus-associated lymphoid tissue (iBALT) is known to contribute to protection against M. tuberculosis and prevent reactivation of latent TB (34). These iBALT structures that formed in the lungs of M. tuberculosis–infected macaques provide an environment for B cell maturation and antigen-specific memory effector T cells within the tissue (35). iBALT structures are highly organized lymphoid aggregates consisting of B cell zones, serving as a germinal center, and T cell zones, harboring CD4+ and CD8+ T cells along with DCs and high endothelial venules (36, 37). CD4+ effector memory T cells residing in these iBALTs are targeted by SIV, resulting in their depletion and poor reconstitution despite successful control of viral replication by ART (38). We have previously demonstrated that iBALT persisted in the lungs of rhesus macaques in the setting of M. tuberculosis/SIV coinfection, even when CD4+ T cells are depleted. Moreover,
iBALT structures occupied a higher percentage of lung area in nonreactivators compared with reactivators, suggesting that the presence of iBALT correlates with protective immunity against *M. tuberculosis* reactivation in the setting of *M. tuberculosis*/SIV coinfection (15, 39). In this study, we examined iBALT structures in the lung using IHC. We observed a predominance of CD68⁺CD163⁺ macrophages (Figure 4A and Supplemental Figure 5, A and B) phagocytosing necrotic SIV-infected cells in the T cell zone, which is typically occupied by CD4⁺ T lymphocytes in lesions from macaques with LTBI. These productively infected macrophages and myeloid cells have been shown to serve as a reservoir for SIV in ART-suppressed macaques and are associated with high levels of immune activation (40). We have previously shown in our model of *M. tuberculosis*/SIV coinfection, through BrdU labeling, that high turnover of macrophages correlates with TB reactivation (20). Studies have shown that CD4⁺ T cell depletion after SIV infection significantly increases viral replication in macrophages and other antigen-presenting cells (41). In the face of ART, these SIV-infected macrophages serve as a reservoir by harboring latent viral genomes and contribute to viral rebound upon ART interruption (40). High turnover of macrophages contributes to macrophage persistence as antigen-presenting cells and drives chronic immune activation (42). Furthermore, a recent study also suggested that synergy between *M. tuberculosis* and SIV within lung granulomas facilitates *M. tuberculosis* bacterial dissemination and growth, thereby contributing to TB reactivation (43). Thus, our work suggests a significant dysregulation in the reconstitution of iBALT structures proximal to granulomas despite ART, and this may be one of the major mechanisms by which immune function remains impaired in *M. tuberculosis*/HIV–coinfected individuals.

Further investigation of macrophages and myeloid-derived cells in the T cell zone of BALT showed that these cells expressed indoleamine 2,3-dioxygenase (IDO1) (see IDO1-expressing macrophages in granulomatous lesions in Figure 4B). Our previous studies in the macaque model of *M. tuberculosis* infection showed IDO-expressing cells in the macrophage-rich layer of granulomas, which likely serves to prevent optimal interactions between CD4⁺ T cells and *M. tuberculosis*-infected antigen-presenting cells. Moreover, increased expression of IDO1 correlated with *M. tuberculosis* bacterial burden, and IDO1 expression was also associated with poorly formed iBALT. Our laboratory has previously reported that inhibition of IDO1 activity resulted in reorganization of granulomas and granuloma-associated iBALT structures, resulting in improved *M. tuberculosis* clearance (44). IDO1 is an immunoregulatory enzyme known to induce apoptosis of effector CD4⁺ T cells and promote the activation of regulatory T cells. Thus, expression of IDO in macrophages (Figure 4C), CD141⁺ tolerogenic DCs (Figure 4D), and other myeloid-lineage cells in ART-treated animals may promote dysregulated homing of CD4⁺ T cells in the T cell zone of iBALT and poor restoration of CD4⁺ T cells in the lung interstitium. Furthermore, our model can serve as a resource to
test the efficacy of various IDO1-modulating agents in preventing TB reactivation and ameliorating chronic immune activation.

Discussion

Although the macaque model of *M. tuberculosis*/HIV coinfection has been extensively used, ours is the first report to our knowledge on the implementation of ART in this model system. Our results indicate that ART significantly reduces SIV viral loads in all tissue compartments. Furthermore, significant restoration of CD4+ T cell levels was observed in the periphery, the alveolar compartment, as well as in extrapulmonary tissues. We clearly show, however, that ART failed to reconstitute CD4+ T cells in lung tissue during the shorter duration of ART administered in this study. We believe this demonstrates the strength of our model system, since extensive examination of the lung tissue is virtually impossible in patients. Accordingly, ART was unable to prevent SIV-induced reactivation of LTBI in the early phase of treatment. As in humans, the *M. tuberculosis*/SIV coinfection/ART model in rhesus macaques recapitulated the efficacy of ART in reducing viral load and reconstituting CD4+ T cells. A meta-analysis reported an 18% pooled incidence of TB-associated IRIS in PLHIV initiated on ART (45). Thus, although ART prevents TB in many PLHIV, a substantial minority of such individuals go on to develop TB. Our results suggest that imprecise or dysregulated restoration of CD4+ T cells in the lung may play an important role in this process. Our success in being able to model a more widespread failure of LTBI maintenance may be due to the use of a system in which high doses (300 TCID₅₀) of the virus were used for coinfection via the systemic (intraocular) route. The use of a physiologically relevant low dose and a mucosal route for SIV coinfection, along with extremely low doses of *M. tuberculosis* sufficient to generate LTBI in our model would, however, render our model cost-prohibitive and statistically underpowered. Although in our model of *M. tuberculosis*/SIV coinfection, we challenged LTBI macaques with SIV to study the role of SIV infection in *M. tuberculosis*-specific CD4+ T cell immunity, we understand that the sequence of infection can be reversed in areas where there is an HIV and TB syndemic. Our findings of TB reactivation with SIV challenge 9 weeks after *M. tuberculosis* infection are in accord with other studies, in which SIV challenge given 8–10 months (16) after *M. tuberculosis* infection resulted in TB reactivation. Also, our model may be of value for the study of recurrent TB in PLHIV, as it explains the cause of relapse after anti-TB treatment completion (46) and the increased susceptibility to exogenous *M. tuberculosis* reinfection after curative anti-TB therapy (47).

Our findings are consistent with available human data suggesting that initiation of ART after *M. tuberculosis*/HIV coinfection may not prevent reactivation of LTBI in all individuals (48, 49). It is, however, conceivable that coupling ART with either isoniazid preventive therapy (IPT) or isoniazid plus rifampine 3-month therapy (3HP) may completely or substantially prevent TB reactivation. In this regard, it is important to note that we have developed a model for LTBI treatment in macaques using 3HP (18). Furthermore, as novel treatment regimens for LTBI with greater efficacy are developed, our model could serve as the key resource to validate them. As such, our results have the potential to inform treatment approaches for the syndemic resulting from the 2 most important infectious diseases of humanity.

Methods

Animal studies. Sixteen Indian-origin rhesus macaques (*Macaca mulatta*) were infected with a low dose of approximately 10 CFU *M. tuberculosis* CDC1551 (BEI Resources, catalog NR13649) via the aerosol route. The animals were enrolled from a specific pathogen-free colony maintained at TNPRC and were tested and found to be free from SPF-4 (simian retrovirus D, SIV, STLV-1, and herpes B virus) and TB pathogens. PCR-based molecular typing was performed to study the expression of the MHC class 1 alleles MAMU A*01, B*08, B*17, though enrollment in the study was independent of MAMU status. All animals had a positive TST after 3 weeks of exposure, confirming infection. The animals were then examined weekly for body temperature and weight and subjected to a physical examination by a board-certified veterinary clinician. Blood examination, consisting of hematology and serum biochemistry, was performed weekly, whereas BAL assessment was performed biweekly in mice under general anesthesia. LTBI was confirmed if the animals did not show signs of reactivation in the form of fever, greater than 20% weight loss, anorexia, labored breathing, and/or increased CRP levels up to week 8 after *M. tuberculosis* exposure. Nine weeks after *M. tuberculosis* infection, 12 LTBI-confirmed animals were challenged with 300 TCID₅₀ *SIVmac239* (provided by the Preston Marx Laboratory, TNPRC, Covington, Louisiana, USA) via the intravenous route, whereas 4 animals served as LTBI controls. Once infection with SIV was confirmed with plasma SIV RNA viral load, the animals were randomized to the control (ART-naive) group (*n* = 8) or the treatment (ART) group (*n* = 4). Thirteen weeks after *M. tuberculosis* infection, animals in the treatment group were placed on a CART regimen. All animals were euthanized by week 24, which was a predetermined study endpoint, or at earlier time points if the animals were clinically unwell and/or showed signs of TB reactivation, as determined by veterinarians, in accordance with humane endpoints criteria.

ART drug formulation. PMPA and FTC were obtained from Gilead Sciences and DTG from ViiV Healthcare. These antiretroviral drugs were administered in a formulation of a 3-drug cocktail dissolved in the vehicle kleptose following previously published doses for each drug. Each milliliter of formulation contained the reverse transcriptase inhibitors PMPA (20 mg/mL) (50–52) and FTC (30 mg/mL) (53) and the integrase inhibitor DTG (2.5 mg/mL) (54, 55).

Plasma and BAL SIV viral load. To determine the efficacy of cART, BAL samples were collected biweekly by vigorous infusion of 50 mL sterile PBS through an orotracheal tube and aspiration of as much of the instilled volume as possible. The procedure was performed by trained veterinarians. The aspirated fluid was mixed with 10% FBS (v/v) during the transit. BAL supernatant (acellular) was stored at –80°C until analysis. Plasma and BAL SIV viral loads were determined in acellular BAL supernatant by RNA extraction and subsequent reverse transcription quantitative PCR (RT-qPCR) using a probe targeting the gag gene of SIV. Plasma and BAL SIV viral load measurements were performed at the National Institute of Allergy and Infectious Diseases (NIAID), Division of AIDS (DAIDS), Nonhuman Primate Core Virology Laboratory for AIDS Vaccine Research and Development at Duke University, Durham, North Carolina, USA (contract HHSN27201800003C). The lower limit of quantification for SIV copies in the RNA in this assay was 100 copies/sample.

ISH assay–RNAscope. Rhesus macaque lungs, BrNLNs, and splenic tissues were collected during necropsy immediately following euthanasia. Serial sections were prepared from formalin-fixed, paraffin-
embedded (FFPE) tissue blocks and used for ISH with the RNA-seq 2.5 HD Reagent Kit (catalog 322350, Advanced Cell Diagnostics) exactly according to the manufacturer’s instructions. A SIV-mac239-specific probe (catalog 312811, Advanced Cell Diagnostics) containing 83 ZZ pairs complementary to the transcripts coded by the viral genome regions 1251–9420 (GenBank: D01065.1) (56), which code for multiple SIV proviral genes (gag, vif, env, vpx, vpr, nef, rev), was used. An identical assay using a probe for the bacterial DalP8 gene (catalog 320751, Advanced Cell Diagnostics), which is not expressed in mammalian tissues, was used as a negative control. Positive signals were detected by counting the number of dots per cell labeled with red dye, and images were captured using an Olympus BX46 microscope and an Olympus DP27 camera (Olympus America). A semiquantitative scoring system, based on the manufacturer’s recommendation (57), was used to compare gene expression levels.

Confocal microscopy. FFPE sections from the lung, spleen, and lymph nodes harvested at the study endpoint were stained with H&E, and fluorescence IHC and ISH were performed as described previously (44, 58, 59).

Tissue processing and flow cytometry. High-parameter flow cytometry was performed on whole blood and BAL samples collected at weeks 3, 7, 11, 15, and 19, and also on lung, BrLN, spleen, and granuloma tissues harvested at specific study endpoints, as described previously (15, 18, 21). Briefly, T cell phenotypes were studied using the following antibodies: anti-CD3 (clone SP34-2), anti-CD4 (clone L200), anti-CD8 (clone RPA-T8), anti-CD69 (clone FN50), anti-CXCR3 (clone IC6/CXCR3), anti-CCR6 (clone 11A9), anti-HLA-DR (clone L243) (all purchased from BD Biosciences), and anti-PD-1 (clone EH12.2H7, BioLegend). Flow cytometric data were analyzed with the FlowJo platform using previously described gating strategies (15, 18, 21).

Statistics. Graphs were prepared and statistical comparisons applied using GraphPad Prism, version 8 (GraphPad Software). Various statistical comparisons were performed using a 2-tailed Student’s t test or 1- or 2-way ANOVA with Tukey’s or Holm-Šidák’s multiple comparisons tests when applicable. Statistical differences between groups were considered significant when the P value was 0.05 or less. Data are presented as the mean ± SEM.

Study approval. All animals were housed in the Animal Biosafety Level III (ABS3) at TNPRC, where they were treated according to standards recommended by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and the NIH Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011). This study was approved by the IACUC of TNPRC (protocols P0247R, P0324, and P0295R).

Author contributions
SRG, ANB, SAK, SM, JR, and DK designed the studies. SRG, ANB, AC, DKS, AF, THL, BS, andXA conducted the experiments and acquired the data. SRG, RS, US, VV, SM, SAK, JR, andDK analyzed and interpreted the data and contributed to writing of the manuscript.

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