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Title: Anti-retroviral therapy does not reduce tuberculosis reactivation in a tuberculosis-HIV co-infection model

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Abbreviations: cART, combination Anti-retroviral Therapy; ATB, active TB; BAL, bronchoalveolar lavage; CFU, Colony Forming Unit; CRP, C-reactive protein; CXR, thoracic radiograph; iBALT, inducible bronchus-associated lymphoid tissue; LTBI, latent tuberculosis infection; Mtb, Mycobacterium tuberculosis; NHP, non-human primate; PBMC, peripheral blood mononuclear cell; BrLN, Bronchial lymph node; PLHIV, People living with HIV; SIV, Simian Immunodeficiency Virus; TB, tuberculosis; TST, tuberculin skin test.
Abstract.
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 employed *Mtb*/Simian Immunodeficiency Virus (SIV) co-infected macaques to model *Mtb*/HIV co-infection and study the impact of ART on TB reactivation due to HIV-infection. While ART significantly reduced viral loads and increased CD4+ T cell counts in blood and 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 iBALT likely contributed to dysregulated T cell homing and impaired lung immunity. Thus, while ART is indispensable in controlling viral replication, CD4+ T cells restoration, and preventing opportunistic infection, it appears inadequate in reversing clinical signs of TB reactivation during the relatively short duration of ART administered in this study. This warrants modeling 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 *Mtb*/HIV co-infection.

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 co-morbidities such as tuberculosis (1) contribute significantly to all-cause mortalities in ART-adherent populations (2, 3) and the World Health Organization (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* (*Mtb*). Despite scale-up efforts to facilitate ART accessibility to around 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 tuberculous 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 four to seven-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 HIV co-infection and ART treatment will provide insights into improving treatments for TB and HIV co-infection.

Previously, we have shown that a nonhuman primate (rhesus macaque) model of *Mtb*/SIV co-infection effectively recapitulate many aspects of human disease, including productive SIV infection, CD4+ T cell depletion, *Mtb* reactivation, and chronic immune activation (15-20). Here we extend this model to study the impact of ART on viral replication, CD4+ T cell restoration in various tissue compartments, chronic immune activation, and TB reactivation. Our results indicate that while ART effectively and rapidly controls SIV replication in co-infected macaques, leading
to CD4+ T cell restoration, it does not decrease SIV-induced TB reactivation during the period studied.

Results.

Clinical correlates of TB reactivation in ART-treated NHPs with Mtb/SIV co-infection:

Sixteen Indian-origin mycobacteria- and SPF-4-naïve rhesus macaques (Macaca mulatta) that were infected with ~10 CFUs of Mtb CDC1551 via aerosol (15, 21), converted to positive tuberculin skin tests (TSTs) after 3 weeks of Mtb exposure (Table S1), confirming Mtb infection and remained devoid of signs and symptoms of TB disease for up to 9 weeks after Mtb infection were considered to have latent TB infection (LTBI), and used for further perturbations (Additional details in Supplemental Methods and Table S1). A subset of the animals with established LTBI (n=12) was challenged with 300 TCID_{50} SIVmac239 via the intravenous route (15, 17, 22). Infection with SIV was confirmed by plasma viral load performed at longitudinal time points, and at 13 weeks post-Mtb infection, four animals were initiated on WHO-recommended ART regimen consisting of dolutegravir 2.5 mg ml\(^{-1}\) (DTG) and two nucleoside reverse transcriptase inhibitors tenofovir disoproxil 20 mg ml\(^{-1}\) (PMPA), and emtricitabine 30 mg ml\(^{-1}\) (FTC) once daily through a single subcutaneous injection (23). The three experimental groups namely, LTBI, ART-naïve, and ART (Fig 1A) were studied longitudinally for their clinical and pathological features and their immune responses.

ART-treated animals showed rapid and significant (~3-log) decline (p < 0.00001) in plasma SIV viral loads compared to untreated controls within weeks of ART initiation (Fig 1B). Despite the decline in SIV viral loads, however, there was no improvement in survival with a relative risk of
TB reactivation in the ART-treated group (RR 1.23, 95% CI 0.97-1.5) compared to ART-naïve controls (Fig 1C). None of the animals in the LTBI group (n=4) showed any clinical or pathological signs of TB at any point during the length of the study and were euthanized at week 24. Clinical parameters that were monitored weekly, such as changes in weight and body temperature (Fig S1A-B), showed no significant differences between the groups. We have previously shown that elevated serum CRP levels correlated with lung *Mtb* burdens irrespective of *SIV* infection status (20). Serum CRP was not detected in the LTBI group, consistent with the low bacterial burdens in these animals. However, CRP levels were significantly elevated (p = 0.0248) in both ART-naïve and ART-treated groups with *Mtb*/SIV co-infection at the necropsy endpoint relative to the pre-*SIV* infection time point (week 9). Furthermore, no differences in serum CRP levels were observed in the ART-naïve and ART groups (p = 0.9073) at necropsy (Fig 1D). Viral loads in the acellular BAL fluid, obtained at necropsy, also recorded significantly decreased viral loads (p = 0.0353) relative to controls (Fig 1E). Using the RNAscope *in situ* hybridization (ISH) assay, which detects intracellular SIV RNA targets, and performed a semi-quantitative analysis based upon the counting of the number of cells with discrete intracellular punctate red dots. The semi-quantitative scores were (mean±SEM) 0.833±0.16, and 0±0 for lungs, 4±0, and 2.4 ± 0.4 for spleen, 2.83±1.16, and 2.1±0.29 for lymph node in ART-naïve (n=3) and ART (n=3) groups respectively, and were not significantly different. We found that ART-naïve animals displayed a high density of SIV vRNA+ cells in lymphocyte-rich structures such as germinal centers in spleen and bronchial lymph node (BrLN) and periarterial lymphatic sheaths in the spleen (Fig 1F). However, there were few vRNA+ cells in lung tissue, 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 two weeks after ART therapy initiation, there were substantially lower numbers of SIV vRNA+ cells reported in germinal centers of spleen and BrLN, with minimal change in the lung.

**Mtb burden and associated lung pathology during ART therapy in Mtb/SIV co-infection:**

The Mtb burdens in LTBI, ART-naïve, and ART-treated animals by plating BAL, lungs, BrLN, and spleen tissue collected at necropsy. BAL samples from the LTBI group had no detectable tubercle bacilli, however, significantly higher Mtb burdens (p = 0.0014) were present in SIV co-infected animals irrespective of ART treatment. Similarly, SIV co-infected animals from both ART-naïve and ART-treated groups had higher Mtb burdens in lungs (p = 0.0464), BrLN (p = 0.0067) and spleen (p = 0.0402) compared to the LTBI group, with no significant differences between ART-naïve and ART-treated groups (p = 0.8568, p = 0.6577, p = 0.2437, respectively) (Fig 2A-E). While granulomas are a hallmark of TB pathology, lung granulomas are known to be heterogeneous for cellular composition, bacterial burden, and gross pathology (26). At necropsy, lung resected from the macaques were manually dissected to macroscopically identify granulomas and study their gross, mycobacterial, and immune characteristics. We found a significantly higher bacterial burden (p = 0.0002) in granulomas from SIV co-infected animals compared to LTBI animals with ART offering no significant reduction (p = 0.1348) in granuloma Mtb CFU. Using the grid overlay technique described previously (27), serial lung sections were assessed for the number of lesions in each field to generate an arbitrary score corresponding to the percentage of lung involvement (Fig 2F). Animals with Mtb/SIV co-infection showed significantly higher numbers of lesions (p= 0.0240) compared to LTBI with ART-treated animals having scores comparable to ART-naïve controls (p= 0.99). Gross pathology (Fig 2G) shows that reactivated
animals harbored numerous, large granulomas and H&E staining shows confluent granulomas with necrotic cores in both ART-naïve and ART-treated groups.

Differential CD4+ T cell restoration in alveolar and interstitial compartments after ART therapy in Mtb/SIV co-infection 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 studied the effect of ART on CD4+ T cells in various tissues using multiparameter flow cytometry. Compared to ART-naïve controls, ART-treated animals showed significantly higher CD4+ T cells in whole blood (p = 0.0427), BAL (p = 0.0009), BrLN (p = 0.0229) and spleen (p = 0.0174) (Fig 3A-E), indicating that ART was able to significantly restore CD4+ T cells in these compartments. In contrast, minimal restoration of CD4+ T cells was observed in the lung interstitium (supporting tissue that includes alveolar epithelium, pulmonary capillary endothelium, along with perivascular, and perilymphatic tissues) following ART (Fig 3C, F, 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 cell in the lungs of ART-treated animals in our study, is consistent with reactivation to TB that occurred despite ART.

Chronic immune activation is associated with HIV and TB/HIV co-infection and can result in delayed functional recovery of the immune system and accelerate progression to AIDS (29). We found no significant differences in the frequencies (Fig S2A-F and S3A-B) of HLA-DR+ CD4+ T cells, PD-1+ CD4+ T cells, CXCR3+ CD4+ T cells, and CCR6+ CD4+ T cells, in blood and BAL samples from ART-naïve and ART treated groups. However, CD69+CD4+ T cell levels, a marker of early activation in response to Mtb antigen (21, 30), were higher in BAL samples of the ART-treated group compared to ART-naïve and LTBI groups. There was a significant increase in the
frequency of CXCR3+ CCR6+ CD4+ T cells in ART treated animals. CD4+ T cells co-expressing CXCR3 and CCR6 are reported to be preferentially enriched with HIV DNA in PLHIV on HAART (31). There was a selective expansion of CXCR3+CD4+ and CCR6+CD4+ T cell populations, within 2 weeks of cART initiation Fig (S4A-B). Our findings are in line with a recent report suggesting an increase in expression of CXCR3+CCR6+CD4+ T cells are associated with the onset of TB-IRIS in HIV patients recently initiated on HAART (32).

We observed an increased presence of CD68+CD163+ macrophages in the lung section of Mtb/SIV co-infected animals, though the differences between ART-naïve and ART groups were not significant (p=0.2654) (Fig S6). This increase in myeloid population can be due Type I IFN mediated proliferation and trafficking of Mtb-permissive innate immune cells contribute to the exacerbation of TB disease (33).

Dysregulation in homing of CD4+ T cells to iBALT in lung interstitium of Mtb/SIV co-infected macaques.

The presence of effector bronchus-associated lymphoid tissue (iBALT) is known to contribute towards protection against Mtb and prevent reactivation of latent TB (34). These iBALT structures, formed in the lungs of Mtb infected macaques, provide an environment for B cell maturation, 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 dendritic cells 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 *Mtb/SIV* co-infection even when CD4+ T cells are depleted. Moreover, iBALT structures occupied a higher percentage of lung area in non-reactivators compared to reactivators, suggesting that the presence of iBALT correlates with protective immunity against *Mtb* reactivation in the setting of *Mtb/SIV* co-infection (15, 39). In this study, we examined iBALT structures in the lung by using immunohistochemistry. We observed a predominance of CD68+CD163+ macrophages (Fig 4A) phagocytosing necrotic SIV infected cells in the T cell zone, which is typically occupied by CD4+ T lymphocytes in lesions from macaques with LTBI. The presence of 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 *Mtb/SIV* co-infection, through BrdU labeling, that high turnover of macrophages correlates with TB reactivation (20). Studies have shown that CD4+ T cell depletion, post SIV infection, significantly increase in 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 cell and drives chronic immune activation (42). Furthermore, a recent study also suggests of the synergy between *Mtb* and SIV within lung granuloma facilitate *Mtb* bacterial dissemination and growth thus 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 *Mtb/HIV* co-infected individuals.

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

**Discussion.**

While the macaque model of *Mtb*/HIV co-infection has been extensively utilized, ours is the first report of implementing ART in this model system. Our results suggest that ART significantly reduces SIV viral loads in all tissue compartments. Furthermore, significant restoration of CD4$^+$ T cell levels is 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 the lung tissue during the shorter duration of ART administered in this study. This exhibits a strength of our model system, since extensive interrogation of the lung tissue is virtually impossible in human patients. Accordingly, ART was unable to prevent SIV-induced reactivation of LTBI in the early phase of
treatment. As in humans, the *Mtb/SIV* co-infection/ART model in rhesus macaques recapitulated the efficacy of ART in viral load reduction and CD4+ T cell reconstitution. A meta-analysis reports, 18% pooled incidence of tuberculosis-associated IRIS in PLHIV initiated on ART(45). Thus, while ART prevents TB in many PLHIV, yet a significant minority of such individuals develop TB. Our results suggest that imprecise or dysregulated restoration of CD4+ T cells to the lung may play a significant role in this process. Our success in being able to model a more wide-spread failure of LTBI maintenance may be due to the use of a system where high doses (300 TCID50) of the virus were used for co-infection via the systemic, intravenous route. The use of a physiologically relevant low dose and mucosal route for SIV co-infection along with the use of extremely low-doses of *Mtb* for infection necessary to generate LTBI in our model would, however, render our model cost-prohibitive and statistically underpowered. Although in our model of *Mtb/SIV* co-infection, we challenged latent tuberculosis macaque with SIV to study the role of SIV infection on *Mtb* specific CD4+ T-cell immunity, we understand that the sequence of infection can be reverse in areas with HIV and TB syndemic. Our findings of TB reactivation with SIV challenge at nine weeks post-*Mtb* infection are in tune with findings of other groups where SIV challenge followed after 8-10 months(16) after *Mtb* infection, developed TB reactivation. Also, our model may be of value at studying recurrent TB in PLHIV, explaining the cause of relapse after anti-TB treatment completion (46) and increased susceptibility to exogenous *Mtb* reinfection after curative anti-TB therapy (47).

Our findings are consistent with available human data suggesting that initiation of ART after *Mtb/HIV* co-infection 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 Rifapentine three-month therapy (3HP), may completely or significantly 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 two most important infectious diseases of humanity.

Methods:

Study approval. All the animals were housed in the Animal Biosafety level III (ABSL3) at Tulane National Primate Research Center where they were treated as per the standards recommended by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and the NIH Guide for the Care and Use of Laboratory Animals. The study was approved by the IACUC of the Tulane National Primate Research Center (TNPRC) (protocols P0247R, P0324, P0295R).

Animal studies

Animal studies were performed following approved IACUC protocols at TNPRC (Covington, LA). Sixteen Indian-origin rhesus macaques (Macaca mulatta) were infected with low dose ~10 colony-forming units (CFUs) of Mtb CDC1551 (BEI resources, Cat# NR13649, Manassas, VA) via an aerosol route. Animals were enrolled from a specific pathogen-free colony maintained at TNPRC and were tested free from SPF-4 (simian retrovirus D, SIV, STLV-1 and herpes B virus) and tuberculosis pathogens. A PCR-based molecular typing was performed to study the expression of major histocompatibility class MHC class 1 alleles, MAMU A*01, B*08, B*17, though
enrollment in the study was independent of the MAMU status. All animals reported positive tuberculin skin test (TST) after 3 weeks of exposure, confirming infection. Animals were then followed by weekly physical examination, including body temperature and weight collection and physical examination by a board-certified veterinary clinician. Blood examination, consisting of hematology and serum biochemistry, was performed weekly whereas bronchoalveolar lavage (BAL) was performed biweekly under general anesthesia. Latent TB infection was confirmed if the animals did not show signs of reactivation viz. fever, >20% weight loss, anorexia, labored breathing, and/or raised CRP until week 8 post of *Mtb* exposure. At week 9 post *Mtb* infection, LTBI confirmed animals (n=12) were challenged with 300 TCID<sub>50</sub> *SIVmac<sub>239</sub>* (Preston Marx Lab, TNPRC, Covington, LA) via intravenous route while (n=4) served as LTBI controls. Once infection with SIV was confirmed with plasma SIV RNA viral load animals were randomized to control (ART-naïve) group n=8 and treatment (ART) group n=4. At 13 weeks post *Mtb* infection, treatment group animals were administered combined antiretroviral therapy (cART) regimen. All animals were euthanized by week 24, which was a predetermined study endpoint or at earlier timepoints if the animals were clinically unwell and/or showed signs of TB reactivation, as determined by veterinarians, adhering to humane endpoints criteria.

**ART drug formulation**

PMPA and FTC were obtained from Gilead Sciences (Foster City, CA) and DTG from ViiV Healthcare (Research Triangle, NC). These antiretroviral drugs were administered in a formulation of 3 drug cocktail dissolved in a vehicle kleptose following published doses for each drug. Each ml of formulation contained two reverse transcriptase inhibitors 20 mg ml<sup>-1</sup>, tenofovir disoproxil (PMPA) (50-52), 30 mg ml<sup>-1</sup> emtricitabine (FTC) (53) and an integrase inhibitor 2.5 mg ml<sup>-1</sup> dolutegravir (DTG) (54, 55).
**Plasma and BAL SIV viral load**

To determine the efficacy of cART, bronchoalveolar lavage (BAL) samples were collected biweekly by vigorously infusing 50 ml of sterile PBS through an orotracheal tube and aspirating as much of instilled volume as possible. The procedure was performed by trained veterinarians. The aspirated fluid was mixed to 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-polymerase chain reaction (RT-qPCR) using probe targeting the gag gene of SIV. Plasma and BAL SIV viral loads were performed at the NIAID DAIDS Nonhuman Primate Core Virology Laboratory for AIDS Vaccine Research and Development at Duke University, Durham, NC (contract # HHSN27220180003C). The lower limit of quantification for SIV copies in the RNA in this assay is 100 copies/sample.

**In Situ Hybridization Assay-RNAscope**

Rhesus macaque lungs, bronchial lymph nodes, and spleen tissues were collected during necropsy immediately following euthanasia. Serial sections were prepared from formalin-fixed, paraffin-embedded tissue blocks and used for In Situ Hybridization (ISH) using RNAscope 2.5 HD reagent kit (Catalog # 322350, Advanced Cell Diagnostics, Newark, CA) exactly as per the manufacturer’s instructions. A SIVmac239 specific probe (Catalog # 312811, Advanced Cell Diagnostics) containing 83 ZZ pairs complementary to the transcripts coded by viral genome region 1251 – 9420 (GenBank: D01065.1) (56) which code for multiple SIV proviral genes (gag, vif, pol, tat, env, vpx, vpr, nef, rev) was used. An identical assay using a probe for bacterial DapB 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 dyes, and images were captured using Olympus BX46 microscope with Olympus DP27 camera (Olympus America, Center Valley, PA). A semi-quantitative scoring system based on the manufacturer’s recommendation (57), was used to compare the gene expression.

**Confocal microscopy**

Formalin-fixed and paraffin-embedded (FFPE) sections from the lung, spleen, and lymph nodes harvested at endpoint were stained with H&E, fluorescent immunohistochemistry, and in-situ hybridization as described (44, 58).

**Tissue processing and flow cytometry**

High parameter flow cytometry was performed on whole blood and BAL samples collected on timepoints weeks 3, 7, 11, 15, 19 and also on lung, BrLN, spleen, granuloma tissues harvested at endpoints, as previously described (15, 18, 21). Briefly, T cell phenotypes were studied using antibodies: CD3 (clone SP34-2), CD4 (clone L200), CD8 (clone RPA-T8), CD69 (clone FN50), CXCR3 (clone 1C6/CXCR3 ), CCR6 (clone 11A9), HLA-DR (clone L243), PD-1 (clone EH12.2H7, Biolegend, San Diego, CA, USA ) all purchased from BD Biosciences (San Jose , CA, USA) unless specified. Flow cytometry data was analyzed using gating strategies described previously using FlowJo platform (Ashland, OR,USA).

**Statistical analyses**

Graphs were prepared and statistical comparisons applied using GraphPad Prism version 8 (La Jolla, CA). Various statistical comparisons were performed viz. 2-tailed Student’s t-test, one-way or two-way analysis of variance (59) with Tukey’s or Sidak’s multiple comparisons tests wherever
applicable and as described in the figure legends. Statistical differences between groups were reported significant when p-value is less than or equal to 0.05. The data are presented in mean ± SEM.


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Figure 1. Clinical correlates of TB reactivation in ART-treated NHPs with *Mtb/SIV* co-infection: (A) Study outline. Sixteen Indian origin rhesus macaques infected with low-dose *Mtb* CDC1551 via aerosol route developed latent TB. At week 9 post *Mtb* challenge, some of these NHPs (n=12) were challenged with intravenous infection of *SIVmac239*. At week 13, of the twelve *Mtb/SIV* co-infected NHPs (n=4) received ART regimen while n=8 served as ART-naïve controls. Animals with TB reactivation and signs of disease had to undergo an early necropsy to meet the humane endpoints criteria set by TNPRC IACUC committee, while animals with no signs of active disease were necropsied by week 24 (B) Plasma viral loads were measured at peak of SIV infection i.e. around week 11 of infection and at the endpoint. (C) Survival curve, X-axis displays times in weeks post *Mtb* infection and Y-axis displays percent survival. (D) Serum C-reactive protein (CRP) levels measured at week 11 and the endpoint. (E) BAL SIV RNA load measured at necropsy (F) RNAscope in-situ hybridization assay was used to examine the presence of SIV RNA in tissues like lung, BrLN, and spleen. ART treatment substantially reduced the viral particles, vRNA+ cells (red), in spleen and BrLN tissue whereas the changes were not appreciable in the sparsely infected lung parenchyma. The three groups studied are *Mtb* infection only i.e. LTBI (n=4, green), *Mtb/SIV* co-infection i.e. ART-naïve (n=8, red) and *Mtb/SIV* co-infection with ART treatment i.e. ART (n=4, blue); (B, D, E) data represented as mean±SEM; (C) log-rank test (Mantel-Cox) used to compare survival curves; (D) two-way ANOVA with Sidak’s multiple comparison test and (B, E) two-tailed Student’s t-test; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. The error bars in the dot plot represent the standard error of the mean (SEM)
Figure 1.
Figure 2. *Mtb* bacterial burden and lung pathology. *Mtb* bacterial burden was obtained by normalizing the CFU counts to log-transformed CFU per gram of tissue. (A) *Mtb* CFUs in total BAL sample (cellular + acellular component); (B) *Mtb* CFU count normalized to per gram of lung tissue collected at necropsy; (C) Multiple granulomas (n= 1-6) per animal were grouped as per the experimental classification of the animal; each granuloma was weighed and its CFU count normalized to per gram of granuloma tissue; (D) BrLN; (E) Spleen; (F) percent lung involvement was calculated by pathologists through extensive analysis of serially cut fresh lung samples and counting no. of lesions in low power magnification. (G) Gross pathology and H&E staining portray the large granulomatous and necrotic lesions in animals with SIV induced *Mtb* reactivation while minimal pathology seen in samples from latent TB animals. The three groups studied are *Mtb* infection only i.e. LTBI (n=4, shown in green), *Mtb*/SIV co-infection i.e. ART-naïve (n=8, shown in red) and *Mtb*/SIV co-infection with ART treatment i.e. ART (n=4, shown in blue). (A-F) data represented as mean±SEM; (A-F) One-way ANOVA with Tukey’s multiple comparison test; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. The error bars in the dot plot represent the standard error of the mean (SEM)
Figure 2.
Figure 3. Differential CD4+ T cell restoration in alveolar and interstitial compartments after ART therapy in Mtb/SIV co-infection of NHPs. Multiparameter flow cytometry was performed on single-cell suspension of various tissue samples and whole blood collected at necropsy from Mtb/SIV co-infected rhesus macaques treated with ART. The three groups studied are Mtb infection only i.e. LTBI (n=4, green), Mtb/SIV co-infection i.e. ART-naïve (n=8, red) and Mtb/SIV co-infection with ART treatment i.e. ART (n=4, blue). CD4+ T cell frequency was analyzed in (A) whole blood, (B) BAL, (C) lung, (D) Br. LN, (E) spleen. (F) confocal microscopy of formalin-fixed and paraffin-embedded (FFPE) sections from lungs harvested at the endpoint of LTBI (n=3), ART-naïve (n=6), and ART (n=3) treated macaques reports the CD4+ T cells (CD4/nuclei) in lung tissue sections, counted using HALO™ image analysis software. (G) represent CD4+ T cell (red), CD68+CD163+ macrophage (green), nucleus (grey), autofluorescent RBCs (yellow), and white arrowheads indicate macrophages phagocytosing CD4+ T cells in the lungs of LTBI, ART-naïve and ART groups respectively. (A-F) data represented as mean±SEM; (A-F) one-way ANOVA with Tukey’s multiple comparison test; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. The error bars in the dot plot represent the standard error of the mean (SEM).
Figure 3.
**Figure 4. Dysregulation in homing of CD4+ T cells to iBALT in lung interstitium of Mtb/SIV co-infected macaques.** Immunohistochemistry staining and confocal imaging of formalin-fixed, paraffin-embedded (FFPE) lung sections from Mtb/SIV infected macaques with/without ART. The figure is representative of three experimental replicates. (A) Nuclei/DAPI (grey), SIV RNA+ (red), CD3+ T lymphocytes (blue) and CD68+CD163+ macrophages (green) identify macrophages phagocytosing vRNA+ cells present in the iBALT. (B) Nuclei/DAPI (grey), IDO1 expressing cells (red), CD20+ B lymphocytes (blue) and CD68+CD163+ macrophages (green) identify well-organized B cell zones of iBALT and presence of IDO1 expressing macrophages in the T cell zone of the iBALT. (C) Nuclei/DAPI (grey), IDO1 expressing cells (red), and CD68+CD163+ macrophages (green) of granuloma in Mtb/SIV co-infected animal showing that majority of the IDO1 expression is seen in CD68+CD163+ macrophage rich layer of granulomas in macaques with TB reactivation. (D) Nuclei/DAPI (blue), IDO1 expressing cells (green), and CD141+ tolerogenic dendritic cells (red), shows IDO1 expression by dendritic cells. The IHC staining was performed on sections of the lungs from macaques with Mtb infection only i.e. LTBI (n=3), Mtb/SIV co-infection i.e. ART-naïve (n=3) and Mtb/SIV co-infection with ART treatment i.e. ART (n=3) groups.
Figure 4.